

Toxicity of *Microcystis* Species Isolated from Natural Blooms and Purification of the Toxin

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Microcystis strains (2 toxic and 18 nontoxic to mice) were isolated from toxic waterblooms that had been collected from Lake Kasumigaura, Ibaraki Prefecture, Japan, in August 1985. Thirteen of the strains (2 toxic and 11 nontoxic) were *Microcystis aeruginosa*, 2 (nontoxic) were *Microcystis wesenbergii*, and the other 5 were difficult to identify. Six (1 toxic and 4 nontoxic *M. aeruginosa* and 1 *M. wesenbergii*) of these 20 strains were established as axenic cultures. A toxic and axenic strain of *M. aeruginosa*, K-139, was used to study the relationship between growth conditions and toxicity. Cells in early-to-mid-log phase showed the highest toxicity (50% lethal dose, 7.5 mg of cells per kg of mouse), and maximum toxicity was not affected by growth temperatures between 22 and 30°C. Purification and characterization of the toxins from K-139 cells were also conducted, and at least two toxins were detected. One of the toxins (molecular mass, 980 daltons) has not been reported previously. The main target of the toxin in mice was the liver. Marked congestion and necrosis in the parenchymal cells around the central veins of the liver were observed microscopically in specimens that had been prepared from the mice with acute toxicity after injection with the toxin.

Waterblooms of cyanobacteria appear widely in nutrient-rich fresh and brackish waters. Several of the common bloom-forming species are known to produce toxins (3, 5). *Microcystis aeruginosa* is the most common toxic cyanobacterium, and the toxicity of the blooms seems to be mainly associated with this species (12, 16). *Microcystis viridis* is also highly toxic and contributes to the toxicity of the blooms (17). The toxin(s) produced by *Microcystis* species has been isolated and designated as microcystin or cyanoginosin, which is composed of cyclic heptapeptides (1, 3). The toxins are cytotoxic and cause extensive hemorrhage in the liver (5, 6, 9). Mice exposed to the toxin die within 3 h.

The distribution of toxic *Microcystis* species in fresh water has been investigated only occasionally, and the ecology of the toxic and nontoxic species is unclear. Furthermore, few physiological, genetic, and taxonomic studies on the toxic *Microcystis* species have been conducted because axenic strains have been difficult to establish. Recently, we have developed a technique for isolating clones of *Microcystis* spp. from waterblooms by using solid media with agarose and have succeeded in obtaining axenic strains (13). In this paper, we will describe the establishment of toxic or nontoxic clones and axenic strains of this genus, the purification of the toxins from a toxic strain, and the histopathologic observations of acutely intoxicated mice after an intraperitoneal injection of the toxin.

MATERIALS AND METHODS

Media and culture. Liquid CB medium (20) and the solid form of this medium with 0.4% agarose (FMC Corp. Marine Colloids Div. Rockland, Maine) were used for culture (13). *Microcystis* cells were grown in 100 ml of liquid CB medium in 200-ml flasks or in solid CB medium in plates in an

inverted position under 2,000 lx illumination of fluorescent light (cool white) at 30°C. The cells in the broth cultures were separated by a sonifier (model B-2200J-4; Branson Sonic Power Co., Danbury, Conn.) and a vortex mixer, and the cell density was optically measured at 660 nm with a spectrophotometer (model 101; Nissei Sangyo Co., Tokyo, Japan).

Isolation of unicellular clones. Waterbloom samples were collected from Tsuchiura Bay in Lake Kasumigaura in Ibaraki Prefecture on 24 September 1985. Microscopically, the *M. aeruginosa* cells were the most abundant in the blooms. After the samples were vigorously spun with a vortex mixer to break the blooms, the samples were diluted in sterilized, deionized and distilled water and plated on the solid media. At 2 weeks after incubation, 20 of 150 colonies grown on the first plates were arbitrarily selected and established as clones. The isolation procedure for unicellular and axenic strains was described in a previous report (13). The strains isolated from the blooms were identified by following the procedure of Komárek (10).

