PATHOGENESIS OF *CLOSTRIDIUM BOTULINUM: IN VIVO* FATE OF *C. BOTULINUM* TYPE A SPORES

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ABSTRACT.—C. botulinum Type A spores contain sufficient toxin to produce fatal botulism in experimental animals. After 4 hours, i.p. injected spores undergo conversion from a heat-resistant spore to a heat-sensitive cell, probably a germinated spore or vegetative cell. This conversion appears to be prerequisite for liberation of spore bound toxin, since no botulinal toxin was noted before loss of heat-resistance. Within 0.5 minutes after i.p. injection, spores enter the bloodstream, liver, spleen and kidneys. The process of spore clearance and germination appear to be retarded by Type A antitoxin binding to spores rendering them non-phagocytizable. A significant number of viable heat-sensitive cells of *C. botulinum* are found in peritoneal exudates at later stages of survival in both antitoxin-protected and non-protected animals, indicating the capability of *in vivo* spore germination in the peritoneal cavity. No significant numbers of vegetative cells are found in liver, kidneys, or spleen of antitoxin protected mice. This implies that *C. botulinum* spores are trapped by the splanchnic mechanism, and may not be able to germinate in these organs.

Early works (Orr, 1922) on botulism pathology report *C. botulinum* spore dissemination into various tissues and production of toxin after oral challenge. Coleman and Meyer (1922) extended this work in demonstrating invasion of all organs of the body regardless of the mode of administration of spores. They were first to implicate toxin production *in vivo* from toxin-free spores.

Following intramuscular (i.m.) challenge, Keppie (1951) found clusters of heterophil leukocytes gathered at the site of injection which resulted in engulfment of spores and transportation away from the original site of injection. *In vivo* spore germination was rejected on the theorectical basis that spores could not germinate in the presence of oxygen. Keppie (1951) concluded that the toxin was released from spores by phagocytic digestion.

Delayed infections by latent spores of *C. botulinum* has been observed in clinical cases. Assuming no recurrent exposure to the toxin, these cases may have resulted from the ingestion of foods heated sufficiently to destroy toxin, but not heat-resistant spores. Therefore, ingestion of *C. botulinum* spores as an etiological agent in botulism food poisoning must be considered.

Two possibilities have been offered (Grecz and Lin, 1967) to explain spore toxicity: 1. *in vivo* spore germination and subsequent release of toxin from vegetative cells, and 2. degradation of the spore releasing its bound toxin.

The purpose of this paper is to demonstrate that *C. botulinum* spore do, in fact, germinate *in vivo*, and to define *in vivo* loci of mice which are conducive to this germination.

Materials and Methods

Culture methods: Clostridium botulinum Type A strain 33A was obtained from Dr. W. E. Perkins, National Canners Association, Berkeley, California. The culture was grown at 30 C in 5% Trypticase (BBL), 0.5% peptone (Difco) and 0.1% sodium thioglycolate. Within 6 days, abundant sporulation had occurred, at which time, the spores were harvested in a refrigerated Sorvall RC-2 continuous centrifugation system and cleaned with trypsin and lysozyme by method of Grecz, *et al.* (1962). The spores were washed twice, resuspended in 0.87% NaCl and stored at 4 C until used.

Heat-shocking procedure: The spore inoculum was heated in a screw-cap tube for 15 minutes at 80 C denaturing any free botulinal toxin in the medium. *C. botulinum* spores are able to survive this treatment.

Colony counts: The number of viable organisms of *C. botulinum* were determined by subculture in Wynne's broth (Wynne, *et al.*, 1955) plus 0.75% agar. One ml portions of serial dilutions were transferred to oval flat tubes and melted sterile Wynne's agar was added. To achieve anaerobiosis, an additional layer (2 cm.) of sterile Wynne's agar was poured. The tubes were plugged with foam rubber stoppers and incubated at 30 C. Colonies were counted after 96 hours.

Mice: White Swiss mice raised for 10 generations in a closed colony at the Illinois Institute of Technology were utilized in all experiments. They were fed and watered *ad libitum,* and attained a weight of 25 grams before experimentation.

