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Factors affecting the toxicity of rotting carcasses containing *Clostridium botulinum* type C

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SUMMARY

Mice killed shortly after receiving 1300–3000 spores of *Clostridium botulinum* type C *per os* were incubated at one of four chosen temperatures together with bottles of cooked meat medium seeded with a similar inoculum. After incubation the rotting carcasses were homogenized. Sterile membrane filtrates of the homogenates (10–20.8%, w/v) and pure cultures were then titrated for toxicity. A temperature of 37 °C produced less toxicity in most carcasses than in cultures. At 30 °C, however, toxicity (often 2×10^5 to 2×10^6 mouse intraperitoneal LD/g or ml) was roughly similar in both systems, and some carcasses and cultures were still toxic (2×10^4 to 2×10^5 LD/g or ml) after 349 days. Surprisingly, at 23 °C, though greatly reduced in the cultures, toxicity was high in many carcasses for at least 28 days. Little or no toxin was produced in either system at 16 °C. Unfiltered homogenates (17·8–22·5%, w/v; dose 0·25 ml *per os*) of toxic carcasses incubated at 30 °C for 7 days invariably produced death from botulism, often within as little as 4 h, but a 1 in 10 dilution produced less than 100% mortality.

INTRODUCTION

Of the seven immunological types of *Clostridium botulinum* two (C and D) are notorious for producing botulism in animals but, for reasons that are not understood, rarely if ever affect man. Human botulism, although well known, is fortunately uncommon. The disease occurs much more frequently in animals, however, largely because they often relish spoiled foodstuffs that would be repugnant to man.

Rotting carcasses represent one of the commonest sources of toxin for animals. Avian botulism sometimes occurs through the intermediary of sarcophagous maggots. Toxic carrion must inevitably contain living *C. botulinum*; death from the ingestion of such material will, in the absence of safe carcass disposal and presence of a suitable ambient temperature, be followed by a predictable chain of events – putrefaction, post-mortem invasion of the body tissues by *C. botulinum* from the gut, and the production of further lethal quantities of toxin. A similar chain of events may occur when animals die from causes other than botulism in areas where type C and D spores are prevalent in the environment. In South Africa where, before the introduction of vaccination, *ca* 100000 cattle died annually from

carrion-transmitted botulism, virtually every piece of rotting flesh in some districts was said to become toxic (Sterne & Wenzel, 1952).

Despite its importance in the causation of animal botulism, toxigenesis in putrefying carcasses has attracted little study other than that of Notermans, Dufrenne & Kosaki (1980). They found toxicity not exceeding 5×10^4 mouse intraperitoneal LD50/g in the tissues of Pekin ducks dosed orally with *C. botulinum* spores (type B, C or E) and then killed and incubated at 25 °C for 3–4 days. There is no information, however, on the factors that influence the concentration of toxin produced in the mixed microbial environment of putrefying tissue.

The purpose of this study was to gain information on the effects of different temperatures on the production of type C toxicity in rotting mouse carcasses and in pure broth cultures over different periods of time.

MATERIALS AND METHODS

C. botulinum *spore suspension*

The type C strain no. FH6513 (see Graham, 1978) was preserved at −20 °C in sealed glass tubes containing small volumes of the liquid phase of a sporulated culture in Cooked Meat Medium (Difco). On plating, this particular culture yielded colonies of which 60–70% were capable of giving rise to toxic subcultures, the remainder being non-toxigenic due presumably to spontaneous loss of bacteriophage as reported by Inoue & Iida (1970) and Eklund & Poysky (1974).

Preparation of toxic mouse homogenates

By means of a 1 ml syringe and curved Animal Feeding Needle (18-gauge, 2 inch; Harvard Apparatus Ltd, Edenbridge, Kent) mice were given *per os* a dose of 1300–3000 spores (dose volume 0·25 ml) prepared from frozen spore suspension by diluting appropriately in distilled water. The mice were then killed within 10 min by cervical dislocation and individually wrapped in three sealed plastic bags before being incubated at one of several chosen temperatures. Anaerobic jars were not used. After incubation each putrefying carcass was weighed, cut into about five pieces and homogenized in 100 ml of gelatin phosphate buffer (Bowmer, 1963) to make a 11·2–20·8% (w/v) suspension; or, in later experiments, in sufficient to make a 10% (w/v) suspension. The homogenization was effected by four 15 s top-speed bursts of an Atomix Emulsifier (MSE Scientific Instruments, Crawley, West Sussex). The material was then passed through a Whatman No. 4 filter paper, partly cleared by centrifugation for 10 min at 3000 rpm, sterilized by means of a Millex-HA Filter Unit (Millipore UK Ltd, Harrow, Middlesex), and designated 'filtered mouse homogenate' (FMH); or merely passed through muslin and designated 'unfiltered mouse homogenate' (UMH). In interpreting the experiments it must be borne in mind that the 'lethality' of UMH, as shown by the intraperitoneal injection of doubling dilutions into mice, was 2–8 (mean 4) times greater than that of FMH. This was probably due to incomplete release of toxin from the tissues by homogenization, but some contribution to lethality by living *C. botulinum* organisms cannot be ruled out.