Bioassay of toxicity. After three consecutive cycles of freeze-thawing, the cells were disrupted (model 185 sonifier; Branson) at 50 W for 3 min. The disrupted cell suspension was centrifuged at 30,000 × g for 15 min, and the resulting supernatants were used for the assay of toxicity. Female C57BL/6 mice, 12 to 15 weeks old, were used for the preliminary assay to determine cell toxicity. The cells were scored as toxic if one or more in a group of four mice was killed after being given an injection of 262 mg (dry weight) of test material that had been prepared from the cloned cells per kg of mouse. In other cases, female C3H/HeJ mice, 8 to 10 weeks old, were used. These mice were given intraperitoneal injections with the test samples and were scored for the number of deaths within 3 h, as acute toxic death. The

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TABLE 1. Established strains of *Microcystis* sp. and their toxicity

Toxicity ^a	Species	Strain (mortality) ^b
Toxic	<i>M. aeruginosa</i>	K-70 (2/4), K-139 ^c (4/4)
Nontoxic	<i>M. aeruginosa</i>	K-1, K-17, ^c K-18, K-20, K-37, K-54, ^c K-61, K-62, K-63, K-79, ^c K-81 ^c
	<i>M. wessenbergii</i>	K-36, K-55 ^c
	Unidentified	K-6, K-7, K-66, K-75, K-106

^a Dose, 262 mg (dry weight) of cells per kg of mouse.

^b Four mice per group were used for testing toxicity, and the mortality of nontoxic strains was 0/4.

^c Axenic strain.

50% lethal dose (LD₅₀) mg (dry weight) per kg of mouse was calculated by the method of Reed and Muench (11).

Purification of toxin. Two different procedures were used for the purification of toxin. The first procedure was the method of Brooks and Codd (2). Briefly, crude toxin was extracted with *n*-butanol-methanol-water from lyophilized cells and was purified through a Bond Elut C₁₈ cartridge (Analytichem International, Harbor City, Calif.) and then fractionated by Sephadex G-25 chromatography. The toxic peak was followed by reversed-phase high-pressure liquid chromatography (HPLC) over a column of Inertsil ODS (4.6 by 250 mm; Gasukuro Kogyo, Inc., Tokyo, Japan) in place of the Sep-Pak C18 cartridge or a phase column of Hypersil 50DS (4 by 250 mm). The mobile phase for HPLC was 26% acetonitrile in 10 mM ammonium acetate. The second procedure was the method of Harada et al. (8). Briefly, toxin was extracted with 5% aqueous acetic acid from the lyophilized cells and then purified by using a Baker 10 C₁₈ cartridge (J. T. Baker, Phillipsburg, N.J.) and HPLC with a Nucleosil 5C₁₈ column (4.6 by 150; Chemco Scientific Co., Osaka, Japan) under reversed-phase isocratic conditions with a mobile phase of methanol-0.05 M phosphate buffer (pH 3) (58:42 [vol/vol]).

Standard microcystins. Microcystins LR, YR, and RR were isolated and purified from *M. aeruginosa* M-228 and *M. viridis* TAC-44 (8).

Mass spectrometry and ¹H nuclear magnetic resonance. The molecular weight of the toxin was determined by secondary ion mass spectrometry by using a double-focusing mass spectrometer (model M-80B; Hitachi) with a high-field magnet, a secondary ion mass spectrometry source, and a M-0101 data system. The sample was dissolved in methanol

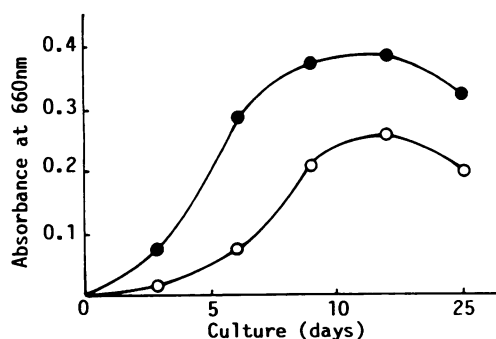


FIG. 1. Growth curves of *M. aeruginosa* K-139. Strain K-139 was grown statically at 22°C (○) or 30°C (●) under 2,000 lx.

