Microcystin-LR Toxicodynamics, Induced Pathology, and Immunohistochemical Localization in Livers of Blue-Green Algae Exposed Rainbow Trout (*Oncorhynchus mykiss*)

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With this retrospective study, we investigated the temporal pattern of toxin exposure and pathology, as well as the topical relationship between hepatotoxic injury and localization of microcystin-LR, a potent hepatotoxin, tumor promoter, and inhibitor of protein phosphatases-1 and -2A (PP), in livers of MC-gavaged rainbow trout (*Oncorhynchus mykiss*) yearlings, using an immunohistochemical detection method and MC-specific antibodies. H&E stains of liver sections were used to determine pathological changes. Nuclear morphology of hepatocytes and ISEL analysis were employed as endpoints to detect the advent of apoptotic cell death in hepatocytes. Trout had been gavaged with lyophilized cyanobacteria (*Microcystis aeruginosa,* strain PCC 7806) at acutely toxic doses of 5700 μg microcystin (MC) per kg of body weight (bw), as described previously (Tencalla and Dietrich, 1997). Briefly, 3 control and 3 test animal were killed 1, 3, 12, 24, 48, and 72 h after bolus dosing, and livers were fixed and paraffin embedded for histological analysis and later retrospective histochemical analyses. The results of the immunohistochemistry reported here revealed a time dependent, discernible increase in MC-positive staining intensity throughout the liver, clearly not concurring with the kinetics of hepatic PP inhibition observed in the same fish and reported in an earlier publication by Tencalla and Dietrich (1997). After 3 h, marked and increasing MC-immunopositivity was observed in the cytoplasm, as well as the nuclei of hepatocytes. Apoptotic cell death could be detected after 48 h, at the very earliest. These data suggest that accumulation of MC and subsequent changes in cellular morphology, PP inhibition, and hepatocyte necrosis represent the primary events in microcystin induced hepatotoxicity and appear to be associated with the reversible interaction of MC with the PP. In contrast, apoptotic cell death, as demonstrated here, seems to be of only secondary nature and presumably results from the covalent interaction of MC with cellular and nuclear PP as well as other thiol containing cellular proteins.

Key Words: **apoptosis, fish, histopathology, immunohistochemistry, liver, microcystin, protein phosphatase-1 and -2A, serum, toxin.**

Microcystins (MC) constitute a family of toxins that are produced by several cyanobacterial taxa. These cyclic heptapeptide molecules contain both L- and D-amino acids and an unusual hydrophobic C_{20} D-amino acid commonly termed ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid). In most of the more-than-60 presently known toxin congeners, the 5 D-amino acid components are maintained while the two L-amino acids are variable (Botes *et al.*, 1985). Microcystin-LR, containing L-Leu and L-Arg, is one of the most commonly occurring (Watanabe *et al.*, 1996) and at the same time most toxic congeners, defined as having the highest capacity for protein phosphatase (PP) -1 and -2A inhibition (see below) of this class of substances (Rinehart *et al.*, 1994).

The toxicity of MC in mammals is characterized by fulminant intrahepatic hemorrhage, followed by hypovolemic shock or hepatic insufficiency and death of the animals (Carmichael, 1992, 1994). Microcystins reabsorbed from the gastrointestinal tract are believed to be taken up from the blood into the hepatocytes via a multispecific bile acid transport system (Eriksson *et al.*, 1990; Hooser *et al.*, 1991b; Runnegar *et al.*, 1995a, 1981, 1991). At acutely toxic doses, rounding of hepatocytes occurred concurrently with the loss of normal hepatic architecture. The latter pathological changes are considered to result from the interaction of MC with serine/threonine protein phosphatases-1 and -2A (PP), essential for maintaining the monomerization (phosphorylation)/polymerization (dephosphorylation) equilibrium of the cytoskeletal intermediate filaments (Eriksson *et al.*, 1992, 1989; Falconer and Yeung, 1992). Through MC-mediated inactivation of PP, this equilibrium is shifted towards monomerization and dissociation of the cytoskeleton (Eriksson *et al.*, 1992, 1989; Falconer and Yeung, 1992). Analogous, however not identical, pathological changes to those reported for mammals were also observed in fish treated with purified MC or cyanobacterial material (Phillips *et al.*, 1985; Råbergh *et al.*, 1991; Tencalla and Dietrich, 1997).

Microcystin-LR, representative for other MC congeners, was shown to interact with the catalytic subunit of PP (PPc) in a two-step mechanism (Craig *et al.*, 1996). This biphasic

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reaction involves a rapid, reversible binding (usually within minutes to a few hours) and inactivation of PPc followed by a much delayed covalent interaction (requiring several h) (Craig *et al*., 1996; MacKintosh *et al*., 1995; Runnegar *et al*., 1995b). The reversible interaction with PP is of hydrophobic and ionic nature, involving ADDA and Masp/Glu residues (Bagu *et al*., 1997; Nishiwaki-Matsushima *et al*., 1991; Rinehart *et al*., 1988; Stotts *et al*., 1993). This reversible interaction, believed to optimally position the MC-LR molecule to PPc, is considered prerequisite for covalent binding of MC-LR to the PPc. The question, however, remains whether the pathological changes observed in the hepatocytes of mammals and fish result from the rapid but reversible interaction of MC with PP or whether covalent binding to PP, and therefore long-term PP inhibition, is necessary for the initiation of the subsequent cascade of changes. Circumstantial evidence supporting the hypothesis that the reversible binding of MC-LR/MC to the PPc is sufficient for PP inhibition and development of liver pathology is provided by the fact that mice and rats experimentally dosed with MC die within 1 to 3 h following exposure (Kaya, 1996; Kaya and Watanabe, 1990), i.e., a time frame in which only a small amount of MC would be expected to have undergone covalent interaction with the PP. In addition, since MC was reported to be rapidly eliminated via biliary excretion (Sahin *et al*., 1996) following conjugation to glutathione and endogenous thiol containing proteins (Kondo *et al*., 1996; Pflugmacher *et al*., 1998) PP-MC reversible binding appears to compete with conjugation (detoxifying) and binding reactions to other thiol-containing proteins. If indeed the detoxifying reactions compete with PP for MC and the reversible reaction (MC-PP binding) is responsible for the reported PP inhibition, then the inhibition of PP, measured as total PP activity in the livers of exposed animals, should at least in part be reversible also. The latter hypothesis would also suggest that covalently bound MC should be immunohistochemically detectable in livers of exposed animals much later than the concurrent inhibition of hepatic PP.

