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Severe Hepatotoxicity Caused by the Tropical Cyanobacterium (Blue-Green Alga) *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raju Isolated from a Domestic Water Supply Reservoir

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***Cylindrospermopsis raciborskii*, a tropical blooming species of cyanobacterium (blue-green alga), was isolated from the domestic water supply reservoir on Palm Island, a continental island off the tropical northeast coast of Australia. This species, not previously known to be toxic, was shown to be severely hepatotoxic for mice. The 50% lethal dose at 24 h after injection was found to be 64 ± 5 mg of freeze-dried culture per kg of mouse. The principal lesion produced was centrilobular to massive hepatocyte necrosis, but various degrees of injury were also seen in the kidneys, adrenal glands, lungs, and intestine. The possible implication of this finding in relation to an incident of hepatoenteritis in humans living on the island is discussed.**

The occurrence of blooms or dense growths of cyanobacteria (blue-green algae) in water storage bodies is of increasing importance and related to the problem of eutrophication of rivers and streams (A. R. B. Jackson, M. T. C. Runnegar, I. R. Falconer, and A. McInnes, *in* R. F. Keeler, A. A. Seawright, L. F. James, and M. Megarty [ed.], Proceedings of the 2nd Australian-U.S. Symposium on Poisonous Plants, in press). Many of these blooms are toxic and have caused significant deaths of livestock and wildlife, as well as being implicated in human illnesses (4, 6, 11). Although the distribution of freshwater cyanobacterial blooms is worldwide, most toxic species occur in temperate rather than tropical waters (15).

In November 1979 an outbreak of hepatoenteritis at Palm Island, in northern Queensland, involved 148 people, mainly children, the majority of whom required hospitalization (3). The outbreak occurred a few days after treatment of Solomon Dam, a drinking-water supply for the island, with copper sulfate to control a dense algal bloom (2). Copper sulfate at 1 ppm (1 μg/ml) causes lysis of cyanobacteria within a few hours and the release into the water of any toxic cellular components. An epidemiological investigation of the incident revealed that only people in households connected to the reticulated water supply from Solomon Dam were affected, and others on alternative water supplies were spared. From all available evidence it was, retrospectively, postulated that the sickness was related to algal toxicity (1, 2), and a detailed study of the water quality of the reservoir was commissioned by the Queensland Department of Local Government.

In the course of this study two species of cyanobacterium were identified as regular components of the phytoplankton (P. R. Hawkins and D. J. Griffiths, Report to the Queensland Government on "Chemical and biological monitoring of water quality at Solomon Dam, Palm Island," January 1985). These were two varieties of *Anabaena circinalis*, which when tested proved to be nontoxic (M. T. C. Runnegar, unpublished results), and *Cylindrospermopsis raciborskii*. This paper reports the severe toxicity of the *C. raciborskii* isolate, a species not previously known to be toxic.

MATERIALS AND METHODS

The blue-green alga *C. raciborskii* (Woloszynska) Seenaya and Subba Raju (12) was isolated from Solomon Dam, Palm Island (18°45′S, 146°35′E), a tropical continental island about 15 nautical miles (ca. 28 km) off the northeast Australian coast. The organism was identified as *C. raciborskii* by J. Komárek of the Institute of Botany, Třeboň, Czechoslovakia. The organism was isolated by being streaked onto agar plates (0.5% Bacto-Agar [Difco Laboratories]) made from CHU-10 medium (5) supplemented with BG-11 trace metal mix (16) at 1 ml/liter.

