J. Pharm. Pharmacol. 1979, 31: 295–299 Received September 8, 1978

The protection of primates against soman poisoning by pretreatment with pyridostigmine

P. DIRNHUBER, M. C. FRENCH, D. M. GREEN*, L. LEADBEATER AND J. A. STRATTON

Procurement Executive, Ministry of Defence, Chemical Defence Establishment, Porton Down, Salisbury, Wiltshire SP4 0JQ U.K.

The effectiveness of pyridostigmine pretreatment against soman poisoning has been determined in rhesus monkeys and marmosets receiving atropine therapy. Pretreatment with the maximum sign-free dose (200 μg kg^{-1}, i.v.) raised the subcutaneous LD50 of soman by a factor of 28 in rhesus monkeys and 15 in marmosets. The protection afforded by a quarter of the sign-free dose of pyridostigmine was not significantly less. These levels of protection are higher than any reported in non-primate species.

Poisoning by soman (1,2,2-trimethylpropyl methylphosphonofluoridate), an organophosphorus anticholinesterase agent, does not respond to treatment with atropine and an oxime (Loomis & Salafsky 1963; Heilbronn & Tolagen 1965). However, pretreatment with certain carbamates, in conjunction with atropine therapy, confers considerable protection against soman poisoning although there is a marked species variation in the efficacy of the treatment in the order guinea-pig > dog > rabbit > mouse > rat (Berry & Davies 1970; Gordon et al 1978). Gordon et al (1978) found pyridostigmine (3-dimethylcarbamoyloxy-1-methyl-pyridinium bromide) to be one of the most effective of the carbamates that were tested in guinea-pigs.

The present study was undertaken to assess quantitatively the effectiveness of pyridostigmine pretreatment in non-human primates (rhesus monkeys and marmosets) in order to provide a firmer basis for the extension of this treatment to man.

MATERIALS AND METHODS

Compounds

Pyridostigmine iodide and soman were synthesized in this Establishment. Atropine sulphate, ketamine (Vetalar) and pentobarbitone sodium (Sagatal) were purchased from BDH Limited, Parke Davis Limited and May and Baker Limited respectively. Each of the drugs, with the exception of Vetalar and Sagatal, was dissolved in sterile physiological saline and injected in a volume of 0·5 ml kg^{-1}.

Animals

Rhesus monkeys (*Macacca mulatta*, 2·7–3·4 kg) and marmosets (*Callithrix jacchus*, 215–355 g) of either sex were used.

* Correspondence.

Estimation of whole blood cholinesterase (ChE) time activity profile after intravenous (i.v.) administration of pyridostigmine

Rhesus monkeys were housed in a metal press cage and tranquillized with 5 mg kg^{-1} intramuscular (i.m.) injection of ketamine so that they could be safely removed from the cage and restricted. A sterile 18 gauge polythene cannula (Argyle-medicut) was inserted into the right saphenous vein to allow sequential blood sampling. One hour after injection of ketamine (the time required in the rhesus monkey to recover from the tranquillizing effect of ketamine) pyridostigmine was injected into the left saphenous vein and blood samples taken at intervals up to 5 h. Two animals were used for each dose of pyridostigmine administered.

Marmosets were anaesthetized with 40 mg kg^{-1} intraperitoneal sodium pentobarbitone since it was not practicable to take sequential blood samples from this small species when conscious. Polythene cannulae were inserted into the left carotid artery and femoral vein, the former being used for sequential blood sampling and the latter for injection of pyridostigmine. One animal was used for each dose of pyridostigmine. Animals were killed (i.v. pentobarbitone sodium) immediately after the experiments.

ChE activity was measured at 30 °C by the spectrophotometric method of Ellman et al (1961). The reaction was measured at 412 nm with a Pye–Unicam SP500 coupled to a Weyfringe ADCP-2 digital printer. An optimal substrate concentration of 1mM acetylthiocholine was used. The reaction rate was linear for 2 min and therefore no decarbamoylation was occurring during the assay. The term ChE refers to total acetylthiocholine hydrolase activity of the blood. Mean values for acetylthiocholine hydrolase activity in whole blood of un-

treated rhesus monkeys and marmosets were 5·65 (s.d. 1·62) and 2·32 μmol min^{-1} ml^{-1} respectively.

