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Prevention of Soman Toxicity After the Continuous Administration of Physostigmine

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LIM, D. K., Y. ITO, Z. J. YU, B. HOSKINS AND I. K. HO. *Prevention of soman toxicity after the continuous administration of physostigmine.* PHARMACOL BIOCHEM BEHAV 31(3) 633–639, 1988.—Protective effects of continuous administration of physostigmine alone, or in addition to scopolamine, against soman-induced toxicity were studied in guinea pigs. The results clearly demonstrated that treatment with physostigmine continuously via implanted mini-osmotic pumps for 4 or 7 days prior to soman exposure significantly protected from soman-induced mortality. In vehicle-infused guinea pigs, tremors, convulsions and loss of righting reflex occurred prior to their deaths induced by soman. Although all of the guinea pigs which received physostigmine pretreatment for 4 days prior to soman administration also displayed soman-induced tremors and convulsions, the onsets of these symptoms were significantly delayed. When animals continuously treated with physostigmine received injections of scopolamine 10 min prior to soman injections, there was a decreased incidence of all three toxicity symptoms as well as an increase in the latency to onset of tremors. Scopolamine was also able to reverse toxicity symptoms when soman was administered earlier. In animals which had been continuously treated with physostigmine via mini-osmotic pumps, the protective action against soman-induced toxicity was still apparent. On the contrary, acute physostigmine administration failed to protect against soman lethality. The present results suggest that the prophylactic uses of physostigmine via mini-osmotic pumps might be more useful than the acute bolus administration of physostigmine.

Physostigmine Scopolamine Soman Behaviors Mortality

ORGANOPHOSPHATES, such as DFP and soman, irreversibly inhibit cholinesterase enzymes by phosphorylation. Poisoning by organophosphates results in acetylcholine accumulation at the synaptic junction and overactivity of the parasympathetic cholinergic system. Carbamate pretreatment in combination with atropine has been found to offer protection against poisoning by organophosphates (2, 8, 9, 15). The prophylactic action of these carbamates is believed to be due to their reversible inhibition of cholinesterase, thereby preventing phosphorylation and aging of the enzyme by organophosphates (10,13).

Deshpande *et al.* (2) have reported that pretreatment with pyridostigmine does not protect against sarin-induced mortality, while pretreatment with physostigmine does. Pyridostigmine is a quaternary compound which does not readily penetrate the CNS (12), whereas physostigmine readily crosses into the brain. Thus far, the prophylatic uses of physostigmine against the toxicity of organophosphates have been in acute situations. Since the duration of action of physostigmine is very short (21), it is practically difficult to decide when physostigmine should be administered. Recently, we have reported on the usefulness of mini-osmotic pumps for continuous administration of physostigmine. Since scopolamine has less effect than atropine upon respiration (22), scopolamine may be used as additional therapy against soman toxicity.

The present study was designed to determine whether the continuous administration of physostigmine has a protective effect against soman toxicity and if the addition of scopolamine enhances protection against soman toxicity.

METHOD

Animals and Materials

Male Hartley guinea pigs weighing 200–250 grams were obtained from Charles River Breeding Laboratories, Wilmington, MA. Upon arrival, they were housed two to a cage (9×29×7 inches) and maintained in a room with controlled humidity of 55%, temperature-regulated at 74±2°F, 100% fresh air continually circulating and 12 hour light/dark cycles. The guinea pigs were given constant access to food and water.

Drug Solutions and the Implantation of Mini-Osmotic Pumps

The solvent system or vehicle used for delivery of

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physostigmine consisted of: 20% propylene glycol, 10% ethanol and 70% water (1 part glacial acetic acid in 2000 parts water, pH 4). In accordance with other laboratories which use the same model (U.S. Army MRDC, personal communication), the concentration of propylene glycol was reduced to half of that used previously (18).

