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ACUTE BOTULINUM-LIKE INTOXICATION BY TETANUS NEUROTOXIN IN MICE

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SUMMARY. Intravenous injection of purified tetanus toxin(1000-0.06 μg) killed mice within minutes(20-450 min), causing flaccid paralysis indistinguishable from that in botulinum intoxication: a linear relation was found between the log of the toxin dose and that of death time(survival time). The dose and route dependences of the manifestations of the spastic paralysis typical of classical tetanus and of the acute botulinum-like flaccid paralysis were studied in relation to the death time. Treatment of the toxin with trypsin or gangliosides did not affect its acute botulinum-like toxicity. Theophylline delayed the time of acute death due to the botulinum-like intoxication in mice caused by tetanus toxin and provided some protection.

Tetanus toxin is known to have two apparently different actions, central and peripheral(1). Its action on the central nervous system(CNS) mainly elicits hyperactivity of the motor system, producing signs of spastic paralysis typical of tetanus in man and animals. On the other hand, as peripheral effects of tetanus toxin, flaccid paralysis or block of neuromuscular transmission has been observed in some clinical cases of tetanus(2), experimental local tetanus(3, 4), rabbit iris muscle following intraocular injection(5) and in goldfish(6), hen(7) and a phrenic nerve-hemidiaphragm preparation(8). Under particular experimental conditions, after early administration of antitoxin(9) or a large amount of toxoid(10), tetanus toxin causes predominantly flaccid paralysis. However, the mechanism by which tetanus toxin, a single toxin protein, produces two different signs of paralysis and the extent to which the peripheral effects participate in tetanus intoxication in the whole animal have not been clarified because no suitable model system was available. While studying generalized tetanus in mice, we found that intravenous(iv) injection of a large amount of purified tetanus toxin caused rapid death of mice after flaccid paralysis indistinguishable from that caused by botulinum toxin, which

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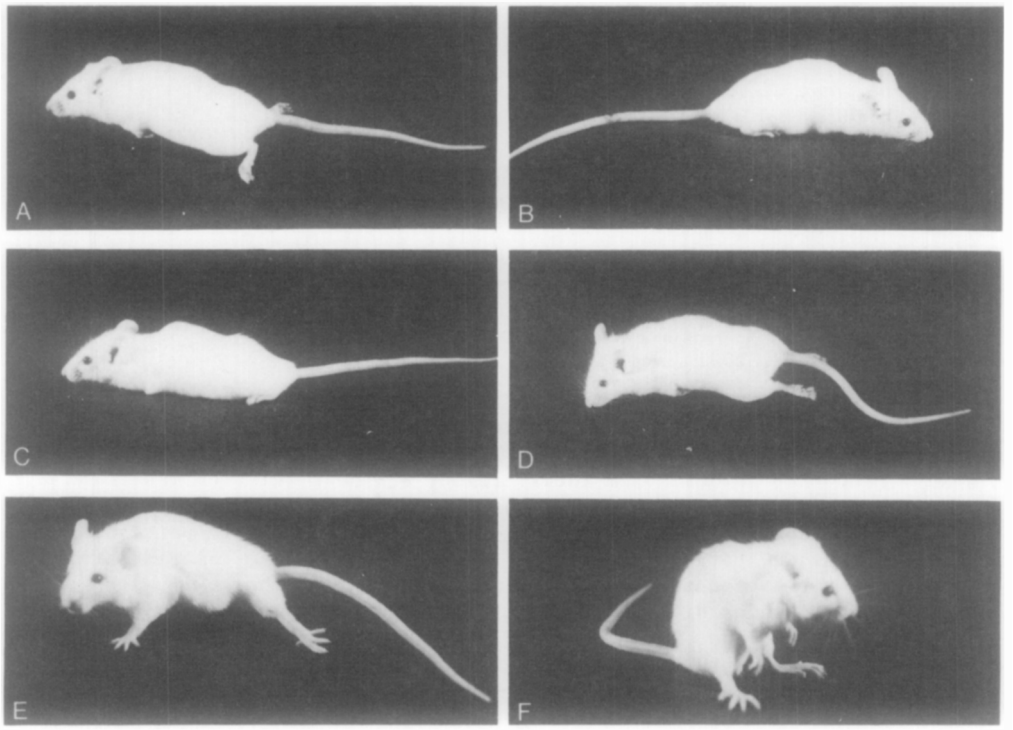

Fig. 1. Flaccid paralysis of a mouse after iv injection of a large dose (200 μg) of purified tetanus toxin, at various stages(early stage to death), (A-D) of acute intoxication. The mouse died 34 min after the injection. Spastic paralysis after intramuscular(E) and intravenous(F) injections of small amounts(0.00632 μg and 0.002 μg, respectively) of the toxin.

blocks peripherally neuromuscular transmission(11). This paper reports studies on acute botulinum-like intoxication by tetanus toxin in mice.