In order to test the above hypothesis, a retrospective immunohistochemical study was conducted using yearling rainbow trout that were administered acutely toxic doses of the microcystin-producing cyanobacteria *Microcystis aeruginosa* PCC 7806 via gavage (Tencalla and Dietrich, 1997). Uptake of the toxin into the liver was monitored with anti-MC antibodies and compared to the onset of PP inhibition reported in an earlier publication by Tencalla and Dietrich (1997). In addition, the pathogenic time course of microcystin-induced hepatotoxicity and form of cell death (necrosis vs. apoptosis) was investigated.

MATERIALS AND METHODS

For this retrospective study, paraffin-embedded liver samples taken from an earlier study by Tencalla and Dietrich (1997) were employed. Briefly, in that earlier study, yearling rainbow trout ($n = 36$) with a mean weight of 96 ± 11 g, purchased from a local fish hatchery, had been orally dosed with approximately 1 ml suspension of freeze-dried *M. aeruginosa* and dechlorinated tap water. The bolus doses, amounting to an equivalent of 5700 μg microcystin-LR per kg body weight, had been directly applied into the fish stomachs using blunt-tip gavage syringes (Asic, Denmark). Three test and 3 control fish had been killed at 1, 3, 12, 24, 48, and 72 h after bolus dosing, and liver and blood samples collected.

In order to achieve a more complete understanding of the events involving microcystin-induced liver pathology, the results obtained in this retrospective study were combined and compared with the data on PP inhibition and blood enzyme parameters of the same fish published earlier by Tencalla and Dietrich (1997).

Anti-MC Antibodies

Aminoethyl-MC (AE-MC) was synthesized as described by Mikhailov *et al*. (manuscript submitted). Briefly, conjugation of AE-MC with the carrier proteins (50 μg of MCLR per mg of soybean trypsin inhibitor [SBTI, Sigma, U.S.]) was accomplished with 2% glutaraldehyde at pH 8.7, followed by multiple dialysis to ensure the absence of free toxin. Polyclonal antibodies were raised in white New Zealand rabbits (both females and males) by immunization with conjugates of the AE-MCLR with SBTI. The rabbit polyclonal antibodies were affinity purified.

Histopathology

Tissues were processed in a standard fashion. Briefly, liver samples were fixed in 4% neutral buffered formalin for several h, dehydrated, paraffin embedded, and archived. Sections of 3–5 μm were mounted on aminopropyltriethoxysilane-coated slides (APTS, A-3648, Sigma, USA). Following deparaffinization in 3 xylene baths, sections were rehydrated, stained with hematoxylin and eosin (H&E), and mounted with Crystal/Mount™ (Biømeda, USA) for later pathological assessment. Liver samples were also fixed in osmium tetraoxide for electron microscopic analyses.

Immunohistochemistry

Paraffin-embedded sections were deparaffinized, rehydrated, and incubated with type XIV bacterial protease (P-5147, Sigma, USA) in PBS for antigen retrieval at 37°C for 15 min. Endogenous peroxidase was blocked with 3% H_2O_2 for 15 min. Endogenous biotin was blocked with a specific blocking kit (Avidin/Biotin blocking kit, Vector Inc., USA). Microcystin-LR antiserum was applied in a humidified atmosphere for 16–20 h at 4°C. Antigen-primary antibody complex visualization was achieved using biotin-conjugated secondary antibodies, HRP-labeled streptavidin and AEC-chromogen (3-amino-9-ethylcarbazole) (Super Sensitive™, BioGenex, USA). Sections were counterstained for 15 sec with hematoxylin, rinsed in tap water and mounted with Crystal/Mount™ (Biømeda, USA).

Detection of Apoptotic Cells

Recognition of apoptotic nuclei/bodies in the paraffin-embedded tissue sections was achieved either by morphological evaluation of H&E-stained slides or by histochemical fragment end labeling of DNA (ISEL). The latter was performed using an *in situ* DNA-fragment end labeling kit (FragEL™; Oncogene, USA) according to the manufacturer's instructions. A semiquantitative apoptotic index (apoptotic cells/field) was determined by counting ISEL-positive cells of 7 randomly chosen fields. Fields were defined by a 10×10 grid ($10 \times$ ocular) and a $20 \times$ objective ($= 200 \times$ magnification).

Statistics

The Shapiro-Wilks and Bartlett's tests were used to assess the normality of the data distribution and the homogeneity of variance, respectively, and the semiquantitative apoptotic index was analyzed using ANOVA followed by