TABLE 2. Effects of duration of culture and temperature on toxicity of *M. aeruginosa* K-139

Duration of culture (day)	LD ₅₀ (mg/kg)	
	22°C	30°C
4	NT ^a	7.3
7	7.3	37.9
13	37.0	35.3

^a NT, Not tested.

at a concentration of 10 µg/ml, and the dissolved sample (0.5 to 1.0 µl) was loaded onto the mass spectrometer. About 1 µl of glycerol-1 N HCl (1:1) matrix was added to the sample on a silver target. The toxin (10 mg) was dissolved in methanol-d₄, and the spectrum of ¹H nuclear magnetic resonance was measured with a 400-MHz spectrometer (model GHX-400; JEOL Ltd., Tokyo, Japan) by using tetramethylsilane as an internal standard.

Histopathologic investigation. Mice were examined within minutes after death or after they were killed by cervical dislocation at the indicated times after the injection of toxic materials. After visual inspection of the organs such as the liver, spleen, kidneys, adrenal glands, mesenteric lymph node, pancreas, brain, small intestine, stomach, omentum, peritoneum, heart, lungs and thymus, these organs were removed, fixed in 10% Formalin solution, sectioned, and stained with hematoxylin-eosin. These specimens were then examined with a transmitted-light microscope.

RESULTS

Isolation and toxicity of *Microcystis* strains. The LD₁₀₀ of the waterblooms (starting material) to mice was 220 mg/kg. The cloned cells isolated from the waterblooms were grown in liquid CB media and harvested in late log phase. The toxicities of the cells were examined. As shown in Table 1, 2 toxic and 11 nontoxic strains of *M. aeruginosa*, 2 nontoxic strains of *M. wessenbergii*, and 5 unidentified strains were established. Of the 20 strains, 6 strains (1 toxic *M. aeruginosa* [K-139], 4 nontoxic *M. aeruginosa* [K-17, K-54, K-79, and K-81] and 1 *M. wessenbergii* [K-55]) were axenic.

Effects of culture period and temperature on cell toxicity. *M. aeruginosa* K-139 cells, one of the established axenic strains, obtained maximum growth at day 9 at 30°C or by day

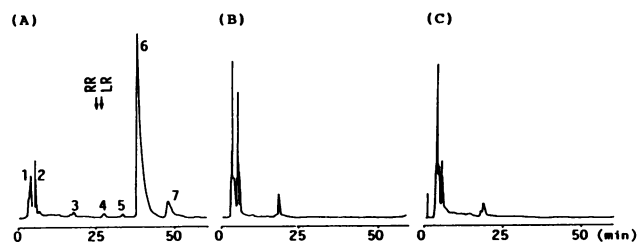


FIG. 2. High-performance liquid chromatograms of extracts from toxic and nontoxic strains. Samples were prepared by the method of Brooks and Codd (2). Column, Inertsil C₁₈ ODS (4.6 mm by 250 mm); mobile phase, 26% acetonitrile in 10 mM ammonium acetate; flow rate, 1 ml/min; detector wavelength, 240 nm. (A) *M. aeruginosa* K-139 (toxic); (B) *M. aeruginosa* K-17 (nontoxic); (C) *M. aeruginosa* K-81 (nontoxic). RR, microcystin RR; LR, microcystin LR.