Preparation of toxic pure cultures for comparison with mouse homogenates

In each experiment, McCartney bottles (25 ml) containing about 22 ml of boiled

and cooled cooked meat medium were seeded with an inoculum identical with that administered to the mice and incubated alongside the mouse carcasses. After incubation each bottle was inverted several times and allowed to settle. The supernate was then removed, partly cleared by centrifugation for 10 min at 3000 rpm, sterilized by filtration (as above) and designated 'filtered culture' (FC); or removed and, without further treatment, designated 'unfiltered culture' (UC). Filtration of liquid culture reduced its mouse intraperitoneal toxicity by a factor of only 1–2 (mean 1·6). In this respect, culture differed strikingly from mouse homogenate (above).

Toxin assay

Toxic preparations derived from cultures of putrefying carcasses were usually examined immediately, but occasionally they were first stored at −20 °C – a procedure shown to have no appreciable effect on the results. The preparations were titrated by injecting decimal dilutions made in gelatin phosphate buffer into single mice intraperitoneally in volumes of 0·5 ml. The results, which were always clear-cut, were based on death from botulism within 4 days and expressed as lethal doses (LD) per g or ml. Concentrations of <200 LD/g or ml may have been due merely to the toxicity of the spore inocula. On a number of occasions throughout the study the rotted tissues of untreated control mice were examined for toxin, invariably with negative results. When administered *per os*, toxic preparations were given in dose volumes of 0·25 ml by the method already described; mice similarly treated with non-toxic control inocula were apparently unaffected by the procedure. Deaths produced by intraperitoneal or oral inoculation could be readily prevented by the administration of *C. botulinum* type C antitoxin.

RESULTS

Toxicity of mouse carcasses and pure cultures incubated at 30 °C

Toxicity after incubation for different periods

Fig. 1 shows the concentrations of toxin in filtrates (FMH and FC) prepared from putrefying mouse carcasses and broth cultures incubated at 30 °C for 1, 4, 7, 14 and 21 days. Apart from the failure of the occasional carcass to produce a detectable increase in the small amount of toxin administered with the spore inoculum, the production of toxin in rotting cadavers was comparable with that in pure cultures. The results suggested (days 1 and 4) that the initiation of toxigenesis was slightly earlier in the cultures, but that at day 7 carcasses were at least as toxic as cultures if not more so. Later (day 21), there appeared to be a tendency for toxin in putrefying mice to decline.

After incubation for 131 days carcasses were in general less toxic than cultures (Table 1). After 257 and 349 days, however, cultures and carcasses resembled each other in that some had lost much or all of their toxicity while others were still decidedly toxic.

Administration of filtrates of mouse homogenates and cultures per os

Mice in groups of six were dosed *per os* with the toxic filtrates (30 FMHs and 25 FCs) already titrated intraperitoneally (see Fig. 1). As is seen in Table 2,

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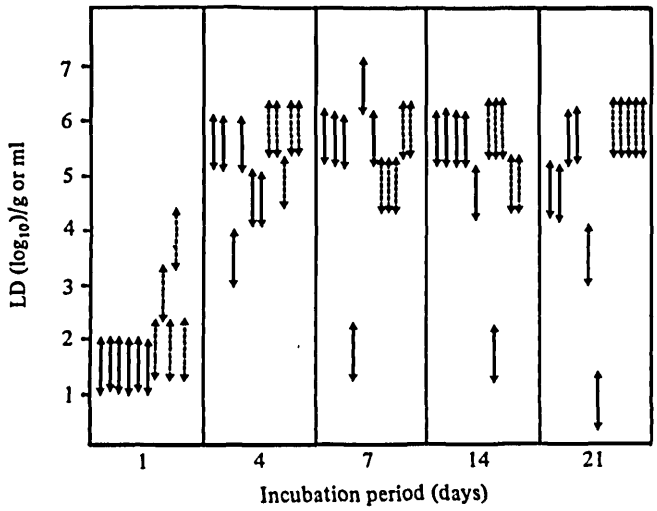

Fig. 1. The toxicity of mouse carcasses and broth cultures seeded with *C. botulinum* and incubated at 30 °C for different periods. Toxicity is expressed as mouse intraperitoneal LD/g or ml. Each solid vertical line represents a mouse carcass and shows the range within which its toxicity fell. Each interrupted vertical line represents a cooked meat broth culture.

