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Efficacy of Atropine/Pralidoxime/Diazepam or Atropine/HI-6/Prodiazepam in Primates Intoxicated by Soman

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LALLEMENT, G., D. CLARENCON, G. BROCHIER, D. BAUBICHON, M. GALONNIER, G. BLANCHET AND J. C. MESTRIES. *Efficacy of atropine/pralidoxime/diazepam or atropine/HI6/prodiazepam in primates intoxicated by soman.* PHARMACOL BIOCHEM BEHAV **56**(2) 325–332, 1997.— We performed an experiment to characterize the toxicity of soman in cynomolgus monkeys when the organophosphorus intoxication was followed by a treatment with either the three-drug therapy atropine/pralidoxime/diazepam or the association atropine/HI-6/prodiazepam. Clinical, electrophysiological and histological approaches were combined. Our data demonstrate that the protection afforded against soman toxicity was better with the combination atropine/HI-6/prodiazepam compared to atropine/ pralidoxime/diazepam. This was observed transiently in term of vigilance and respiratory function of intoxicated animals, but particularly in term of their EEG- and ECG disturbances. Moreover, compared to those treated with atropine/pralidoxime/diazepam, animals treated with atropine/HI-6/prodiazepam recovered slightly sooner and did not exhibit prostration 2 days after intoxication although their rapidity of movements was not totally restored. The final recovery observed 3 weeks after intoxication was similar for the two groups. The value of the combination of atropine/HI-6/ prodiazepam vs atropine/pralidoxime/diazepam to counteract soman toxicity was also confirmed in term of brain neuroprotection since greater lesions were observed with the second three drug treatment three weeks after intoxication.

Soman Epilepsy Atropine Diazepam Oximes Primates

SOMAN, an organophosphorus (OP) compound is a potent irreversible inhibitor of acetylcholinesterase (AChE) in both the central and peripheral nervous systems, which can cause severe incapacitation and death. Soman-induced clinical symptoms include salivation, diarrhea, lacrimation, tremors, convulsions and seizures. Recent pathophysiological studies have revealed that exposure to soman may result in CNS and myocardial lesions (15).

The currently recommended therapy against OP intoxication is based on pretreatment with pyridostigmine, a reversible inhibitor of AChE that undergoes a spontaneous reactivation, followed by treatment with a three-drug regimen consisting of a) atropine, an anticholinergic drug, b) pralidoxime, a reactivator of OP-inhibited AChE, and c) diazepam, a benzodiazepine anticonvulsant.

Atropine is a basic component of the three-drug therapy since it antagonizes acetylcholine action at muscarinic sites, alleviating tracheobronchial and salivary secretion, bronchoconstriction, miosis, intestinal hypermotility and bradycardia.

Nucleophilic compounds, such as oximes (RCH-NOH), can reactivate the phosphorylated enzyme if administered prior to its irreversible inactivation named "aging." The oxime widely used to reactivate organophosphate-inhibited AChE is pralidoxime. Over the years, several oximes have been synthetized that exhibit a greater potency for AChE reactivation, especially in the case of organophosphate-inhibited enzyme. Such

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a reactivator, HI-6, bispyridinium oxime, has been proposed as a possible substitute for pralidoxime within the choice of therapy for OP intoxications in man. Indeed, HI-6 has a relatively low toxicity (2,9), and has been shown to be efficient in the treatment of soman intoxication (5,20), which is relatively refractory to treatment with pralidoxime in rodents (2,7,13). However, the choice of HI-6 as a replacement for pralidoxime is critically dependent on the eventual demonstration of an improved efficacy in the treatment of soman intoxication in non human primates.

Consequently, we performed experiments to characterize the toxicity of soman in cynomolgus monkeys pretreated with pyridostigmine when the organophosphorus intoxication was followed by a treatment with either the present three-drug therapy (atropine/pralidoxime/diazepam) or the new combination atropine/HI-6/prodiazepam. Prodiazepam (lysyl, peptido-aminobenzophenone diazepam pro-drug) was experienced since it constitutes, unlike diazepam, a water soluble drug which, in vivo, undergoes a rapid hydrolysis by an aminopeptidase to give lysine and diazepam (14,19).

