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Long-Term Study of Brain Lesions Following Soman, in Comparison to DFP and Metrazol Poisoning

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The long-term histopathological effects of acute lethal (95 μg kg^{-1}) and sublethal (56 μg kg^{-1}) doses of soman were studied in rats and were compared to lesions caused by equipotent doses of either another cholinesterase (ChE) inhibitor, DFP (1.8 mg kg^{-1}), or a non-organophosphorus convulsant, metrazol (100 mg kg^{-1}). Severe toxic signs were noted following one LD_{50} dose administration of all the compounds, yet only soman induced brain lesions. Moreover, even when administered at a sublethal dose (0.5 LD_{50}), soman induced some histological changes without any clinical signs of intoxication.

Soman-induced brain lesions were assessed quantitatively using a computerized image analyser. The analysis was carried out for up to 3 months following administration, and a dynamic pattern of pathology was shown. The cortical thickness and area of CA1 and CA3 cells declined significantly as early as 1 week post-exposure. No pathological findings were detected following DFP and metrazol administration. It is therefore suggested that brain lesions are not common for all ChE inhibitors and that convulsions *per se* are not the only factor leading to brain damage following the administration of soman. The degenerative process (found also with the sublethal dose of soman) might be due to a secondary effect, unrelated to soman's clinical toxicity, but leading to long-term brain injuries.

Introduction

Soman (pinacolyl methylphosphonofluoridate), is a potent, rapidly acting irreversible cholinesterase (ChE) inhibitor which causes, at near LD_{50} doses, both peripheral and central clinical symptoms of intoxication.

Brain injuries following soman poisoning have been found in a variety of species, where a consistent pattern of neuronal degeneration and necrosis was found in anatomically defined brain regions.[1-3] Behavioural alterations were also found following lethal doses of soman.[4,5] Although the descriptive neuropathology induced by soman is now fairly well established, there is still a considerable debate as to the mechanism(s) underlying these lesions. It is not decided yet whether the injury to the brain is the result of soman itself on specific neurons, or whether it is due to indirect effects such as convulsions, or ChE inhibition, which are components of soman poisoning. Soman-induced brain injury has been assumed to be associated with seizures, since, lesions are mainly found following convulsive doses of soman[2,6-8] and since the extent of brain damage is minimal following anticonvulsant treatments.[9,10] Cholinergic hyperactivity due to ChE inhibition, has been suggested as a major factor in the pathogenesis of soman-induced neuronal necrosis.[11] It is thought to occur via a mechanism similar to that of acetylcholine (ACh)-induced myopathy.[12]

The present study was undertaken in order to characterize the long-term effects of soman on the central nervous system (CNS) up to 3 months following administration, and to isolate the relative contribution of either convulsions or ChE inhibition to the specific morphological changes induced by soman.

For this purpose, the pathological effects of soman (1 LD_{50}) were compared to those of either metrazol (pentylentetrazol), which is a non-organophosphate (OP) convulsant, to DFP (diisopropyl phosphorofluoridate), which is another OP cholinesterase inhibitor, and to a non-convulsive dose (0.5 LD_{50}) of soman itself, which might separate the specific role of this compound on nervous tissues. Another objective of this study was to quantitatively assess the morphological brain damage following a lethal dose of soman. An established quantitative histological model of OP-induced brain lesions will serve in evaluating the efficacy of therapeutic mixtures in preventing these lesions.

Methods

Animals

Male Sprague-Dawley rats (Charles River, England), weighing 250–300 g, were used throughout this study. The animals were maintained on a 12 h light–dark cycle and were provided with rat purina chow and water *ad libitum*.

Chemicals

Soman (pinacolyl methylphosphonofluoridate) was kept as a stock solution (20 mg ml^{-1} in propylene glycol) at –20°C. Likewise, DFP (diisopropyl phosphorofluoridate, Sigma, USA) was kept as a stock solution (40 mg ml^{-1} in propylene glycol) at –20°C. Metrazol (pentylentetrazol, Sigma USA) was dissolved in saline (200 mg ml^{-1}).

