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17. ABSTRACT (Continue on reverse side if necessary and identify by block number) Botulism is caused solely by the protein neurotoxins produced by <i>Clostridium botulinum</i> . These toxins act by inhibiting acetylcholine release at neuromuscular junctions. Agents which stimulate the efflux of neurotransmitter, such as 3,4-diaminopyridine (3,4-DAP), could be useful in the treatment of botulism. Type C botulism affects a variety of species, but is especially severe in waterfowl, causing massive die-offs each year. To evaluate 3,4-DAP as a potential therapy for type C botulism, mice were injected i.p. with 10, 20			

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or 40 LD<sub>50</sub> of type C toxin. After 3 hr, when symptoms of botulism were apparent therapy with 3,4-DAP was begun for half of each group of mice. Mice were injected i.p. with 8 mg/kg of the drug hourly. This treatment with 3,4-DAP did not significantly increase the survival times of mice receiving type C toxin. However, therapy with 3,4-DAP, administered at the same concentration and according to the same dosage schedule, significantly prolonged the survival times of mice that had received 20 LD<sub>50</sub> of type A botulinum neurotoxin. The difference in the effectiveness of 3,4-DAP against type A and C botulinum toxins may be due to variations in the mechanism of action of these neurotoxins at the molecular level.

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EFFICACY OF 3,4-DIAMINOPYRIDINE AS A THERAPY  
FOR TYPE C BOTULISM

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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## ABSTRACT

L. S. SIEGEL and J. I. PRICE. Efficacy of 3,4-Diaminopyridine as a Therapy for Type C Botulism. *Toxicon* \_\_\_\_\_, \_\_\_\_\_-\_\_\_\_\_, 198\_\_\_\_. Botulism is caused solely by the protein neurotoxins produced by *Clostridium botulinum*. These toxins act by inhibiting acetylcholine release at neuromuscular junctions. Agents which stimulate the efflux of neurotransmitter, such as 3,4-diaminopyridine (3,4-DAP), could be useful in the treatment of botulism. Type C botulism affects a variety of species, but is especially severe in waterfowl, causing massive die-offs each year. To evaluate 3,4-DAP as a potential therapy for type C botulism, mice were injected i.p. with 10, 20 or 40 LD<sub>50</sub> of type C toxin. After 3 hr, when symptoms of botulism were apparent, therapy with 3,4-DAP was begun for half of each group of mice. Mice were injected i.p. with 8 mg/kg of the drug hourly. This treatment with 3,4-DAP did not significantly increase the survival times of mice receiving type C toxin. However, therapy with 3,4-DAP, administered at the same concentration and according to the same dosage schedule, significantly prolonged the survival times of mice that had received 20 LD<sub>50</sub> of type A botulinum neurotoxin. This difference in the effectiveness of 3,4-DAP against type A and C botulinum toxins may be due to variations in the mechanism of action of these neurotoxins at the molecular level. ←

## INTRODUCTION

*Clostridium botulinum* produces seven immunologically distinct protein neurotoxins, designated types A, B, C<sub>1</sub>, D, E, F, and G (SUGYAMA, 1980). Types A - F neurotoxins cause botulism in humans, animals and/or birds. *Clostridium botulinum* synthesizing type G neurotoxin were isolated from soil in Argentina (GIMENEZ and CICCARELLI, 1970) and from humans at autopsy (SONNABEND *et al.*, 1981), but this toxin has not been associated with illness in any species.

The neurotoxin produced by *Clostridium botulinum* type C causes botulism in birds, particularly in waterfowl (ducks, geese and swans) (SMITH, 1982). Avian botulism has been reported worldwide ( FRIEND *et al.*, 1985). Loss of over a million birds in a single outbreak at one location has been reported; outbreaks involving the death of 50,000 or more birds are relatively common (FRIEND *et al.*, 1985). Die-offs occur almost yearly, but losses vary from species to species and from year to year ( FRIEND *et al.*, 1985). Outbreaks of type C botulism also occur in pheasants on game farms, and in chickens and turkeys (HARIHARAN and MITCHELL, 1977). The disease also causes economically important losses of cattle, sheep, horses, and mink (SMITH, 1977). Other species reportedly affected by type C botulism include ferrets, pigs and dogs (HARIHARAN and MITCHELL, 1977), as well as lions (GREENWOOD, 1985) and turtles (SMITH, 1982).

