Pharmacology Biochemistry & Behavior, Vol. 35, pp. 561–565. © Pergamon Press plc, 1990. Printed in the U.S.A. 0091-3057/90 $3.00 + .00

On the Development of Behavioral Tolerance to Organophosphates III: Behavioral Aspects

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Received 5 July 1989

WOLTHUIS, O. L., I. H. C. H. M. PHILIPPENS AND R. VANWERSCH. *On the development of behavioral tolerance to organophosphates III: Behavioral aspects.* PHARMACOL BIOCHEM BEHAV **35**(3) 561–565, 1990.—As part of a study on the mechanisms underlying behavioral tolerance to cholinesterase-inhibiting organophosphates (OP's) the present investigation was focussed on behavioral procedures affecting the development of tolerance. The effects of chronic administration of the OP's DFP (600 μg/kg SC) and soman (60 μg/kg SC) were compared in rats. These doses do not cause detectable effects upon close observation of the animals. As was found before, behavioral tolerance developed following DFP, but not following soman. Repeated behavioral testing affected the development of tolerance. Cross-tolerance between these two inhibitors was not found. Surprisingly, when DFP was administered 48 hr after soman, all animals were observationally normal, and when soman was given 48 hr after DFP the majority of the animals died. This indicates that the sequence in which these inhibitors were administered was of major importance. It is concluded that practice-related and/or state-dependent factors are important for the development of behavioral tolerance and that one should be careful in making generalizing statements about tolerance to cholinesterase-inhibiting OP's.

DFP Soman Cholinesterase Inhibitors Behavioral tolerance

UPON repeated injections in rats of low doses of the irreversible cholinesterase-inhibiting organophosphates (OP's) DFP and soman, behavioral tolerance was found after DFP, but not after soman (14). Measured 1 hour after the injections, performance decrements after DFP gradually subsided in the course of weeks, whereas these decrements after soman remained. Twenty-four hours after each injection of each of these OPs' performance was virtually normal.

These differences between the effects of the two OP's could *not* be explained: 1) by differences in the progressive inhibition of acetylcholinesterase activity to very low values or by de novo synthesis of this enzyme, both measured in various tissues (CNS, muscle and blood), 2) by differences in the down-regulation of muscarinic receptors in the CNS or nicotinic receptors in the striated muscles, 3) by differences in phosphorylphosphatase (DFP-ase or soman-ase) activity in blood, plasma or liver. Recent results (15) indicate that the differences can also not be explained on the basis of differences in the inhibition of carboxylesterases. So far, the only significant findings are differences in the electrophysiological effects of these OP's at the neuromuscular synapse (11); electrophysiological experiments with hippocampal slices are in progress.

The aim of these studies is to elucidate the mechanism(s) whereby an intoxicated subject can survive—and even function relatively normal—notwithstanding a very low AChE activity. Knowledge of these mechanisms may provide cues for a possible pharmacological induction or mimicking of a state of "instant" tolerance which could be therapeutically useful following acute intoxications with these compounds. An argument against this may be that, after all, no signs of behavioral tolerance were found in behavioral tests 1 hour after the injection of soman [in contrast to DFP, see (13) and present results]. However, it should be noted that 24 hours after soman, shuttlebox performance was virtually normal in the presence of a profound inhibition of AChE. Hence, the effects of soman found 1 hour after the injection might not be due to AChE inhibition, but to another effect of this compound. Soman may be an exception, since tolerance phenomena have been found with many other cholinesterase inhibitors (4, 7, 12). In this respect it might be relevant that soman acts preferentially in the CNS (15,16) and may have a different effect on the receptor ionic-channel sites than DFP (1,2). In addition, it should be kept in mind that soman is a far more specific inhibitor of cholinesterases than, e.g., DFP; the latter compound inhibits many other serine-esterases (3).

In the attempts to elucidate the mechanisms underlying these tolerance phenomena, behavioral factors should not be ignored. The group of Bignami, in particular, has shown in chronically OP-treated animals that repeated testing contributes to the development of tolerance (4,5). They refer to this as "the behaviorally augmented (practice-related) component of tolerance" (6). Hence, as part of a study on the development of tolerance, attention was focussed on behavioral factors. In addition, attempts were made to see if cross-tolerance between DFP and soman could be detected. Finally, it was attempted to investigate whether the sequence in

which the two compounds were given made any difference; i.e., first DFP and then soman, or alternatively first soman and then DFP.