MATERIALS AND METHODS

Eighteen male Cynomolgus monkeys (*Macaca fascicularis*, Maurice Island origin) weighing 4–8 kgs were used. Animals of equivalent weights were distributed in two groups for the determination of the efficacy of atropine/pralidoxime/ diazepam (group A, 9 animals) versus atropine/HI-6/prodiazepam (group B, 9 animals). Animals were housed individually until the day of the experiment. One month before the soman challenge, 5 animals from each group, respectively called "group A- and group B- implanted," were deeply anaesthetized with ketamine (20mg/kg; IM) and prepared for electroencephalographic recordings (EEG) as previously described (16,17). Briefly, gold electrolyzed monopolar screws were implanted over the somatomotor, somesthesic and visual areas. Screws were connected to a 19 contact connector and were embedded with dental cement. After a 4 weeks recovery period, during which systemic antibiotic (totapen 1g/day during 10 days) and local antiseptic (hibitane 5%) post surgical treatments were performed, the animals were placed on a contention seat and connected to an EEG recorder (ALVAR 16 channel polygraph, France). EEG frequency spectrum characteristics were processed on a computer which allowed a real time power spectral analysis based on a Fast Fourier transform algorithm. EEG power was estimated in the following 5 frequency ranges (0.5–5 Hz, 5–10 Hz, 10–16 Hz, 16–48 Hz). The animals were also prepared for electrocardiographic recording (ECG) according to our previously published method (17) using 4 electrodes placed into the acromial apophysis and into the tibias. Experiments were carried out in a temperature regulated room (24 ± 1°C).

Animals of ''group A- and group B implanted,'' placed on the contention seat, were pretreated 1 hour before soman intoxication with pyridostigmine bromide (0.2mg/kg; IM). In a previous study, it has been established that such a dose of pyridostigmine induces a blood cholinesterase inhibition of about 30–40% at the time of soman injection, i.e., a range of inhibition usually adopted for the pretreatment of OP intoxication (8). This inhibition was then stable during one hour. Animals were then injected with 8 LD_{50} of soman (30μg/kg; IM (21)) in the right leg. The 5 soman-challenged animals of "group-A implanted" were treated with a combined injection of atropine sulphate (0.5mg/kg; 0.72μmol/kg), pralidoxime (30mg/kg; 139μmol/kg) and with a single injection of diazepam (0.2mg/kg ; 0.7μmol/kg) by IM route 1 min after intoxication. At the same time, the 5 animals of "group-B implanted" received a combined injection of atropine (same dose as above), HI-6 (50mg/kg; 139μmol/kg) and prodiazepam (0.35mg/kg; 0.7μmol/kg) by IM route. Treatment was given in the left leg. The doses of atropine, oximes and diazepam adopted in our study were similar to those previously used by others in toxicological studies (4). It is worthy of mention that the doses of atropine, oximes and diazepam used, expressed in μmol/kg, were identical in both combined therapies. Animals were monitored (EEG, ECG) during the first 6 hours after intoxication. They were also closely and continuously observed for these first 6 hours. Acute signs of toxicity specifically monitored included muscle fasciculations, tremors, clonic jerks, convulsions, respiratory disturbance, unconsciousness, hyperreactivity in response to sensory stimuli. These signs were noted during 9 time intervals after soman intoxication (2–5 min, 10 min, 15 min, 30–45 min, 1–2 hours, 2–3 hours, 3–4 hours, 4–5 hours, 5–6 hours). Recovery was appreciated at the same times by visual tracking and feeding. After this 6 hour phase of observation, animals of "group A- and group B- implanted" were removed, without anaesthetic treatment, from the contention seat, put in their cages and observed as for the others animals of groups A and B (i.e. animals non implanted, see below) from 1 day to 3 weeks after intoxication.

Non implanted, freely moving animals of groups A and B (4 in each group) were pretreated, intoxicated and treated 1 min after soman in the same conditions as, respectively, animals of "group A- and group B- implanted." Signs of toxicity identical to those previously mentioned were noted at the same 9 time intervals from 2–5 min to 6 h after intoxication and also 1, 2, 3, 4 days, 1, 2, 3 weeks after soman administration. Moreover for these freely moving animals, at each time of observation, a) toxicity was also appreciated by the eventual prostration of the animal and b) recovery was evaluated using 7 criteria including, as for "group A- and group B- implanted," visual tracking and feeding but also eyelid reflex, biting reflex, grasping, sitting position, walking and climbing.