MATERIALS AND METHODS. Randomly bred OF1 strain white mice of both sexes weighing 22-30 g were used. Tetanus toxin was prepared and highly purified from bacterial extracts ("intracellular" toxin) as described in our previous report(12). Samples of 0.1 ml of toxin in phosphate buffered saline, pH 7.0, containing 0.2% gelatin(PBS-G7) were injected iv into a tail vein, subcutaneously(sc) or intramuscularly(im) into the right hind leg of mice. Groups of at least 5 mice were used for each dose of toxin. The mice were observed continuously at 25°C and cardiac arrest was taken as the criterion of death. Toxin(2.5 mg/ml) was treated with trypsin(EC 3.4.4.4; Sigma, ×2 crystallized) at 37°C for 30 or 60 min in 0.05 M Na acetate buffer, pH 6.0(trypsin to toxin, 1:50). Then trypsin inhibitor(Sigma, twice the amount of trypsin) was added, incubation was continued for a few minutes and then the reaction mixture was diluted with an equal volume of PBS-G7. The effects of gangliosides in detoxification of tetanus toxin were examined by the method reported for botulinum toxin(13), using gangliosides(Sigma, Type II, III; Spelco Co., GT1). Theophylline was purchased from Nakarai Chemicals, Kyoto. Rabbit anti-purified tetanus toxin was obtained by the method described previously(14).

RESULTS AND DISCUSSION

Clinical signs of acute intoxication in mice after intravenous injection of tetanus toxin: After iv injection of 200 μg of purified tetanus toxin, the

