Symptoms and Pathology Produced By Toxic Microcystis Aeruginosa NRC-1 In Laboratory and Domestic Animals

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RÉSUMÉ

Des épreuves de toxicité ont été faites avec M. aeruginosa NRC-1 lyophilisé. Dans ces épreuves, on a utilisé des souris, des lapins, des poulets, des canards, deux veaux et un agneau.

Les symptômes et les changements pathologiques sont décrits. A poids égal la dose léthale "per os" requise pour les grands animaux et les oiseaux, a du être de trois à cinq fois plus grande que pour les animaux de laboratoire. Les symptômes étaient moins prononcés et le temps de survie plus long chez les animaux plus résistants. L'hypertrophie et la congestion du foie accompagnées de nécrose des cellules hépatiques étaient constantes et pathognomoniques. En général, ces constatations concordent avec les observations des autres chercheurs qui ont fait des traveux sur la toxicité des fleurs d'eau Microcystis naturelles.

Les toxicités et les structures du microcystin et des six autres polypeptides cycliques qui sont biologiquement actifs sont énumérées dans un tableau. Les effets pathologiques produits par le microcystin chez les animaux de laboratoires et chez les animaux domestiques concordent avec ceux produits chez l'homme mais ils sont differents de ceux produits chez les animaux par les peptides toxiques de Amanita phalloides.

SUMMARY

Toxicity tests with lyophilized M. aeruginosa NRC-1 cells have been conducted using mice, guinea pigs, rabbits, chickens, ducks, two calves and one lamb as the test animals.

The symptoms and pathological changes are described. On an equivalent weight basis it required three to five times the oral dosage to kill the large animals and birds as it did to kill the laboratory animals. The symptoms were less pronounced and the survival times were longer in the more resistant animals. Enlargement and congestion of the liver with necrosis of the hepatic cells were constant and pathognomonic. These findings are in general agreement with the observations of other workers who have examined the toxicity of naturally occurring Microcystis waterblooms.

The toxicities and structures of microcystin and of six other biologically active cyclic polypeptides are summarized. The pathological effects produced by microcystin in laboratory and domestic animals resemble those produced in man but differ from those produced in animals by the toxic peptides of Amanita phalloides.

Symptoms and pathological changes in livestock and laboratory animals caused by waterblooms consisting predominantly of species of *Microcystis* have been described by several workers. Fitch *et al* (1) were among the first to give a comprehensive account of observations made during out-

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breaks of algal poisoning in large domestic animals in Minnesota. Steyn (2, 3) has also reported severe losses among cattle, sheep and other animals in South Africa following ingestion of water heavily contaminated with blooms of the alga *M. toxica* Stephens. Other investigators using laboratory animals have examined the effects of the fresh and frozen waterblooms (4), algal extracts (5) and purified toxin (6). Gross and histopathological changes produced in rats by intraperitoneal injection of an aqueous extract of dried *M. aeruginosa* have been described in detail by Ashworth and Mason (7). Other reports (8-13) also describe symptoms and pathological changes associated with algal poisoning but many of these either lack a clear description of the algal composition of the blooms or involve other genera than *Microcystis*.

Recently a toxic strain of *M. aeruginosa* NRC-1 has been isolated and mass cultured. Symptoms and some gross pathological changes produced by parenteral and oral administration of the freeze-dried cells in mice have been described (14-17). *Microcystis* Fast Death Factor (FDF) (14), the toxin responsible for these effects, has been identified as a cyclic polypeptide (18) and henceforth will be called "microcystin". Since these observations were confined to tests with mice it was decided to extend the study to larger species of laboratory animals, domestic animals and birds.

Some of the results reported in this study have been briefly described and cited as unpublished in review articles (15-17).

Materials and Methods

M. aeruginosa NRC-1 and the methods used for its isolation, mass culture, freeze-drying of cells and bioassay of toxicity with mice have been described previously (14, 15, 17, 18, 19). One lot of cells (No. 16) having an intraperitoneal (IP) lethal dose for mice (LD_{100} (IP, mouse)) = 80 mg. (dry wt.) per kg. (body wt.) was used in all experiments but one. In the latter a pool of Lots 17 and 18 (1.7:1) having an LD_{100} (IP, mouse) = approx. 160 mg./kg. was used. Suspensions of freeze-dried cells were prepared by trituration with distilled water in a mortar.