Injections: Intraperitoneal (i.p.) injections into mice were made with 26 gauge, 2.5 ml disposable syringes.

Preparation of Exudates for Viability Analysis: Mice were injected i.p. with 2 x 10^8 spores. At selected time intervals, the peritoneal cavity was washed with 1 ml of sterile 0.87% saline, and exudates withdraw using a 26 gauge syringe. The peritoneal exudate was diluted 1:10 with sterile distilled water. Serial dilutions in distilled water for viable cell count in Wynne's agar were performed as described above.

Preparation of Tissues for Viability Analysis: Following removal of peritoneal exudates from mice, the animals were etherized and the peritoneal cavity surgically opened. The body cavity was rinsed with sterile saline, and the liver, kidneys and spleen removed.

The organs were placed in sterile plastic centrifuge tubes and washed with sterile saline by agitation on a vortex mixer. Washing was continued until the saline supernates became clear. Each organ, regardless of volume, was placed in 20 ml of sterile saline in a second sterile plastic centrifuge tube, and sonicated (Branson Sonifier, Model S-125, 8 amps) for 2-4 minutes to rupture the cells and release all spores.

The tubes were centrifuged (300 x g, 1 hour), and the supernatant discarded. Appropriate decimal dilutions of the pellet were analyzed for the number of viable spores in Wynne's agar.

Examination of Blood for Viable Spores and Vegetative Cells: Blood was removed from the mouse by two methods: (i) the animal was etherized and the abdominal and pleural

cavities were surgically opened using sterile techniques. A 26 gauge, 2.5 ml syringe was inserted into the heart and the blood was withdrawn; (ii) a direct cardiac puncture was made into a mouse slightly stunned by ether. Decimal dilutions of the blood were made and examined for the number of viable organisms in Wynne's agar.

Results

Peritoneal Cavity: After i.p. injection of 2 x 10^8 heat-shocked spores of *C. botulinum* Type A, only 6.6 x 10^5 heat-resistant spores could be recovered from the peritoneal cavity at the start of the experiment (Table 1). This apparent 300 fold reduction in number of spores was due to: (i) dilution of spores by body fluids; or (ii) rapid distribution of the spores throughout the animal body.

The first line of Table 1 shows that the intraperitoneal cavity of control mice receiving no spore inoculum contained some spores (6.0 x 10^2) as well as some heat-sensitive cells (4.4 x 10^3). This relatively low level of contamination was considered as "background" flora. Examination of Table 1 showed that the number of spores recovered from the animal body after initial i.p. injection gradually but consistently declined so that by 48 hours the number of spores recovered was approximately 100 fold lower than the initial level. Furthermore, at 48 hours, the number of spores recovered was only slightly higher than "background" spore load in control mice.

Incubation beyond 3 hours rapidly increased the number of heat-

Table 1.—Number of viable spores recovered from the peritoneal cavity of mice after intraperitoneal injection of 2 x 10^8 heat-shocked spores of *Clostridium botulinum* 33A.

Time After Intraperitoneal Injection Hours	Number of Animals Challenged	Number of Viable Organisms Recovered	
		Heat-Shocked (Spores)	Heat Sensitive Cells (Total Minus Spores)
Control[a]	3	6.0 x 10^2	4.4 x 10^3
0	3	6.6 x 10^5	4 x 10^3
1	2	5.0 x 10^5	4 x 10^4
2	3	1.0 x 10^5	1.0 x 10^3
3	3	2.0 x 10^5	5 x 10^4
4	3	1.0 x 10^5	2 x 10^5
8	3	2.0 x 10^4	1.5 x 10^5
24	3	2.0 x 10^4	1.8 x 10^5
		4.0 x 10^4	5.0 x 10^4
36	1	2.0 x 10^4	4.3 x 10^5
	1	1.0 x 10^4	1.0 x 10^4
	1	5.0 x 10^2	2.5 x 10^3
	1	1.0 x 10^3	3.0 x 10^3
48[b]	2	7.0 x 10^3	5.0 x 10^5
	1	1.0 x 10^3	2.0 x 10^5
	1	3.0 x 10^3	1.0 x 10^5
	1	2.0 x 10^3	5.0 x 10^3