Observations of clinical signs of toxicity and recovery were scored based on the incidence of each sign, observed in group A and in group B, during each of the 16 time intervals from 2–5 min to 3 weeks after soman intoxication. For each time, the incidences observed in the two groups of animals (atropine/pralidoxime/diazepam and atropine/HI-6/prodiazepam) were then compared using the non parametric Kolmogorov-Smirnov's test (two sample test, Statview, Abacus concept, USA). Significance was set at $p < 0.05$.

Three weeks after soman-challenge all animals were sacrificed by IV injection of a high dose of sodium pentobarbital (25mg/kg). Entire brain and heart were collected for histopathological examination. Tissues were immersed in 10% neutral formalin during 4 weeks. They were then processed by using routine paraffin embeding methods. Sections 5 to 8μm thick were stained with hemotoxylin and eosin. Brain sections included frontal cortex, entorhinal cortex, amygdala, caudate, hippocampus, thalamus, midbrain, pons, medulla and cerebellum.

RESULTS

Clinical Signs of Soman Toxicity

Severe signs of soman toxicity were seen in each animal. In general, muscle fasciculations and tremors generally occured within 1-2 minutes after soman exposure (1.2 ± 0.4 min;

TABLE 1

INCIDENCES IN PERCENTAGE OF SIGNS OF TOXICITY OBSERVED IN INTOXICATED ANIMALS TREATED EITHER WITH ATROPINE/HI-6/PRODIAZEPAM (WHITE COLUMNS) OR WITH ATROPINE/PRALIDOXIME/DIAZEPAM (GRAY COLUMNS)

Time after Intoxication	Fasciculations		Tremors		Clonies		Convulsions		Coma		Respiratory Disorders		Hyperreactivity		Prostration	
2–5 min	88	100	88	100	77	100	77	100	44	77	11	33	0	11	0	0
10 min	11	0	44	33	0	0	0	0	100	100	55	100	0	0	0	0
15 min	11	0	33	22	0	0	0	0	44†	100	33*	88	0	11	0	0
30–45 min	11	11	44	44	0	0	0	0	22	44	11	22	22	22	0	25
1–2 h	22	11	55	77	11	11	22	22	0	0	11	11	33	44	0	25
2–3 h	11	11	33	22	0	11	0	0	0	0	0	0	22	44	0	25
3–4 h	0	0	22	22	0	0	0	0	0	0	0	0	22	44	0	25
4-5 h	0	0	11	22	0	0	0	0	0	0	0	0	22	33	0	25
5–6 h	0	0	11	0	0	0	0	0	0	0	0	0	0	33	0	25
24 h	0	0	0	0	0	0	0	0	0	0	0	0	0	0	22	0
48 h	0	0	0	0	0	0	0	0	0	0	0	0	0	0	22†	100
3 days	0	0	0	0	0	0	0	0	0	0	0	0	0	0	22	0
From 4 days to 3 weeks	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

The incidences of each sign observed in the two groups of animals during each of the time intervals were compared using the non parametric Kolmogorov–Smirnov's test; *$p < 0.05$, †$p < 0.01$.

TABLE 2

INCIDENCES IN PERCENTAGE OF SIGNS OF RECOVERY OBSERVED IN INTOXICATED ANIMALS TREATED EITHER WITH ATROPINE/HI-6/PRODIAZEPAM (WHITE COLUMNS) OR WITH ATROPINE/PRALIDOXIME/DIAZEPAM (GRAY COLUMNS)

Time after Intoxication	Eyelid Reflex		Biting Reflex		Grasping		Visual Tracking		Sitting Position		Walking Climbing		Feeding	
2–5 min	25	25	25	0	25	25	55	44	25	0	0	0	0	0
10 min	25	25	0	0	0	0	33	22	0	0	0	0	0	0
15 min	75	75	25	25	0	50	55	33	0	0	0	0	0	0
30–45 min	100	100	75	75	50	75	77	44	75	0†	75	0†	11	0
1–2 h	100	100	75	75	50	100	77	55	100	75	100	75	55	22
2–3 h	100	100	100	50	100	50	88	77	100	75	100	50	55	33
3–4 h	100	100	75	25	75	25	100	88	75	75	75	50	66	33
4–5 h	100	100	50	25	75	25	100	100	100	75	75	50	66	55
5–6 h	100	100	50	25	75	50	100	100	100	75	100	50	66	55
24 h	100	100	100	44†	100	77	100	100	100	100	88	88	100	88
48 h	100	100	88	0†	100	0†	100	100	100	88	88	0†	100	100
3 days	100	100	88	44	88	88	100	100	100	100	100	88	100	100
From 4 days to 3 weeks	100	100	100	100	100	100	100	100	100	100	100	100	100	100

