Toxic cyanobacteria in drinking water supplies can cause serious public health problems. In the present study we analyzed the time course of changes in lung histology in young and adult male Swiss mice injected intraperitoneally (ip) with a cyanobacterial extract containing the hepatotoxic microcystins. Microcystins are cyclical heptapeptides quantified by ELISA method. Ninety mice were divided into two groups. Group C received an injection of saline (300 µl, ip) and group Ci received a sublethal dose of microcystins (48.2 µg/kg, ip). Mice of the Ci group were further divided into young (4 weeks old) and adult (12 weeks old) animals. At 2 and 8 h and at 1, 2, 3, and 4 days after the injection of the toxic cyanobacterial extract, the mice were anesthetized and the trachea was occluded at end-expiration. The lungs were removed en bloc, fixed, sectioned, and stained with hematoxylin-eosin. The percentage of the area of alveolar collapse and the number of polymorphonuclear (PMN) and mononuclear cell infiltrations were determined by point counting. Alveolar collapse increased from C to all Ci groups (123 to 262%) independently of time, reaching a maximum value earlier in young than in adult animals. The amount of PMN cells increased with time of the lesion (52 to 161%). The inflammatory response also reached the highest level earlier in young than in adult mice. After 2 days, PMN levels remained unchanged in adult mice, while in young mice the maximum number was observed at day 1 and was similar at days 2, 3, and 4. We conclude that the toxins and/or other cyanobacterial compounds probably exert these effects by reaching the lung through the blood stream after ip injection.
age to the tissue, which may lead to death or, in a chronic intoxication, may promote hepatic tumors (3). Microcystins in the liver can also induce hepatocytes to produce arachidonic acid metabolites such as prostacyclin (6-Keto F$_1$α) and thromboxane (TXB$_2$) by stimulation of the cyclooxygenase pathway (4). This observation suggests that microcystins can promote inflammation, which may contribute to the hepatic shock that leads to death.

There are some reports from different regions of the world of human intoxication due to toxic cyanobacteria ingestion (5,6). In Brazil, more than 100 chronic renal patients were intoxicated in the town of Caruaru in 1996 and 52 died due to intravenous exposure to water containing microcystins during dialysis treatment (7-9).

In addition to the oral and intravenous routes of intoxication, human beings can be exposed to cyanotoxins through inhalation. This may occur in recreational water containing cyanobacteria or their toxins. Fitzgeorge et al. (10) observed extensive necrosis of the epithelium of both olfactory and respiratory zones in mice that received a purified microcystin-LR (MCYST-LR) by the intranasal route. Through this route, the LD$_{50}$ of this toxin was the same as that observed after administration by the intraperitoneal (ip) route. These investigators suggested that the phenomenon results from the necrosis of the nasal epithelium, which facilitates the absorption of the toxin into the blood stream through the nasal capillaries.

However, there are few reports analyzing the effects of microcystins on the lungs. Turner et al. (11) reported a case of two recruits in England who developed severe pneumonia after contact with water containing toxic Microcystis aeruginosa, and Slatkin et al. (12) detected pulmonary thrombosis in mice injected with lethal doses of microcystins. It has been already demonstrated that this toxin can reach the lung, and its distribution after oral and intratracheal administration has been well described by Ito et al. (13,14).

The aim of the present study was to analyze the time course of changes in lung histology in young and adult mice injected ip with a cyanobacterial extract containing microcystins.

A toxic M. aeruginosa strain (NPJB-1) was cultured in ASM-1 medium as described by Ferrão-Filho and Azevedo (15). The culture was not axenic, but the concentration of bacteria was considered to be very low. At the exponential growth phase (between 15 and 20 days) the cells were harvested, concentrated with a Pellicon Cassette System (Millipore, Billerica, MA, USA) that uses a tangential flow multiple filter technique, re-suspended in deionized water, and disrupted by cycles of freeze-thawing. Particulate organic matter was removed from the solution by filtration through fiberglass filters and the extract containing dissolved microcystins was used in the experiments. This strain has already been described as a producer of two types of microcystins: MCYST-LR and MCYST-LF (16). For this study, the total microcystins present in the strain extract were quantified by ELISA using the Envirologix Inc. (Portland, ME, USA) commercial plate kits, according to the manufacturer’s protocol.