Fresh saline solutions of the above chemicals were prepared for each experiment. The toxicity of soman and DFP was tested on a small group of mice (n=8–10) before the beginning of the experiment, to assure the quality of these solutions.

Experimental design

Animals were assigned to one of the following treatment groups: soman (95 μg kg^{-1}, i.m.: n=80), DFP (1.8 mg kg^{-1}, i.m.: n=50), metrazol (100 mg kg^{-1}, s.c.: n=50) and saline (injected s.c.: n=30). In an additional group, soman was injected at a sublethal, 0.5 LD_{50} dose (50 μg kg^{-1}, i.m.: n=30).

Histology

For histological evaluation, animals were sacrificed by decapitation 1, 3, 7, 30 and 90 d following administration (5–7 rats per group). Brains were rapidly removed from the skull, immersed in 4% neutral buffered paraformaldehyde at 4°C and processed routinely for paraffin embedding. Coronal sections, 6 μm thick, were cut serially at 100 μm intervals. Selective brain sections were stained with haematoxylin and eosin (H&E) for general histology, and with Von Kossa staining for the identification of calcium deposits.[13]

Morphometry

Quantitative evaluations of some morphometric parameters were performed using a computerized image analysis system (Olympus Cue 2, Galai, Israel). The system consisted of a photomicroscope fitted with a CCD video camera and a power unit that transmitted the microscopic images on a Sony colour monitor.

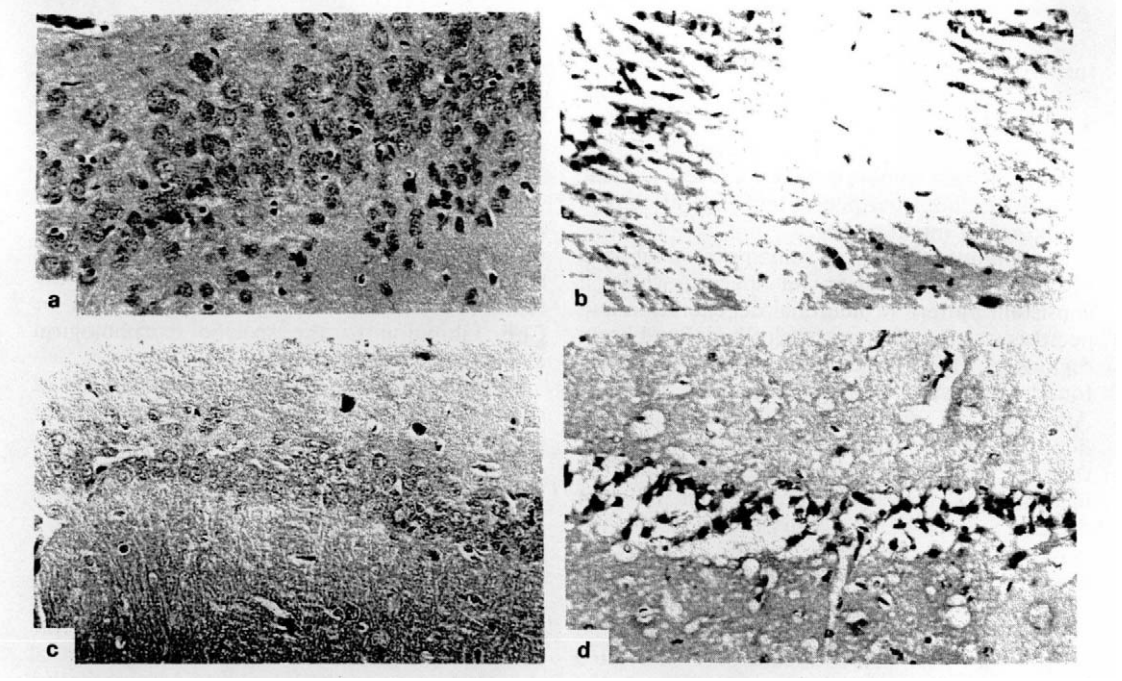

Figure 1 Brain sections taken from the piriform cortex (A,B) and hippocampal CA1 region (C,D) 24 h post-DFP, soman and metrazol exposure (1 LD_{50}). Note normal morphology following DFP (A) and metrazol (C) injections, compared to brain damage seen following soman (B,D) (H&E staining; original magnification ×100).