Mini-osmotic pumps (Model 2001, Alza Corp., Palo Alto, CA) were used to deliver either the vehicle above or physostigmine salicylate in the vehicle at a rate of 0.06 mg/kg/hr, the "low dose" of physostigmine, or at a rate of 0.12 mg/kg/hr, the "high dose" of physostigmine. The pumps were implanted under the skin on the backs of the animals after local anesthesia had been produced via topical application of lidocaine HCl as described previously (18).

Normal saline (0.9% NaCl) was used as the vehicle for IM injections of scopolamine (0.1 mg/kg) and SC injections of soman (60 μg/kg, twice the LD_{50} dose). The soman used was 99.1% pure (w/v).

Drug Treatment Regimens

Three separate experiments were planned as follows:

1) The guinea pigs were implanted with the physostigmine-containing mini-osmotic pumps (delivering 0.12 mg/kg/hr) and blood was collected from a front leg 1, 3 and 7 days after the implantation. Acetylcholinesterase activity in the blood was determined. On the seventh day, the guinea pigs were sacrificed and brain AChE activity was determined.

2) The guinea pigs were divided into three main treatment groups: Vehicle Group (I) those that were implanted with mini-osmotic pumps which delivered the vehicle for 7 days; Low Dose Group (II) those that were implanted with mini-osmotic pumps that delivered the "low dose" of physostigmine (0.06 mg/kg/hr) for 7 days; and High Dose Group (III) those that were implanted with mini-osmotic pumps which delivered the "high dose" of physostigmine (0.12 mg/kg/hr) for 7 days. On the seventh day after pump implantations, the Vehicle Group (I) was further subdivided into 4 groups in order for us to determine the efficacy of acute pretreatment with physostigmine. One group (Ia) was injected with saline 10 minutes prior to soman administration. This group served as the control group. Two groups received single, acute injections of physostigmine (0.12 mg/kg); whereas one of these groups (Ib) was injected with physostigmine 10 minutes prior to soman administration, the other group (Ic) was injected with scopolamine 10 minutes prior to administration of soman. Finally, one group (Id) received both scopolamine and physostigmine 10 minutes prior to the soman injection. The Low Dose Group (II) was injected with scopolamine 10 minutes prior to soman administration. The High Dose Group (III) was further subdivided into (IIIa) those that received saline, IM, 10 minutes prior to SC injections of soman, (IIIb) those that received scopolamine (0.1 mg/kg, IM) 10 minutes prior to SC injections of soman and (IIIc) those that received soman 25 minutes prior to IM injections of scopolamine.

3) The guinea pigs were divided into 2 main treatment groups: (I) those that were implanted with mini-osmotic pumps which delivered the vehicle for 4 days; and (II) those that were implanted with mini-osmotic pumps that delivered physostigmine (0.12 mg/kg/hr) for 4 days. Group II was further subdivided as follows: on day 4, some of these animals (Group IIa) were administered saline followed 10 minutes later by SC injections of soman; other physostigmine-treated animals were administered scopolamine (0.1 mg/kg, IM) followed 10 minutes later by the soman injections (Group IIb); still other physostigmine-treated animals were injected with soman followed 25 minutes later by scopolamine (Group IIc).

Following soman administration, the guinea pigs were monitored for: incidence of and latency of onset of tremors, convulsions and loss of righting reflex, incidence and time of deaths.

TABLE 1

ACETYLCHOLINESTERASE ACTIVITY IN RED BLOOD CELLS DURING MINI-OSMOTIC PUMP IMPLANTATION WITH PHYSOSTIGMINE SALICYLATE

Days of Continuous Physostigmine Treatment		
1	3	7
42.7 ± 6.2*	45.5 ± 4.0*	81.9 ± 7.6

Blood was collected on the indicated days after pump implantation (0.12 mg/kg/hr).

The values are % of control AChE activity and are means ± S.E. of determinations on 5 animals. The AChE activity of control as 0.347 ± 0.027 (fmoles AThCh hydrolyzed/RBC/min, N = 12).