Seven species of animals were tested: mouse, guinea pig, rabbit, lamb, calf, chicken and domestic duck. The ranges in animal weights and the doses administered by the IP or oral routes are given in Tables I-III. Multiples of the LD_{100} (IP, mouse) were used for the dosages of the other animals, as indicated. Prior to oral administration, the animals were fasted for periods varying from 18 hrs. for mice to 60 hrs. for calves, since previous tests with mice had indicated that the LD_{100} (Oral) was lower and less variable if stomachs were empty. With few exceptions the animals dosed intraperitoneally were similarly fasted. For oral administration a blunted No. 14 gauge needle was used for mice and catheters or a stomach tube were used for the larger animals. The oral doses given to ducks were, with one exception, lower than the oral doses given to mice because the ducks regurgitated or excreted large volumes of cell suspension soon after administration. All survivors were observed for 48 hrs. or longer.

Necropsies were made immediately or as soon as possible after death. Portions of the principal organs including lung, heart, liver, spleen, kidney and, in some instances, adrenal gland were fixed in 10 per cent formol saline for histopathological studies. Tissue sections were stained with haematoxylin and eosin.

Samples of blood plasma from four species of experimental animals were tested for prothrombin activity according to the method of Quick (20). Oxalated blood samples were collected before oral dosage with algal cells and again after the appearance of well-marked toxic symptoms. The blood samples were centrifuged immediately to obtain the plasma. One calf was bled four times at intervals after administration of the algal cells. Simultaneously with the coagulation tests, haemoglobin determinations and erythrocyte counts were made for the two calves and the lamb.

Results

Toxicity and Symptoms

Mouse — The intraperitoneal LD_{100} was about 80 mg./kg. in two of three serial tests (Table I). When pregnant mice were used in a third experiment, 160 mg./kg. were required for the same effect. The oral LD_{100} was approximately 3.2 g./kg. or 40 times the intraperitoneal lethal dose.

Symptoms developing after lethal dosages by either route were similar but onset was more rapid in groups injected intraperitoneally. In these mice the asymptomatic period varied from 0.2 to 0.5 hrs.

TABLE I

Survival times, in hours, of three species of laboratory animals dosed with M. aeruginosa NRC-1 cells

Species and weights	Animals per dose	Dosage							
		IP, mg./kg.				Oral, g./kg.*			
		40	80	160	320	1.6	3.2	6.4	12.8
Mouse (g.):									
17–20	4	S**	1.0–3.6	0.8–1.3	—	—	1.8–>6***	1.8–4	1.2–2.4
24–27	3	S	2.5–3.8	1.5–1.8	—	—	—	—	—
23–40 (pregnant)	3	S	S	1.5–1.6	—	—	2.7–4.7	1–>4	2.8–>4
Guinea pig (kg.)									
0.5–0.7	2	—	3.2–>4	3.3–3.5	2.0	8–S	10–12+	8–12	8–9
Rabbit (kg.):									
2.0–2.6	2–4††	—	—	—	—	5–S	2.5–30	1.3–>4	1.2

* Representing multiples of 20, 40, 80 and 160 respectively, of LD^{100} (IP, mouse) = 80 mg./kg., administered in volumes of 0.8–1.5 ml. (mouse), 10.5–38 ml. (guinea pig) and 35-85 ml. (rabbit).
** S = survived and returned to normal.
*** > = greater than number of hours indicated but less than 18 hrs.
\+ One animal, 2.4 g./kg. dosage.
†† Two, four, three and one animal per dose for 1.6, 3.2, 6.4 and 12.8 g./kg., respectively.

TABLE II

Survival times of two species of ruminants dosed orally with different lots of M. aeruginosa NRC-1 cells

Species and Age	Body Wt., kg.	Lot No.	Dosage		Hours of Survival
			g./kg.*	Vol., l.	
Lamb (2 mo.)	10.5	16	16.0	0.7	18–19
Calf (7 mo.)	86.8	16	9.6	5.6	28**
(4 mo.)	78.2	17+18	32.0	6.9	9

*Representing multiples of 200 and 120, respectively of LD^{100} (IP, mouse) = 80 mg./kg. (Lot 16) and a multiple of 200 for LD^{100} (IP, mouse) = 160 mg./kg. (Lots 17 and 18).
**Killed in moribund condition.