Specific on-line images were frozen and captured by means of a frame-grabber, and on these the morphometric analysis was performed. The morphological parameters that were chosen to represent brain damage were: cortical thickness as an indicator of brain atrophy, and the number and area of cells within the hippocampus as an expression of cellular damage. Hippocampal cells were selected for analysis because of their relation to cognitive function.[14]

Cortical thickness was evaluated on the basis of measurements in both hemispheres in three serial sections for each animal.

The mean individual surface area of cells in CA1 and CA3 subfields was calculated from measurements of at least ten cells in each hemisphere in two serial sections from each animal. Cells, selected at random in the same defined subregions, were measured using a ×25 microscopic objective.

The number of cells was evaluated in a frame of $2 \times 10^5\ \mu m^2$ in both hemispheres in three serial sections from each animal.

All measurements were performed in sections corresponding to Figure 32 in Paxinos and Watson's Rat Brain Atlas.[15]

Data analysis

The morphometric data were subjected to one-way analysis of variance (ANOVA). Whenever statistical significance was observed ($P<0.05$), further analysis of the various experimental groups was carried out using Scheffe test.

Results

Animal observations

Animals injected with 1 LD_{50} of either soman (95 $\mu g\ kg^{-1}$) or DFP (1.8 $mg\ kg^{-1}$) developed typical symptoms of cholinergic toxicity. While DFP-treated animals displayed tremors and muscle fasciculations, the soman-injected rats also experienced persistent convulsions. No clinical signs were observed following 0.5 LD_{50} of soman. Rats administered with 1 LD_{50} of metrazol (100 $mg\ kg^{-1}$) displayed intensive convulsions, mainly during the first hour post-exposure.

Throughout the 3-month experimental period, only animals injected with 1 LD_{50} of soman were highly irritable and had recurrent epileptic episodes. These rats were very difficult to handle and exhibited behavioural symptoms similar to those observed following septal lesions.[16]

Histology

No neuronal lesions or any other histological alterations were found in control rats, in DFP or in metrazol-injected animals, up to 3 months post-administration.

Following 1 LD_{50} of soman, bilateral symmetric lesions were observed 24 h after injection. The damage was most severe in selected populations of neurons in the piriform cortex, hippocampus, frontal cortex and dorsolateral thalamic nuclei (Figure 1).

The soman-induced lesions progressed with time and spread to brain areas which were not initially involved. While the necrosis of CA1 pyramidal cells occurred shortly after intoxication (Figure 1-D), CA3 cells were not affected until 1-month later, when they appeared shrunken and had pyknotic nuclei.

Three months following 1 LD_{50} of soman, brain injury was characterized by enlarged ventricles, axonal degeneration, and an almost complete disappearance of the hippocampal CA1 cell layer. Cellular damage was found also in the septum, in both lateral and medial nuclei, in the amygdala and in the entorhinal cortex.

In addition, at this time-interval, calcification sites were detected in the thalamus, mainly in ventromedial nuclei (Figure 2). Following 0.5