Unialgal cultures for toxicity testing were grown in modified CHU-10 medium buffered with 0.02 M HEPES (*N*-2′-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH adjusted to 7.55 before autoclaving). A batch technique with aeration was used with 1 liter of medium in each of 31 Erlenmeyer flasks. Starter cultures (100 ml in 250-ml flasks) were grown to the late exponential phase and then added to the 31 vessels. All cultures were incubated at 25 ± 1°C with continuous illumination from cool white fluorescent tubes at an intensity of about 90 $\mu E \cdot s^{-1} \cdot m^{-2}$ (LI-COR 188B quantum meter). The cultures were harvested at the late exponential phase by centrifugation (15 min at 10,000 × *g*) and freeze-dried. A known weight of freeze-dried algae was extracted in 0.9% (wt/vol) NaCl by ultrasonication at 4°C and centrifuged to clear the extract of cell debris, and the supernatant was tested for toxicity. Male white 3-month-old mice were inoculated intraperitoneally. Preliminary experiments were done with a wide range of concentrations, and mice were autopsied at death or when killed between 1 and 5 days after injection. A quantitative determination of the toxicity was done with eight groups of five mice each inoculated intraperitoneally with 5.25 to 336 mg of freeze-dried culture per kg of mouse; an additional group was left

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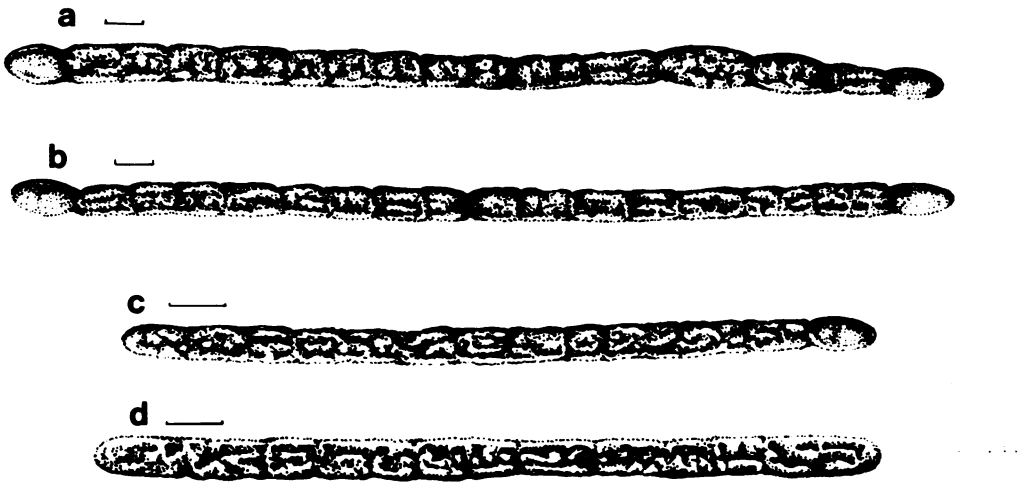

FIG. 1. *C. raciborskii*. (a) Trichome with mature akinetes; development is always close to or adjacent to the heterocysts. Gas vacuoles are indicated by darkly shaded regions in vegetative cells. (b) Sterile trichome with heterocysts at both ends; this is not an uncommon form. (c) Sterile trichome with a single heterocyst, always terminal. (d) Sterile trichome without heterocysts. Bar, 10 μm.

uninoculated. Selected tissues from all mice were preserved in 10% buffered Formol-saline. Paraffin sections were stained for histological examination with hematoxylin and eosin and phosphotungstic acid-hematoxylin. Sections for the demonstration of fat were prepared by the osmium tetroxide postfixation method (10). The 50% lethal doses were calculated by probit analysis with a Genstat program.

RESULTS

C. raciborskii (Fig. 1) has pointed terminal heterocysts occurring at one or both ends of the trichome. Akinetes are oval or barrel shaped and often contain globular inclusions. The akinetes form near the ends of the trichome, either one or several in a series. Septa of vegetative cells are indistinct, and the cells contain prominent gas vacuoles. The species has been known under a number of synonyms (9): *Anabaena* (*Anabaenopsis*) *raciborskii* (Woloszynska), *Anabaenopsis raciborskii* (Woloszynska) Elenkin, *Anabaenopsis seriata* Prescott, *Anabaenopsis maksimilianii* Obuchova, *Anabaenopsis koganii* Obuchova, *Anabaenopsis wustericum* Obuchova, *Cylindrospermum kaufmanii* (Schmidle) Huber-Pestalozzi, and *Cylindrospermum doryphorum* (Schmidle) Bruhl and Biswas.