The incidences of each sign observed in the two groups of animals during each of the time intervals were compared using the non parametric Kolmogorov–Smirnov's test; *$p < 0.05$, †$p < 0.01$.

mean ± sem; $n = 18$). These were the first toxic signs which were associated in some animals with chewing (4/9 in group A and 3/9 in group B).

Animals then had convulsions associated with tonic-clonic episodes or opisthotonus and protusion of the tongue. The latency to convulsions was the same in the two treated groups (2.8 ± 1.1 min; mean ± sem; $n = 18$). During this acute phase 4 animals of group A and 5 of group B exhibited cyanosis. The incidence and the duration of muscle fasciculations, tremors and convulsions during the post soman-challenge period was variable between group A and B (see Table 1). However, no statistical differences was seen between the two groups.

All animals of group A became rapidly unresponsive and unconscious within 5.5 ± 2.2 min (mean ± sem; $n = 9$). Anarchic respiratory abdominal dyspneic movements were observed in these animals throughout the comatose state. In 4 animals this was accompanied by bronchial secretions. The loss of consciousness in the group A animals persisted between 20 min (5 animals) and 40 min (4 animals). Animals of group B also became unconscious within 4.9 ± 1.4 min (mean ± sem; $n = 9$). However, the incidence of coma, 15 min after soman intoxication, in group B was significantly lower compared to group A (Table 1). Indeed for 5 animals of group B the comatose state lasted only 10–12 min. On an other hand, dyspneic movements were observed in only 5 animals of group B 10 min after intoxication. Fifteen min after soman treatment, the incidence of this respiratory disorder was significantly lower compared to group A. However, these differences between groups A and B, observed 15 min after soman administration in terms of incidences of coma and respiratory disorders, were very transient since they were no apparent as from 30 min after intoxication.

In both groups A and B, some animals (3 to 4 in each group) were hyperreactive to sound or tactile stimulation during the first 6 h after intoxication. Each stimulation led to a reappearence of fasciculations or tremors.

Recovery of Soman Intoxicated Animals

After the initial convulsive and comatose episodes animals recovered. The incidences of eyelid reflex, biting reflex, grasping and visual tracking were equivalent between the two groups during the first 6 h post intoxication. The recovery of the animals, as determined by the ability to sit and to walk and climb, was significantly more rapid in group B compared to group A, 30–45 min after intoxication (Table 2). At this time, no animal of group A was able of sitting and walking compared to 75% in group B. However, groups A and B were then equivalent as from 1 hour after intoxication.

Twenty four h after intoxication, all animals were able to walk, grasp and climb. At this time, only 44% animals of group A exhibit biting reflex. Comparatively group B recovered sooner since, from 1 day after intoxication, almost all animals of this group were able to walk, grasp and also to bite. At this time the biting reflex was significantly elevated compared to group A. Two days after treatment all animals of group A remained prostrated without reactivity while the majority of the animals of group B were able to walk, grasp, bite and climb. The incidences of biting, grasping, walking and prostration were thus significantly in favor of group B compared to group A, 2 days after intoxication. However, the rapidity for walking and climbing of group B animals was considered, by visual examination, as reduced, compared to non intoxicated animals. Three days after soman administration, group A animals recovered and appeared in the same clinical state as 1 day after intoxication. No significant difference was noted compared to group B. From 4 days to 3 weeks after treatment, all animals in both group A and B had a total recovery.

Eighty eight to 100% of animals, whatever the group observed, were able to feed from 24 h after soman-challenge.