Protection experiments

i. *Rhesus monkeys.* The animals were housed in a metal press cage so that injections could be given without removal of the animal from the cage. Pyridostigmine was administered into a saphenous vein before subcutaneous (s.c.) injection of soman into the flank; atropine sulphate was administered i.m. into the thigh after the soman.

Details of the times of administration and doses of drugs used are given in Results.

ii. *Marmosets.* The animals were held by a gloved hand and i.v. injections given into a tail vein. The sites of s.c. and i.m. injections were the same as in rhesus monkeys as was also the pretreatment and therapeutic drug regimen.

Mortalities were recorded over a period of 7 days. The LD10, LD50 and LD90 values were estimated by probit analysis (Finney 1971; Natoff & Rieff 1970). The slopes of the log dose-probit mortality lines were calculated by the method of least squares. Parallelism between the log dose-probit mortality lines was examined by analysis of chi-squared. The probit analysis and statistical calculations were performed with a computer.

RESULTS

Time – ChE activity profiles after i.v. administration of pyridostigmine

The time-activity profiles were determined in order to define the optimum time for challenge with soman, i.e. at the time when the blood ChE was maximally carbamoylated. The intravenous route for the administration of pyridostigmine was used to obtain reproducible inhibition-time profiles. Pyridostigmine produced a dose-related inhibition of blood ChE in the rhesus monkey and marmoset. The time to peak carbamoylation occurred within 10–20 min after dosing. In each species the maximum sign-free dose was 200 μg kg^{-1}—higher doses produced marked muscle fasciculations (localized mainly over the head and shoulders) and miosis. At the maximum sign-free dose of drug the maximum blood ChE inhibition was 54 and 61% in the monkey and marmoset respectively and at a quarter of that dose the corresponding inhibitions were 30 and 33%.

Protection experiments with pyridostigmine in rhesus monkeys

1. *Evaluation of degree of protection.* The maximum sign-free dose was used in one series of experiments and 25% of this dose (50 μg kg^{-1} i.v.) in another series. The animals were pretreated with pyridostigmine 15 min (time to peak inhibition) before poisoning with soman. All the animals were given atropine sulphate (4 mg kg^{-1} i.m.) 15 s after soman.

The 7 day mortality figures together with calculated LD10, LD50 and LD90 values of soman alone and in the presence of treatment with the two dose levels of pyridostigmine are given in Table 1. To conserve animals the lethality of soman in untreated animals was determined from data of Fukuyama and Ashwick (unpublished work 1963) supplemented by data from 4 animals to confirm consistency with the current studies. The s.c. LD50 of soman (Table 1) was found to be 13·0 μg kg^{-1}.

There was a statistically significant ($P < 0{\cdot}01$) reduction in the slopes of the log – dose probit mortality curves in animals receiving the antidotal regimens compared with the slope obtained with soman alone (Table 1). The 200 and 50 μg kg^{-1} dose of pyridostigmine raised the LD50 of soman by a factor of 28 and 13 respectively (Table 2). When the log – dose probit mortality curves obtained with the two doses of pyridostigmine were compared statistically, there was no significant deviation from parallelism and a potency ratio of 2·1 was calculated; this ratio was not statistically significant at $P < 0{\cdot}05$.

2. *Signs of poisoning produced under treatment.* The severity of signs of poisoning depended on the dose of soman given: for convenience in reporting the results the dose ranges of soman have been classified as (i) low—where no mortalities occurred, (ii) medium—about 50% mortalities and (iii) high—100% mortalities. The signs of poisoning in animals receiving the maximum sign-free dose of pyridostigmine are summarized below:

i. *Low range.* In animals given 5·0–6·0 LD50 soman initial signs of poisoning consisting of muscular weakness occurred within 1–4 min. The animals then became prostrate with brief episodes of convulsions. The animals did not completely lose consciousness (corneal reflex still present) and there was only a low incidence of respiratory depression. About 15 min after becoming prostrate there was no convulsive activity and the animals began to make efforts to right themselves. They were able to sit up about 1 h after poisoning and were eating and drinking within 24–48 h. During the period 5 h–3 days after poisoning, movements were co-ordinated but were moderately slow and gross behaviour was slightly depressed. All animals completely recovered after 3–4 days.

Table 1. Subcutaneous toxicity of soman in untreated rhesus monkeys and in rhesus monkeys pretreated with 50 or 200 μg kg^{-1} i.v. pyridostigmine given 15 min before and atropine (4 mg kg^{-1} i.m.) given 15 s after soman.