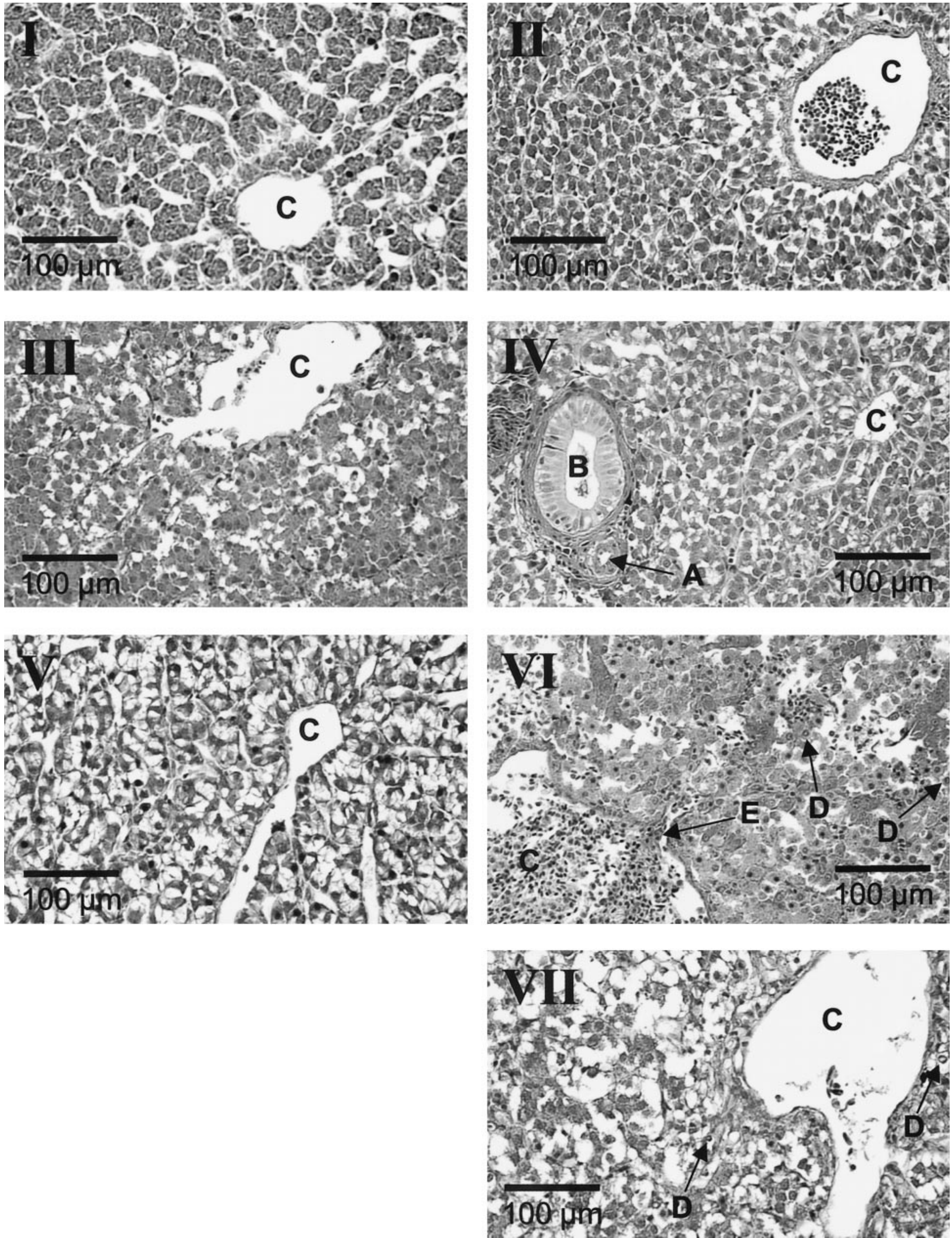

FIG. 1. H&E-stained sections of livers from rainbow trout gavaged with toxic cyanobacteria equivalent to 5.7 mg MC $\times$ kg^{-1} bw at the following times after dosing: I, control section; II, 1 h; III, 3 h; IV, 12 h; V, 24 h VI, 48 h, and VII, 72 h. A, arteriole; B, bile duct; C, central vein; D, condensed chromatin, typical of apoptosis; E, ruptured vessel with hemorrhage.

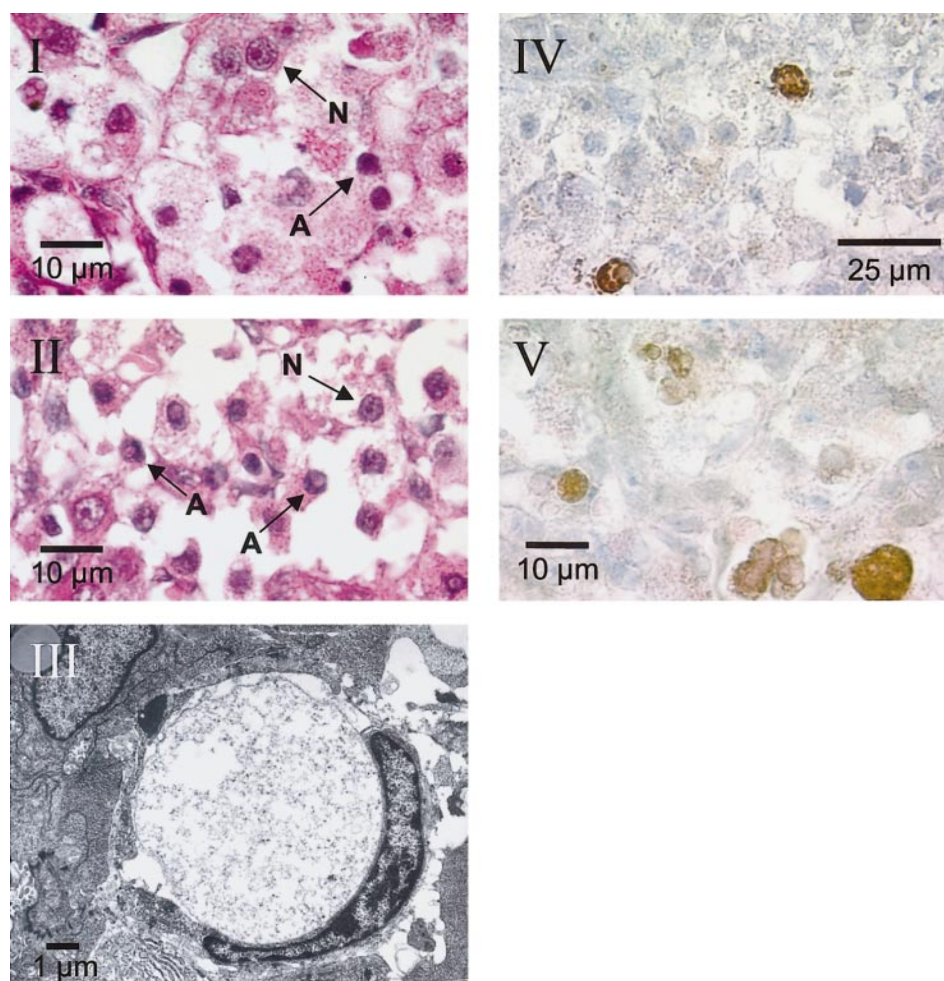

FIG. 2. Apoptotic cells in rainbow trout livers 72 h after gavage with toxic cyanobacteria equivalent to 5.7 mg MC $\times$ kg^{-1} bw. I, H&E-stained section showing moderate pathology and II, showing severe pathology; III, transmission electron micrograph of condensed chromatin in hepatocyte nucleus; IV, ISEL-stained section 600 $\times$ magnification and V, ISEL-stained section 1000 $\times$ magnification. A, condensed chromatin, typical of apoptotic cells; N, normal-appearing nuclei.