METHOD

Animals

Male Small Wistar rats were used with a starting body weight of 150–170 g. They were bred in the laboratory under SPF conditions, i.e., hysterectomy derived, bacteriologically controlled and kept under sterile conditions. All animals were experimentally naive.

Procedures

Training and testing occurred by active avoidance in a two-way shuttlebox as described before (14). Briefly, the method was as follows. Animals that received 20 trials a day at time intervals of 1 min + 20% (random) were trained to avoid footshock (250 μA, constant current principle) by moving into the other compartment within 10 sec after a light stimulus was presented. It took usually 4–6 days of training to reach the criteria, which was 80% or more correct avoidance responses (CARs).

In Experiments I and II all animals were first trained and thereafter randomly assigned to the different treatment/test groups, all animals were subcutaneously injected (injection volume: 1 ml/kg) on Monday, Wednesday and Friday. Performance tests 24 hr after injection took place on Tuesday and Thursday, not on weekends. The different treatment and test-groups were:

Experiment I consisted of 5 groups, each of 8 animals:
a. Saline 3× per week. Performance tested 1 hr and 24 hr after injection.
b. DFP 600 μg/kg 3× per week. Performance tested 1 hr and 24 hr after injection. On session 31 these animals, which had become tolerant to DFP, were injected with 60 μg/kg *soman* instead of DFP and performance was tested 1 hr and 24 hr later.
c. Saline 3× per week. Performance not tested, except 1 hr and 24 hr after injection of saline in session 31.
d. DFP 600 μg/kg 3× per week. Performance not tested, except 1 hr and 24 hr after injection of DFP in session 31.
e. DFP 600 μg/kg 3× per week. Performance was tested 6 hr (!) and 24 hr after injection.

Experiment II consisted of 4 groups; at the start of the experiment each group consisted of 8 animals:
a. Saline 3× per week. Performance tested 1 hr and 24 hr after injection.
b. Soman 60 μg/kg 3× per week. Performance tested 1 hr and 24 hr after injection. On session 36 these animals were injected with DFP 600 μg/kg.
c. Saline 3× per week. Performance not tested, except 1 hr and 24 hr after injection of saline in session 36.
d. Soman 60 μg/kg 3× per week. Performance not tested, except 1 hr and 24 hr after the injection of soman in session 36.

Experiment III consisted of 4 groups of 6 animals each, which were going to be injected with soman (60 μg/kg) or DFP (600 μg/kg) according to the following schedules:

		Injections:		
Monday	Tuesday	Wednesday	Thursday	Friday
a. soman	—	DFP	—	soman
b. DFP	—	soman	—	DFP
c. soman	DFP	soman		
d. DFP	soman	DFP		

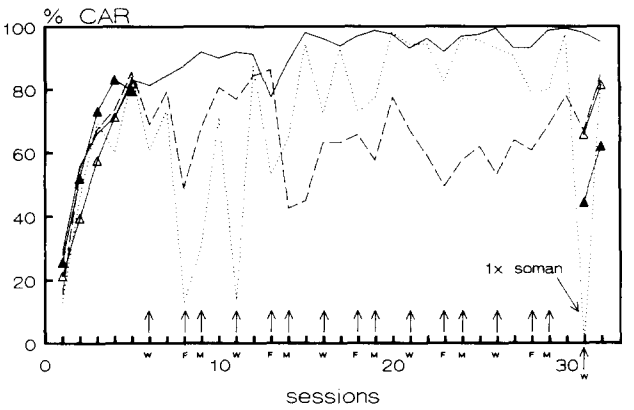

FIG. 1. The effects of repeated SC injections (see arrows) with saline (1 ml/kg) or DFP (600 μg/kg) on shuttlebox performance of rats, defined by the percentage correct avoidance reactions (% CAR). Injections were given on Monday, Wednesday and Friday. Performance in two groups (saline: — and DFP: ····) were tested 1 hr and 24 hr after the injections (except on weekends). At the end of the experiment, when the DFP-treated animals had become behaviorally tolerant, they were injected with soman (60 μg/kg) and tested 1 hr and 24 hr later. In an additional DFP-treated group (----) performance was tested 6 hr and 24 hr after injection. Two other groups were trained and subsequently injected with saline (△) or DFP (▲), but were not tested until 1 hr and 24 hr after the last injection in session 31. The mean performance of groups of 8 rats is shown, for the sake of clarity standard errors have been omitted. M, W and F indicates Monday, Wednesday and Friday respectively. It can be seen that repeated behavioral testing, as well as the time interval between injection and testing, affect the development of tolerance. Moreover, cross-tolerance between soman and DFP is absent.