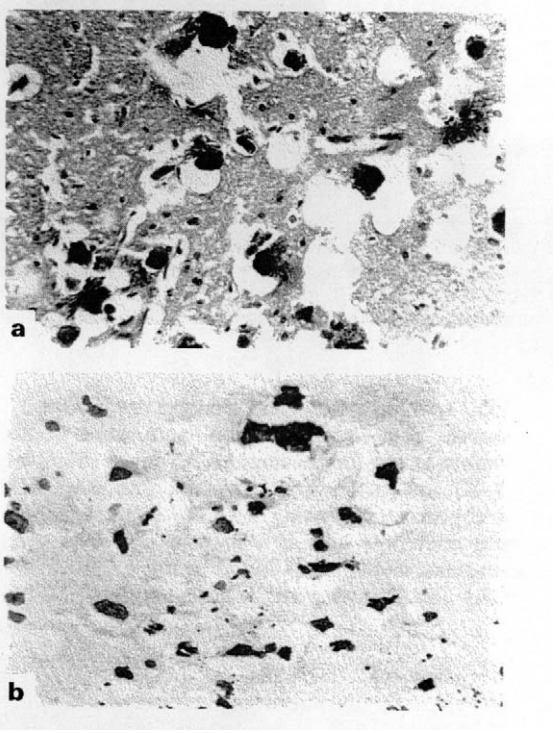

Figure 2 Brain sections taken from thalamic ventromedial nuclei. 3 months following soman (1 LD_{50}) exposure, showing calcification. A; H&E staining, B; Von Kossa staining (original magnification ×100).

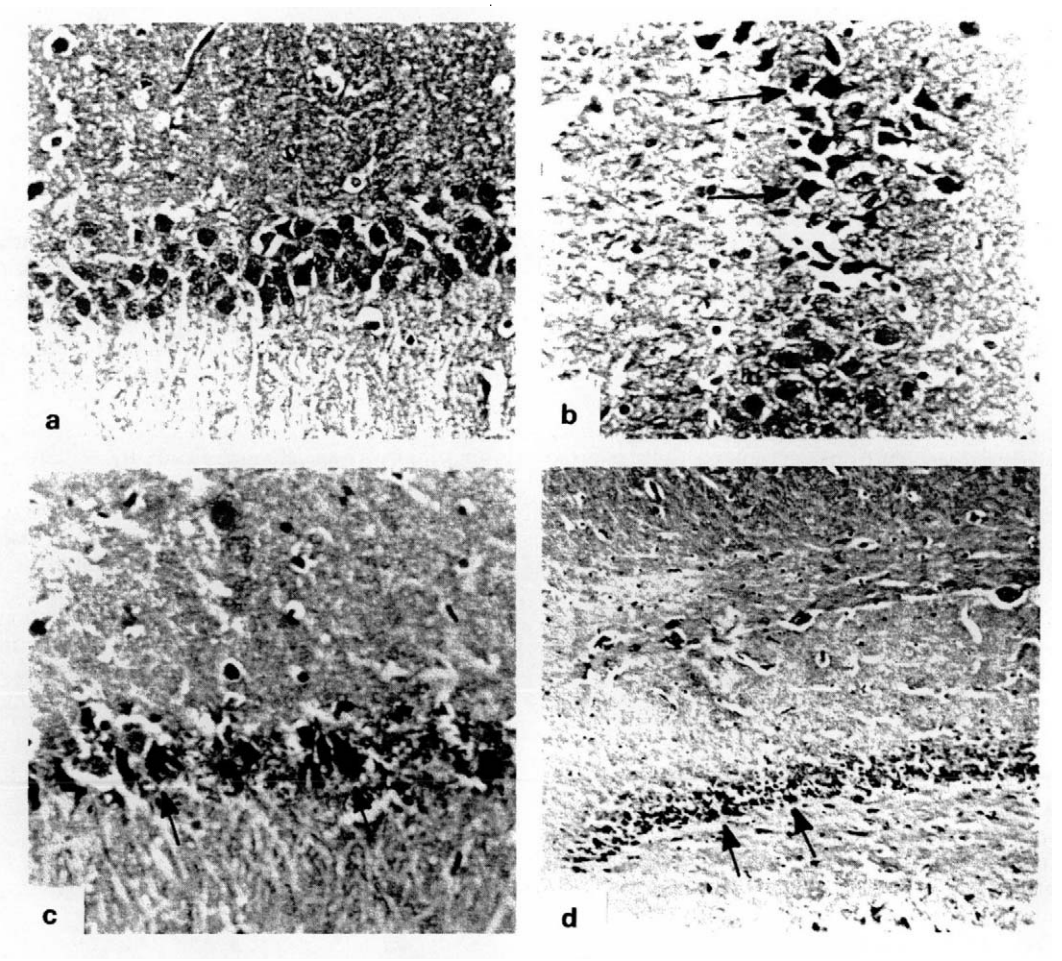

Figure 3 Brain sections through the hippocampus taken from rats injected with 0.5 LD_{50} soman (50 μg kg^{-1}), 1 (A,B) and 3 months (C,D) post-administration. While at 1 month, morphological changes can be noted only in the CA1 region (B, arrows), at 3 months post-exposure, pyknotic cells are shown in CA3 (C, arrows) and in the dentate gyrus (D) (H&E staining. Original magnification ×100 for A-C, ×40 for D).