*$p<0.05$ compared to the control AChE activity.

TABLE 2

EFFECT OF CONTINUOUS ADMINISTRATION OF PHYSOSTIGMINE SALICYLATE ON AChE ACTIVITY IN BRAINS AND DIAPHRAGMS OF GUINEA PIGS

	Acetylcholinesterase Activity (nmole AThCh hydrolyzed/mg protein/min)		
	Striatum	Frontal Cortex	Diaphragm
Control	335.8 ± 27.5	47.1 ± 3.0	3.44 ± 0.2
Physostigmine	242.1 ± 10.3*	38.4 ± 2.6	3.28 ± 0.3

Guinea pigs were sacrificed 7 days after the implantations.

The values are the mean ± S.E. of determinations on 5 animals.

*$p<0.05$ compared to the respective control value.

Determination of Acetylcholinesterase (AChE) Activity

Striatum and frontal cortex were dissected out according to the procedure of Glowinski and Iversen (7). The AChE activity was determined according to the method of Ellman *et al.* (5). The tissues were homogenized in ice-cold sodium phosphate buffer (0.1 M, pH 8.0) at a concentration of approximately 20 mg wet weight/ml buffer. Enzyme activity was expressed as nmol of acetylthiocholine hydrolyzed/mg protein/min. Acetylcholinesterase activity in red blood cells was also determined according to the Ellman method (5). Red blood cell numbers were estimated using a Coulter counter (Coulter Electronics Limited). Enzyme activity in red blood cells was expressed as fmol of acetylthiocholine hydrolyzed/RBC/min.

Protein concentration was measured according to the method of Lowry *et al.* (19).

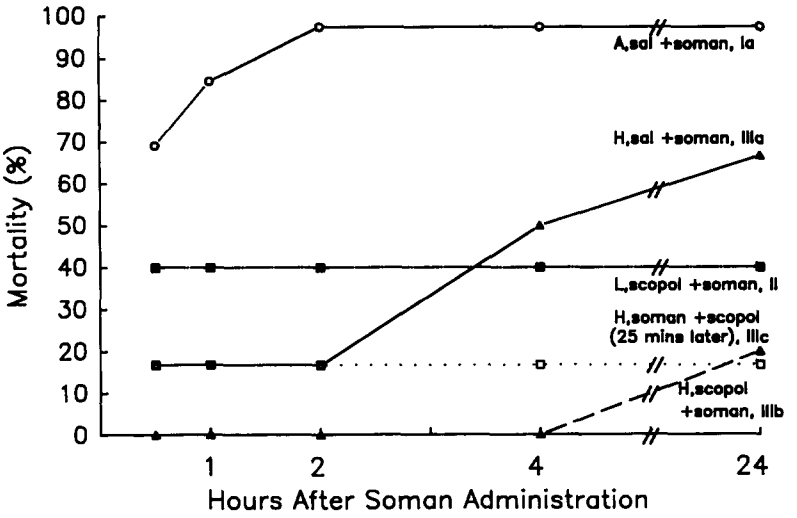

FIG. 1. Cumulative mortality after soman administration to guinea pigs pretreated for 7 days with physostigmine. Mortalities were monitored after soman administration ($2LD_{50}$). A, L and H represent vehicle, low dose of physostigmine and high dose of physostigmine, respectively. The group numbering system is explained in the Method section.

Statistics

When applicable, data were analyzed for significance by Student's *t*-test; a p value <0.05 between two means was considered significant.

RESULTS

AChE Activity During the Infusion of Physostigmine

Table 1 shows that whereas acetylcholinesterase activity was significantly inhibited after 1 day or 3 days of physostigmine treatment, it was no different from activity in red blood cells from controls after 7 days.

