

THE SUSCEPTIBILITY OF THE MALLARD DUCK (*ANAS PLATYRHYNCHOS*) TO *CLOSTRIDIUM BOTULINUM* C₂ TOXIN

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SUMMARY: Most strains of *Clostridium botulinum* type C, after having lost their capacity to produce their dominant toxin (C₁) as a result of being "cured" of their prophages, continue to produce C₂, a trypsin-activable toxin reported by other investigators. While of relatively low toxicity when administered perorally to the adult mallard duck (*Anas platyrhynchos*), it was highly toxic when given parenterally. By the intravenous route, for example, it was more than 1,000 times as toxic as C₁ toxin by the same route, when compared on the basis of mouse intraperitoneal toxicity. The cause of death in every instance was massive pulmonary edema and hemorrhage rather than the respiratory paralysis that occurs in C₁ intoxication.

INTRODUCTION

A toxic component produced by *Clostridium botulinum* types C and D in laboratory cultures, after they were "cured" of their prophages by treatment with acridine orange or ultraviolet light, was reported by Eklund and Poysky (1972). Distinct from the predominant toxins, C₁ and D, this component in most of the strains tested was demonstrable only after treatment with trypsin. Its identity with the C₂ toxin described by Jansen (1971) was established by immunological methods (Eklund et al., 1972).

Our interest in C₂ toxin, as it may relate to disease in wild aquatic birds, derived from the proclivity of some strains of *C. botulinum* type C to lose toxigenicity in the course of a variable, but unpredictable, number of serial transfers in laboratory media. This characteristic, first reported by Bengtson in 1922 [cited by Bengtson (1924)], is well known to investigators working with the type C bacterium. Eklund et al. (1972), however, demonstrated that strain X220B2, after having lost its capacity to produce C₁ toxin as a result of serial transfers, still produced C₂ toxin.

Although proof is lacking, we suspect that a similar loss of C₁ toxigenicity occurs in avian botulism epizootic areas, perhaps by way of a series of "transfers" through invertebrate carcasses (Jensen and Allen, 1960) or other suitable naturally occurring media. If so, there may be many *C. botulinum*-like bacteria in waterfowl marshes, unidentifiable on the basis of C₁ toxin production but still capable of producing C₂ toxin. Nakamura et al. (1978) recently reported the production of C₂ toxin by eight soil isolates of *C. botulinum* type C previously assumed to

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Investigation of the possible hazard of C_2 toxin to aquatic birds was the purpose of the study reported here. Brief summaries of our earlier observations on the toxicity of C_2 toxin for the mallard duck (*Anas platyrhynchos*) appeared earlier (Disease and Parasites of Fish and Wildlife, 1974; Migratory Birds, 1978). Miyazaki and Sakaguchi (1978) recently compared the potency of several botulinum toxins, including C_2 , administered to chickens by four routes. Differences between the responses of ducks and chickens to C_2 toxin administered perorally and parenterally will be discussed.

MATERIALS AND METHODS

Cultures: Type C strains A028 and A022, isolated from C_1 -producing strains 468C and 6812, respectively, after they had been grown in the presence of acridine orange by Eklund et al. (1972), produced C_2 but no C_1 toxin. Toxicogenic strain X220B2 (isolated from the liver of a duck at the Bear River Research Station), its C_1 toxigenicity lost after 19 serial transfers in LYA broth, still produced C_2 toxin.

Toxin production: All strains of type C were grown in 8-liter LYA broth cultures [Lactalysate (Baltimore Biological Laboratories, Cockeysville, MD), 3% yeast autolysate (Inolex Corporation, Glenwood, IL), 2%; glucose, 0.5%; sodium citrate, 0.35%; pH 7.0 after autoclaving], according to the cellophane tube procedure described by Sterne and Wentzel (1950). All crude cultures to be used in oral toxicity tests in ducks were examined microscopically for the presence of spores, but spore counts were not made. Viability of these cultures after heat shocking (80 C, 15 min) was demonstrated by subculturing in LYA broth.

Cells were removed from crude cultures by centrifugation and passage through 0.2 μ m grid membranes (Nalge Co., Rochester, NY).

Partial purification of C_1 (strain X220B2) and C_2 (strains A022 and A028) crude culture filtrates (CCF) was accomplished by twice precipitating them with 60% saturated $(NH_4)_2SO_4$, taking up the precipitate each time with 0.1 M phosphate buffer at pH 6.5, and finally dialyzing them against additional volumes of the same buffer for 48 hr. Mouse toxicity equal to that of CCF was attained by adjusting the volumes of C_2 solutions with phosphate buffer.

The C_2 toxin in both CCF and partially purified filtrates (PPF) was activated by 0.25% trypsin (Difco, 1:250) at pH 6.5 for 1 hr at 37 C (Eklund et al., 1972).