Untreated		Treated			
			Pyridostigmine 50 μg kg^{-1} i.v.		Pyridostigmine 200 μg kg^{-1} i.v.
Dose soman μg kg^{-1}	Mortality (7 days)	Dose soman μg kg^{-1}	Mortality (7 days)	Dose soman μg kg^{-1}	Mortality (7 days)
8*	0/3	32·5	0/3	65	0/1
11*	1/4	65·0	0/4	78	0/2
15*	3/4	92·3	1/3	130	2/7
17	1/2	130·0	3/4	260	3/7
20*	3/3	183·0	2/4	520	3/6
33	2/2	366·6	2/4	1040	3/4
		733·2	4/4	4160	4/4
Lethal dose (μg kg^{-1})	95% Conf. Lts	Lethal dose (μg kg^{-1})	95% Conf. Lts	Lethal dose (μg kg^{-1})	95% Conf. Lts
LD10 9·7	(1·9–12·1)	LD10 51	(3–97)	LD10 71	(12–161)
LD50 13·0	(9·1–18·1)	LD50 176	(90–418)	LD50 378	(170–1098)
LD90 18·8	(15·2–76·7)	LD90 608	(300–14719)	LD90 2014	(812–152468)
Slope = 8·90 ± s.e. 3·69		Slope = 2·38 ± s.e. 0·84		Slope = 1·76 ± s.e. 0·63	

*Data obtained from Fukuyama and Ashwick unpublished work (1963).

ii. *Medium range.* Animals receiving 10–80 LD50 soman became prostrate with violent convulsions within 0·5–1 min and lost consciousness within 1–11 min. Respiration became slow, shallow and laboured (dyspnoea) and the animals appeared very close to death. In animals surviving this stage respiration rate and depth gradually increased together with return of consciousness within 10–30 min after poisoning. Within 2–4 h after poisoning the animals were able to crawl about the cage and sit up although they quickly became fatigued after a small amount of exertion. Some animals relapsed, collapsing to an immobile state together with respiratory depression which lasted for 2–3 h but then the animals gradually recovered. The signs during the recovery period 5 h–3 days were similar to animals poisoned with the low dose range of soman; complete recovery occurred after 3–4 days. One animal that survived 80 LD50s was observed for a period of 8 weeks and no chronic signs of poisoning were apparent.

Table 2. Computed protection ratios obtained in rhesus monkeys pretreated with 50 or 200 μg kg^{-1} i.v. pyridostigmine given 15 min before and atropine (4 mg kg^{-1} i.m.) given 15 s after soman (95% confidence limits in brackets).

Dose	I	II	III	IV
50 μg kg^{-1}	5·24 (1·89–14·52)	13·01 (7·24–23·36)	32·03 (10·47–99·6)	2·7 (1·01–7·34)
200 μg kg^{-1}	7·33 (2·0–26·88)	28·03 (14·05–55·84)	107·05 (24·9–460)	3·78 (1·04–13·7)

I = LD10 treated/LD10 untreated. II = LD50 treated/LD50 untreated. III = LD90 treated/LD90 untreated. IV = LD10 treated/LD90 untreated.

iii. *High range.* Within 1 min after receiving 320 LD50 soman the animals collapsed into a state of unconsciousness accompanied by violent convulsions and marked dyspnoea. In animals surviving this stage consciousness returned together with increase in rate and depth of respiration 10–30 min after poisoning. The animals then made attempts to crawl about the cage but relapsed after about 1 h and died. It is worthy of note that 3 out of 4 animals regained consciousness after the initial crisis period and lived for 1–2 h after poisoning (see Table 3).

Throughout the experiments observations were made on the presence and duration of muscle fasciculations. In some animals marked fasciculations were produced but in others little or no fasciculation occurred irrespective of the dose of soman given. Generally when fasciculations were present they occurred immediately after poisoning and then disappeared, together with body tremors, as the animal showed signs of recovery by attempting to crawl or right themselves. There was no marked salivation or miosis and only a small incidence of diarrhoea throughout all experiments.

The signs of poisoning and their durations of action in soman-poisoned monkeys pretreated with 200 μg kg^{-1} pyridostigmine are recorded in Table 3. The course of soman poisoning was similar in monkeys

Table 3. Signs of poisoning, with their duration of action, produced in rhesus monkeys given low, medium and high dose levels of soman: animals pretreated with 200 μg kg^{-1} i.v. pyridostigmine given 15 min before and atropine (4 mg kg^{-1} i.m.) 15 s after soman.