TABLE III

Survival time, in hours, of two species of birds dosed with M. aeruginosa NRC-1 cells

Species and weight	Animals per dose	Dosage				
		IP, mg./kg.		Oral, g./kg.*		
		80	320	2.2	8	16
Chicken 0.5–0.9 kg	4	9.5–>12**	—	—	>5–S***	>12–24.5
Duck 1.9–3.2 kg	2	S	>12–S†	S	—	S††

*Multiples of 27.5, 100 and 200 respectively, of LD^{100} (IP, mouse) = 80 mg./kg.
** > = greater than number of hours indicated but less than 20 hrs.
***S = survived and returned to normal.
†Four anmials.
††Rectal excretion and regurgitation of part of dose during first 2 hrs.

with death in 0.8 to 3.8 hrs. The orally dosed mice behaved normally for 0.5 to 1.5 hrs. and about 27 per cent survived longer than 4 hrs. The first symptoms were huddling together, slightly ruffled coats and increased frequency and depth of respiration. Later, muscular twitchings of the head and neck and peculiar convulsive jumping movements were observed. These periods of hyperexcitability alternated with quiet periods. The animals gradually weakened and eventually lay motionless. Prior to death respiration became slow and laboured and occasionally bicycling movements of the hind legs occurred. In a few cases weakness developed rapidly and the animals exhibited incoordination and partial paralysis of the hind limbs. Pallor of the ears, iris and tail generally accompanied the other symptoms.

Guinea pig — The LD_{100} for both the intraperitoneal and oral route in guinea pigs was essentially the same as that determined for mice, but survival times were longer (Table I). The asymptomatic periods varied from 0.3 to 1 hr. (IP) to 3 to 5 hrs. (Oral). Symptoms resembled those observed in mice, but rapidly progressing weakness and partial paralysis were noted more frequently. Muscular twitchings occurred shortly before death only in guinea pigs which received high intraperitoneal doses.

Rabbit — The oral LD_{100} for rabbits (Table I) was similar to that for guinea pigs. The asymptomatic periods ranged from 0.8 to 3 hrs. with deaths usually occurring in 1.2 to 5 hrs. One of the four animals which received 3.2 g./kg. remained normal for over 6 hrs. and succumbed only after 30 hrs.

In general, dyspnea and tachycardia were first observed. Partial paralysis commenced in the neck muscles producing tilting of the head, then affected the hind legs and later the front legs, progressing to almost complete lack of muscle tone. However, fibrillar muscle tremors were perceptible by palpation. Peripheral anaemia developed concurrently with loss of muscle tone. As death approached, respiration became slow, irregular and occasionally gasping. Heart action became extremely rapid, resembling fibrillation, and spasmodic contractions of the hind legs occurred.

In the rabbit which survived 30 hrs. symptoms developed more slowly. By 21 hrs. after dosage the animal showed only loss of muscle tone and depression, but no notable peripheral anaemia. Partial paralysis of the leg muscles developed 2 hrs. before death. During the last quarter hour short, violent, convulsive muscular spasms produced slashing movements of the hind legs and opisthotonos. The ears were cyanotic; the conjunctiva hyperaemic.

Lamb and calf — The algae were lethal for these large animals but survival times were relatively longer and the toxic effects less pronounced than in the laboratory animals (Table II).

The lamb showed slight blanching of the oral mucosa and conjunctiva, signs of muscle weakness, slightly shallower respiration and accelerated heart action commencing about 2 hrs. after dosing. During the subsequent 4 hrs. these symptoms became more pronounced and muscle weakness was indicated by tilting of the head and difficulty in supporting the body weight. Death occurred during the night, approximately 18 hrs. after dosage.