[a]/Control mice received 1 ml sterile saline but no spores.
[b]/Recovered from surviving animals; only 10% of the injected animals survived 48 hrs.

sensitive cells and reached a plateau of approximately 2 x 10^5 cells which seemed to be relatively stable between 8 to 48 hours (the upper limit of this experiment). Within the precision of these experiments, the number of heat-sensitive (germinated) cells was essentially equivalent to the initial number of spores which could be recovered from the peritoneal cavity of the mouse during the first 8 hours. Thus, these results suggest that after injection into the mouse, heat-resistant spores were essentially all converted into heat-sensitive (probably germinated) forms within 4-8 hours. The heat sensitive forms appeared not to be destroyed to a detectable degree in the mouse body for up to 48 hours.

The cells recovered from the peritoneal cavity of injected mice were shown to produce specific Type A toxin as determined by mouse toxicity tests of subculture. Control mice in which the animals were challenged with 1 ml of sterile saline without spores contained low levels of viable cells. The organisms recovered from the controls were nontoxic.

It is important to note that mice

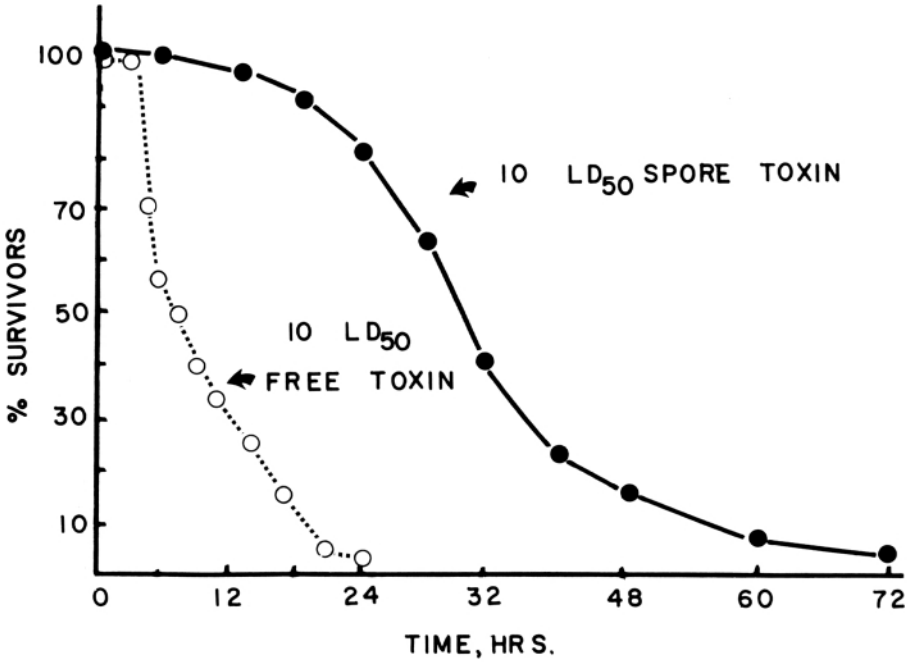

FIGURE 1. Rate of death of mice injected with spores of *C. botulinum* as compared with mice injected with free type A botulinum toxin.

●——● Mice injected i.p. with 2 x 10^8 heat-shocked spores containing 10 LD_{50} of type A toxin.

○·····○ Mice injected i.p. with a dilution of pure type A botulinum toxin.

injected with 2 x 10^8 spores (containing the equivalent of 10 LD_{50} botulinal toxin by mice assay) did not show any typical symptoms of botulism for the first 8 hours. Fifty-percent of the animals died 32 hours after injection (Figure 1). Normally, mice injected with 10 LD_{50} of botulinum toxin expire within 8-18 hours, with 50% expiring within 10 hours. Presumably, the increased expiration time with spores is needed for *in vivo* spore germination. Millipore Filtrate (0.22 μ) of the spore suspension were not lethal to any mice injected.

These observations suggest that germination of *C. botulinum* spores occurs previous to toxin release *in vivo*.