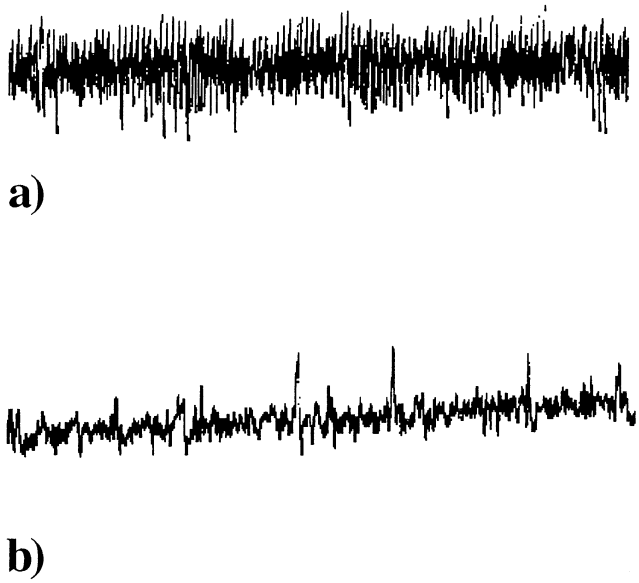

FIG. 1. EEG epileptic activity induced by soman. The animal was treated one minute after soman challenge with atropine/pralidoxime/diazepam. a) Three min and b) 15 min after intoxication. Note the monomorphic aspect of the EEG pattern 15 minutes after intoxication with low amplitude and a persistent spiking activity.

ECG Recordings

Electrocardiographic recordings showed extrasystolic episodes in three of the five animals of "group A implanted." These extrasystolic episodes appeared 4–5 min after injection of soman and lasted about 20 min. These perturbations of cardiac rhythm were never observed in "group B implanted." In both groups the cardiac frequency observed before soman intoxication was about 130 pulses/min. Three to 4 min after intoxication the frequency increased to about 200–250 pulses/min in both groups A and B. This increase was observed over 2 h, the cardiac frequency then gradually declined to reach normal values 3 hours after intoxication.

EEG Recordings

EEG recordings obtained during the first 6 h after intoxication showed, in all animals examined, a status epilepticus activity appearing 2–3 min after soman intoxication and lasting until loss of conciousness i.e over 2 to 3 min. Thereafter, EEG showed a monomorphic aspect all the 6 h period long with low amplitude. Besides this monomorphic aspect, spikes were observed on EEG recordings in all animals of "group A implanted" during the first 6 h after intoxication. This was observed in parietal and occipital EEG derivations. Comparatively spiking activity was also observed in all animals of "group B implanted" but only during some min (6 to 8) after intoxication (Figure 1). Band frequency analysis demonstrated that EEG energy was mostly distributed in the 16–48 Hz band all the 6 hour observation period long (data not shown).