Toxicity tests: Levels of C_1 and C_2 toxicity upon which duck toxicity tests were based were established in white mice of the Rocky Mountain Laboratory strain. A 0.1-ml volume of an appropriate number of twofold dilutions was injected intraperitoneally into each of eight mice in each test group. The mouse LD_{50} of the toxins used in duck toxicity tests was estimated by the method of Reed and Muench (1938). Four mice were injected intravenously for comparative pathologic examinations.

Commercially bred adult mallard ducks, *Anas platyrhynchos* (average weight,

1,224 g), were used in toxicity measurements of both C₁ and C₂ preparations. Equal numbers of male and female birds were inoculated perorally (by means of a pipette passed into the proventriculus), intravenously (v. cutaneaulnaris), and intraperitoneally. In order to simulate the exposure of wild aquatic birds to C₂ intoxication as closely as possible, we used only whole crude cultures (cells not removed) in peroral toxicity tests. Precise titrations of A028 CCF and PPF were run only by the iv route. After inoculation, all birds were observed at frequent intervals for the notation of signs of intoxication.

Four mallards were given peroral doses of 5×10^4 mouse LD₅₀ of C₁ toxin (X220B2 whole culture) mixed with 3,200 mouse LD₅₀ of C₂ toxin (A028 whole culture) for the purpose of elucidating any effect C₂ toxin might have on the rate of absorption or lethality of C₁ toxin. A second group of four birds given C₁ toxin alone served as controls.

RESULTS

Although LYA broth supported excellent growth of all strains and high levels of C₁ toxin (1.2×10^7 mouse ip LD₅₀ per ml by X220B2), levels of C₂ toxin comparable to those reported by Eklund et al. (1972) were not attained. The highest C₂ toxicity recorded for CCF (A028 and A022) before trypsinization was 50 mouse ip LD₅₀, as compared with 320 after activation.

Signs of intoxication: Mice injected intraperitoneally or intravenously became weak and listless, but we made no attempt to find evidence of true paralysis that might have been provided by, for example, electrical stimulation of intoxicated nerve-muscle preparations. Respiratory difficulty was sometimes obvious, but the "wasp waist" (caused by paralysis of the phrenic nerves) of C₁ neurointoxication was not seen.

None of nine ducks given 10-ml peroral doses of whole crude culture of strain A028 (cells not removed; 50 mouse ip LD₅₀ per ml) showed ill effects within a 10-day observation period, nor did any of a second group of nine ducks given an equal volume of the same culture after trypsinization (320 LD₅₀ per ml). However, one of two birds dosed perorally with four 10-ml volumes of unactivated whole crude culture given at 1-hr intervals developed signs of intoxication 19 hr after the fourth dose. It sat with its neck thrown back and to one side, but it could stand and hold its head erect when disturbed. Just before it died 1.5 hr later, it stood reluctantly and could still hold up its head; fluid dripped from the nares. It died in convulsions moments later. The second bird, started on a series of 10.0-ml doses at the same time, exhibited no signs of intoxication after a total peroral dose of 120 ml (6,000 mouse ip LD₅₀; potentially 38,400 after trypsin activation) of whole crude culture given over a period of 30 hr.

Mallards injected intravenously with C₂ toxin (either A022 or A028) showed a more dramatic response than did birds intoxicated by any other route (Table I). Death time varied considerably, but those given doses of eight to 32 mouse ip LD₅₀ commonly died within 1.5 to 4 hr. When smaller lethal doses were given,

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TABLE I

Toxicity of C. botulinum C₂ toxin, crude culture filtrate and partially purified filtrate, for the mallard duck by the iv route

| Dose (Mouse ip LD ₅₀) | Mortality (Deaths/Number tested) | |
|--------------------------------------|-------------------------------------|-------|
| | CCF ¹ | PPF |
| 0.8 | 0/16 | 0/16 |
| 1.6 | 4/16 | 6/16 |
| 3.2 | 12/16 | 10/16 |
| 6.4 | 16/16 | 16/16 |

¹ The mallard iv LD₅₀ was 2.4 mouse ip LD₅₀ for both CCF and PPF.

death time was as short as 6 hr or as long as 32 hr (observations were not routinely made between 7 and 20 hr after inoculation; death time for birds dying within that period was recorded as the interval between the time of inoculation and 8:00 o'clock the following day).