Table 1. *Toxicity of mouse carcasses and cultures inoculated with* C. botulinum *and incubated at 30 °C for protracted periods*

No. of days incubation at 30 °C	Specimen (n =)	Number of carcasses or cultures showing the stated LD (log 10) of toxin per g or ml respectively				
		< 2·3	2·3–3·3	3·3–4·3	4·3–5·3	5·3–6·3
131	M (6)	—	—	—	5	1
	C (4)	—	—	—	1	3
257	M (5)	—	1	1	3	—
	C (4)	1	—	1	—	2
349	M (5)	1	2	—	2	—
	C (3)	2	—	—	1	—

M, Mouse carcass; C, culture.

Table 2. *Administration of filtrates of mouse homogenates and cultures* per os

Inocula	Dilution of preparations inoculated was 1 in	Total of mice that died after being inoculated in groups of 6 with individual FMHs or FCs prepared after incubation for (days)				
		1	4	7	14	21
FMHS*	1	1/36	4/36	11/36	4/36	5/36
FCs	1	1/30	30/30	30/30	29/30	30/30
	10	ND	9/30	7/30	21/24	18/30

* These preparations represented 11·2–20·7 % (w/v) dilutions of the original mouse carcasses; and, in addition, filtration reduced the lethality of the mouse homogenates much more than that of the cultures (see Materials and Methods). ND, Not done.

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Table 3. *Toxicity of mouse carcasses and cultures inoculated with* C. botulinum *and incubated at 37 °, 23 ° and 16 °C*

Incubation			Number of carcasses or cultures showing the stated LD (log 10) of toxin per g or ml respectively				
Temperature	Duration (days)	Specimen (n =)	< 2·3*	2·3–3·3	3·3–4·3	4·3–5·3	5·3–6·3
37 °C	7	M (18)	1	3	3	4	7
		C (18)	—	—	—	3	15
	14	M (6)	2	—	1	3	—
		C (6)	—	—	—	3	3
	19	M (6)†	—	—	2	2	1
		C (6)	—	—	—	1	5
	21	M (6)	1	—	2	2	1
		C (6)	—	—	—	1	5
23 °C	7	M (6)	—	—	—	—	6
		C (6)	—	2	4	—	—
	14	M (6)	—	—	—	1	5
		C (6)	—	—	5	1	—
	19	M (6)‡	—	—	—	2	3
		C (6)	—	—	1	5	—
	28	M (6)	2	—	—	—	4
		C (6)	—	—	1	5	—
16 °C	7	M (6)	6	—	—	—	—
		C (6)	6	—	—	—	—
	14	M (10)	10	—	—	—	—
		C (12)	10	1	1	—	—
	28	M (9)	9	—	—	—	—
		C (12)	10	—	2	—	—
	42	M (6)	5	1	—	—	—
		C (6)	6	—	—	—	—

M, mouse carcass; C, culture.
* Toxin at this low concentration may merely represent the toxicity of the spore inoculum.
† One mouse gave the result < 3·3. ‡ One mouse gave the result < 4·3.

undiluted FCs differed from FMHs in producing many more deaths, but this was probably due to the factors mentioned in the Table footnote. It is apparent that when mice were dosed with FCs diluted as little as 1 in 10 mortality was much reduced.

The high oral toxicity of unfiltered mouse homogenate

Because of the comparatively low oral toxicity of FMH, both UMH and FMH (17·8–20·2 %, w/v) were prepared from each of five toxic carcasses rotted for 7 days at 30 °C. The 10 preparations were each given *per os* to six mice. Each UMH produced 100 % mortality from botulism within 20 h, many of the deaths occurring in <4 h. The FMHs produced mortality ranging only from 33 to 67 %.

In a further experiment a single UMH (22·5 %, w/v), also produced from a carcass incubated for 7 days at 30 °C, was given to three groups of six mice in dilutions of 1 in 1, 10 and 100; mortality was 100 %, 50 % and 33 % respectively, showing that the high oral toxicity of UMH was rapidly reduced by moderate dilution.