There is little data to support the contention that type C botulism occurs in humans. Although there are two reports of human cases of

botulism attributed to type C, type C toxin was not demonstrated in the serum or feces of the patients nor in suspect food (SMITH, 1977). However, non-human primates are susceptible to type C botulism. BENGTON (1922) reported the first isolation of type C *Clostridium botulinum* and experimentally demonstrated its toxicity in monkeys (genus and species not specified). Experimental intoxication of *Macacus rhesus* (GUNNISON and MEYER, 1930) and of *Macaca mulatta* (WAGENAAR *et al.*, 1953) with type C toxin has been reported. Natural outbreaks of type C botulism in primates have occurred in zoos (SMART *et al.*, 1980; SMITH *et al.*, 1985).

Botulism can be prevented by immunization with inactivated toxin (toxoid). In South Africa and Australia, immunization of cattle and sheep with toxoid has been a common practice for many years (SMITH, 1977). Due to the potential for economic losses from the disease, most mink raisers immunize their animals (SMITH, 1977). However, immunization of waterfowl and many other species at risk is impractical.

Treatment of type C botulism by injection of the corresponding antitoxin is efficacious, if started early in the course of the disease (SMITH, 1977). This therapy has been used for mink (SMITH, 1977) and for birds (FRIEND *et al.*, 1985; SMITH *et al.*, 1985). Because treating sick birds with antitoxin is expensive, and supplies of antitoxin are limited, this therapy is normally used only for endangered species.

Since botulinum toxins block the release of acetylcholine at the neuromuscular junction and thereby produce paralysis (BURGAN *et al.*, 1949), agents known to increase neurotransmitter release have been investigated for potential therapeutic use. It has been demonstrated previously that

3,4-DAP is an effective treatment for type A botulism in mice, but not for type B, E or F (SIEGEL *et al.*, 1986). Therefore, we investigated the effect of 3,4-DAP treatment on the survival times of mice injected with type C botulinum toxin and compared it with that of type A (as a reference standard for drug action).

## MATERIALS AND METHODS

### *Botulinum toxins.*

Toxin lethal activity was determined as previously described (SIEGEL *et al.*, 1986). All doses of toxin reported here are expressed as mouse i.p. LD<sub>50</sub>/ml. The stock solution of type A botulinum toxin, prepared as reported earlier (SIEGEL *et al.*, 1986), contained  $2.0 \times 10^7$  LD<sub>50</sub>/ml. For the production of type C toxin, the Stockholm strain of *Clostridium botulinum* type C was grown in a medium containing 2.0% casein hydrolysate, 2.0% yeast extract, 1.0% glucose, and 0.05% sodium thioglycolate (pH 7.6). After incubation at 37°C for 5 days, the toxin in the culture was precipitated by adjusting the pH to 4.0 with 3 N H<sub>2</sub>SO<sub>4</sub>, with the addition of RNA at 0.4 mg/ml as a precipitation aid (IWASAKI and SAKAGUCHI, 1978). The precipitate was washed with distilled water, and the toxin extracted with 0.2 M phosphate buffer at pH 6.0. The extract, containing  $2.2 \times 10^5$  LD<sub>50</sub>/ml, was divided into aliquots and stored at -70°C.

### *Experimental protocol.*

Studies were conducted as described previously (SIEGEL *et al.*, 1986). An aliquot of stock toxin was thawed and immediately diluted in cold gel-phosphate buffer such that 0.2 ml contained 10, 20 or 40 LD<sub>50</sub>. At zero time, each mouse in a group of at least 30 mice was injected i.p. with either 10, 20 or 40 LD<sub>50</sub> of type C botulinum toxin. A group of mice (30 or more) injected with 20 LD<sub>50</sub> of type A toxin served as a positive control for

the efficacy of 3,4-DAP therapy. After 3 hr, when the mice showed signs typical of botulism, treatment with 3,4-DAP was begun for half of each group of mice. The drug, in 0.2 ml sterile saline, was injected i.p. at hourly intervals, at a dose of 8 mg/kg. A control group of mice did not receive toxin, but were injected with 3,4-DAP according to the same schedule. All mice were checked for survival at 15 min intervals.