Tukey's multiple comparison test (Toxstat, 1991) via log base-10 transformation of the original data.

RESULTS

Antibodies

Polyclonal antisera did not react with free BSA or Tris-blocked ReactiBind™ plates nor with free SBTI adsorbed on polyvinylchloride plates (Falcon 3912, MicroTestIII Flexible Assay Plate, Becton Dickinson, USA).

Mortality and Gross Morphology

As reported by Tencalla and Dietrich (1997), no mortality occurred either during gavage or throughout the test duration (72 h). Visual inspection of the gastrointestinal tract at the respective time points during the experiment had demonstrated a progressive development of yellowish discoloration in livers of treated fish. However, no increase in either liver size or liver weight had been observed, when compared to the respective controls.

Retrospective Histopathology

Changes in the cord-like organization of hepatocytes could already be seen 1 h post application of the bolus dose. These changes appeared primarily in the pericentral region of the liver (Fig. 1:II) and were characterized by the appearance of hepatocytes with condensed cytoplasm (Figs. 1:II–1:VII). Between 3 and 12 h, the latter changes became progressively more pronounced involving larger areas of the liver. In addition, small hemorrhages could be detected where sinusoids appeared to be ruptured (Fig. 1:VI). An involvement of the entire liver was observed as of 24 h post-dosing. Overt lysis of hepatocyte membranes (necrotic cells) was observed, as of 48 h post-dosing, in many cases associated with pyknotic nuclei (Fig. 1:VI and 1:VII).

Apoptosis

Morphological alterations of nuclei consistent with those typical for apoptosis (Wyllie *et al*., 1980), were observed in the H and E stained sections and electron microscopic micrographs of liver as of 48 h post-dosing, however not in all of the

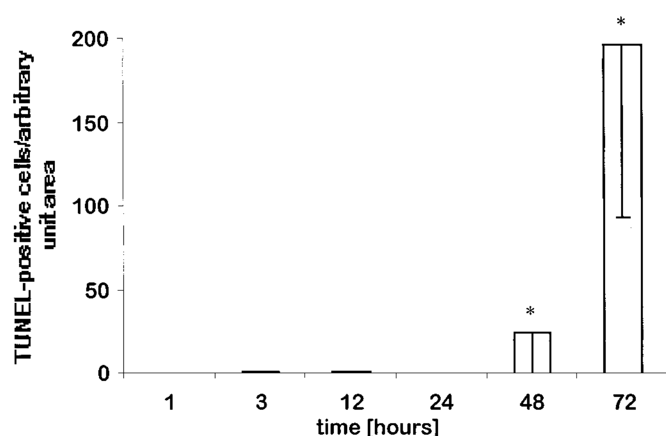

FIG. 3. Semiquantitative numbers of ISEL-positive cells in liver sections of rainbow trout gavaged with toxic cyanobacteria equivalent to 5.7 mg MC × kg^{-1} bw at 1, 3, 12, 24, 48, and 72 h after dosing. ANOVA followed by Tukey's multiple comparison test were used for statistical analysis. *Statistically significant at $p < 0.05$.

exposed fish (Figs. 2: I–2:III). As of 72 h post-dosing, livers of all fish exhibited apoptotic nuclei. Histochemical fragment end-labeling of DNA (ISEL) revealed a similar pattern of appearance of apoptotic nuclei (Figs. 2:IV and 2:V), i.e., partial positivity at the 48 h time point and ISEL positivity in all sections at 72 h. Semiquantitative evaluation of apoptotic nuclei in the liver sections corroborated the visual impression described above, in that significantly higher numbers of apoptotic nuclei were detectable as of 48 h post-dosing only, dramatically increasing at the 72 h time point (Fig. 3).

Immunohistochemistry

MC immunopositive staining was observed as of 12 h post-dosing. At this time point, the immunopositive staining did not involve the whole liver section but rather was restricted to isolated cells (Fig. 4). Positive staining increased progressively in the number of cells involved as well as in intensity of staining with increasing time post-dosing, culminating in strong cytoplasmic as well as nucleic staining of hepatocytes throughout the liver section of fish killed at the 72 h time point (Fig. 4).

DISCUSSION

Rainbow trout gavaged with lyophilized microcystin-producing cyanobacteria, *M. aeruginosa* PCC 7806, demonstrated characteristic pathological changes in the liver, including necrosis and apoptosis of hepatocytes, as well as intrahepatic hemorrhage (Fig. 1). These changes are comparable to those reported in mammals (Hooser *et al*., 1989; Lovell *et al*., 1989; Solter *et al*., 1998; Yoshida *et al*., 1998, 1997). The sequence of hepatic cell death, with necrosis appearing very early and apoptosis rather late in the development of the liver damage, has also been observed in mice treated with acutely toxic doses of microcystin (Yoshida *et al*., 1998). These observations therefore suggest that the sequence of pathological events underlying microcystin-induced liver damage is highly comparable in mammalian and fish species. Consequently, this also implies that the biochemical reactions leading to this sequence of events are similar or identical in the two classes.