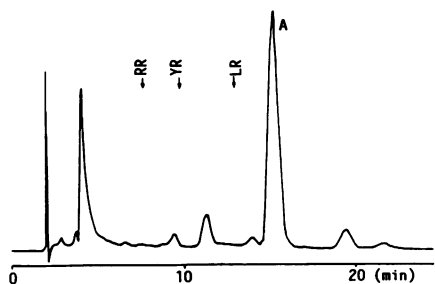


FIG. 3. High-performance liquid chromatogram of *M. aeruginosa* K-139 extract. Samples were prepared by the method of Harada et al. (8). Column, Nucleosil 5C₁₈ (4.6 by 150 mm); mobile phase, methanol-0.05 M phosphate buffer (58:42 [pH 3.0]); flow rate, 1 ml/min; detector wavelength, 238 nm. RR, Microcystin RR; YR, microcystin YR; LR, microcystin LR.

12 at 22°C (Fig. 1). The cell yield at 22°C was two-thirds of that at 30°C. There were striking differences in the toxicities among the test materials. The cells in the early-to-mid-log phase were more toxic than those in late log or stationary phase (Table 2 and Fig. 1). However, the maximum toxicities of the cells grown at 22 and 30°C were apparently the same.

Purification of toxins and their toxicity. The toxic peak fractionated by Sephadex G-25 chromatography was subjected to HPLC, and seven peaks were detected, as shown in Fig. 2. The toxicity test showed that peaks 6 and 7 represented toxins; the LD₅₀ of the toxin represented by peak 6 was 0.33 mg of dry weight per kg but that of peak 7 could not be determined because of insufficient material. Preliminary analysis of the amino acid composition of this toxin (peak 6 in Fig. 2) showed that it contained alanine, glutamic acid, leucine, arginine, aspartic acid, β-methylaspartic acid, and small amounts of other amino acids.

Since the HPLC step in the first purification procedure did not effectively resolve the different microcystins, we attempted a second purification method that consisted of

extraction with 5% aqueous acetic acid, a cleanup with octadecylsilinized silica gel, and separation by HPLC (8). Figure 3 shows the HPLC of the toxic fraction, in which peak A represented a toxin and corresponds to peak 6 in the chromatogram of Fig. 2. The secondary ion mass spectrometry spectrum of the isolated toxin with a mixture of glycerol and 1 N hydrochloric acid as the matrix gave predominantly a protonated molecule, (M+H)⁺ at *m/z* 981 (Fig. 4), indicating that the toxin has a molecular weight of 980. Figure 5 shows the ¹H nuclear magnetic resonance spectrum (400 MHz) of the toxin, and this pattern is very similar, but not identical, to those of other microcystins (19). In contrast, no peaks in the vicinities of peaks 6 and 7 and LR, RR (Fig. 2), and YR (data not shown) were detected in nontoxic K-17 and K-81 strains (LD₅₀ > 1 g/kg of mouse).

Pathologic changes. No gross pathologic changes were seen in any of the organs examined except the liver after injection of toxic materials, including that represented by peak 6. The livers appeared dark, swollen, and engorged. However, all the organs, including the livers, in the mice that were given injections of nontoxic materials were normal in appearance when examined postmortem at 3 h after injection.

No obvious findings were observed by histopathologic examination on hematoxylin-eosin-stained specimens of any organs, except the livers. The liver revealed a marked congestion around the central veins, with occasional hemorrhage, and hepatic parenchyma was compressed by sinusoids that were filled with blood (Fig. 6A). The survivors that had been given an injection of 0.25 mg of the peak 6 fraction per kg were killed and examined at 12, 24, or 69 h postinjection. Occasionally, well-circumscribed, necrotic foci were present in the liver of the mice killed at 12 h. Nuclear debris and neutrophil infiltration in the necrotic foci became very pronounced at 24 h (Fig. 6B). Macrophage infiltration in and around the necrotic foci and phagocytosis of the necrotic debris were observed at 69 h. Livers of the surviving mice given injections of 0.11 mg of the peak 6 fraction per kg showed similar findings, with smaller necrotic foci.