Activity of AChE was determined in striata, frontal cortices and diaphragms of five guinea pigs which had been implanted with mini-osmotic pumps which continuously delivered physostigmine (0.12 mg/kg/hr) for 7 days. Table 2 shows that activity of AChE was inhibited only in striata, whereas samples of cortex and diaphragm had activity that was not different from that found in tissues from control guinea pigs.

From these AChE activity results, day 4 was chosen for further study as a day when AChE was actively inhibited and day 7 was likewise chosen as a day when AChE was not inhibited.

The Effect of Soman Administration on Toxicity and Lethality 7 Days After Mini-Osmotic Pump Implantations

Figure 1 shows that animals in treatment Group I suffered over 90% mortality within 2 hours of soman administration. By contrast, the guinea pigs which had received continuous exposure to physostigmine for 7 days prior to soman administration were protected from soman-induced mortality. Only 16.7% of the animals which had received the high dose of physostigmine for 7 days prior to soman administration (Group IIIa) died within 2 hours of soman administration and the final total mortality of this group was 67%, i.e., mortality was delayed and decreased. When animals like those in Group IIIa were treated with scopolamine 25 minutes after soman administration (Group IIIc), total survival as well as survival time was further increased. When soman administration was preceded by scopolamine in the group which had received the high dose of physostigmine for 7 days (Group IIIb), total mortality was only 16.7%. Continuous exposure to the low dose of physostigmine for 7 days followed by scopolamine treatment (Group II) also offered significant protection from soman-induced mortality.

Figure 2 shows that when physostigmine was administered acutely 10 minutes before injections of soman (Group Ib, total survival (1 out of 6) appeared to increase, compared to the survival level of 1 out of 13 in Group Ia. Survival time, however, was obviously increased by acute pretreatment with physostigmine. When only scopolamine was given 10 minutes prior to soman administration (Group Ic), survival was not markedly increased. Finally, when guinea pigs were administered both physostigmine and scopolamine 10 minutes prior to soman administration (Group Id), no mortality occurred.

When symptoms of soman-induced toxicity were monitored in the various treatment groups (Table 3), continuous pretreatment with physostigmine generally delayed the onset of tremors, whether or not the animals were also treated with scopolamine prior to injections of soman. In this regard, the low dose of physostigmine, when supplemented with scopolamine 10 minutes prior to soman administration (Group II), was as effective as the higher dose of physostigmine without supplemental scopolamine administration (Group IIIa). The incidence of tremors was not substantially altered by any of the pretreatment regimens except in the group of animals which, having received the high dose of physostigmine for 7 days, were administered soman followed 25 minutes later by scopolamine on the seventh day (Group IIIc), in which case, there were no incidences of tremor, convulsions or loss of righting reflex. The data also indicate

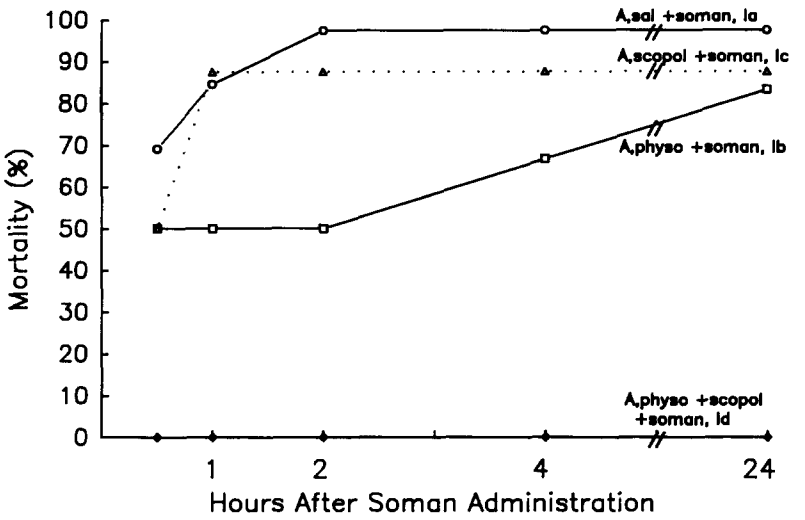

FIG. 2. Cumulative mortality after soman administration to guinea pigs infused with vehicle and pretreated acutely with the high dose of physostigmine (0.12 mg/kg).