Dose of soman (n × LD50)	n	Animal No.	Onset of first signs (min)	Onset of prostration* (min)	Time span (min) Unconsciousness	Time span (min) of fasiculations	Resp. dep.	Time to regain 'sitting up' posture (min)	Eating and drinking	Time to regain normal agility	Mortality (7 days)
Low range	5	1	4	4·5	—	4·5–7·5		49	48 h	3 days	
	6	2	1	1·5	—	—	4–11	46	24 h	2 days	0/3
	6	3	3	4·5	—	4·5–6		60	24 h	3 days	
Middle	40	1	0·5	1	1–20	—	(9–20) (48–60)	240	48 h	3 days	
range	40	2	0·75	0·75	2–15	60–65	(6–27) (220–300)	65	48 h	3 days	2/4
	40	3	1	1	1–17	2–3	3–7+				
	40	4	0·75	1	1–25	—	7–25+				
High range	320	1	0·5	0·5	0·5–15	—	0·5–15+				
	320	2	0·25	1	1–10	0·25–10	1–68+				
	320	3	0·5	0·5	1–9	—	(1–30) (80–115+)				4/4
	320	4	0·5	0·5	0·5–31	—	(3–17) (35–65+)				

* Commonly associated with onset of convulsive activity. + Time to death.

receiving 50 μg kg^{-1} pyridostigmine although those animals were challenged with lower doses of soman.

Protection experiments with marmosets

Only an approximate estimation of the protection afforded by pyridostigmine against soman poisoning could be made due to the limited number of animals available. The 7 day mortality figures together with approximate values of LD50 obtained with soman given alone and in the presence of treatment with 50 and 200 μg kg^{-1} doses of pyridostigmine are given in Table 4. The pyridostigmine was given 10 min before challenge of soman to correspond with peak carbamoylation of blood ChE. The s.c. LD50 of soman in untreated marmosets was about 8 μg kg^{-1}. Pretreatment with 50 and 200 μg kg^{-1} pyridostigmine raised the LD50 of soman by factors of about 12·5 and 15 times respectively thus giving similar protection factors to those obtained in rhesus monkeys and again demonstrating little difference in the degree of protection afforded between the two dose levels of pyridostigmine. The signs of poisoning were very similar to those produced in the corresponding rhesus monkey experiments with the exception that survivors regained consciousness and recovered more quickly without relapse. Complete recovery occurred about 24 h after poisoning.

DISCUSSION

The results show that pretreatment with pyridostigmine combined with atropine given therapeutically affords a high degree of protection in rhesus monkeys and marmosets poisoned with soman. When the degree of protection is calculated on the basis of elevation of LD50 then pretreatment with the maximum sign-free dose of pyridostigmine raised the LD50 of soman by a factor of 28 and about 15 in rhesus monkeys and marmosets respectively. These protection factors are higher than those reported in non-primate species receiving a more

Table 4. Subcutaneous toxicity of soman in untreated marmosets and in marmosets pretreated with 50 or 200 μg kg^{-1}, i.v. pyridostigmine given 10 min before and atropine (4 mg kg^{-1}, i.m.) given 15 s after soman.

Untreated		Treated			
Dose soman μg kg^{-1}	Mortality (7 days)	Dose soman μg kg^{-1}	Pyridostigmine 50 μg kg^{-1} i.v. (mortality 7 days)	Dose soman μg kg^{-1}	Pyridostigmine 200 μg kg^{-1} i.v. (mortality 7 days)
6·5	1/4	58·5	0/3	78	0/4
9·1	3/4	117	2/3	156	3/4
13	4/4				
LD50 (μg kg^{-1})		LD50 (μg kg^{-1})	LD50 protection ratio	LD50 (μg kg^{-1})	LD50 protection ratio
ca 8		ca 100	ca 12·5	ca 120	ca 15

complex supporting therapy (Gordon et al 1978). Inclusion of oxime in the drug treatment increases the protection afforded in laboratory animals by pyridogstigmine and atropine alone (Gordon et al 1978) and will probably provide similarly enhanced protection to primates. The protective factors determined in the present study were for animals receiving a single therapeutic dose of atropine. The factors could probably be raised if additional therapy were given. Rhesus monkeys challenged with massive doses of soman (320 LD50s) survived for 1–2 h, adequate time for additional therapy to be given.