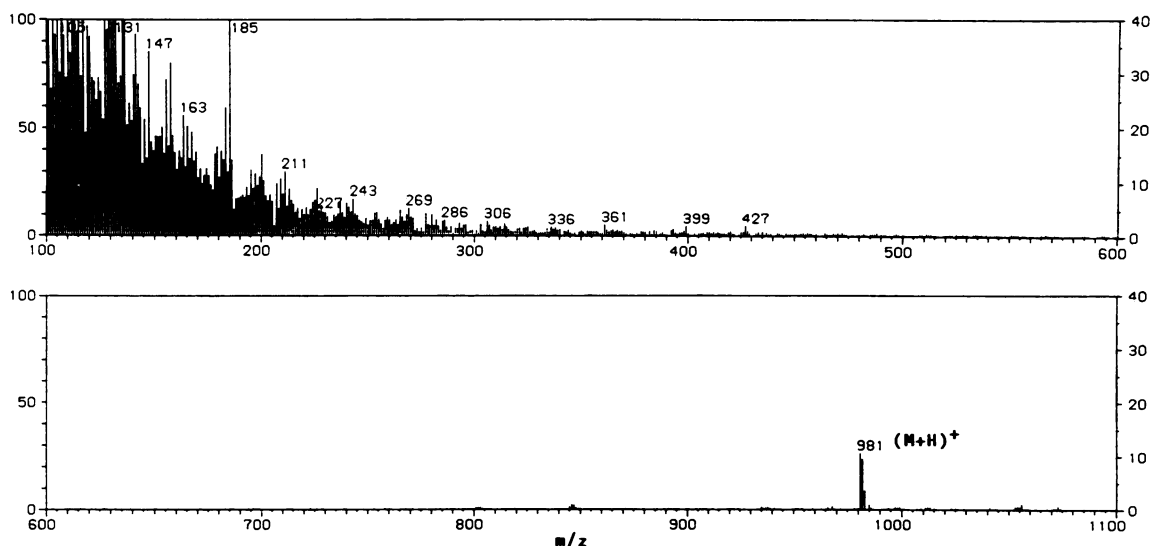


FIG. 4. Secondary ion mass spectrum of *M. aeruginosa* K-139 toxin.

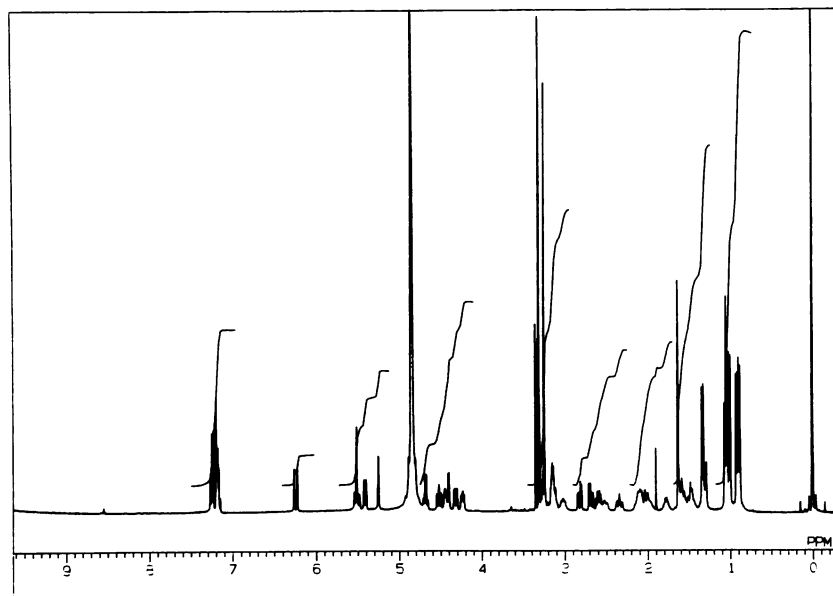


FIG. 5. ^1H nuclear magnetic resonance spectrum of *M. aeruginosa* K-139 toxin.