Extracts of the *C. raciborskii* cultures proved to be toxic to mice. The 50% lethal dose at 24 h after intraperitoneal inoculation was 64 ± 5 mg of freeze-dried culture per kg of mouse. A dose of 168 mg/kg killed all mice 6 to 9 h after inoculation. Increasing that dose by 2 to 10 times caused a prolongation in survival time to 10 to 12 h. At doses near the 50% lethal dose (64 mg/kg), acutely poisoned mice died in up to 19 h; if not sacrificed, some surviving mice subacutely affected died in up to 5 days.

Affected mice were huddled, anorexic, and often had slight diarrhea, and those dying in the minimum time (6 to 9 h) terminally showed a slow gasping respiration and occasional limb paddling. Autopsy findings were related to dose. The livers of mice receiving in excess of 168 mg/kg were pale. In the range of 84 to 168 mg/kg, the livers were swollen and mottled red by a lobular pattern of congestion and hemorrhage, the lungs, kidneys, and adrenal glands were also congested, and the small intestine was congested and edematous. At a dose of 63 mg/kg or lower, the livers were pale, sometimes with white foci, particularly on the margins; this change was often associated with the presence of focal hemorrhages in the lungs.

Histopathologically, the livers showed hepatocellular coagulative necrosis (Fig. 2). This ranged from centrilobular at the lowest dose causing injury (10.5 mg/kg) to massive (involving all hepatocytes) at 168 mg/kg. These more severely affected livers showed sinusoidal congestion and often hemorrhages associated with the presence of eosinophilic material and necrotic cells in the central veins. Surviving hepatocytes showed lipidosis, a particularly marked feature of the livers of mice surviving up to 5 days. As doses exceeded 168 mg/kg, hepatocyte necrosis became more variable, at first sparing some of the centrilobular hepatocytes until, at 5 to 10 times that dose, only scattered small groups of cells were obviously necrotic, and surviving

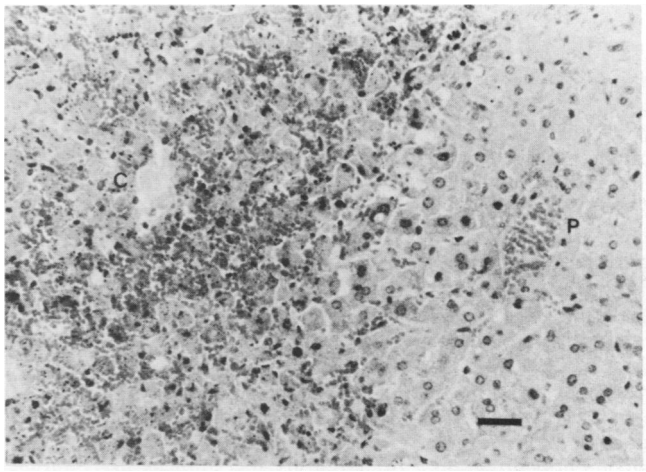

FIG. 2. Liver tissue from a mouse that died 15 h after intraperitoneal inoculation with a *C. raciborskii* extract (63 mg/kg) showing necrosis of central to midzonal hepatocytes and associated hemorrhage. C, Central vein; P, portal vein. Hematoxylin and eosin stain. Bar, 50 μm.

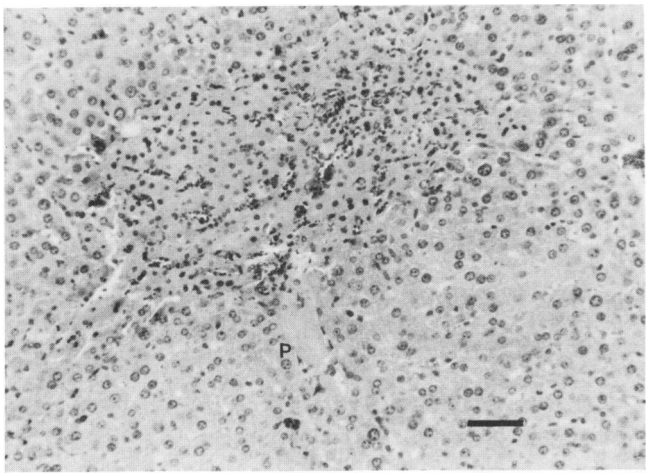

FIG. 3. Liver tissue from a mouse killed 30 h after intraperitoneal inoculation with a *C. raciborskii* extract (50 mg/kg) showing a focus of infarction associated with thrombosis of a portal vein (P). Hematoxylin and eosin stain. Bar, 50 μm.

hepatocytes were swollen and showed cytoplasmic basophilic streaking.