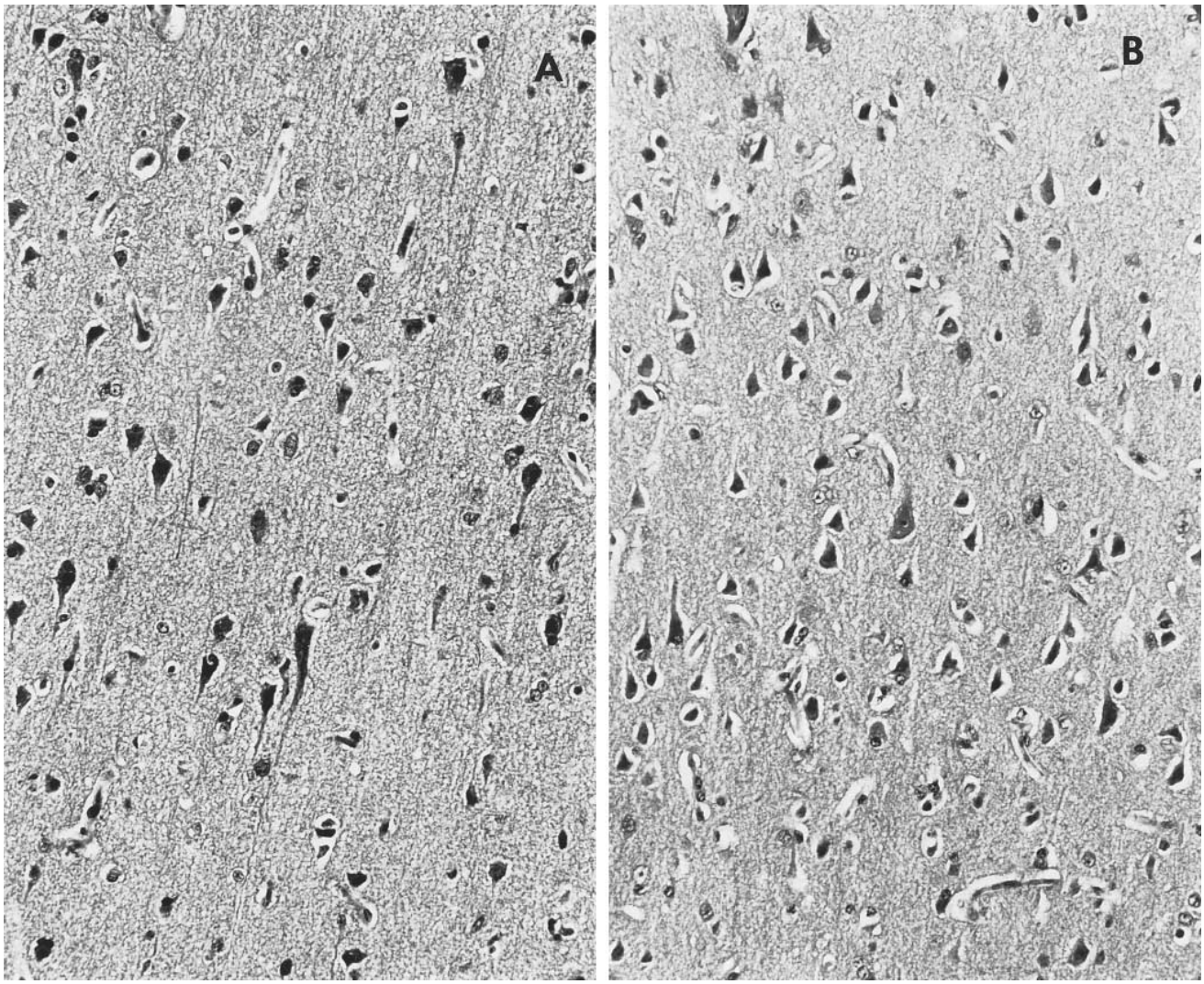

FIG. 2. Hematoxylin/eosin stain of the frontoparietal cortex three weeks after soman exposure and treatment with either atropine/pralidoxime/diazepam (A) or atropine/HI-6/prodiazepam (B). Note the reduced neuronal density in (A) compared to (B). Magnification ×200.

Histopathological Examination

Three weeks after soman-challenge neuronal rarefaction accompanied with punctiform hemorrhages was observed in the frontoparietal cortex of all animals of group A (Figure 2). Moreover, there was a disappearence of the cerebellar Purkinje cells in these animals (Figure 3). In one animal of this group, a neuronal pycnosis in CA_1 hippocampal area and a spongiform change of the neuropil of piriform cortex (layers II, III) leading to a disruption of neuronal tissue integrity was also observed (data not shown). Comparatively a histopathological alteration consisting of neuronal necrosis was observed in the frontoparietal cortex of only one animal of group B. Moreover, only a discrete rarefaction of the Purkinje cells was observed in the cerebellum of all animals of this group (Figure 3).

Myocardial degeneration was never observed in both groups A and B. However, one animal of group A exhibited a lesional syndrom in the epicardium characterized by an invasion of this tissue with large plasmodic cells surrounding adipocytes with crystalline inclusions (data not shown).

DISCUSSION

The objective of this study was to compare the beneficial effects against soman of two three-drug therapies including either atropine/pralidoxime/diazepam (group A) or atropine/HI-6/prodiazepam (group B). This constitutes the first investigation on non human primates of the protective effects of combined treatments against soman reporting, in the same study, signs of toxicity, recovery after challenge, EEG and ECG recordings and histopathological examinations of the heart and brain of the intoxicated animals. Indeed, until now, most of the research has focused on survival rate of animals after OP intoxication and treatment with varied combined therapies (5,6,20).