Spores in the Blood: Table 2 demonstrates that heat-resistant spores penetrate into the blood stream within 0.5 minutes after i.p. injection. It can be seen that the cells remain heat-resistant for at least 70 minutes, a fact which is in accordance with lack of spore germination or loss of heat-resistance during the first 3 hours as pointed out in Table 1. The number of spores entering the blood appears to be somewhat lower than the number of spores recovered from peritoneal exudate during comparable time intervals. However, the data definitely establish the fact that spores penetrate extremely rapidly into the bloodstream. Background flora bacteria, upon penetration into the bloodstream are effectively destroyed by phagocytes in the bloodstream.

TABLE 2.—Number of Viable Spores in the Blood of Mice Injected Intraperitoneally with 2 x 10^8 Spores.

Time of Withdrawal (min.)	Method of Withdrawal*	Number of Heat-Shocked Spores (per ml.)	Number of Non-Heat-Shocked Spores (per ml.)
0	A	0	0
.5	A	1×10^4	1×10^4
15	B	3×10^5	5×10^4
20	A	6×10^3	6×10^3
	A	3×10^2	3×10^4
30	B	7×10^4	1×10^3
	B	2.5×10^4	5×10^3
40	A	1×10^3	1×10^3
	A	2×10^3	2×10^3
60	A	2.1×10^3	2×10^3
70	A	7×10^2	5×10^2

* Method A—direct cardiac puncture while animal etherized.
Method B—animal sacrificed, body opened, cardiac puncture.

Spores in Liver, Kidney and Spleen: Table 3 shows that spores of *C. botulinum* 33A injected i.p. are rapidly disseminated to the liver, kidneys and spleen between 0.5 and 4 hours. The spores persist in the liver and kidneys at a relatively constant level up to 36 hours at which time a rapid decrease in the number of viable organisms occurred. In the spleen, spore clearance initiated at 8 hours and continued for 24-48 hours. The number of heat-sensitive (germinated) spores or vegetative cells was usually approximately 5 fold higher than the number of heat-resistant spores.

As with the peritoneal exudate, control colonies from organ preparations were not toxic to mice. The Colonies in 10 experimental plates proved to be toxic as determined by mice i.p. assay (antitoxin-protected mice survived). Moreover, organs from healthy animals, ground and injected into mice, did not cause ill effects. These controls substantiate the presence of *C. botulinum* spores in the organs.

RES Clearance of Spores in Mice Protected by Antitoxin: In order to obviate the effect of spore toxin, a series of samples similar to those in the preceding experiment were analyzed, with the exception that 0.1 ml of antitoxin sufficient to protect the animal from death was administered with each spore inoculum. In this way, it was possible to evaluate the survival of spores *in vivo* for extended periods of time.

In the peritoneal exudate of passively immunized mice, appearance of a heat-sensitive element could not

TABLE 3.—Number of viable cells recovered from organs of mice after intraperitoneal injection of 2 x 10^8 heat-shocked spores of *Clostridium botulinum* 33A. (Mice received no antitoxin).

Time After Intraperitoneal Injection (hrs.)	Viable Cells Recovered From Organs[a]					
	Livers		Kidneys		Spleens	
	Spores	Heat Sensitive Cells	Spores	Heat Sensitive Cells	Spores	Heat Sensitive Cells
0 (controls)	1×10^2	2×10^4	3×10^1	1.7×10^2	3×10^1	1.7×10^2
1/2	2×10^3	8×10^4	1×10^3	1×10^4	3×10^3	2.7×10^4
1	1×10^4	1×10^5	3×10^4	2×10^3	8×10^3	7.2×10^4
2	5×10^4	3×10^4	2×10^3	3×10^4	3×10^4	1.7×10^5
3	7×10^4	1×10^5	2×10^3	2.8×10^4	2×10^4	1.8×10^5
4	7×10^4	2×10^5	2×10^3	2.8×10^4	7×10^4	1.3×10^5
8	5×10^4	1.6×10^5	1×10^4	2×10^4	1×10^5	1×10^5
18	7×10^4	2.3×10^5	.....	.....	3×10^4	7×10^4
24	1×10^5	3×10^5	3×10^4	7×10^4	3×10^3	2.7×10^4
36	1×10^5	2×10^5	3×10^4	2×10^4	1×10^3	8×10^3
48	2×10^3	5×10^4	1×10^3	2×10^3	2×10^3	1.5×10^3

