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THE EFFECTS OF CLOSTRIDIUM BOTULINUM TOXIN TYPE C_β GIVEN ORALLY TO GOATS

By

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FJØLSTAD, MORTEN: *The effects of Clostridium botulinum toxin type C_β given orally to goats.* Acta vet. scand. 1973, *14*, 69—80. — Feeding experiments with Clostridium botulinum toxin type C_β were carried out using 11 goats. The toxin, which originated partly from the liver of a cat carcass and partly from broth inoculated with Cl. botulinum type C_β isolated from the cat liver, was given to the goats through a rubber stomach-tube. Symptoms of botulism usually occurred on the second or third day after the challenge. Death was caused by doses of toxin as small as 0.5 MMLD (minimum mouse lethal dose) per g body weight of goat, and 1 MMLD per g was considered to be lethal for goats when they are fed hay and concentrated fodder. When the animals were grazing or received ensilage they seemed to tolerate somewhat higher doses. A cumulative effect was shown to take place when the doses of toxin were given in the course of 8 days. A sensitizing effect of many small doses could not be demonstrated. On the contrary, animals which had received several small doses of toxin later on tolerated doses which caused death in inexperienced animals. One of these animals tolerated a dose approx. 3 times higher than a dose usually causing death. Serum from such goats, however, did not give demonstrable protection in mice challenged to botulinum toxin type C_β. Toxin could not be demonstrated in samples of serum from goats showing symptoms of botulism. In 2 of 9 animals toxin could be demonstrated in samples from the liver, but only after the liver had remained in the carcass for approx. 20 hrs. after death.

botulinum toxin; ruminants; cumulative effect.

Botulism in ruminants is quite a common disease in some parts of the world and is caused primarily by Clostridium botulinum toxin types C (C_β) and D. Little is known, however, about the amount of toxin needed to cause death, or symptoms of illness, in ruminants. Feeding experiments have been carried out (*Seddon* 1922, *Bennetts & Hall* 1938), but the amount of toxin used was not expressed in biological units such as mouse lethal doses. *Müller* (1961), however, performed one experiment in

which he fed approx. ½ million mouse lethal doses type C toxin to a cow weighing approx. 600 kg. The cow showed symptoms of illness 2 days later and died 4 days after the feeding.

The amount of a certain type of botulinum toxin needed to cause symptoms of illness or death when administered orally, varies from species to species (*Stableforth & Galloway* 1959). This may also be the case with various species of ruminants.

The purpose of the present investigation was 1) to determine the minimum dose of toxin, expressed as minimum mouse lethal doses (MMLD), needed to cause death, or symptoms of illness, in goats when the toxin was administered orally, 2) to investigate whether the kind of fodder the animals received at the time of challenge could interfere with the effect of the toxin, 3) to see if several small toxin doses, given at certain intervals, could have a cumulative effect or could sensitize the animal, and 4) to try to detect botulinum toxin in samples from animals showing symptoms of, or dying from, botulism.

MATERIALS AND METHODS

Toxin

The botulinum C_β-toxin used for animals I, II, III, IV and V (except the last dose to animal V) originated from the liver of a cat carcass which had previously been the cause of botulism in a herd of ruminants (*Fjølstad & Klund* 1969). In the remainder of the experiments toxin produced by the Cl. botulinum type C_β-strain isolated from the liver of the cat carcass was used. The liver of the cat was ground in a mortar containing sterile sand and saline and the suspension filtered through gauze. The filtrate was used as supply of toxin. Production of the toxin was carried out by cultivating the isolated Cl. botulinum strain in 4 500-ml flasks containing 400 ml Robertson's cooked meat medium (*Robertson* 1915—1916) to which was added glucose to a final concentration of 0.2 %. Prior to inoculation the medium was boiled for 20 min. and then cooled under running tap water. After inoculation, the flasks were incubated anaerobically at 37 °C for 5 days. The broth culture was filtered through gauze and the filtrate used as supply of toxin. The toxin (from both the liver suspensions and from the broth cultures) was frozen at —20 °C in small aliquots, so that each dose given to experimental animals was thawed only once after the activity of the toxin had been

determined. The toxin concentration was determined once a week during the experimental periods.