LD_{50} soman, histological changes were noted 1 month after administration and were found mainly in the hippocampal CA3 layer (Figure 3 A–B) and in the frontal cortex. The degenerative process, following 0.5 LD_{50} soman, continued with time and at 3 months post-exposure, neuronal damage was found in the hippocampal CA1 and dentate gyrus regions (Figure 3 C–D).

Morphometry

Quantitative morphometric analysis of the effect of soman (1 LD_{50}) on cortical thickness as a function of time is presented in Figure 4. A significant decrease (P <0.001) in cortical thickness, compared to baseline values, was found 1 week after injection. Cortical thickness declined throughout the first week, but no further change was found thereafter, up to 3 months post-exposure.

Analysis of the cells within the hippocampus revealed a specific effect of soman on both cell area (Figure 5) and cell number (Figure 6) in the CA1 and CA3 regions. A significant decrease in cell area (P <0.001) was found during the first week post-exposure. No further decline was found after the first week.

While the effect of soman on cell area was apparent during the first week in both types of hippocampal pyramidal cells (Figure 5), the time–response effect on cell number differed between the CA1 and CA3 cells. The number of CA1 cells decreased significantly from the first week (P <0.001) and the process continued up to

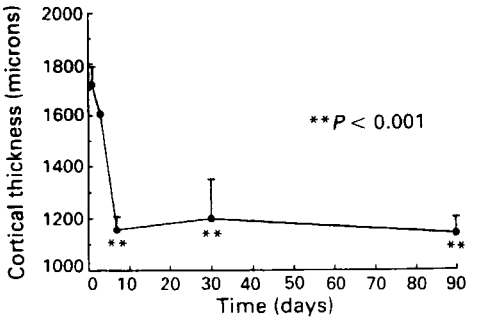

Figure 4 Morphometric analysis of cortical thickness following soman administration (1 LD_{50}). Data are mean ± s.d. of measurements taken at both hemispheres in three serial sections (7 animals per time point).

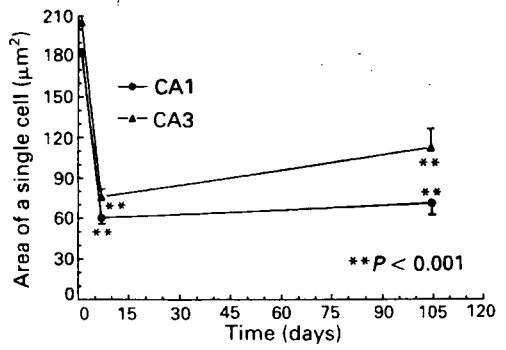

Figure 5 Morphometric analysis of a single-cell surface area in the hippocampal CA1 and CA3 subfields of rats following soman injection (1 LD_{50}). The mean surface area ± s.d. of a single cell in each experimental group was calculated from approximately 200 measurements (40 cells from each animal and 5–7 animals per time point).

3 months. The response of CA3 cells was less pronounced and the number of CA3 cells only decreased significantly at 3 months (P <0.001).

Discussion

The results presented in this 3-month follow-up study, indicate that the single administration of soman to rats at a dose of 1 LD_{50}, results in distinct, progressive lesions in various brain structures. However, no brain injuries were found after the administration of similar (1 LD_{50}) doses of either a potent convulsant (metrazol) or a different anti-ChE compound (DFP).