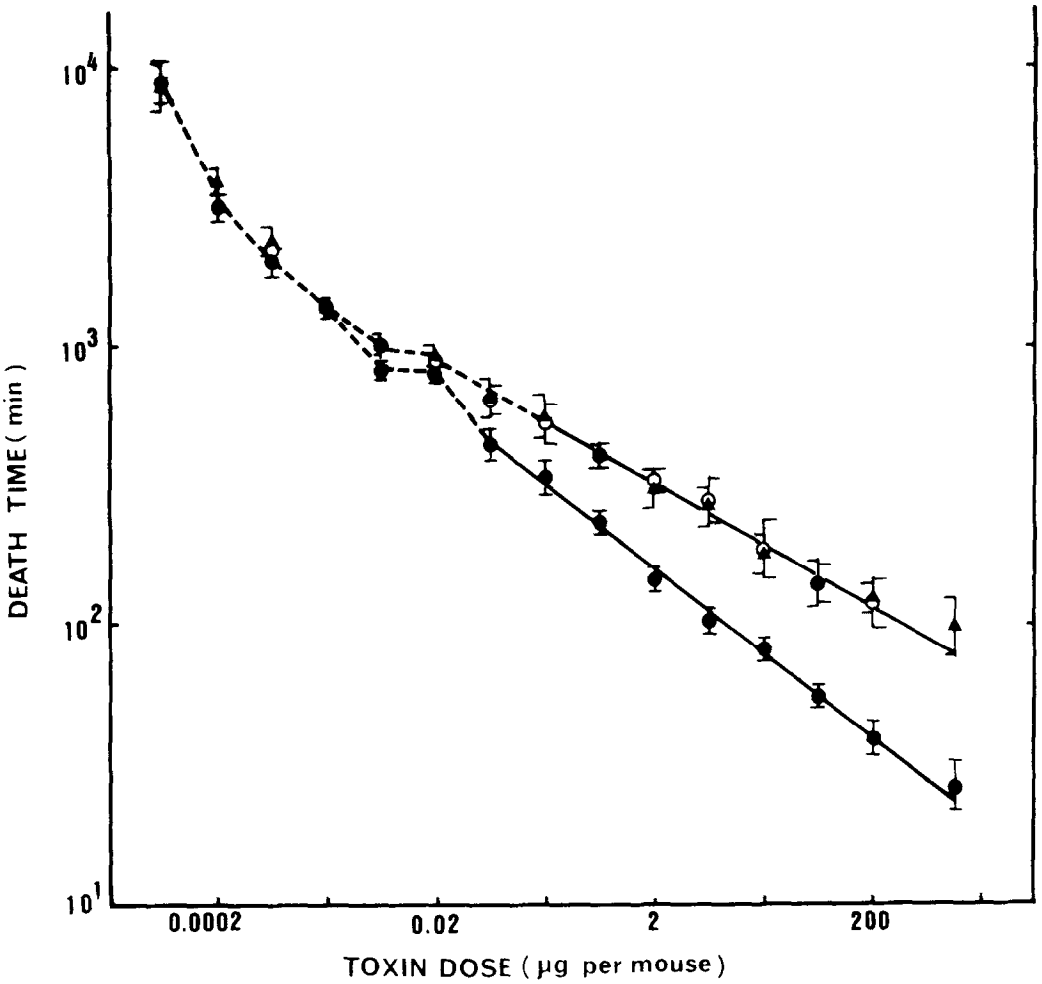

Fig. 2. Logarithmic relation of tetanus toxin dose and mean survival time (death time) of mice after intravenous(●), intramuscular(▲) or subcutaneous(○) injection of toxin. Solid lines show the appearance of flaccid paralysis, dotted lines that of spastic paralysis. Mean death times for 5 mice with S.D. are plotted.

first detectable signs, consisting of a waddling gait, followed by dragging of the hind legs appeared in about 20 min. As muscular flaccidity increased, the fore and hind legs all became paralytic(Fig. 1A), and the animals became too weak to stand(Fig. 1B). Animals remained motionless with roughed up fur, a limp tail and hollow flanks and their breathing became labored(Fig. 1C). They developed marked exophthalmus, sometimes with lacrimation. A few minutes before death, a bouncing gait followed by a spell of generalized convulsions with urination occurred and then complete flaccid paralysis of the whole body with curling of the body(Fig. 1D). Death occurred in 40.3 ± 6.2 min. Respira-

TABLE 1. Effect of trypsin treatment on the acute botulinum-like toxicity

Time of treatment	Death time*
None	50.0 ± 4.9
30 min	59.0 ± 9.8
60 min	49.5 ± 4.2

Toxin(2.5 mg/ml) was treated with trypsin(trypsin to toxin, 1:50) at 37°C for 30 or 60 min in 0.05 M Na acetate buffer, pH 6.0 as described in MATERIALS AND METHODS. *Values are means for 5 mice ± S.D. injected with 125 μg of toxin.

tion stopped about 1 min before cardiac arrest. These characters were indistinguishable from those in death from botulinum intoxication. Classical signs of tetanus(15), such as rigidity of the muscles, an extensor position with constant muscular spasms, tetanic convulsions, stiffness of the tail and closed eyes, were never observed.

Dose response of mice to tetanus toxin injected by various routes: Figure 2 shows the relations of the log dose of tetanus toxin to the mean survival time(death time) of mice after iv, im or sc injection of toxin. Injection iv of a large dose(1000-0.0632 μg) of toxin caused acute death with flaccid paralysis and a linear correlation was observed between the log dose and log death time. Similar responses and linear correlations were obtained on injection im or sc of 1000-0.2 μg of toxin, but iv injection resulted in more rapid death and a smaller standard deviation(S.D.) of the death time. Injection iv of 1000 μg of toxin caused death in 20 min. With doses of less than 0.2 μg, signs of spastic paralysis became evident with decrease in the dose, and with less than 0.02 μg, spastic paralysis was observed predominantly, though minor local flaccid paralysis of the toes of injected limbs remained after im or sc injection of over 0.002 μg. In contrast to the im and sc routes, which produced more or less asymmetrical(more severe on the injected limb and more asymmetrical at smaller doses) signs(spastic(Fig. 1E) or flaccid) at doses of less than 6.32 μg, iv injection always caused symmetrical signs and at small doses(<0.02 μg) symmetrical spastic paralysis provoked a "kangaroo like posture"(Fig. 1F). With doses of from 0.02 μg(which roughly corresponds to the highest toxin dose causing predominantly spastic paralysis)

TABLE 2. Effect of treatment of toxin with gangliosides on acute botulinum-like toxicity

Treatment	Death time* (min)
None	53.8 ± 5.9
gangliosides Type II	51.8 ± 5.2
gangliosides Type III	55.1 ± 8.0
ganglioside GT1	57.0 ± 7.3