The comparison of the retrospective study presented here with the biochemical data of the original study (Tencalla and Dietrich, 1997) (Fig. 5), suggests some incoherence with regard to the mechanism underlying the development of microcystin-induced liver pathology. Indeed, a rapid uptake of microcystin from the gastrointestinal tract into the blood, concurrent with high levels of extractable microcystin in the liver and almost complete PP inhibition within 3 h following gavage, was reported in the original study (Tencalla and Dietrich, 1997), whereas the concentrations of microcystin in the blood and liver decrease and PP activity recuperates to almost 50% the original value by end of the study (Fig. 5). These observations contrast the early onset of necrosis which progressively increased in severity toward the end of our study period (72 h, Fig. 1), as well as to a rather late appearance of immunohistochemically detectable microcystin in the tissue sections (Fig. 4), as reported in the retrospective study here. These seemingly contradictory findings, however, indicate that rapid MC uptake is responsible for the early onset of PP inhibition and necrosis. Methanol-extractable (free or non-covalently bound) hepatic MC, also reaches peak levels simultaneously with the almost complete inhibition of endogenous hepatic PP activity (3 h post gavage). The subsequent decrease in free or non-covalently-bound hepatic MC and concurrent recuperation of endogenous PP activity, suggests that rapid PP inhibition is (1) directly dependent on the concentration of available unbound MC; and (2) a result of this reversible interaction of MC with the catalytic subunit of the protein phosphatases-1 and -2A (PPc). The latter interpretation is supported by several observations:

- Hydrophobic and ionic interactions of MC occur with PPc involving Adda and Masp/Glu residues (Bagu *et al*., 1997; Nishiwaki-Matsushima *et al*., 1991; Rinehart *et al*., 1988; Stotts *et al*., 1993);
- These are considered to be essential primary events in the biochemical mechanism of MC-induced PP inhibition;
- PP inactivation was suggested to precede covalent modification of PPc;
- MC-LR was shown to interact with PP *in vitro* in a 2-step mechanism (Bagu *et al*., 1997; Craig *et al*., 1996; MacKintosh *et al*., 1995) involving rapid binding and inactivation of the PPc occurring within minutes, followed by a slower covalent binding within hours (Craig *et al*., 1996);
- Last but not least, endogenous hepatic PP were already recuperating, i.e., retrieving some of their activity, when MC was first immunohistochemically detectable in the livers.

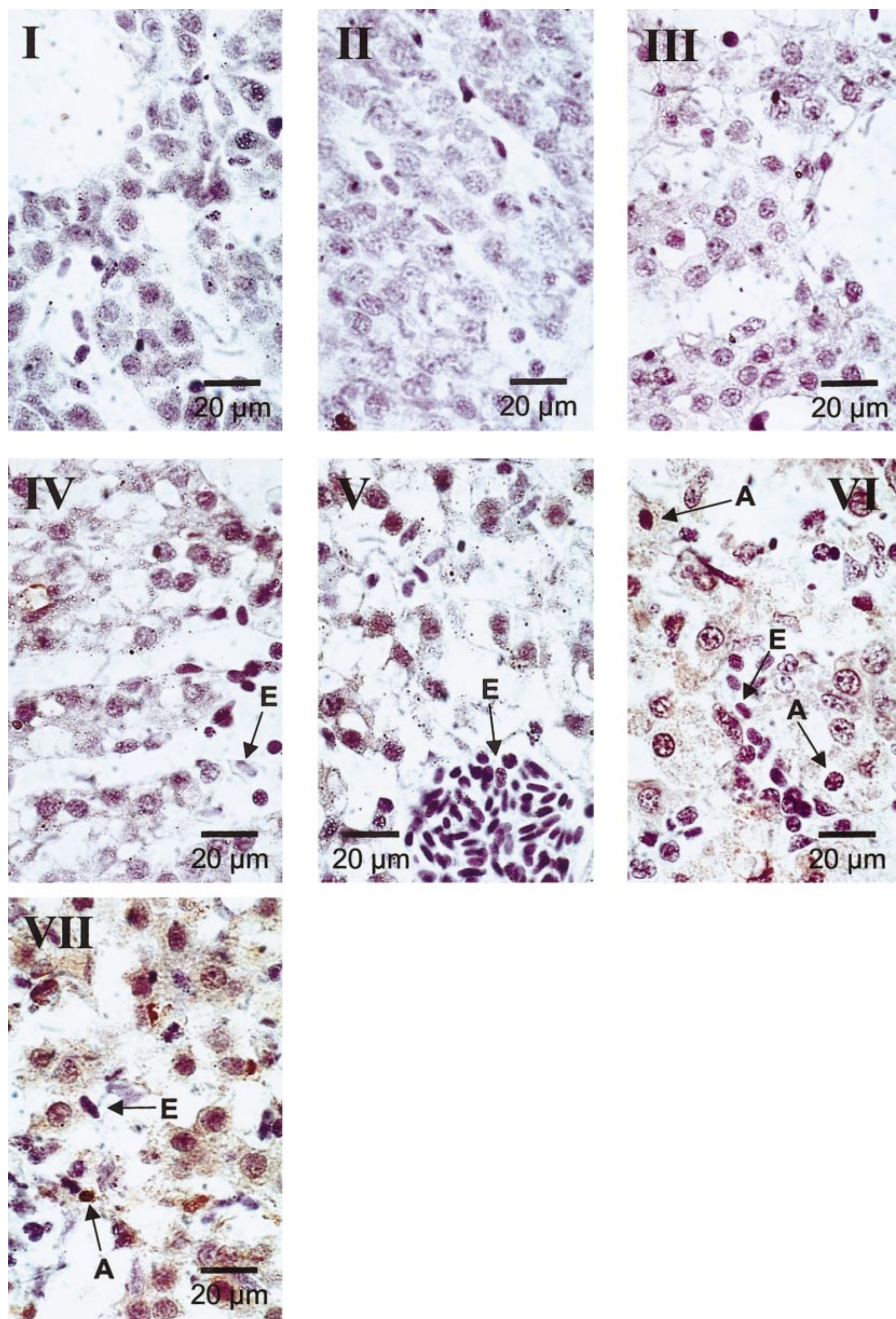

FIG. 4. Immunohistochemical staining with anti-MC antiserum in livers of rainbow trout gavaged with toxic cyanobacteria equivalent to 5.7 mg MC $\times$ kg^{-1} bw at the following times after dosing: I, control section; II, 1 h; III, 3 h; IV, 12 h; V, 24 h; VI, 48 h, and VII, 72 h. A, condensed chromatin, typical of apoptotic cells; E, erythrocyte.