The present data demonstrated that both treatments (group A or group B) were unable to prevent acute signs of toxicity including fasciculations, tremors and convulsions. Similarly, neither group A or group B animals were protected against status epilepticus in spite of the presence of a benzodiazepine anticonvulsant in each treatment. However, the duration of the epileptic activity was very short since, in all animals,

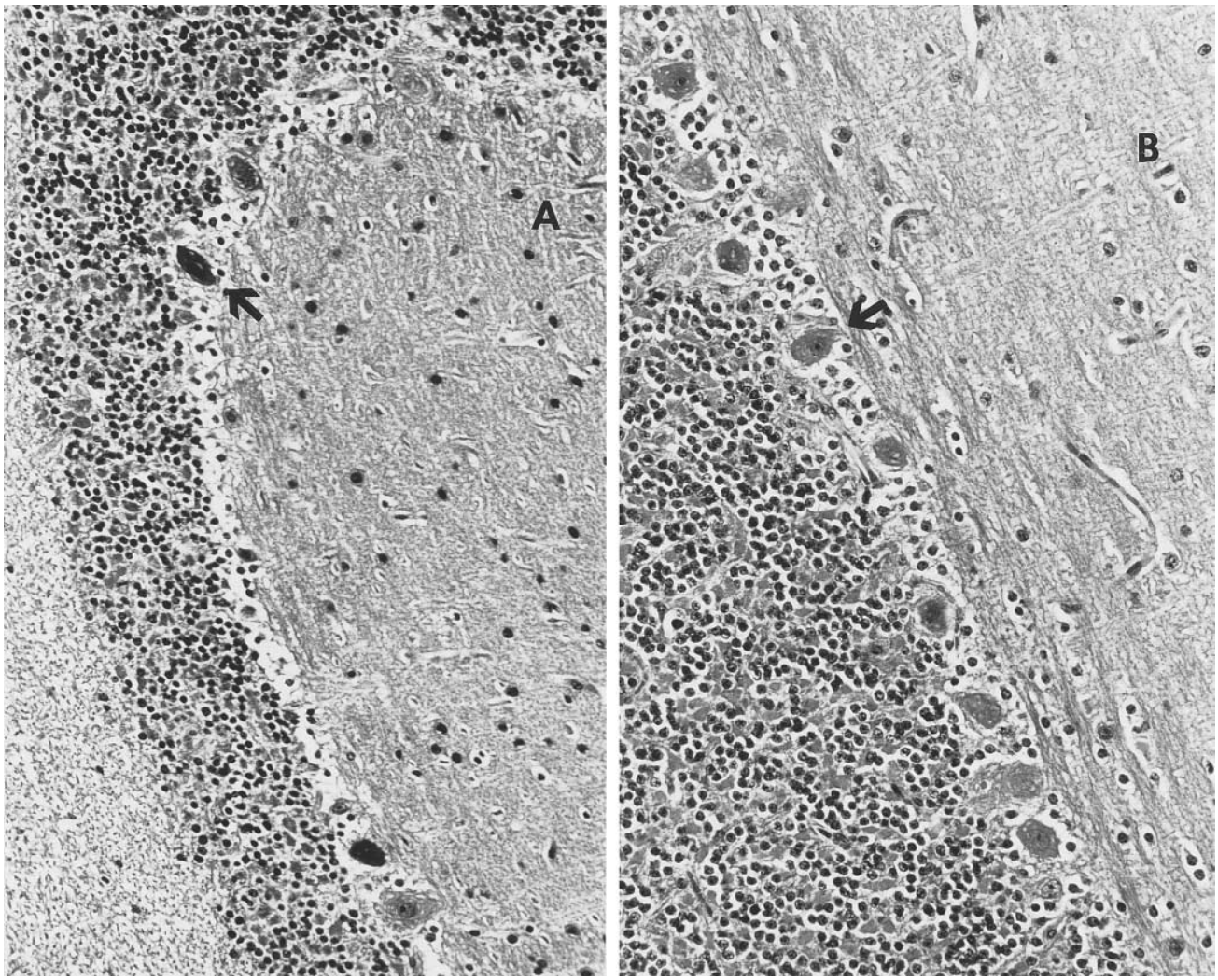

FIG. 3. Hematoxylin/eosin stain of the cerebellum three weeks after soman exposure and treatment with either atropine/pralidoxime/diazepam (A) or atropine/HI-6/prodiazepam (B). Note (see arrows) the severe disappearance and the shrinking aspect of Purkinje cells in (A) compared to the discrete neuronal damage in (B). Magnification ×200.

it did not exceed 2–3 min in duration. It is very likely that, without the anticonvulsant, considering the results of experiments on rodents, this status epilepticus would have continue for a long period (some hours in the case of rodents (1,12)).