The 2 suspensions made from cat liver were diluted in saline so that they contained 10,000 and 20,000 MMLD per ml. The pooled broth cultures contained 30,000 MMLD per ml. Calculations, according to the method of Reed & Muench (*Davis et al.* 1968), were also carried out in order to estimate the number of LD50 in the toxin preparations. The ratio between the dilutions immediately above and below that causing 50 % mortality was used as the number of fold dilution in the formula. The number of LD50 (adjusted to nearest 100 doses) was 12,500 and 25,900 per ml in the suspensions of cat liver containing 10,000 and 20,000 MMLD respectively, and 36,300 per ml in the broth culture. Calculations show that 1 MMLD corresponds to between 1.2 and 1.3 LD50.

Animal experiments

The toxin concentrations in the cat liver suspensions and broth culture were determined by inoculating 0.2 ml of serial dilutions (1:1000, 1:2000, 1:3000, 1:4000 etc.) in saline intraperitoneally into white mice (NMRI/BOM) weighing approx. 20 g. Each group comprised 4 mice. The highest dilution that killed all the mice in one group was defined as containing 1 minimum mouse lethal dose per 0.2 ml (1 MMLD) (*Davis et al.*). For the detection of toxin in samples (serum and liver) from experimental animals, doses of 1 ml were inoculated into mice. To investigate whether serum from goats had any protective effect against botulinum toxin, samples of 1.5 ml serum were inoculated subcutaneously into mice, and 18 hrs. later they received 1.5 MMLD toxin type C_β intraperitoneally.

Eleven goats (Norwegian breed), weighing from 23 kg to 56 kg, were used in the toxicological experiments. Some of the animals had been grazing or fed ensilage prior to the experiment, and some were also fed ensilage and concentrated feed during the experimental period, while others were fed hay and concentrated feed. The type of feed mentioned in Table 1 was used for at least 14 days prior to the challenge with toxin. The toxin-containing material was diluted in saline and given orally using a rubber stomach-tube. When an animal showed symptoms of botulism after administration of toxin, it was allowed to recover

Table 1. Botulinum toxin

Goat	Weight	Number of minimum mouse lethal doses (MMLD)																			
	(kg)	0	1	2	3	4	6	14	21	22	23	24	25	26	27	28	29	36	43	44	47	50
I ♀	40	0.5																				
II ♀	46	1.3																				
III ♀	35	0.9								0.9												
IV ♀	56	1														1						
V ♂	38	0.5							0.5									0.5	0.5		0.5	0.5
VI ♀	42	0.9						0.9														
VII ♀	31	0.9																				
VIII ♀	27.8	0.5						0.5								0.5						
IX ♀	37.5	0.08	0.08	0.08	0.08	0.08				0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	← 0.1 each day for				
X ♂	24.2	0.5					0.9															
XI ♂	23	0.5					0.9															

* \+ weak symptoms (depressed, reduced appetite, some difficulties in chewing hay, lowered tonus in the tongue and some salivation).

\+ \+ more pronounced symptoms (able to stand on feet and to take some concentrated fodder).

\+ \+ \+ still more pronounced symptoms (unable to stand on feet but rested on the chest, could take some concentrated fodder).

\+ \+ \+ \+ symptoms inevitably leading to death (lying on the side, not able to eat, drink or stand on the feet).