*Statistical analysis.*

A computer statistics program, Biomedical Data Program 1L, was used to calculate mean, standard error of the mean, and median survival time. The pattern of survival of treated versus untreated groups of mice was compared by using the Mantel-Cox statistic. A P value of 0.05 or less was considered statistically significant.

*Chemicals.*

Three, four-diaminopyridine (98% pure) was obtained from Aldrich Chemical Company, Inc., Milwaukee, WI. Casein hydrolysate (N-Z amine A) was purchased from Humko Sheffield Chemical Co., Memphis, TN, and yeast extract was a product of Difco Laboratories, Detroit, MI. RNA (Type III from bakers yeast) and sodium thioglycolate were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals used were reagent grade products.

## RESULTS

SIEGEL *et al.* (1986) previously reported that the survival times of mice injected with type A botulinum toxin can be prolonged by treatment with 3,4-DAP. In the studies reported here, a group of mice injected with 20 LD<sub>50</sub> of type A toxin served as a positive control for the efficacy of 3,4-DAP. As shown in Table 1, the difference in the pattern of survival of the treated versus untreated groups of mice for three such experiments is statistically significant ( $P \leq 0.05$ ). When tested under identical conditions, treatment with 3,4-DAP failed to prolong the survival time of mice injected with 10, 20 or 40 LD<sub>50</sub> of type C botulinum toxin (Table 1). In two experiments, administration of 3,4-DAP actually shortened the time to death (Table 1). The control group of mice, which did not receive toxin but were injected with 3,4-DAP according to the same dosage schedule, did not display any apparent adverse effects.

## DISCUSSION

Since botulinum neurotoxins inhibit acetylcholine release at cholinergic nerve terminals (BURGAN *et al.*, 1949), drugs that increase the liberation of the neurotransmitter may be useful in the therapy of botulism. Four-aminopyridine (4-AP) has been shown to enhance acetylcholine release from nerve terminals (MOLGO *et al.*, 1975; YEH *et al.*, 1976), and its efficacy as a treatment for type C botulism has been evaluated in mice and rats (MORRISON and KRYZHANOVSKY, 1985). In those studies, mice were injected i.m. with varying doses (0.1-0.2  $\mu$ g) of type C toxin. Administration of 4-AP (5 mg/kg i.p.) within 3-5 min of toxin injection significantly prolonged the survival times of mice that had received 0.12  $\mu$ g of toxin. A 2 mg/kg dose of 4-AP administered twice daily produced the maximum increase in survival times. If 4-AP (2 mg/kg) was given after local paralysis had developed (within 1 day after toxin injection), survival times were significantly increased. Rats were totally paralyzed and showed signs of respiratory insufficiency within 3 days of injection with 50  $\mu$ g of type C toxin, but within 5-6 min of injection of 4-AP (5 mg/kg), the animals were able to lift their heads and to move about. The duration of the improvement was 1 to 1.5 hr. Further injections of 4-AP again resulted in a reduction of signs in the rats. Although the results reported were noteworthy, 4-AP was tested only at very low doses of botulinum toxin (1-2 LD<sub>50</sub>, according to the authors), and very slight increases in toxicity within that range dramatically reduced the effectiveness of the drug.

Three, four-diaminopyridine (3,4-DAP) is reportedly less convulsant than 4-AP in laboratory animals (VOHRA and PRADHAN, 1964), and is six to seven times more effective than 4-AP in reestablishing neuromuscular transmission in rat muscles previously paralyzed by type A botulinum toxin (MOLGO *et al.*, 1980). Three, four-diaminopyridine has been used to treat type C botulism in two circus lions (GREENWOOD, 1985). Improvements in the condition of both lions were transient, lasting for only about 30 min, and treatment with 3,4-DAP was discontinued. Unfortunately, treatment with 3,4-DAP was not initiated until late in the course of the disease, and only very low doses of the drug were used. In our studies, therapy with 3,4-DAP was not effective against type C botulinum neurotoxin (Table 1). However, treatment with the same concentration of 3,4-DAP (8 mg/kg) according to the same dosage schedule significantly prolonged the survival times of mice that had received type A botulinum neurotoxin (Table 1).