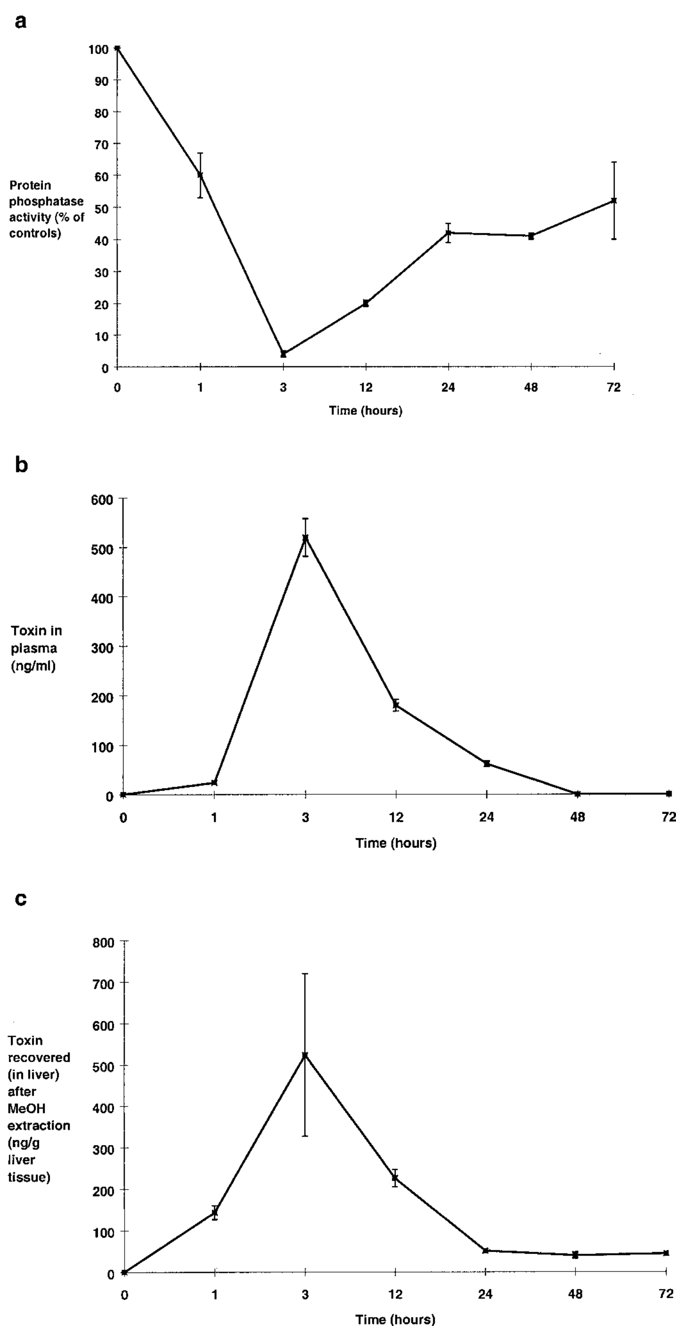

FIG. 5. (a) Hepatic protein phosphatase activities in trout after gavage with toxic *Microcystis aeruginosa* PCC 7806 at the equivalent dose of 5.7 mg MC $\times$ kg^{-1} bw. (b) MC concentrations found in plasma and (c) extractable MC concentrations in liver tissue. All points represent averages $\pm$ SD (reprinted from Tencalla and Dietrich, 1999. Biochemical characterization of microcystin toxicity in rainbow trout (*Oncorhynchus mykiss*). *Toxicon* **35,** 583–595, with permission from Elsevier Science).

However, the question still remains whether the reversible inhibition of PP is sufficient or even solely responsible for the induction of the observed hepatocyte necrosis. Previous *in vitro* studies with mouse, rat, and fish hepatocytes demonstrated that MC-induced cytoskeletal alterations occur within minutes (mammalian hepatocytes) (Eriksson *et al*., 1989; Hooser *et al*., 1991a; Toivola *et al*., 1997) to hours (fish hepatocytes) (Fladmark *et al*., 1998) following MC exposure and resulted in necrotic or apoptotic death of the hepatocytes. If indeed non-covalently bound MC was solely responsible for hepatic necrosis, then one would have to assume that once the level of unbound MC in the liver had decreased (as shown by Tencalla and Dietrich (1997) (Fig. 5) hepatic necrosis would stagnate. The time course of hepatic MC concentrations measured via methanol-extraction as reported by Tencalla and Dietrich (1997) (Fig. 5) most likely does not represent the true concentrations of MC present in the liver. Indeed, two factors simultaneously and independently may contribute to the apparent decrease of extractable MC after 3 h. One is biliary excretion of MC or its metabolites (Sahin *et al*., 1996), the second being the slow covalent addition of MC to the catalytic subunit of PP and other thiol-containing cellular proteins (Hitzfeld *et al*., 1999) e.g., glutathione (Kondo *et al*., 1996). While the former mechanism (biliary excretion) would progressively reduce the amount of free hepatic MC, covalent binding of microcystin to the PP would prevent extractability of MC and therefore contribute to the underestimation of actual amounts of toxin present in the liver. This interpretation is consistent with previous data showing that a covalent complex between PP and the toxin is resistant to organic solvents (Craig *et al*., 1996). The above observations suggest that large amounts of non-covalently bound MC are present in the liver at 3 h, which are capable of rapidly but reversibly inhibiting PP activity while being excreted at the same time. Biliary excretion of biochemically active MC also peaked 3 h after gavage in rainbow trout (Sahin *et al*., 1996). This rapid but reversible PP-MC interaction is also subject to association-dissociation kinetics. While dissociated (unbound) MC can undergo conjugation and biliary excretion, associated MC can slowly form a covalent bond with the PP, thus forming an increasingly higher amount of irreversibly inhibited hepatic PP. The PP inhibition time course, as reported by Tencalla and Dietrich (1997) (Fig. 5), thus represents the sum of the reversible and irreversible interactions of MC with hepatic PP. This would therefore explain why protein phosphatase activities recovered (Fig. 5) after having come to near complete inhibition within 3 h after dosage, and suggests that MC covalently bound to PP also contributes to the progressive necrosis observed *in vivo*.

The observed increase in ISEL positive and morphologically apoptotic hepatocytes clearly is a late event in the sequence of pathological changes observed in livers of trout exposed to MC. Apoptotic hepatocytes appear even later than the first MC-immunopositive hepatocytes. This suggests that a covalent interaction of MC with PP or other thiol-containing proteins and peptides may be necessary to induce the observed apoptosis. Particularly, the inhibition of PP-2A is assumed to play a role in the aetiology of apoptosis in hepatocytes following MC exposure. Indeed, MC-induced apoptotic cell death was reported in rat hepatocytes *in vitro*, whereas exposure to inhib-

itors 1 and 2 (specific inhibitors of PP1) do not induce apoptotic morphology (Mellgren *et al*., 1993).