DISCUSSION

The phytoplankton blooms collected from Lake Kasumigaura in this investigation were toxic, and *M. aeruginosa* was the most dominant species. A total of 2 out of 20 isolates were found to be toxic and were identified as *M. aeruginosa* (Table 1). *M. aeruginosa* is the most common toxic cyanobacterium. Scott (12) reported that the toxicity of waterblooms was always associated with *M. aeruginosa* forma *aeruginosa* and that nontoxic strains of *M. aeruginosa* forma *aeruginosa* were not common in such blooms. On the other hand, Watanabe et al. (17) reported that *M. viridis* was one of the toxic species in the blooms, because the toxic blooms that had been collected from Lake Kasumigaura in August 1984 consisted of 35 to 72% *M. viridis* cells. All five cultured strains of *M. viridis* were toxic, while eight out of nine *M. aeruginosa* forma *aeruginosa* were nontoxic. In our investigation, *M. viridis* was not among the isolated strains, and toxic and nontoxic *M. aeruginosa* were the dominant strains in the blooms at the time of collection. The toxicity of the cells in culture varies according to the growth phase (Table 2). Previously (14), we demonstrated that there were toxic and nontoxic blooms in this lake. In another lake study, Carmichael and Gorham (4) reported that the phytoplankton assemblages as well as their toxicity varied greatly depending on where and when the blooms were collected. These parameters changed even on a daily basis. Because Lake Kasumigaura is a relatively big lake (168 km²; average depth, 3.9 m), the ecology of the blooms in this lake, i.e., occurrence, species composition, and toxicities of blooms, might be quite changeable depending on where and when the blooms were collected. The discrepancy between our findings and those of Watanabe et al. (17) might be explained by the above reasons.

No special morphological difference was seen among the isolated toxic and nontoxic *M. aeruginosa* strains. Moreover, morphological changes of the cells occurred while they were maintained in culture in our laboratory. Therefore, it is not possible to study toxic *Microcystis* spp. on the basis of

their morphological characteristics alone. Establishment of axenic strains of *Microcystis* is necessary for physiological, biochemical, and genetic studies. We succeeded in establishing six axenic (one toxic and four nontoxic *M. aeruginosa* and one nontoxic *M. wesenbergii*) *Microcystis* strains (Table 1).

The microcystins so far isolated were cyclic heptapeptides with molecular masses ranging from 909 to 1,044 daltons (3). An axenic strain K-139 of *M. aeruginosa* produces at least two toxins (Fig. 2 and 3), and one of them has a molecular weight of 980. Desmethylated microcystin LR, with a molecular weight of 980, which processes aspartic acid instead of β -methylaspartic acid in microcystin LR, has been isolated by Carmichael (3) and Harada et al. (K.-I. Harada, K. Matsuura, M. Suzuki, M. F. Watanabe, S. Oishi, A. M. Dahlem, V. B. Beasley, and W. W. Carmichael, Toxicon, in press). The detailed HPLC and thin-layer chromatography analyses indicated that our isolated toxin is not identical with the known desmethylmicrocystin LR. Structural studies on this toxin will be described in detail elsewhere.

The toxicity of *M. aeruginosa* cells was variable depending on the growth phase. Hughes et al. (9) reported that the toxicity of the *M. aeruginosa* NRC-1 strain was greatest in the mid-log phase (under normal-growth conditions) or in the late-log phase (under the slow-growth condition). Watanabe et al. (18) found that the toxicity of *M. aeruginosa* cells was the highest at the beginning of the stationary phase but low in the mid-log phase. In our experiments, the greatest toxicity of K-139 cells was observed in the early-to-mid-log phase. Considering these results, the toxicity of toxic *Microcystis* cells is variable, dependent upon the strains used and the culture conditions.

Histopathologic findings indicate that in the mouse the liver is the primary site in the acute toxicity of this toxin. The parenchymal cells and the endothelial cells in blood vessels in the liver were severely damaged. This observation agrees with those of Hughes et al. (9), Falconer et al. (6), and our previous observation (14).