The 7-month-old calf exhibited signs of muscle weakness after an elapse of 6 hrs. and remained recumbent or moved about with an unsteady gait when aroused. Muscle fibrillations were noted in the region of the rumen. The conjunctiva, initially slightly hyperaemic, became somewhat anaemic. These symptoms became more pronounced 22 to 28 hrs. after dosage and the animal was unable to support its weight. Muscle fibrillations over shoulder and flank were almost continuous, alternating with sudden, short contractions. Heart action was accelerated and cardiac ausculation indicated an increased incidence of systolic murmurs. Respiration became slightly laboured and abdominal. At this advanced stage of intoxication the animal was killed for pathological examination.

The 4-month-old calf which was given a large dose of less toxic cells produced similar, but more rapidly progressing symptoms. Muscle weakness and inertia appeared about 5 hrs. after treatment and reached a peak 3 hrs. later. Muscular fibrillation was not observed. Death occurred in 9 hrs.

Chicken and duck — These species, particularly the ducks, exhibited a somewhat greater tolerance to the toxin than the other laboratory animals tested (Table III). In chickens, oral doses corresponding to those received by the large domestic animals were required for lethal effect. The intraperitoneal LD_{100} for ducks was four times higher than that of the laboratory animals and they survived the highest oral

TABLE IV

Prothrombin times of plasma, red blood count and haemoglobin from selected animals which received oral doses of M. aeruginosa NRC-1 cells

Species	Oral Dosage, g./kg.	Time after dosage, hr.	Mean prothrombin time* (with S.D.), sec.	R.B.C. X10⁶/cmm.	Hbg. g./100cc.
Guinea pig	12.8	0 2.3	30.0 ± 1.0 44.7 ± 1.2	—	—
Rabbit	3.2	0 4.8	10.5 ± 1.3 13.3 ± 0.6	—	—
Rabbit	6.4	0 3.5	8.3 ± 0.6 9.0 ± 1.4	—	—
Rabbit	6.4	0 3.2	11.5 ± 1.0 14.3 ± 0.6	—	—
Calf (7 mo.)	9.6	0 3.8 6.0 22.0 28.0	17.7 ± 0.6 15.7 ± 1.2 15.0 ± 1.0 31.0 ± 1.7 38.0 ± 1.0	7.2 — 6.7 3.9 3.9	8.7 8.2 7.5 4.8 5.0
Calf (4 mo.)	32.0	0 5.0	20.3 ± 0.6 21.7 ± 0.6	9.1 11.7	10.5 13.5
Lamb	16.0	0 5.5	29.7 + 1.5 45.7 + 1.5	9.3 8.9	10.5 9.0

*Method of Quick.–Tests replicated three or four times.

dose of 16 g./kg. For 5 to 6 hrs. after treatment all birds were normal in behaviour or only slightly quieter than usual. A limited number observed during the last hour prior to death, showed well-marked somnolence progressing to almost complete paralysis.

Gross Pathology

The principal effects of the toxic algae were abnormalities in the blood supply of the tissues. The peripheral circulatory system appeared to be drained of blood, causing anaemia of ears, eyes and tail. Concentration of blood in the viscera was largely confined to the liver.

Liver — This organ in all species showed the most striking and pathognomonic change. Livers of mice were enlarged and hyperaemic and blood oozed freely from the cut surface. The organ was often mottled with punctate haemorrhages bordered by yellowish-brown degeneration. When death was delayed these areas of degeneration were more extensive. In rabbits engorgement with blood was pronounced but was not marked in guinea pigs unless death was delayed for 8 to 12 hrs. Congestion and mottling were particularly marked in livers of the lamb and the calf which received the large dose. In the lamb there was a striking "nutmeg" appearance.

Heart — Petechial haemorrhages were found in the coronary fat of the lamb and in the myocardium of the birds. Hearts of other species appeared normal. Residual blood in the heart and large vessels of the guinea pigs and rabbits was only partly coagulated.

Lung — The lungs of the mice and rabbits appeared pale. In the other species findings varied from anaemia to some congestion but were not pronounced.

Spleen — This organ was normal or only slightly congested in all species.

Kidney — The chickens and ducks showed some renal congestion but kidney tissue of other animals was not remarkable.

Gastrointestinal tract — Mice showed slight hyperaemia of the intestinal mucosa and traces of blood in the ingesta of the upper part, particularly in those animals

dosed orally. No changes were found in these tissues of other animals despite the large oral doses given in some instances.