No convincing signs of neurointoxication were observed in ducks injected parenterally. Peracute cases (usually ducks given intravenously 32 or more mouse ip LD₅₀) dying within 2 hr after inoculation commonly showed no evidence of respiratory distress until 15–30 min before death. Moist rales and the dripping of fluid from the nares were consistently the first signs of intoxication. Affected birds vigorously shook their heads, apparently in an effort to expel the fluid from their respiratory passages. In such cases, death in convulsions was the only observed outcome. When iv doses were one-half the approximate duck LD₅₀, moist rales were heard in about one-third of the test birds, but the passage of fluid through the nares was not observed.

While C₂ toxin given by the ip route induced characteristic signs and death in mallards, the lethal dose appeared to be larger than the iv dose, although titrations were not run. Among 10 birds given 800 mouse ip LD₅₀ by the same route, one died in 2.5 hr, one in 6.5 hr, and the remaining eight died sometime within 7 to 20 hr.

Production of antiserum against type C toxins was incidental to the subject of this study, and it is mentioned only because the failure of three of 24 ducks to survive a series of sublethal intramuscular challenge doses of C₂ toxin provided evidence of its toxicity by that route. They exhibited characteristic signs of C₂ intoxication before death.

Necropsy findings: The only consistent gross pathologic changes in ducks dead of C₂ intoxication were massive pulmonary edema and congestion, regardless of the route of inoculation. Lungs were saturated with serous fluid, sometimes blood tinged, sometimes frankly bloody. Usually there was a considerable volume of similar fluid in the thoracoabdominal cavity. Pulmonary edema was only occasionally seen in mice given C₂ toxin by the ip route. However, massive pulmonary edema, congestion, and hemorrhage were found in two mice given

iv doses. More than 1.0 ml of bloody fluid was removed by pipette from the pleural cavity of one so treated.

Histopathological examination confirmed the presence of pulmonary edema and congestion in every fatal case of C₂ intoxication in ducks. Perivascular hemorrhage and edema were also common lung lesions, as was the presence of fibrin or mucus or both in bronchi and parabronchi. Congestion was also a common but not constant finding in the kidneys.

Both the iv and ip LD₅₀ of C₁ toxin (CCF and PPF from strain X220B2) for the mallard duck were about 3,000 mouse ip LD₅₀.

Two of four ducks died after peroral doses of a mixture of C₁ and C₂ toxin, while one of four dosed with C₁ alone died. There were no significant differences in death times of the birds in the two groups, and the difference between mortality rates may have occurred by chance.

DISCUSSION

In view of the widely held and well documented concept that the neurotoxin of *C. botulinum* (any one of the seven known) is "the most poisonous poison" (Lamanna, 1959), the intravenous toxicity of C₂ toxin for the mallard duck is quite remarkable, as demonstrated by these studies. The duck iv LD₅₀ reported here is still considered to be an approximation, because the calculated endpoint changed from 1.6 mouse ip LD₅₀, as reported earlier (Migratory Birds, 1978), to 2.4 as the number of experimental birds increased. Even so, C₂ toxin was still more than a thousand times as toxic as C₁ by that route.

We found no evidence in this study of the toxico-infections (either C₁ or C₂) reported by Miyazaki et al. (1978) in chickens. Such cases occur when *C. botulinum* grows and produces toxin in intestinal contents. In fact, among many hundreds of mallard ducks experimentally intoxicated perorally with whole cultures of *C. botulinum* type C (containing viable spores) at the Bear River Research Station over a period of 20 years, only two that we were aware of may have had toxico-infections as evidenced by delayed appearance of signs of intoxication and persistence of toxin in the blood serum or feces (unpublished). The difference between the findings of Miyazaki et al. (1978) and ours could possibly be explained by age differences; they used young chickens, while we used adult ducks. Perhaps the young bird, like the human infant (Arnon et al., 1977), is more susceptible to toxico-infection, or, perhaps the explanation lies in species susceptibility. We have found wide differences among various species of birds in their susceptibility to type E toxins (unpublished).

Pulmonary congestion and edema, the immediate cause of death in every case of C₂ intoxication of mallards in our study, was not reported in chickens by Miyazaki et al. (1978). Again this discrepancy may be the result of age or species differences of the hosts.

If C₂ toxin has any neurotoxic activity in mallard ducks, it was not evident in our study. Intoxicated birds were usually on their feet until shortly before

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death in violent convulsions, while birds in the final stages of C₁ neurointoxication are almost totally paralyzed and die quietly. We have not, however, ruled out a possible neurotoxic component of C₂ toxin.

The possible role of C₂ toxin as a primary disease agent of aquatic birds was not resolved by this study. Adult mallard ducks appeared not to be highly susceptible to its action when the toxin was administered perorally, but since the iv lethal dose is so small, it is possible that birds whose intestinal mucosa is severely damaged by parasites could absorb a lethal dose through the lesions. The possible symbiotic action of C₂ toxin with C₁ toxin or other agents, perhaps by increasing capillary permeability, needs further study.

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