Samples of 400 µl of toxin(2.5 mg/ml 50 mM Tris-HCl, pH 7.2) mixed with 265 µl gangliosides(Sigma Type II or Type III, 2.5 mg/ml distilled water) and 35 µl of 0.5 M Tris-HCl, pH 7.2, or with 70 µl ganglioside GT1(1 mg/ml distilled water) and 30 µl of 0.5 M Tris-HCl, pH 7.2. After incubation at 37°C for 20 min, 0.3 ml and 0.5 ml, respectively, of chilled PBS-G7 were added to reaction mixtures to make a final volume of 1.0 ml. Samples of 0.1 ml were injected iv into group of 5 mice to determine toxicity. *Values are means for 5 mice ± S.D.

to 0.0632 µg of toxin, there was little difference in the death time(ca. 15 hr). This death time might be considered as the apparent minimum death time(16). Precipitation with antitoxin abolished both types of toxicity of the toxin preparations.

Effects of treatment of tetanus toxin with trypsin and gangliosides on its acute toxicity: Certain types of botulinum toxin are activated by trypsin and inactivated by gangliosides(13). Therefore, we examined the effects of these treatments on the acute botulinum-like toxicity of tetanus toxin. Table 1 and 2 show that unlike botulinum toxin, tetanus toxin was not activated by trypsin nor inactivated by gangliosides.

Effect of theophylline on the acute toxicity of tetanus toxin in mice: Mice were injected intraperitoneally(ip) with 2.5 mg of theophylline 15 min before iv injection of various doses of tetanus toxin, and death times were compared with those of control mice that had not been treated with theophylline. Table 3 shows that theophylline significantly increased the time of death following flaccid paralysis in mice, as reported in the case of botulinum toxin(17); as with the latter, the protective effect of theophylline was temporary. Pre-treatment with theophylline decreased the acute toxicity to about one-tenth of that in control mice at doses of toxin of above 0.632 µg. The protective effect of theophylline was less with doses of the toxin of less than 0.2 µg, and with decrease in the dose signs of spastic paralysis began to appear.

TABLE 3. Effect of theophylline on acute botulinum-like toxicity of tetanus toxin

Toxin dose (μg)	Death time* (min)		Ratio (B/A)
	Untreated mice(A)	Theophylline treated mice(B)	
200	40.3 ± 6.2	72.9 ± 17.3	1.81
63.2	56.5 ± 4.8	85.6 ± 5.1	1.52
20.0	81.9 ± 7.2	135.7 ± 22.7	1.66
6.32	113.4 ± 12.7	195.5 ± 35.6	1.72
2.0	141.1 ± 9.1	250.2 ± 83.6	1.77
0.632	233.7 ± 24.9	354.7 ± 57.1	1.52
0.20	345.7 ± 53.2	406.3 ± 58.6	1.18
0.0632	454.3 ± 62.0	492.3 ± 66.1	1.08

Mice were injected intraperitoneally with 2.5 mg of theophylline(in 0.5 ml of saline) 15 min before iv injection of various doses of tetanus toxin. *Values are means for 5 mice ± S.D.

Tetanus is generally regarded as a spastic disease due to the action of tetanus toxin on the CNS and it is still widely believed that a definite incubation period of at least a few hours or days(3, 4) is required for tetanus toxin to manifest signs of intoxication. The present results clearly show that iv injection of tetanus toxin rapidly produces signs of flaccid paralysis and early death in the same way as botulinum toxin. Death of mice in 60 to 70 min after a massive dose of tetanus toxin by im injection with atypical signs(15) has been reported and 20 min of incubation period after its iv injection was cited in a review(18), but no data have been provided to permit a judgement of the reliability of the latter experiment(1). However, the relatively rapid death has not previously been recognized as being similar to that caused by botulinum toxin. We recently found by electromyography that block of neuromuscular transmission in fact also occurred in acute tetanus intoxication in mice(19). The botulinum-like intoxication described here was so rapid that we could observe the peripheral effect of tetanus toxin before its central effect, which requires a longer latent period to develop. So, this system will be useful for obtaining a clue to understanding the mechanism of action of tetanus toxin as demonstrated by antagonism by theophylline described above. Atypical, flaccid paralysis, rather than spastic paralysis though weak and delayed, has been observed after im injection of low-nitrated

tetanus toxin(20) and a toxin fragment(21). Therefore, we are studying the structure-function relation of the tetanus toxin molecule using our acute intoxication system. The linear log dose-death time relation of the acute botulinum-like intoxication after iv injection of the toxin provides a new bioassay method for tetanus toxin that is much more rapid(several hours) than conventional methods of observing spastic toxicity after im or sc injection, which require several days.

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