[a] Control animals received 1 ml sterile saline without spores
Averages of 4 animals.

be observed as in mice not passively immunized, (Figure 2). The number of spores decreased slightly during the first 5 days, but dropped approximately 100 fold between the fifth and seventh day. Between one

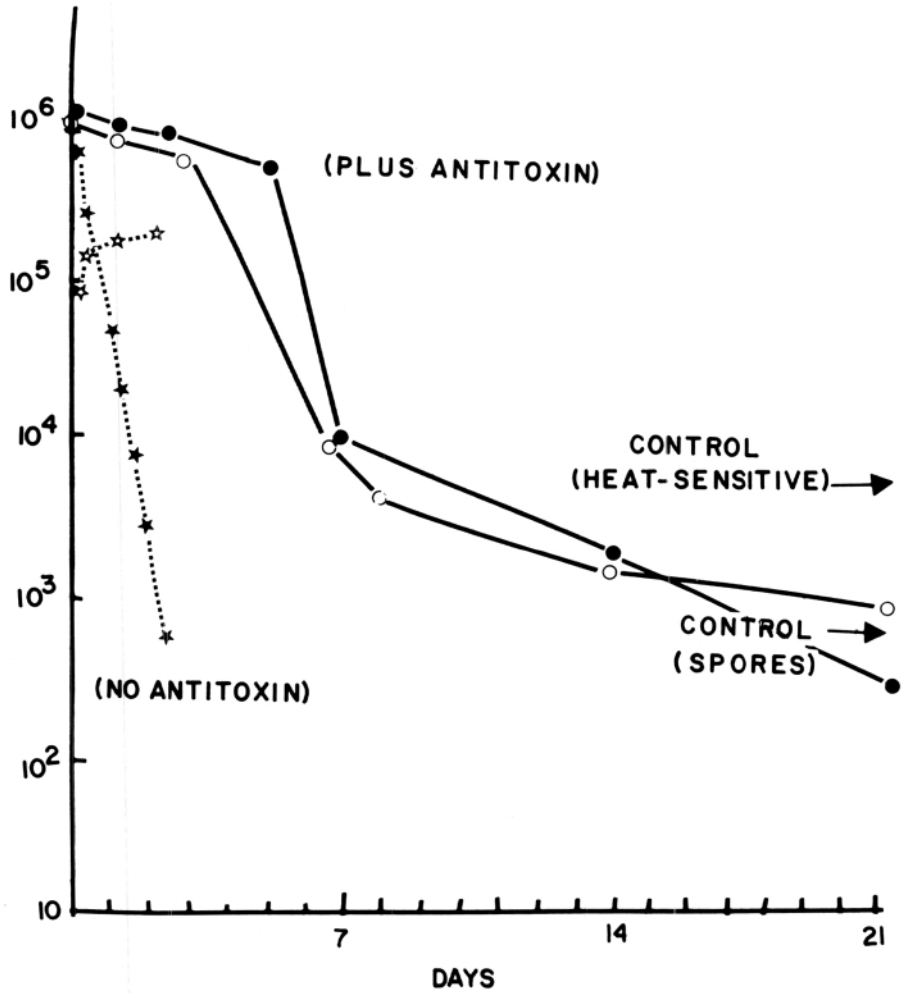

FIGURE 2. Number of Viable Spores in the Peritoneal Cavity of Mice Injected with 2 x 10^8 Heat-shocked Spores of *C. botulinum 33A*.

●—● Spores: 1 ml of cells surviving 80C for 10 min. in type A antitoxin protected mice.

★·····★ Spores: 1 ml of cells surviving 80C for 10 min. without antitoxin.

○—○ Heat-sensitive cells: Cells killed by 80C for 10 min. in type A antitoxin protected mice.