Compounds and Doses

DFP (diisopropylfluorophosphate) and soman (pinacolyl methylphosphonofluoridate) were synthesized by Dr. H. P. Benschop from the Prins Maurits Laboratory TNO. Both compounds were at least 99% pure. The doses chosen were based on preliminary experiments with different dose-levels of DFP or soman. These experiments were carried out preceding our first paper on this topic (14). In these experiments the highest doses of DFP and soman were selected that did not cause overt symptoms upon close observation in the course of 2 weeks, during which period these compounds were injected 3× per week.

Statistics

The test of Welch, including Bonferoni's correction, was used to test the statistical significance of differences (12).

RESULTS

Experiment I

The results obtained with Group Ia (saline) and Group Ib (DFP, up to session 31) shown in Fig. 1 essentially confirmed earlier results (13). These treatments were repeated here to have a simultaneously running control in the same experiment as the other groups. Behavioral tolerance to DFP developed as before. However, when in session 31 soman was injected instead of DFP, performance appeared to have dropped to zero when the animals were tested 1 hr after the injection. Twenty-four hours later performance was practically normal again.

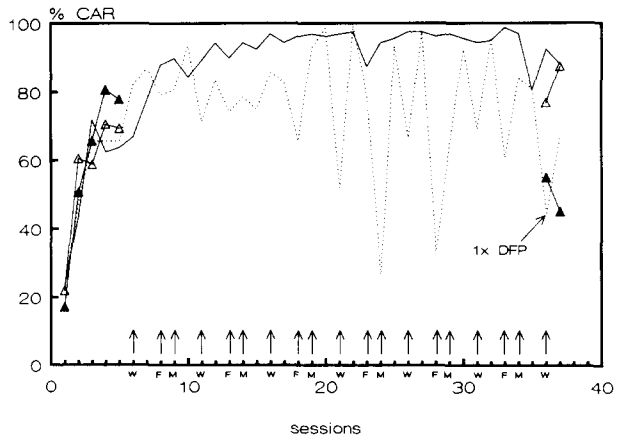

FIG. 2. The effects of repeated SC injections (see arrows) with saline (1 ml/kg) or soman (60 μg/kg) on shuttlebox performance of rats, defined by the percentage correct avoidance reactions (% CAR). Procedures were identical to those shown in Fig. 1. Two groups (saline: — and soman: ····) were behaviorally tested 1 hr and 24 hr after being injected; at the end of the experiment the soman-injected group received one injection of DFP and was tested 1 hr and 24 hr after injection. Two other groups were subjected to the same injection schedule with saline (△) or soman (▲), but were not behaviorally tested until 1 hr and 24 hr after the injection at session 35. M, W and F indicates Monday, Wednesday and Friday respectively. Again, it can be seen that behavioral tolerance to soman hardly develops, although the results are more erratic than previously obtained results. Hence, it is difficult to assess the effect of repeated testing or to determine whether cross-tolerance with DFP exists. The effects of DFP shown here are certainly smaller than those of an injection with soman following repeated doses of DFP, as shown in Fig. 1. The mean performance is shown of 8 animals per group.

Groups Ic (saline) and Id (DFP) received identical injections, but their performance was not tested between sessions 5 and 31. Performance in session 31, tested 1 hr after saline, dropped relative to their performance in session 5, i.e., from 81.9±3.0% to 65.6±9.1% (not significant, $p2>0.05$), which recovered to 81.3±7.9% when the animals were tested 24 hr later. However, in Group Id, 1 hr after an injection of DFP in session 31, performance dropped considerably relative to performance in session 5, i.e., from 79.4±3.5% to 44.4±8.9% (significant, $p2<0.05$). Compared with the results of the saline-treated group in session 31, there was also a drop in mean performance level, but this difference was not significant.