The efficacy of 3,4-DAP as a mode of therapy for botulism caused by type A, B, E or F neurotoxin has been determined (SIEGEL *et al.*, 1986). Treatment with 3,4-DAP was effective in prolonging the survival of mice injected with 10, 20 or 40 LD<sub>50</sub> of type A botulinum toxin, but not against equivalent amounts of type B, E, or F neurotoxin. In addition, 3,4-DAP has been evaluated in isolated rat muscle preparations for its ability to overcome the paralysis previously induced by botulinum toxin. The drug (4  $\mu$ M) effectively restored neuromuscular transmission in muscles paralyzed by type A toxin (MOLGO *et al.*, 1980). Compared to type A, 3,4-DAP (1, 10 and 100  $\mu$ M) was less effective against the paralysis produced by type B (SELLIN

*et al.*, 1983) or type F toxins (KAUFFMAN *et al.*, 1985). However, larger doses of B and F were required to produce an effect comparable to that induced by type A: 5,000 LD<sub>50</sub> of type B produced paralysis equal to that obtained with 20 LD<sub>50</sub> of type A (SELLIN *et al.*, 1983); 200-2,000 LD<sub>50</sub> of type F were needed for an effect equal to 2-3 LD<sub>50</sub> of type A (KAUFFMAN *et al.*, 1985). In rat phrenic nerve-hemidiaphragm preparations, 3,4-DAP (100 μM) increased neuromuscular transmission to a greater extent in muscles previously poisoned with type A botulinum toxin than in those pretreated with type B or E (SIMPSON, 1986).

Thus, based on whole animal studies using equivalent doses of botulinum neurotoxins, 3,4-DAP is an effective therapy against type A toxin (Table 1, SIEGEL *et al.*, 1986), but not against type B, E, or F (SIEGEL *et al.*, 1986), nor against type C (Table 1). These studies suggest that type A botulinum neurotoxin may act by a different mechanism than type B, C, E or F at the molecular level.

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Table 1. EFFECT OF 3,4-DIAMINOPYRIDINE TREATMENT ON SURVIVAL OF MICE INJECTED WITH BOTULINUM TOXIN

Type	LD <sub>50</sub>	Survival Time (hr)				P
		Untreated*		3,4-Diaminopyridine*		
		Mean±SE	Median	Mean±SE	Median	
A	20	10.4 ± 0.72 <sup>†</sup>	9.8	13.3 ± 0.44	>13.8	<0.003
		10.7 ± 0.67	10.0	15.8 ± 0.33	>15.5	<0.001
		10.5 ± 0.54	10.2	15.7 ± 0.16	>15.5	<0.001
C	40	9.2 ± 0.68	8.8	9.1 ± 0.69	8.2	NS <sup>§</sup>
		8.4 ± 0.51	8.2	8.8 ± 0.71	8.2	NS
		9.4 ± 0.43	9.2	7.7 ± 0.55	8.2	[<0.05] <sup>¶</sup>
	20	10.4 ± 0.70	10.0	9.2 ± 0.48	9.2	NS
		12.2 ± 0.63	12.0	11.1 ± 0.66	11.5	NS
		11.3 ± 0.67	11.2	10.2 ± 0.70	10.0	NS
	10	13.2 ± 0.65	14.0	12.3 ± 0.74	12.0	NS
		13.4 ± 0.64	15.0	13.2 ± 0.71	15.0	NS
		14.8 ± 0.54	14.8	12.8 ± 0.53	13.5	[<0.001]

\* Number of mice in each of the treated and untreated groups ≥ 15.

<sup>†</sup> Mean survival time ± standard error of the mean.

<sup>§</sup> Not significant (p > 0.05).

<sup>¶</sup> 3,4-DAP treatment shortened survival time.