Serous cavities — The peritoneum of mice was somewhat anaemic and, in animals injected by this route, the cavity contained a small amount of blood-tinged fluid. This serosanguineous fluid was also found in the peritoneal cavities of the rabbits and in the pericardial sacs and body cavities of the birds. It was abundant in the pleural and peritoneal cavities of the calf and lamb which received large oral doses.

HISTOPATHOLOGY

Tissues from all species except the ducks were examined. The microscopic changes observed were similar to those described in rats by Ashworth and Mason (7).

Liver — Livers in all species studied showed more pronounced changes than other organs. In the mammals hepatic cells in the central zone of each lobule were necrotic or had disappeared and were replaced by a pool of blood. Toward the periphery of the lobule, the cord structure was retained but the hepatic cells showed degenerative changes. Lobules throughout the tissue were uniformly affected. In the chickens the zonal distribution of necrosis and degeneration was not observed, these changes being diffuse in the lobule.

Heart — Myocardial degeneration was slight. The mice, rabbits, lamb and chickens showed slight swelling, loss of cross-striation and some pigmentation of myocardial fibres. The myocardium of the guinea pigs and calves appeared normal.

Lung — Changes were confined to the blood-vascular system. Alveolar capillaries of the rabbits and calves were bloodless. Lungs of all but two of the mice appeared normal while those of the remaining mice and the lamb were mildly hyperaemic. Guinea pig and chicken lungs were acutely congested.

Spleen — This tissue appeared normal in the mice and two of the rabbits. Slight splenic congestion was found in the remaining rabbit, guinea pigs, calves and lamb. Three chicken spleens showed vacuolation of reticulo-endothelial cells but the fourth appeared normal.

Kidney — Glomerular tufts were swollen and devoid of blood. Proteinaceous material was found in the glomerular spaces in two rabbits, the lamb and the 7-month-old calf. Epithelium of the convoluted tubules was swollen and granular. Occasional patchy areas of congestion were found in intertubular capillaries of both cortex and medulla.

Adrenal — No noteworthy changes were observed in the guinea pigs, calves and lamb.

HAEMATOLOGY

The delayed and incomplete clotting of blood, noted at necropsy of some of the experimental animals, suggested a depletion of prothrombin — particularly since severe destruction of liver cells was a constant pathological effect of the algal toxin. The coagulation or prothrombin times for blood plasma of a few experimental animals are recorded in Table IV. A significant prolongation of prothrombin time was observed in the guinea pig, lamb and one calf. In this one calf erythrocyte count and haemoglobin values declined significantly after 22 hrs. The other animals showed no significant changes as a result of dosage.

Discussion

Experiments carried out with mice of a different strain have confirmed previous findings (14, 15) on the intraperitoneal and oral toxic effects of freeze-dried cells of *M. aeruginosa* NRC-1. The effects observed with mice and guinea pigs are in close agreement with those reported by Wheeler *et al* (4) for frozen and thawed blooms of *M. aeruginosa*. The terminal clonic spasms and opisthotonos which were observed in some rabbits have also been noted by Fitch *et al* (1) as symptoms produced in rabbits by a bloom consisting predominantly of *M. aeruginosa* and *Anabaena flos-aquae* with *Aphanizomenon flos-aquae* prevalent.

With the three species of laboratory animals, the oral LD_{100} was approximately 40 times higher than the corresponding intraperitoneal LD_{100}. On the basis of oral dosage per unit weight, it required three to five times as much algae to kill the large animals and birds as it did to kill the laboratory animals. The nervous symptoms and terminal paralysis observed in mice developed more rapidly and were more pronounced than in the guinea pigs while the rabbits occupied an intermediate position in this respect. The more resistant large animals exhibited less obvious symptoms and survived longer, despite larger doses. The relatively high intraperitoneal and oral LD_{100} values for ducks indicate that