It was interesting to note, if DFP-injected animals were not tested 1 hr and 24 hr after, but 6 hr and also 24-hr after injection (Group Ie), that their initial performance decrements measured 6 hr after injection were not only smaller than those of animals tested 1 hr after injection, but also that their average performance tested 24 hr after injection remained suboptimal. This confirmed the results of preliminary experiments in which behavioral testing took place 2, 4 or 6 hr after the injection and in which a trend could be observed that behavioral tolerance did not occur or occurred slower if the time interval between injection and testing was prolonged.

Cross-tolerance between DFP and soman was not found; if soman (60 μg/kg) was injected in session 31 into animals that had become behaviorally tolerant to DFP, performance of all animals dropped to zero (see Fig. 1).

Experiment II

In this experiment (Fig. 2) the animals in Groups IIa (saline) and IIb (soman) performed as expected on the basis of earlier experiments (13), albeit that the responses 1 hr after soman were slightly more erratic than before. Hence, the counter-experiment to detect cross-tolerance between the repeatedly injected soman followed by a single injection of DFP, did not provide evidence against or in favor of cross-tolerance.

In those animals which were repeatedly injected with saline, but were not tested between session 5 and 36, i.e., Group IIc, performance 1 hr after the injection increased somewhat from 69.4±9.3% in session 5 to 76.9±9.5% in session 36 and had increased further to 87.5±4.1% when tested 24 hr later. In the animals repeatedly injected with soman and not tested between session 5 and 36, performance dropped from 77.9±6.3% to 55.0±12.3% (not significant, $p2>0.05$), which decreased further to 45.0±13.3% when tested 24 hours later. The latter performance differed significantly ($p2<0.05$) from that in session 5.

Experiment III

In Experiment IIIa, i.e., soman → no injection day → DFP → no injection day → soman, the animals were observationally normal after DFP, but after the second injection of soman their condition rapidly deteriorated and the animals died.

In Experiment IIIb, i.e., DFP → no injection day → soman → no injection day → DFP, 4 out of 6 animals died after the injection of soman and the remaining 2 animals were in a very bad condition and died immediately after the second dose of DFP was injected.

If the "no injection days" (Tuesday and Thursday) were omitted and the injections were given every day, a similar picture emerged. In group IIIc, i.e., soman → DFP → soman, the animals were given again observationally normal after DFP, but died after the second dose of soman. In group IIId, i.e., DFP → soman → DFP, all animals died after the dose of soman. Consequently, the second dose of DFP could not be given. All animals that died exhibited the classical symptoms of OP poisoning.

The results of these experiments indicate that a dose of soman that causes no symptoms by itself, will be lethal when given 24 hr or 48 hr after DFP, whereas with a similar dose regime, DFP after soman will cause no observable behavioral changes. These results confirmed preliminary findings (2 groups of 4 animals each) in which two doses of DFP followed by soman resulted in a bad condition of the animals (1 out of four died), whereas two doses of soman followed by DFP did not lead to observable behavioral changes. In these preliminary experiments the OP's were given every other day.

DISCUSSION

The results obtained with DFP (see Fig. 1) clearly demonstrate that biochemical factors such as down-regulation of muscarinic receptors or perhaps presynaptic effects [see (14) for references] are not the only factors responsible for behavioral tolerance to DFP. Behavioral, i.e., "practice-related" factors play an important role in the development of behavioral tolerance. Whether chronically DFP-injected animals are behaviorally tested on a day to day basis or not makes quite a difference for the ultimate behavioral test results in session 31 following a DFP injection.

If the temporal link between the injection and testing is made harder to detect, i.e., when the time interval between injection and testing is prolonged from 1 hr to 6 hr, a clearcut behavioral tolerance does not even develop within 30 sessions. Surprisingly, those test results obtained 24 hr after the injections of DFP do not approach control levels and are hardly different from those obtained 6 hr after DFP. Again, the results of this group show that behavioral, and perhaps "state-dependent" factors play an impor-

tant role in the development of the tolerance phenomenon.