TABLE 3

COMPARISON OF DIFFERENT TREATMENT REGIMENS ON INCIDENCE AND LATENCY OF ONSET OF SOMAN-INDUCED TOXICITY SYMPTOMS IN GUINEA PIGS PRETREATED FOR 7 DAYS WITH PHYSOSTIGMINE

		Tremors		Convulsion		Loss of Righting Reflex	
Pretreatment	Treatment[a]	Incidence	Onset (min)	Incidence	Onset (min)	Incidence	Onset (min)
Vehicle	Saline (Ia)	13/13	4.59 ± 0.78	12/13	7.52 ± 1.29	12/13	13.69 ± 3.45
	Physostigmine (Ib)	6/6	5.53 ± 1.13	6/6	7.40 ± 2.03	6/6	13.27 ± 3.91
	Scopolamine (Ic)	4/4	16.35 ± 0.61*	4/4	18.07 ± 0.85*	4/4	30.17 ± 2.27*
	Physostigmine + Scopolamine (Id)	8/8	9.07 ± 1.48*	7/8	11.14 ± 2.06	1/8	3.68
Low Dose	Scopolamine (II)	10/10	9.11 ± 1.03*	10/10	10.80 ± 1.39	6/10	13.12 ± 1.55
High Dose	Saline (IIIa)	12/13	9.80 ± 1.13*	11/13	10.79 ± 1.28	4/13	13.13 ± 2.06
	Scopolamine (IIIb)	3/6	9.67 ± 2.55*	2/6	8.75 ± 6.33	1/6	8.58
	Scopolamine (IIIc)[b]	0/5	—	0/5	—	0/5	—

Guinea pigs were implanted with pumps containing either vehicle or physostigmine.
[a]Seven days after the implantation, soman ($2LD_{50}$) was administered 10 min after the administration of the indicated agents.
[b]Scopolamine was administered 25 min after soman administration.
*$p<0.05$ compared to values for Group Ia.

that acutely, physostigmine had no effects on the time intervals between soman administration and the onset of toxicity symptoms. Interestingly, by far the most pronounced effects upon the latency of onset of toxicity symptoms was observed in the group of guinea pigs treated with scopolamine 10 minutes prior to administration of soman (Group Ic).

The Effect of Soman Administration on Toxicity and Lethality Four Days After the Implantation

Figure 3 shows that the guinea pigs that were not pretreated with physostigmine (Group I) suffered 100% mortality within 30 minutes of soman administration. Those animals which were pretreated with physostigmine for 4 days prior to soman administration (Group IIa) were significantly protected from soman-induced lethality in that one of the nine guinea pigs in this group died within 30 minutes of soman administration and a second death occurred within 24 hours after the soman injection. When scopolamine was administered 10 minutes before soman was injected (Group IIb), there were no deaths. Finally, when scopolamine was administered 25 minutes after soman injections (Group IIc) there were no quick deaths, however, two of the 10 animals in this group died within 24 hours of the soman injections.