In summary, the above data suggest that the rapid PP inhibition, cytoskeletal changes, and early onset of liver necrosis in trout, and possibly in the mammalian species, i.e., the acute symptoms of intoxication, are associated with the reversible interaction of MC with hepatic PP. Hepatocyte necrosis appears to be primarily associated with the reversible and irreversible inhibition of PP1, whereas apoptosis, a late event, is associated with the irreversible inhibition of PP-2A. The importance in the above distinction may lie in the observation that under subchronic exposure conditions, apoptosis, as well as necrosis of hepatocytes, are observed in rats exposed to low doses of MC (Solter *et al*., 1998). Although still mere speculation, the simultaneous occurrence of these two cell death pathways under chronic low-dose exposure, could form the basis for increased hepatic regenerative cell proliferation concurrently with unwarranted survival of pre-apoptotic cells, and thus the beginning of preneoplastic lesions.

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REFERENCES

Bagu, J. R., Sykes, B. D., Craig, M. M., and Holmes, C. F. (1997). A molecular basis for different interactions of marine toxins with protein phosphatase-1—Molecular models for bound motuporin, microcystins, okadaic acid, and calyculin A. *J. Biol. Chem*. **272,** 5087–5097.

Botes, D. P., Wessels, P. L., Kruger, H., Runnegar, M. T. C., Santikarn, S., Smith, R. J., Barna, J. C. J., and Williams, D. H. (1985). Structural studies on cyanoginosins-LR, -YR, -YA, and -YM, peptide toxins from Microcystis aeruginosa. *J. Chem. Soc. Perkin Trans*. **1,** 2747–2748.

Carmichael, W. W. (1992). Cyanobacteria secondary metabolites—the cyanotoxins (Review). *J. Appl. Bacteriol*. **72,** 445–459.

Carmichael, W. W. (1994). The toxins of cyanobacteria. *Sci. Amer*. **270,** 78–86.

Craig, M., Luu, H. A., McCready, T. L., Williams, D., Andersen, R. J., and Holmes, C. F. B. (1996). Molecular mechanisms underlying the interaction of motuporin and microcystins with type-1 and type-2A protein phosphatases. *Biochem. Cell Biol*. **74,** 569–578.

Eriksson, J. E., Brautigan, D. L., Vallee, R., Olmsted, J., Fujiki, H. and Goldman, R. D. (1992). Cytoskeletal integrity in interphase cells requires protein phosphatase activity. *Proc. Natl. Acad. Sci. USA* **89,** 11093–11097.

Eriksson, J. E., Grönberg, L., Nygård, S., Slotte, J. P., and Meriluoto, J. A. O. (1990). Hepatocellular uptake of 3H-dihydromicrocystin-LR, a cyclic peptide toxin. *Biochim. Biophys. Acta* **1025,** 60–66.

Eriksson, J. E., Paatero, G. I. L., Meriluoto, J. A. O., Codd, G. A., Kass, G. E. N., Nicotera, P. and Orrenius, S. (1989). Rapid microfilament reorganization induced in isolated rat hepatocytes by microcystin-LR, a cyclic peptide toxin. *Exp. Cell Res*. **185,** 86-100.

Falconer, I. R., and Yeung, D. S. K. (1992). Cytoskeletal changes in hepatocytes induced by microcystis toxins and their relation to hyperphosphorylation of cell proteins. *Chem. Biol. Interact*. **81,** 181–196.

Fladmark, K. E., Serres, M. H., Larsen, N. L., Yasumoto, T., Aune, T., and Døskeland, S. O. (1998). Sensitive detection of apoptogenic toxins in suspension cultures of rat and salmon hepatocytes. *Toxicon* **36,** 1101–1114.

Hitzfeld, B., Fischer, W., Eriksson, J., Mikhailov, A., Tencalla, F., and Dietrich, D. R. (1999). Toxins of cyanobacteria in fish: Immunohistochemical and immunocytochemical localization in livers and hepatocytes of rainbow trout. *Naunyn-Schmiedeberg's Arch. Pharmacol*. **359,** R159.

Hooser, S. B., Beasley, V. R., Lovell, R. A., Carmichael, W. W., and Haschek, W. M. (1989). Toxicity of microcystin-LR, a cyclic heptapeptide from *Microcystis aeruginosa*, to rats and mice. *Vet. Pathol*. **26,** 246–252.

Hooser, S. B., Beasley, V. R., Waite, L. L., Kuhlenschmidt, M. S., Carmichael, W. W., and Haschek, W. M. (1991a). Actin filament alterations in rat hepatocytes induced *in vivo* and *in vitro* by microcystin-LR, a hepatotoxin from the blue-green alga, *Microcystis aeruginosa*. *Vet. Pathol*. **28,** 259–266.

Hooser, S. B., Kuhlenschmidt, M. S., Dahlem, A. M., Beasley, V. R., Carmichael, W. W., and Haschek, W. M. (1991b). Uptake and subcellular localization of tritiated dihydro-microcystin-LR in rat liver. *Toxicon* **29,** 589–601.

Kaya, K. (1996). Toxicology of microcystins. In *Toxic Microcystis* (M. Watanabe, K. Harada, W. Carmichael, and H. Fujiki, Eds.), pp. 175–202. CRC Press, Boca Raton.

Kaya, K., and Watanabe, M. M. (1990). Microcystin composition of an axenic clonal strain of *Microcystis viridis* and *Microcystis viridis*-containing waterblooms in Japanese freshwaters. *J. Appl. Phycol*. **2,** 173.

Kondo, F., Matsumoto, H., Yamada, S., Ishikawa, N., Ito, E., Nagata, S., Ueno, Y., Suzuki, M., and Harada, K. (1996). Detection and identification of metabolites of microcystins formed *in vivo* in mouse and rat livers. *Chem. Res. Toxicol*. **9,** 1355–1359.