** recovered after 5 weeks.

*** died from mastitis.

ype C_β given orally to goats.

per g body weight of goat given on day:																Symptoms of botulism on day	Severity of symptoms*	Died on day	Kind of feed prior to challenge
52	54	67	68	72	74	83	91	92	93	100	103	110	111	140	223				
																3	+++	**	hay
																2	++++	3	hay
																23	++++	24	pasture hay
																3	++		ensilage
																32	+	44***	hay
0.5	1		0.9	1.4												75	++		pasture hay
						0.8	0.8	0.8	0.8							96	++		hay
											0.8	2.5		3	6	225	++++	226	hay
																16	++++	18	pasture hay
																2	++++	2	ensilage
																30	++++	31	ensilage hay
		24 days→ 0.8								1.6			3.2			30	+(+)		hay
																113	++++	113	hay
																9	++++	11	ensilage
																8	++		hay

completely (as judged by clinical observations) before another dose was given.

Samples from experimental animals

Blood samples were collected from some of the animals when they developed symptoms of botulism and occasionally when they were free of symptoms. Specimens of liver were removed as soon as possible after death, and a new specimen of the liver was taken after the carcass, with the liver in situ, had been stored at approx. 10°C for about 20 hrs. Five g of the liver specimen was inoculated into 400 ml Robertson's medium containing 0.2 % glucose and, after heating at 60°C for 1 hr., these cultures were treated in the same way as that described for toxin production. Another part of the liver specimens was ground in a mortar together with small amounts of saline and filtered through gauze.

RESULTS

Clinical observations

Symptoms of illness usually occurred on the second or third day after administration of the toxin, and death 1—2 days later. One of the first symptoms noticed was a change in the voice to a "hoarser" quality. Other symptoms that developed gradually were loss of appetite, difficulties in chewing (especially hay), some salivation and unwillingness to stand on the feet. When the animals were forced to stand, the muscles of the limbs trembled, and the position was rigid with a slight upward curve of the back. They moved slowly and the hindquarters especially were stiff. When the animals were left alone, they lay down quickly. Some showed increased abdominal respiration, and some also seemed to be hypersensitive to light as they tended to hide the head in the darkest corner of the room.

Toxicological investigations

The results of the feeding experiments are shown in Table 1. Doses of 0.5 MMLD per g body weight of goat were sufficient to cause severe symptoms of botulism (animal I) and even death (animal VIII). A dose of 0.9 MMLD per g caused death in 1 goat fed ensilage (animal VII), but in other cases this dose was not sufficient to even cause symptoms of illness when the animals were grazing or fed ensilage prior to the challenge (animals III,

Table 2. Investigation of blood samples collected at various intervals after challenge.

Goat	Blood samples collected on day	Number of minimum mouse lethal doses (MMLD) per ml serum		Protective effect of serum against botulinum toxin type C_β in mouse
I	9	0		none
II	3	0		none
III	1	0		none
IV	0*	—**		none
V	53	0		none
	82	0		none
	102	0	***	none
	132	—		none
	144	0		none
	226	0		none
VI	18	0		none
IX	73	0		none
	110	0		none

* collected prior to challenge.
** — not investigated.
*** the mice injected did not show even symptoms of botulism.

Table 3. Toxicological investigations of liver from animals dying of botulism.

Goat	Number of minimum mouse lethal doses (MMLD) per ml inoculated material demonstrated in			
	specimen of liver taken soon after death		specimen of liver remained in situ for 20 hrs.	
	directly from liver	from liver specimen incubated in Robertson's medium at 37°C for 5 days	directly from liver	from liver specimen incubated in Robertson's medium at 37°C for 5 days
II	0	0	—*	—
III	0	0	400	25,000
IV	0	—	1	50,000
V	—	0	—	0
VI	0	0	—	0
VII	—	—	0	0
VIII	0	0	0	0
IX	0	0	0	0
X	0	0	0	0

* — not investigated.

VI). In these latter cases, however, the same dose caused death when repeated 2—3 weeks later, after the animals had received hay and concentrated fodder in the interval between challenges.