Table 4 shows that 100% of the guinea pigs which did not receive physostigmine pretreatment (Group I) displayed all three symptoms of soman-induced toxicity prior to their deaths. The onset of these symptoms was quite rapid. Although all of the guinea pigs which received physostigmine pretreatment for 4 days prior to soman administration (Group IIa) also displayed soman-induced tremors and convulsions, the onsets of these symptoms were significantly delayed and the incidence of loss of righting reflex was decreased such that only 2 of the 9 guinea pigs in this group

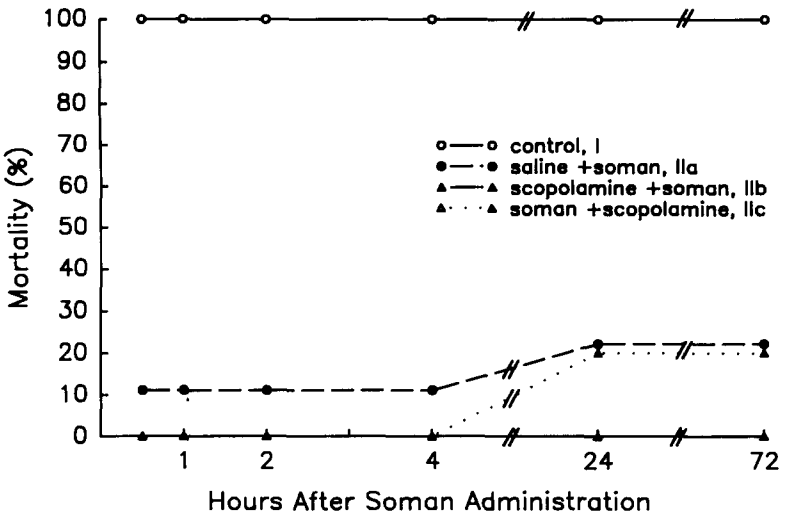

FIG. 3. Cumulative mortality after soman administration to guinea pigs pretreated for 4 days with physostigmine.

TABLE 4

COMPARISON OF DIFFERENT TREATMENT REGIMENS ON INCIDENCE AND LATENCY OF ONSET OF SOMAN-INDUCED TOXICITY SYMPTOMS IN GUINEA PIGS PRETREATED FOR 4 DAYS WITH PHYSOSTIGMINE

	Tremors		Convulsions		Loss of Righting Reflex	
Groups	Incidence	Onset (min)	Incidence	Onset (min)	Incidence	Onset (min)
Control(I)	10/10	3.71 ± 0.43	10/10	4.88 ± 0.43	10/10	6.60 ± 0.36
Saline-Soman(IIa)[a]	9/9	5.97 ± 0.60*	9/9	7.18 ± 0.81*	2/9	5.18 , 4.83
Scopol+Soman(IIb)	8/10	11.38 ± 1.19†	1/10	6.23	0/10	—
Soman-before	10/10	6.06 ± 0.60*	10/10	7.85 ± 0.96	4/10	9.05 ± 1.53
Scopol-after(IIc)[b]	0/10	—	0/10	—	0/10	—

Guinea pigs were implanted with pumps containing either vehicle or physostigmine salicylate. Four days after the implantations, soman ($2LD_{50}$) was administered as described in the Method section and behaviors were monitored.

[a]The convulsions had disappeared within 2 hr of soman administration.

[b]The convulsions had disappeared within 30 min of scopolamine administration.

*$p<0.05$, †$p<0.01$ compared to the respective control values.

showed loss of righting reflex. The animals which received injections of scopolamine 10 minutes prior to soman injections (Group IIb) had a decreased incidence of all three toxicity symptoms as well as a three- to four-fold increase in the latency to onset of tremors. After soman administration to some of the physostigmine-pretreated guinea pigs, these animals were monitored for toxicity symptoms for the 25-minute interval prior to their being injected with scopolamine (Group IIc). During this interval of time, all of these animals displayed tremors and convulsions while 40% of them also showed loss of righting reflex. Following scopolamine administration to these guinea pigs, all symptoms of soman toxicity were reversed within 30 minutes.

DISCUSSION

The present results clearly demonstrate that treatment with physostigmine for a number of days prior to soman exposure significantly protects from soman-induced mortality. This protection is evident by decreases in the number of deaths and by increases in the time between soman exposure and death. However, acute physostigmine administration 10 min before soman exposure does not protect against soman lethality (based on 24-hr mortality).