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Toxigenesis in rotting mouse carcasses and pure cultures at 37, 23 and 16 °C

Table 3 shows the concentrations of toxin in filtrates (FMH and FC) prepared from putrefying carcasses and liquid cultures incubated at temperature other than 30 °C for periods ranging from 1–6 weeks.

In contrast to the temperature of 30 °C (see above), 37 °C was distinctly less favourable for the production of toxicity in rotting carcasses than in cultures. For examples, 78% of cultures generated toxin at a concentration of 2×10^5 to 2×10^6 LD/ml whereas 75% of carcasses produced less – often much less – than 2×10^5 LD/g.

At 23 °C, however, this state of affairs was reversed, most carcasses containing high concentrations of toxin – comparable with those produced at 30 °C (Fig. 1) – but pure cultures containing considerably less.

The temperature of 16 °C was apparently unsuitable for toxin production except in occasional cultures or carcasses in which small amounts were formed after a few weeks.

DISCUSSION

Monkeys are susceptible experimentally by any route to sufficient doses of *C. botulinum* type C toxin (Lamanna, 1970) and natural outbreaks in New World monkeys and gibbons have been described (Smart *et al.* 1980; Smith *et al.* 1985). It seems paradoxical therefore that type C botulism, which is well known in many animal species, occurs rarely if ever in man. There are many possible explanations, one being that in nature high concentrations of type C toxin are produced mainly in putrescent material, acceptable to animals as food but repellant to man. This notion, together with the obvious lack of research on the important topic of toxic carrion as a source of botulism in animals, led to the present study.

The net toxicity of a rotting carcass containing *C. botulinum* depends on both the generation and destruction of toxin. These in turn are likely to be influenced one way or the other by the mixed microflora of carrion. Two variables – temperature and duration of incubation – were studied for their effect on the toxicity of mouse carcasses, comparisons being made with the toxicity of pure cultures in cooked meat medium.

A temperature of 37 °C was distinctly less favourable for toxigenesis in carcasses than in cultures (Table 3). At 30 °C, toxicity was roughly similar in both systems for up to 14 days' incubation, but there may have been some loss of toxicity in the carcasses at 21 days (Fig. 1). Some carcasses and cultures retained considerable toxicity for up to 349 days at 30 °C, but others appeared to lose much or all of it (Table 1). The results at 23 °C were surprising: toxicity was greatly reduced in the cultures but remained high in most carcasses for at least 28 days (Table 3). Little or no toxin was produced at 16 °C in either system, even after incubation for 42 days.

In carcasses and cultures the highest titres recorded fell within the range 10^5–2×10^6 mouse intraperitoneal LD/g or ml, except for one carcass that, after incubation at 30 °C for 7 days, contained between $1{\cdot}2 \times 10^6$ and $1{\cdot}2 \times 10^7$ LD/g. Haagsma, cited by Notermans, Dufrenne & Kozaki (1980), found up to 10^7 LD of type C toxin per g in putrefying waterfowl cadavers. It is conceivable that the carcasses of different species vary in their ability to generate toxin.

Botulism in wild and domesticated animals, though well known in temperate climates, is rarely recorded in the tropics – a matter that invites investigation. Obvious possible explanations include failure to report or diagnose the disease in tropical regions. It is conceivable however that, as in our experiments, the production of toxic carrion is favoured less by the temperatures encountered in the more extreme tropical climates than by those of a warm English summer.

The LD50 of a broth culture filtrate of the type C strain FH6513 was demonstrated previously to be 10^3–10^4 times greater orally than intraperitoneally (Smith, 1986). Table 2 shows that broth culture filtrate, although producing virtually 100% mortality in mice given 0·25 ml *per os*, lost much of its lethality when diluted only 1 in 10. The mortality produced by oral inoculation of 0·25 ml of filtrates of toxic mouse homogenates was always below 100%. This was probably because (1) inevitably, the homogenates represented dilutions (11·2–20·7%, w/v) of the original carcasses, and (2) the membrane filters reduced the lethality of the mouse homogenates much more than that of the cultures. When unfiltered mouse homogenate (17·8–22·5%, w/v) was used, not only was the mortality invariably 100% but many of the deaths occurred in less than 4 h. The presence of viable *C. botulinum* probably played little or no part in the unfiltered homogenates' striking lethality, though the possibility cannot be entirely excluded. The results leave no doubt, however, as to the dramatic effect that would have been observed had each mouse ingested 0·25 g of original rotting carcass.

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