☆···☆ Heat-sensitive cells: Cells killed by 80C for 10 min. without antitoxin.

week and 3 weeks the numbers of spores continued to decrease further. In the presence of antitoxin, heat-sensitive *C. botulinum* cells did not appear in numbers greater than normal background contamination, i.e. approximately 4 x 10^3, indicating that destruction of spores *in vivo* after germination must have been extremely rapid.

In the liver, spleen and kidneys, no situation was found which could be attributed to the presence of vegetative cells in presence of antitoxin, since heat-resistant and heat-sensitive counts were always essentially equal (Table 4). There was gradual disappearance of spores from all these organs during the three weeks. Controls injected with antitoxin alone, contained 50 to 1000 spores, and 1000 to 10,000 heat-sensitive cells, all of which did not produce toxin in subculture.

Discussion

The significant finding emerging from these studies is the fact that spores injected i.p. — with or without antitoxin — are disseminated within seconds or minutes into the blood, liver, spleen and kidneys of the experimental animal. Subsequent clearance of spores from the RES is very gradual.

The mechanism of intoxication of the animals from spore toxin appears to depend on conversion of the heat-resistant spores to heat-sensitive forms; perhaps germinated spores or vegetative cells. The heat-sensitive cells appear at 4 hours and may persist for over 48 hours after i.p. injec-

Table 4.—Number of viable cells recovered from organs of antitoxin protected mice after intraperitoneal injection of 2 x 10^8 heat-shock spores of *Clostridium botulinum* 33A.

Time After Intraperitoneal Injection	Viable Cells Recovered From Organs					
	Livers		Kidneys		Spleens	
	Spores[a]	Total	Spores	Total	Spores	Total
0	1 x 10^2	2 x 10^3	3 x 10^1	3 x 10^2	3 x 10^1	2 x 10^2
20 hours	2 x 10^6	1 x 10^6	7 x 10^4	5 x 10^4	6 x 10^5	5 x 10^5
42 hours	2 x 10^6	2 x 10^6	7 x 10^5	2 x 10^5	1 x 10^6	6 x 10^5
48 hours	1 x 10^6	3 x 10^6	2 x 10^4	1 x 10^4	4 x 10^5	2 x 10^5
67 hours	9 x 10^5	5 x 10^5	7 x 10^4	3 x 10^4	3 x 10^5	1 x 10^5
120 hours	1 x 10^6	7 x 10^5	2 x 10^4	3 x 10^4	2 x 10^5	1 x 10^5
1 week	9 x 10^5	1 x 10^6	1 x 10^5	1 x 10^5	2 x 10^5	1 x 10^5
2 weeks	1 x 10^5	1 x 10^5	1 x 10^4	9 x 10^3	2 x 10^4	2 x 10^4
3 weeks	2 x 10^4	2 x 10^4	1 x 10^4	1 x 10^4	5 x 10^3	5 x 10^3
	5 x 10^5	5 x 10^5	1 x 10^4	2 x 10^4	5 x 10^4	5 x 10^4

[a]/Spore = cells surviving heating at 80 C for 10 minutes.
No germination, no appearance of heat-sensitive forms.
Control mice inoculated with 0.1 ml antitoxin but no spores contained 50 to 1000 spores and 1000 to 10,000 heat-sensitive cells.

tion. Symptoms of botulism intoxication are observed after time intervals necessary for loss of heat-resistance by injected spores.

The presence of botulinal antitoxin A appears to depress normal spore clearance levels *in vivo* by a factor of 5-10x. By a technique developed by this laboratory (Suzuki, *et al.*, 1971a), botulinal toxin was found not to affect phagocytic clearance (Suzuki, *et al.*, 1971b) which is in agreement with other investigators (Freeman, 1961). We speculate that delayed disappearance of spores *in vivo* when antitoxin is administered may be due to antitoxin binding to spores rendering them dormant. This seems plausible as a host defense mechanism against botulism pathogenesis since Suzuki, *et al.* (1970, 1971c) reported the PMN leukocyte as requisite for *C. botulinum* spore germination and toxin release. Staining techniques of phagocytized *C. botulinum* spores have verified this relationship (Booth, *et al* 1971).

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