When several small doses were given at short intervals, the smallest dose giving symptoms of botulism was a total dose of 0.8 MMLD fed in the course of 8 days (animal IX). Animals fed several small doses could later tolerate doses which usually elicited symptoms of botulism, or even death (animals V and IX). One animal tolerated as much as 3 MMLD per g body weight.

Tables 2 and 3 show the results of the toxicological investigations of samples from experimental animals. None of the blood samples collected at various intervals after the challenge was found to contain enough toxin to be demonstrated by inoculating mice. Table 2 also shows that none of the samples of serum investigated gave demonstrable protection against botulinum toxin type C_β in mice. In the liver specimens, botulinum toxin was demonstrated in 2 cases in which the liver remained in situ for 20 hrs. after death, but not in samples taken immediately after death.

DISCUSSION

The toxin used was prepared in 3 batches and then stored at —20°C in small amounts so that each dose given to experimental animals was thawed only once. These conditions, in addition to the titration of toxin made in mice, ensure that the different doses of toxin given during the experiments are comparable with each other.

The feeding experiments show that doses as small as 0.5 MMLD botulinum toxin type C_β per g body weight of goat may elicit symptoms of botulism and even death, and, in most cases, less than 1 MMLD per g was sufficient to cause death in the animals. Although some differences in the individual tolerances to the toxin have to be taken into account, doses of nearly 1 MMLD per g body weight have to be considered lethal to goats fed hay. As can be seen from the experiments the kind of feed the animal received prior to the challenge seems to be of some importance for the amount of toxin tolerated. In several cases a certain amount of toxin did not elicit any symptoms when the animals were grazing or fed ensilage, but when the feed was changed to hay and concentrated fodder and the same dose repeated 2—3 weeks after the first challenge, symptoms of botulism

occurred and death followed. In 1 experiment (animals X and XI), 1 goat fed hay survived from a dose of toxin which caused the death of another goat fed ensilage. However, the animal receiving ensilage showed symptoms of illness on the third day after the challenge of toxin, while the animal receiving hay showed symptoms already on the second day after the challenge, just like the experimental goats which died from botulism. The survival of this goat may possibly be due to a high individual tolerance to the toxin.

As to the effects of the toxin dose given 2—3 weeks after the first challenge, also other causes than the change in the feed have to be considered. A cumulative effect can not be excluded as we do not know, how long the botulinum toxin can remain in the body of ruminants, before it is inactivated or excreted. Another possibility could be a sensitizing effect (*Matveev* 1959), but 2 feeding experiments (animals V and IX) seem to indicate that such a sensitizing effect had not taken place in the goats. On the contrary, animals which received many small doses during a period of time later on tolerated considerably higher doses than those needed to cause death in inexperienced animals. One of these animals tolerated a dose approx. 3 times higher than a dose usually causing death. The theory of *Matveev,* that after ingestion of a sublethal dose of botulinum toxin, small doses of toxin produced by the botulinum organism in the body can cause symptoms of botulism, does not seem to be valid as far as goats are concerned, as in the feeding experiments all the toxin doses given contained viable organisms of Cl. botulinum type C_β. The probable protective effect of green forage is difficult to explain, but the change of microflora and other conditions in the rumen caused by the change in feed may be of some importance. This, however, needs further investigation.

The kind of resistance that seems to develop after repeated small doses of toxin has not been thoroughly investigated. Serum from such animals gave no protection when inoculated into mice which were later on challenged to toxin. The amount of possible circulating antibodies in the ruminants, however, may be too low to give a demonstrable protective effect in diagnostic animals, but high enough to give the ruminants some protection.

A cumulative effect of many small doses of toxin seems to take place (animals V and IX), if the doses are given within a certain period of time e.g. each day in the course of 8 days, but

as shown in the experiments the total dose was higher than the single dose causing more severe symptoms in other experimental animals. A cumulative effect has been demonstrated in mice and mink by *Loftsgård et al.* (1970). This cumulative effect may be of some importance in cases in which the concentration of toxin in the feed is low and the animals receive this feed for several days.