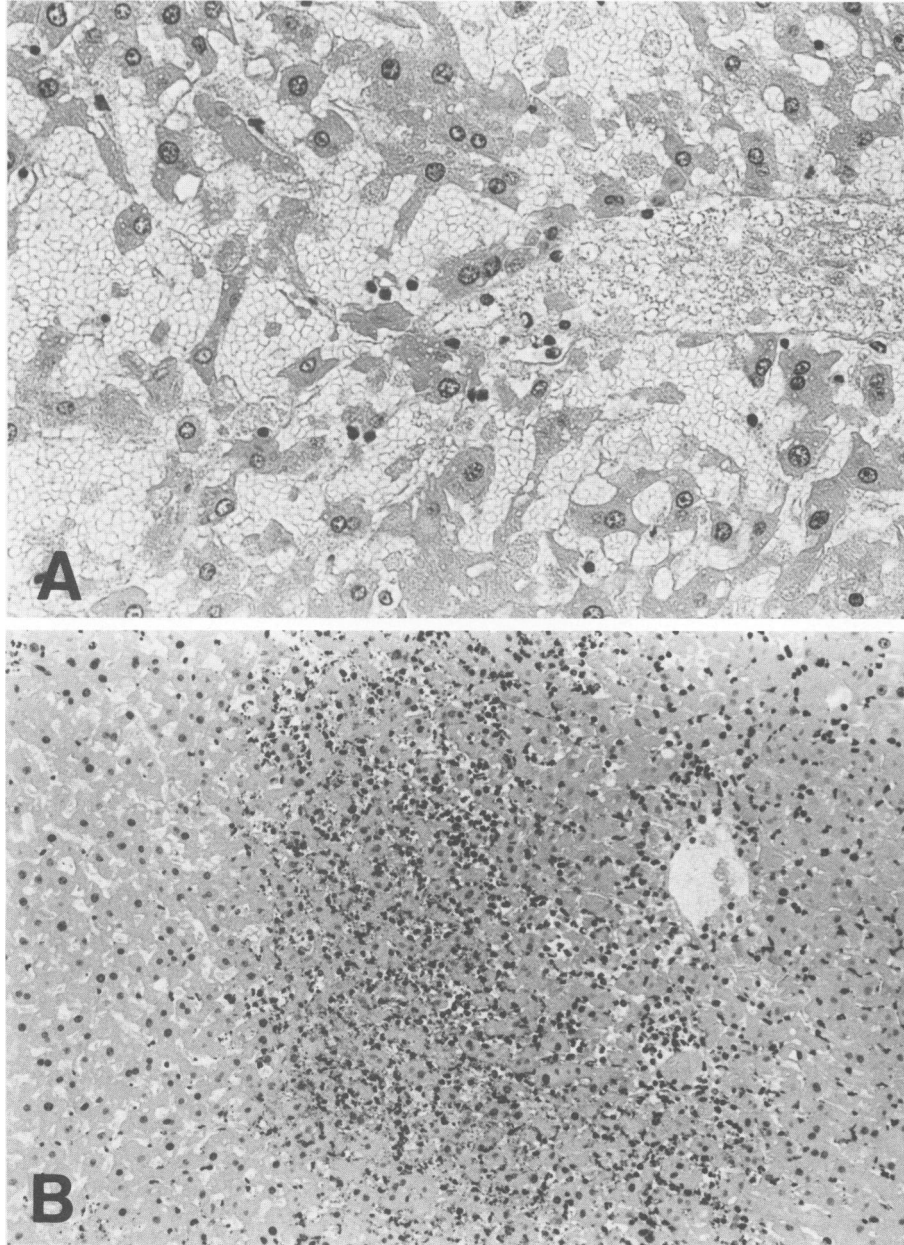


FIG. 6. Photomicrographs of the liver after toxin injection. Mice were injected with peak P6 toxin. (A) Liver of dead mouse 1.5 h after the injection of 0.36 mg toxin per kg (magnification, $\times 350$). (B) Liver of mouse sacrificed at 24 h (magnification, $\times 175$) after injection of 0.24 mg of toxin per kg.

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