TABLE V

Toxicity for mice and structure of some biologically active cyclic polypeptides

Polypeptide	LD^{100} (IV) mg./kg.*	Max. survival time days	Structure
α-Amanitin	0.1 (22)	5 (22)	Hexapeptide: amide of β-emanitin. (24)
β-Amanitin	0.32–p.4 (22)	3 (22)	Hexapeptide: asp., cys., gly., allo-hydroxypro., 2 unknown amino acids. (24)
Phalloidin	1.6-2.0 (22)	2–3 (22)	Hexapeptide: cys., ala., allo-hydroxy-pro., oxindolylala., D-thr., dihydroxy-leu. (23)
Gramicidin (component of tyrothricin)	3.75 (25)	2-7 (25)	Mixture of 3 peptides, each containing 75-80 residues: gly, ala., val., leu., try., phe., tyr. ethanolamine. (26)
Gramicidin S	17** (27)	—	Decapeptide: (val., orn., leu., D-phe., $pro.)_2$ (28)
Bacitracin	435-700 (29)	1-2 (30)	Dodecapeptide: asp., D-asp., D-glu., leu., 3 ileu., D-phe., cys., his., lys., D-orn. (31, 32)
Microcystin	0.47*** (18)	0.5–1.5 hr. (18)	Decapeptide (probable): asp., 2 glu., D-ser., val., orn., 2 ala., 2 leu. (18)

*Based on 25 g. body weight. **LD^{50} (IP) white rat. ***LD^{50} (IP) mouse.

M. aeruginosa poisoning is probably not involved in the syndrome of duck sickness.

The convulsions and rapid death of cattle that have eaten waterblooms of *Microcystis* (1-3) did not occur with domestic animals dosed with *M. aeruginosa* NRC-1. Identical results have been reported by others for natural and experimental *Microcystis* poisonings (2-7).

The frequent occurrence of delayed and incomplete coagulation of the blood in animals treated with cells of *M. aeruginosa* NRC-1 has also been noted by Ashworth and Mason (7). This study shows that the apparent cause is the depression of the prothrombin level resulting from severe liver damage. In progressed cases of poisoning, terminally reduced hemoglobin levels and red blood cell counts were noted similar to the observations by Mason and Wheeler (5).

It is noteworthy that in the current experiments inflammatory changes of the intestinal tract, occasionally reported in field cases (9, 12, 13), were absent. This lends support to the suggestion that other factors, probably of bacterial origin, may be responsible for some of these lesions (17).

From the close agreement in symptoms and pathological changes produced by cells of *M. aeruginosa* NRC-1, by pure microcystin obtained from them, and by waterblooms consisting predominantly of *M. aeruginosa* (including *M. toxica*), it has been concluded that microcystin is one of the principal causes of algal poisoning in nature (16, 17). Other species of blue-green algae have been incriminated in rapid deaths of domestic animals and waterfowl and it has recently been shown (16, 17, 21) that strains of *Anabaena flos-aquae* produce a very fast death factor that is different from microcystin.

In Table V data on the toxicity and structure of the cyclic polypeptides α- and β-amanitin and phalloidin (from the mushroom *Amanita phalloides*) and the antibiotics gramicidin and bacitracin are summarized along with those of microcystin. In 1946, Ashworth and Mason (7) drew attention to the marked similarities in chemical properties, pathological effects and composition of amanita and *M. aeruginosa* toxins and suggested that they might be similar or identical. Amanita toxins resemble microcystin in being cyclic polypeptides of comparable toxicity but survival times and structural composition are different. Phalloidin produces the typical

pharmacological effects of poisoning by *Amanita phalloides* (33). It causes principally the enlargement and fatty infiltration of the liver and haemorrhages in the digestive tract. These effects are different from those produced by microcystin. Amanita poisoning of humans, however, causes necrosis of hepatic cells with loss of cord structure in the central zones of the liver lobules (34) and these effects do resemble those produced in various animals by microcystin. Gramicidins and bacitracin are much less toxic and have different structures from α- or β-amanitin, phalloidin and microcystin.

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Parturition in the Bitch

The author outlines the normal progression of events and a number of the more common abnormalities associated with parturition. Information is presented which is of help to the veterinarian in deciding whether a given case of dystocia should be handled medically, with forceps, or surgically. The dystocias are discussed from both matunal and fetal obstructive etiologies with special emphasis on malpresentations and their correction without surgical intervention. Also included are the uterine inertias with suggestions for medical therapy.

M. C. Yorborough, The Auburn Veterinarian 21: 9, 1965.