Lovell, R. A., Schaeffer, D. J., Hooser, S. B., Haschek, W. M., Dahlem, A. M., Carmichael, W. W., and Beasley, V. R. (1989). Toxicity of intraperitoneal doses of microcystin-LR in two strains of male mice. *J. Env. Pathol. Toxicol. Oncol*. **9,** 221–237.

MacKintosh, R. W., Dalby, K. N., Campbell, D. G., Cohen, P. T., Cohen, P., and MacKintosh, C. (1995). The cyanobacterial toxin microcystin binds covalently to cysteine-273 on protein phosphatase 1. *FEBS Lett*. **371,** 236-240.

Mellgren, G., Vintermyr, O. K., Boe, R., and Doskeland, S. O. (1993). Hepatocyte DNA replication is abolished by inhibitors selecting protein phosphatase 2A rather than phosphatase 1. *Exp. Cell. Res*. **205,** 293–301.

Nishiwaki-Matsushima, R., Nishiwaki, S., Ohta, T., Yoshizawa, S., Suganuma, M., Harada, K.-I., Watanabe, M. F., and Fujiki, H. (1991). Structure-function relationships of microcystins, liver tumor promoters, in interaction with protein phosphatase. *Jpn. J. Cancer Res*. **82,** 993–996.

Pflugmacher, S., Wiegand, C., Oberemm, A., Beattie, K. A., Krause, E., Codd, G. A., and Steinberg, C. E. (1998). Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR: The first step of detoxication. *Biochim. Biophys. Acta* **1425,** 527–533.

Phillips, M. J., Roberts, R. J., Stewart, J. A., and Codd, G. A. (1985). The toxicity of the cyanobacterium *Microcystis aeruginosa* to rainbow trout, *Salmo gairdneri* Richardson. *J. Fish Dis*. **8,** 339–344.

Råbergh, C. M. I., Bylund, G., and Eriksson, J. E. (1991). Histopathological effects of microcystin-LR, a cyclic peptide toxin from the cyanobacterium (blue-green alga) *Microcystis aeruginosa*, on common carp *(Cyprinus carpio L.)*. *Aquat. Toxicol*. **20,** 131–146.

Rinehart, K. L., Harada, K.-I., Namikoshi, M., Chen, C., Harvis, C. A., Munro, M. H. G., Blunt, J. W., Mulligan, P. E., Beasley, B. R., Dahlem, A. M., and Carmichael, W. W. (1988). Nodularin, microcystin, and the configuration of Adda. *J. Am. Chem. Soc*. **110,** 8557–8558.

Rinehart, K. L., Namikoshi, M., and Choi, B. W. (1994). Stucture and biosynthesis of toxins from blue-green algae (cyanobacteria). *J. Appl. Phycol*. **6,** 159–176.

Runnegar, M., Berndt, N. and Kaplowitz, N. (1995a). Microcystin uptake and inhibition of protein phosphatases: effects of chemoprotectants and self-inhibition in relation to known hepatic transporters. *Toxicol. Appl. Pharmacol.* **134,** 264-272.

Runnegar, M., Berndt, N., Kong, S. M., Lee, E. Y., and Zhang, L. (1995b). *In vivo* and *in vitro* binding of microcystin to protein phosphatases 1 and 2A. *Biochem. Biophys. Res. Commun*. **216,** 162–169.

Runnegar, M. T. C., Falconer, I. R. and Silver, J. (1981). Deformation of isolated rat hepatocytes by a peptide hepatotoxin from the blue-green alga Microcystis aeruginosa. *Naunyn-Schmiedeberg's Arch. Pharmacol*. **317,** 268-272.

Runnegar, M. T. C., Gerdes, R. G., and Falconer, I. R. (1991). The uptake of the cyanobacterial hepatotoxin microcystin by isolated rat hepatocytes. *Toxicon* **29,** 43–51.

Sahin, A., Tencalla, F. G., Dietrich, D. R., and Naegeli, H. (1996). Biliary excretion of biochemically active cyanobacteria (blue-green algae) hepatotoxins in fish. *Toxicology* **106,** 123–130.

Solter, P. F., Wollenberg, G. K., Huang, X., Chu, F. S., and Runnegar, M. T. (1998). Prolonged sublethal exposure to the protein phosphatase inhibitor microcystin-LR results in multiple dose-dependent hepatotoxic effects. *Toxicol. Sci*. **44,** 87–96.

Stotts, R. R., Namikoshi, M., Haschek, W. M., Rinehart, K. L., Carmichael, W. W., Dahlem, A. M., and Beasley, V. R. (1993). Structural modifications imparting reduced toxicity in microcystins from *Microcystis spp*. *Toxicon* **31,** 783–789.

Tencalla, F., and Dietrich, D. (1997). Biochemical characterization of microcystin toxicity in rainbow trout (Oncorhynchus mykiss). *Toxicon* **34,** 583–595.

Toivola, D. M., Goldman, R. D., Garrod, D. R., and Eriksson, J. E. (1997). Protein phosphatases maintain the organization and structural interactions of hepatic keratin intermediate filaments. *J. Cell Sci*. **110,** 23–33.

Toxstat Ver. 3.3 (1991). University of Wyoming.

Watanabe, M. F., Harada, K.-I., Carmichael, W. W., and Fujiki, H. (1996). *Toxic Microcystis*, p. 262. CRC Press, Boca Raton, FL.

Wyllie, A. H., Kerr, J. F., and Currie, A. R. (1980). Cell death: The significance of apoptosis. *Int. Rev. Cytol*. **68,** 251–306.

Yoshida, T., Makita, Y., Tsutsumi, T., Nagata, S., Tashiro, F., Yoshida, F., Sekijima, M., Tamura, S.-I., Harada, T., Maita, K., and Ueno, Y. (1998). Immunohistochemical localization of microcystin-LR in the liver of mice: A study on the pathogenesis of microcystin-LR-induced hepatotoxicity. *Toxicol. Pathol*. **26,** 411–418.

Yoshida, T., Makita, Y., Nagata, S., Tsutsumi, T., Yoshida, F., Sekijima, M., Tamura, S.-I., and Ueno, Y. (1997). Acute oral toxicity of microcystin-LR, a cyanobacterial hepatotoxin, in mice. *Nat. Toxins* **5,** 91–95.