These results suggest that the prophylactic uses of physostigmine by the mini-osmotic pump might be more useful than acute bolus administration of physostigmine. The present results also demonstrate that the solvent system used as a vehicle for physostigmine is very important. The lack of inhibition of acetylcholinesterase after 7 days indicates that the drug is not as stable in 20% propylene glycol, 10% ethanol and 70% water, pH=4 as it is in 40% propylene glycol, 10% ethanol and 50% water (18).

It has been reported that acute physostigmine alone or in combination with an antimuscarinic agent, such as atropine, might protect against soman lethality in various animal species (2, 8, 9, 15). Gordon *et al.* (8) have reported that the protection against soman was maximum 30 min after physostigmine pretreatment. Therefore, failure of the acute administration of physostigmine alone to protect against

soman toxicity as in the present study might be due to the dose of physostigmine used and/or the time interval between administration of these two agents.

The protection offered by physostigmine pretreatment is presumably due to protection of cholinesterase by reversible carbamylation, which temporarily renders the enzyme insensitive to irreversible inhibition by organophosphates (13,14). Lennox *et al.* (16) have reported that inhibition of acetylcholinesterase as low as 10% by a carbamate provides some protection against soman toxicity. Although the 4-day pretreatment with physostigmine offers more protection against soman toxicity than the 7-day pretreatment, it appears that the physostigmine-pretreated animals were still protected against soman toxicity even after the termination of physostigmine infusion. Although in our studies, acetylcholinesterase activity in red blood cells was significantly inhibited 3 days, but not 7 days after the mini-osmotic pump implantations, however, the AChE activity in striatum was still inhibited. Yamada *et al.* (23) have reported that repeated administration of physostigmine to guinea pigs does not affect muscarinic receptors. However, recently we have demonstrated that continuous infusion of physostigmine in guinea pigs decreased the density of muscarinic receptors (18).

It has been shown that after subacute treatment with organophosphates, muscarinic and nicotinic receptors become subsensitive to acetylcholine (1, 4, 17, 20). It has been reported that toxic effects of the organophosphates are due not only to irreversible AChE inhibition, but also to other effects, such as abnormal increases in choline and RNA depletion (3, 6, 11). Our results support the contention that soman toxicity does involve other effects. Although the exact mechanisms of the protection against soman toxicity after the termination of physostigmine are unknown, the alleviation of soman toxicity after preexposure with physostigmine might be, in part, due to protection of AChE and the subsensitivity of muscarinic receptors.

The present study indicates that when scopolamine is administered a few minutes before soman exposure, it does not significantly affect the lethality of soman; however, such use of scopolamine in subjects which have undergone subacute physostigmine pretreatment further increases survival to levels above those obtained using the subacute physostigmine prophylaxis alone. The results also demonstrate that scopolamine is able to reverse symptoms of soman toxicity when soman exposure is preceded by continuous physostigmine administration.

Furthermore, although the appearance of tremors, convulsions and loss of righting reflex in guinea pigs treated acutely with scopolamine was significantly delayed when compared to the group which received no scopolamine pretreatment, the mortality of the former group was almost 90% within an hour of soman administration. When the data in Table 4 are compared with data on mortality, it appears that neither the incidence of these symptoms nor their latency of onset will serve as predictors of survival following exposure to lethal doses of soman.

In summary, when physostigmine is used prophylactically, it not only increases survival, but also "buys time" during which other treatments may be used (e.g., artificial respiration) to further enhance survival.

Scopolamine also appears to be quite effective in preventing mortality when it is administered shortly after subjects pretreated subacutely with physostigmine are exposed to lethal doses of soman. In our studies, although the most effective prophylaxis against soman-induced lethality was the regimen in which both scopolamine and physostigmine were administered 10 minutes before soman exposure, the duration of this effectiveness needs to be further studied.

ACKNOWLEDGEMENT

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