Botulinum toxin could not be demonstrated in serum from any of the experimental animals that died, not even from the one that was given doses of 3 and 6 MMLD per g body weight. As stated previously, less than 1 MMLD per g body weight can cause death in goats, and if an even distribution of toxin in the body is assumed, one would expect to find less than 1 MMLD per ml serum and possibly considerably lower concentrations as the blood samples are usually taken at the time when symptoms occur i.e. 2—3 days after the ingestion of toxin. One ml serum is usually the maximum amount which can be inoculated intraperitoneally into a mouse weighing approx. 20 g. The calculations and the results of the investigations indicate that one can not expect to demonstrate toxin in serum from ruminants suffering from botulism caused by type C_β when mice are used as test animals. If, however, the toxin dose ingested is very high, which may be the case in some spontaneous outbreaks, the toxin may possibly be demonstrated in this way.

The results of the investigations of liver from animals which had died from botulism show that of 9 cases, toxin could be demonstrated in only 2, and in both of these only after the liver had remained in the carcass for 20 hrs. after death. This indicates that the toxin demonstrated is formed in the liver post mortem. Some toxin formation may possibly occur during prolonged agony. *Müller* (1967) assumed that the toxin demonstrated in liver probably developed during the last phase of the disease or even post mortem. The present investigations show that the most reliable method for demonstrating toxin in the liver is to let the liver remain in situ for at least 20 hrs. and then incubate a piece of the organ in Robertson's medium. Even with such a procedure toxin seems to be seldom demonstrated. If toxin is demonstrated in this way, however, one can not be sure that this toxin originates from bacteria ingested together with the botulinum toxin. Cl. botulinum type C is not uncommon in nature (*Prévot* 1966) and may be a normal inhabitant of the intestines of ruminants,

and, in animals suffering from some diseases for a time one might expect bacteria to migrate into the liver during agony or post mortem. *Müller* (1967) investigated the liver from cattle in a slaughter-house, and after incubating a piece of liver in broth he found 4 % of the specimens to contain botulinum toxin type C_β. The toxicological investigation of liver from ruminants suspected of botulism seems, therefore, to be of questionable diagnostic value.

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SAMMENDRAG

Virkninger av Clostridium botulinum toxin type C_β gitt pr. os til geit.

Elleve geiter ble brukt til forsøk med Clostridium botulinumtoksin type C_β. Toksinet, som stammet delvis fra leveren i et kattekadaver og delvis fra buljong inokulert med Cl. botulinum type C_β isolert fra samme lever, ble gitt med magesonde. Symptomer på botulisme opptrådte vanligvis etter 2—3 dager. Så små doser som 0,5 MMLD (minimum muselethaldose) pr. gram kroppsvekt geit kunne gi dødelig utgang, og 1 MMLD pr. gram ble ansett å være dødelig for geit som ble fôret på høy og kraftfôr. Hvis dyrene gikk på beite eller fikk silofôr, syntes de å kunne tåle noe høyere doser. En kumulerende effekt viste seg å finne sted når toksindosene ble gitt innen et tidsrom av 8 dager. En sensibiliserende effekt av mange små doser kunne ikke påvises. Det viste seg tvert i mot at dyr som hadde fått mange små doser, senere kunne tolerere toksindoser som var dødelig for andre geiter. En av disse geitene tålte en dose som var ca. 3 ganger høyere enn en dose som vanligvis var dødelig. Serum fra slike geiter ga imidlertid ingen påvisbar beskyttelse hos mus som senere ble sprøytet med botulinumtoksin type C_β. Toksin kunne ikke påvises i serum fra geiter som viste symptomer på botulisme. Hos 2 av 9 geiter kunne toksin påvises i leveren, men bare etter at leveren hadde ligget in situ i ca. 20 timer etter døden.

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