As before (14), a clear tolerance to soman did not develop (Fig. 2) within 35 sessions, although on the basis of the present results it might be possible that ultimately some degree of tolerance may be achieved if the experiment would be prolonged. Whatever the case, it could not be established whether or not regular behavioral testing makes a difference with respect to the performance measured 1 hr after the soman injection in session 35.

A possible difference between the two inhibitors might be that soman may cause brain lesions (10), even in the doses used here (9). This has not been reported for DFP, which might mean that such lesions were not found after administration of this OP. If true, this might help to explain the different effects of these two inhibitors on performance. Therefore, it is of interest to report here that in these animals ($n=5$ of each treatment group), upon a "blind" light microscopical examination of 3 μm slices stained with haematoxilin-eosin, no lesions in the hippocampi or in several cortical areas were found in any of the preparations (see the Acknowledgement section). However, subtle brain damage, such as, e.g., a small but significant reduction in the number neurons, cannot be detected by the methods used so far and have to await cell counts.

If cholinesterase inhibition and the resulting acetylcholine accumulation would be the only mechanisms governing the development of tolerance, one would expect that cross-tolerance would exist between cholinesterase inhibitors. It is obvious from the results that this is not the case. It is true that an inconclusive result is obtained following a DFP injection in session 35 in chronically soman-treated animals because tolerance has not developed (Fig. 2), but Fig. 1 shows clearly that a single low dose of soman in animals made tolerant to DFP has a detrimental effect on performance. It is, therefore, unlikely that cholinesterase inhibition is the only factor.

In the experiments on cross-tolerance it was noted that the performance of those animals in Experiment 1 (see Fig. 1) that received one injection with soman after they had been made behaviorally tolerant to DFP was worse than the animals in Experiment 2 (see Fig. 2) that received one injection of DFP after being repeatedly injected with soman. It was expected that in the absence of cross-tolerance the effect of a single DFP injection into chronically soman-treated animals performance would also drop to a near-zero level, which did not happen. This raised the question whether the sequence in which these two inhibitors were administered might be important. Hence, Experiment 3, in which the effect of the sequence of administration of these two inhibitors was investigated. The results came as a complete surprise; DFP after one or two injections of soman caused no observable effects, whereas soman after one or two injections of DFP was lethal or made the animals very ill. These results are hard to understand. From what we know, the main and prevailing action of both inhibitors at these dose levels is the inhibition of cholinesterase. It is also known that when tested with a whole range of serine-esterases, soman is a far more specific inhibitor for acetylcholinesterase than DFP (3). Yet, soman seems an exception since a whole series of cholinesterase-inhibiting OP's and also carbamates have been shown to induce behavioral tolerance upon chronic administration (4, 7, 13). Are the effects of soman linked to the fact that soman (in comparison with DFP) acts preferentially on the CNS (16,17) or are they due to a different action of these two cholinesterase inhibitors at the acetylcholine receptor sites (1,2)? Or are the sequence-dependent differences in effects of these two OP's (both carboxylesterase inhibitors) due to the fact that the toxic effects of soman are more dependent than DFP on the scavenging action of carboxylesterases? For example, if the majority of the carboxylesterases are inhibited by the larger amount of DFP molecules, a small amount of soman that meets hardly scavenging carboxylesterases in the blood will become rapidly lethal, whereas after a smaller amount of soman molecules, there will be sufficient scavenging carboxylesterase activity left to bind the bulk of the DFP molecules. The answers to these questions are not yet known, but will be investigated.

After this manuscript had been submitted for publication, we became aware of a paper by Fernando *et al.* (8), who injected rats at four-day intervals with soman (90 μg/kg) or sarin (100 μg/kg) and measured lethality, hypothermia, tremors and convulsions. Using these higher doses of soman they found, instead of tolerance, signs of increasing neurotoxicity and lethality. The present results amplify and extend these findings.

In conclusion, the present results show the importance of practice-related or state-dependent factors in the development of behavioral tolerance. They also indicate that one has to be careful in making generalized statements about tolerance to organophosphorous cholinesterase inhibitors, as shown in the results of the experiments on cross-tolerance and in those in which the sequence of administration of two of these inhibitors was varied.

ACKNOWLEDGEMENT

The authors thank the group of Mrs. D. M. G. de Groot (Msc) for their rapid histological assessment of the morphological condition of the various brain areas.

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