In previous studies evaluation of protective effectiveness of carbamate pretreatment has been based on elevation of LD50. In the present study there was a highly significant difference ($P < 0{\cdot}01$) between the slopes of the mortality curves obtained in rhesus monkeys pretreated with pyridostigmine and the slope obtained in untreated animals. Natoff & Rieff (1970) suggested that in protection experiments where shallow mortality curves are obtained, as in the present study, a reduction of the effect of a maximal lethal dose (LD90) to less than a minimal lethal dose (LD10) provided a better quantitative measure of therapeutic efficacy than elevation of LD50 value. Computed ratios of LD10 in treated animals to LD90 in untreated animals gave statistically significant ratios ($P < 0{\cdot}05$) of 3·78 and 2·7 (Table 2) for the 200 and 50 $\mu g\ kg^{-1}$ doses of pyridostigmine respectively. These statistics confirm the high degree of protection afforded by pretreatment with pyridostigmine and indicate that pretreatment with 50 $\mu g\ kg^{-1}$ is as effective as pretreatment with 200 $\mu g\ kg^{-1}$ pyridostigmine as suggested from a straight evaluation of LD50 values.

Although sufficient numbers of marmosets were not available to permit a statistical evaluation of the results obtained, nevertheless they suggest that the effectiveness of pyridostigmine pretreatment in this species is similar to that in the rhesus monkey.

The protective action of carbamates against organophosphorus anticholinesterase poisoning probably depends upon the ability of the carbamate to inhibit acetylcholinesterase forming a semi-stable carbamoylated enzyme which can spontaneously break down to liberate the enzyme (Wilson et al 1960, 1961). The fraction of the enzyme in the tissues that was carbamoylated would be protected against phosphonylation in subsequent poisoning by an organophosphate. The spontaneous decarbamoylation of the enzyme, in parallel with the rapid removal of the organophosphate would release sufficient acetylcholinesterase to maintain life (Berry & Davies 1970). Studies on the effectiveness of pyridostigmine pretreatment in reversing the neuromuscular blockade produced by soman provided evidence which supports this hypothesis (Dirnhuber & Green 1978). In the present investigation the sign-free dose of pyridostigmine and a quarter of that dose gave levels of protection against soman poisoning in the rhesus monkey that were not significantly different even though the corresponding levels of carbamoylation of blood ChE were 54 and 30% respectively. These data are not inconsistent with the proposed mechanism of the protective action of carbamates since it is well established that only a proportion of the tissue acetylcholinesterase is required for normal neuromuscular function (see, e.g. Hobbiger 1976).

The experiments reported in this paper have confirmed that pyridostigmine pretreatment, supported by therapy with atropine, protects primate species against soman poisoning and suggest that this drug treatment would be effective in man.

Acknowledgements

The authors thank Mr S. Callaway for carrying out the statistical analyses and Miss R. A. L. Power for technical assistance.

REFERENCES

Berry, W. K., Davies, D. R. (1970) Biochem. Pharmacol. 19: 927–934

Dirnhuber, P., Green, D. M. (1978) J. Pharm. Pharmacol. 30: 414–425

Ellman, G. L., Courtenay, K. D., Andres, V., Featherstone, H. M. (1961) Biochem. Pharmacol. 7: 88–95

Finney, D J. (1971) Probit Analysis 3rd edn London: Cambridge University Press.

Fukuyama, G. S., Ashwick, W. E. (1963) Unpublished data. Defence Research Establishment Suffield, Canada

Gordon, J. J., Leadbeater, L., Maidment, M. P. (1978) Toxicol. Appl. Pharmacol. 43: 207–216

Heilbronn, E., Tolagen, B. (1965) Biochem. Pharmacol. 19: 927–934

Hobbiger, F. (1976) in: Zaimis, E. (ed.) Handbuch der experimentallen Pharmakologie. Springer, Berlin, Heidelberg. N.Y. New Series, 42: pp 487–581

Loomis, T. A., Salafsky, B. (1963) Toxicol. Appl. Pharmacol. 5: 685–701

Natoff, I. L., Rieff, B. (1970) Br. J. Pharmacol 40: 124–134

Wilson, I. B., Hatch, M. A., Ginsburg, S. (1960) J. Biol. Chem. 235: 2312–2315

Wilson, I. B., Hatch, M. A., Ginsburg, S. (1961) Ibid. 236: 1498–1500