An additional feature was the formation of fibrin thrombi in the portal veins within the livers of some mice that received 42 to 63 mg/kg and survived 24 h. These led to liver infarcts (Fig. 3) being the explanation for the white foci seen upon gross examination. Similar thrombi were also noted in the lungs and kidneys of some of the mice in these same groups. In the lungs of many mice receiving 84 to 168 mg/kg, small arteries and capillaries contained embolic material similar to that seen in the central or hepatic veins of their livers.

Other less specific changes were also noted. The kidneys showed a variable epithelial cell necrosis, usually mild and principally involving the proximal straight tubules, and the adrenal cortices showed variable scattered epithelial cell degeneration and necrosis. The small intestine showed congestion and edema, whereas the stomach, large intestine, brain, thymus, spleen, and heart appeared normal.

DISCUSSION

C. raciborskii was originally found in a lake in Java (18) and was long believed to be confined to the tropics. Skuja (14) described the species as a fundamental component of the Indo-Malayan algal flora. The gradual extension of the range of this species into the temperate zone has been documented by Vinogradska (17), who noted that excessive growth was confined to water bodies in which the temperature exceeded 25°C. It is a truly planktonic species that characteristically forms water blooms in lakes or gently flowing streams which have high concentrations of organic matter (7, 13, 17).

Although the toxicity of a number of cyanobacteria is well documented (4), there have been no such reports for *C. raciborskii*. Toxic cyanobacteria have been shown to contain neurotoxins such as are characteristic of the species *Anabaena circinalis*, *Anabaena flos-aquae*, and *Aphanizomenon flos-aquae* or hepatotoxins such as are characteristic of the species *Microcystis aeruginosa*, *Nodularia spumigena*, and *Oscillatoria agardhei* (Jackson et al., in press). The investigations reported here clearly indicate that *C. raciborskii* is primarily hepatotoxic, although other organs can be involved. The effect on the liver at low and high concentrations is unlike that produced by microcystin, the toxin from *M. aeruginosa* and the most thoroughly studied of the hepatotoxins. Even at low doses (one-sixth of the 50% lethal dose), *C. raciborskii* causes histologically recognizable injury or death to hepatocytes near the central veins, whereas microcystin has a very steep dose-response curve (8). At very high doses the failure of *C. raciborskii* to cause significant histologically recognizable hepatocyte necrosis before the death of the animal is an interesting and unusual finding, as is the prolongation of survival time associated with this phenomenon.

A bloom of *M. aeruginosa* in a city water supply reservoir was shown to cause liver damage in a population supplied with that water (6). In the Palm Island outbreak of hepatoenteritis (2), the organisms in the original bloom in the water supply dam were not identified before treatment with copper sulfate. The alga *C. raciborskii*, which is known to form blooms in tropical environments, was subsequently observed as a seasonally dominant species in Solomon Dam, regularly appearing in the late dry season (October to December) (P. R. Hawkins and D. J. Griffiths, Report to the Queensland Government on "Chemical and biological monitoring of water quality at Solomon Dam, Palm Island," January 1985). Its severe hepatotoxic but also wide-ranging effects in mice make it an organism capable of producing the clinical disease seen at Palm Island. Although largely circumstantial, the evidence quite strongly implicates *C. raciborskii* as being responsible for the outbreak of hepatoenteritis in the human population consuming water from that particular supply. Hepatoenteritis is common in many tropical countries. The evidence presented in this paper suggests that *C. raciborskii* blooms in local water supplies should be considered as one possible cause.

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