Some differences appeared between the two groups tested after the initial episod of convulsions and status epilepticus. Thus, all animals of group A were in coma over 20 to 40 min associated with dyspneic movements while, comparatively, those of group B were slightly less affected 15 min after intoxication since coma and respiratory disorders were more transient in this group. Furthermore, electrophysiological recordings detected long lasting differences between the two groups. Indeed, group A animals exhibited continuous EEG spiking activity over 6 h after intoxication and, for some of them, transient extrasystolic episodes whereas, in group B, spiking activity was detectable only during some min and no extrasystolic episodes were observed after intoxication. Altogether, it thus appeared that the protection afforded against soman toxicity was better, during the first h of intoxication, with the combination atropine/HI-6/prodiazepam compared to atropine/pralidoxime/diazepam. This was observed transiently in term of vigilance and respiratory function of intoxicated animals, but particularly in term of their EEG and ECG disturbances. The fact that, in both cases, EEG energy was mostly distributed in the 16–48 Hz frequency band, could be attributed to the administration of diazepam to the animals.

Treatments were also significantly different in favor of group B in terms of early recovery (first h after intoxication) since sitting position and walking were slightly more rapidly restored in this group compared to group A. However, these differences were very transient since they were observed only 30–45 min after intoxication but not 1 h after. The delayed recovery (from 1 day after soman administration) was also improved in group B since animals treated with atropine/pralidoxime/diazepam exhibited prostration and no reactivity 2 days after intoxication whereas animals treated with atropine/HI-6/prodiazepam did not, even if their rapidity to walk and climb was not totally restored.

The histopathological examination of cerebral tissues confirmed the previous results since a greater protection was

observed with the treatment atropine/HI-6/prodiazepam compared to the group treated with atropine/pralidoxime/diazepam. Indeed, lesions consisting of neuronal rarefaction and cerebellar cell disappearence were found in all animals of this last group. Neuronal alterations were observed mostly in the frontoparietal cortex which is a region where excitotoxic damage is often observed in rodents after soman intoxication (3,11). Since neuropathology due to soman is closely related to seizures (10), its seems interesting to correlate the cortical and cerebellar lesions observed in group A to the persistence of a spiking activity after treatment in these animals while, comparatively, animals of group B, in which cortical EEG activity rapidly normalized after treament, were free of brain damage in the frontoparietal cortex and only slightly affected in the cerebellum.

Epicardiac lesions observed in one animal in group A were surprising since classicaly described cardiac alterations due to soman in rodents consist of myocardial degeneration with microvacuolation, myocytolysis and fibroblastic replacement of damaged cardiac myofibers (18). The present pathology detected in the epicardium of one animal without attack of the myocardium thus remains unclear. It is possible that the crystalline inclusions observed in the epicardiac cells could correspond to a stockage of soman, which is lipophylic, into adipocytes then leading to a proliferation of macrophagic plasmodic cells around them.

In conclusion, it appeared that after a high-dose soman challenge (intoxication with 8 LD50), animals treated with atropine/HI-6/prodiazepam, compared to those treated with atropine/pralidoxime/diazepam, seemed transiently less acutely affected, recovered slightly sooner and had no prostration 2 days after intoxication although their rapidity of movement was not totally restored. Moreover, EEG and ECG disturbances were more pronounced in the group treated with atropine/pralidoxime/diazepam during the first 6 h after intoxication. However, the final recovery observed 3 weeks after intoxication was equivalent between the two groups. As previously mentioned, the doses of atropine, oximes and diazepam used, expressed in μmol/kg, were identical in both combined therapies. The interest of the combination atropine/HI-6/prodiazepam vs atropine/pralidoxime/diazepam to counteract soman toxicity was confirmed in terms of neuroprotection since greater lesions were observed with the second three drug therapy three weeks after intoxication.

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