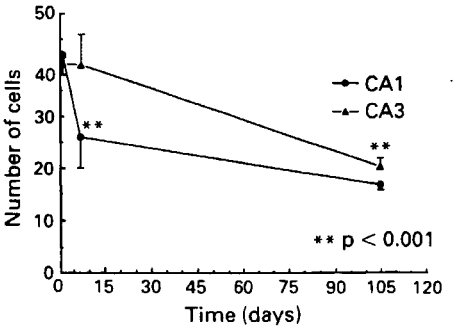

Figure 6 Morphometric analysis of the number of cells in the hippocampus of rats following soman administration (1 LD_{50}). Data represent mean ± s.d. of the number of cells in a frame of 2 × 10^5 μm², in three serial sections from each animal (5–7 animals per time point).

Further, the administration of a non-convulsive dose of soman (0.5 LD_{50}) induced slowly progressive neuronal damage which was less severe, when compared to the lethal 1 LD_{50} dose, but which was accompanied with cognitive impairment (Brandeis R, personal communication, 1990).

Although seizures following soman or metrazol differ in their underlying pathophysiological mechanisms,[17] convulsions do not necessarily predict massive degeneration. According to Switzer *et al.*,[18] only about 50% of the convulsing animals who had been given soman displayed moderate to heavy degeneration. Moreover, in our study, brain lesions were also found following a non-convulsive dose of soman. Neurotoxic effects of sublethal doses of soman have also been shown by other investigators.[19,20]

Recently we have found that a convulsive dose of hyperbaric oxygen did not produce any CNS pathology (Bitterman N and Kadar T, personal communication).

This study does not support the hypothesis that either seizure activity *per se*, or cholinergic hyperactivity by itself are responsible for soman-induced CNS morphological lesions.

However, the duration and pattern of convulsions may play a major role in causing brain lesions. Metrazol produced intense episodes of seizures, with each episode being relatively short in duration, while soman produced persistent seizure activity (status epilepticus) of long duration.[17] Indeed, following metrazol, seizures were observed for up to 30 min following injection, while after soman, convulsions persisted for several hours in the acute phase and thereafter, epileptic episodes were noted for up to 3 months.

In the present study, DFP did not induce any brain lesions at a dose which caused a lethality similar to that of soman. This indicates that not all ChE inhibitors have a similar central neurotoxicity. While both soman and DFP are irreversible ChE-inhibitors, they differ in their propensity to produce either central or peripheral effects.[7] Using the 2-deoxy glucose method, Samson and his colleagues[21] showed that soman had a greater impact on brain regional glucose use than DFP. Furthermore, soman also exhibited different effects with regard to OP-induced myopathy.[22,23] Thus, pharmacokinetic consideration may play a role in the difference between soman and DFP.

Neuropathology following 1 LD_{50} soman, which was clearly observed at 24 h, intensified with time. Selected neurons were severely affected shortly after poisoning while others, such as the CA3 pyramidal cells, septum and thalamic ventromedial nuclei, degenerated in a slower manner and necrosis was noted only 1–3 months after poisoning.

Thus, two separate pathological processes seem to characterize soman's central neurotoxicity. One is the severe rapid necrosis which occurred a few hours after administration and for up to one week and characterized the response of CA1 cells and neurons in the frontal and piriform cortex. The second is the slower, delayed neurotoxic process which progressed over several weeks or months and was exhibited by the response of CA3 cells, as shown by quantitative analysis. Regarding the acute phase, Carpentier *et al.*[24] found that, following a single 1 LD_{50} dose of soman, there was a marked loss of dendrites in the CA1 sector, 1 h post-exposure, representing a rapid degenerative process. The extent of this necrotic process was shown in this study at 24 h.

However, during the 'delayed phase', neurons such as the CA3 cells deteriorated more slowly and neuronal death (expressed by a decrease in cell number) became significant only over a period of months. This slowly progressive neuronal death was also observed in our study following a sublethal dose of soman.

It is concluded that the neuropathological lesions are not common for all ChE inhibitors, or following all forms of convulsions, and might be the result of specific processes initiated by soman itself and which are not mimicked by DFP or metrazol-induced convulsions.

The quantitative evaluation was shown to be valuable in characterizing the magnitude of brain injury and thus, might serve in the future assessment of various treatment modalities aimed at preventing the soman-induced neuropathology.

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