Ninety male Swiss mice were divided into two groups. The control group (C, N = 30 adult animals) received an ip injection of 0.9% NaCl (300 µl) and the test group (Ci) received an ip injection of a sublethal dose of cyanobacterial extract containing microcystins (48.2 µg/kg body weight). The animals of the test group were equally divided into two groups according to age, i.e., young (4 weeks old) and adult (12 weeks old). At 2 and 8 h and at 1, 2, 3, and 4 days after the ip injection of the toxic cyanobacterial extract, five animals from the control and test groups were anesthetized with sevoflurane (2 minimal alveolar concentration), the trachea was occluded at the end of expiration and the
lungs were removed en bloc.

The right lung was quick-frozen by immersion in liquid nitrogen and fixed with Carnoy’s solution (ethanol:chloroform:acetic acid, 70:20:10) at -70°C for 24 h (17). The Carnoy’s solution was replaced with progressively increasing ethanol concentrations at -20°C up to 100% ethanol. The tissue was maintained at -20°C for 4 h, warmed to 4°C for 12 h, and then allowed to reach and remain at room temperature for 2 h. After fixation, the tissue was embedded in paraffin, blocks were cut into 4-µm thick sections with a microtome and the slices were stained with hematoxylin-eosin. Each slide received a code and two investigators who were unaware of the origin of the material performed microscopic examination.

Morphometric analysis was performed using an integrating eyepiece and a 100-point grid consisting of 50 lines of known length coupled to a conventional light microscope (Axioplan, Zeiss, Oberkochen, Germany) (18). The fraction of the area of collapsed alveoli was determined by the point-counting technique at 200X magnification across 10 random non-coincident microscopic fields. Points falling on collapsed alveolar spaces were counted and divided by the total number of points in each microscopic field. Polymorphonuclear (PMN) and mononuclear cells and pulmonary tissue were evaluated at 1000X magnification. Points falling on the tissue area were counted and divided by the total number of points in each microscopic field. Thus, data were reported as the fractional area of pulmonary tissue. The same method was applied to determine the amount of PMN cells. All morphometric parameters determined for all groups were compared by one-way ANOVA and the level of significance was set at 5% in all analyses. Statistical analyses were performed using the SigmaStat software (Jandel Scientific, San Rafael, CA, USA).

The percentages of alveolar collapse and PMN cells are illustrated in Figure 1. The fraction of alveolar collapse was higher in all test groups compared to control, independently of the time of analysis, reaching a maximum value earlier in young than in adult animals. The amount of PMN cells increased with the temporal evolution of the lesion. It is interesting to observe that in young mice the inflammatory response also reached its highest level earlier than in adult animals. After two days, PMN levels remained unchanged in adult mice, while in young mice the maximum level was observed at day 1 and was similar at days 2, 3 and 4 (Figure 2). Additionally, young animals responded with an earlier increase of PMN, followed by collapse whereas adult mice exhibited an inverse profile. These differences could probably be attributed to discrepancies in inflammatory mechanisms, i.e., in young mice, together with PMN infiltration, type II pneumocytes may also be altered very early in the course of lung injury, leading to surfactant dysfunction and alveo-
lar collapse, while in adult mice alveolar collapse peaked at 8 h.

Therefore, we observed that the *M. aeruginosa* extract containing microcystins generated a rapid inflammatory process in mouse lungs, with interstitial edema and recruitment of inflammatory cells that remained stable until day 4, starting earlier in young animals. There was 100% survival until the end of the experiment.

Ito et al. (14) administered a similar sublethal dose of MCYST-LR intratracheally to mice and, using an immunostaining method, observed the presence of this toxin in the lungs until 7 h after injection. After this time, the tissue no longer showed any staining. They also detected a few stained macrophages in the lung and no lesion until the end of the experiment, 2 weeks later. Our results showed that cyanobacterial extracts containing microcystins lead to a rapid inflammatory response in the lung, which continued until the fourth and last day of the experiment. In addition, we observed alveolar collapse that also started rapidly and reached a maximum level earlier in young animals (Figure 1).

According to Naseem et al. (19), MCYST-LR can stimulate alveolar macrophages to produce inflammatory mediators. Nakano et al. (20) observed that TNF-α production by cultured macrophages from mouse peritoneal exudates is stimulated by purified microcystins and toxic and non-toxic extracts of *M. aeruginosa* (a non-lipopolysaccharide producer). They also observed that the toxic extract was more effective than purified microcystins. Therefore, it is important to consider that other cyanobacterial secondary metabolites present in the extract may induce inflammatory responses in lung or act synergistically with microcystins to promote inflammation.

We cannot rule out the presence of bacteria in the extract, which might contribute to those inflammatory effects. However, bacterial contamination of the *M. aeruginosa* culture was found to be very low and therefore negligible in the final volume of *M. aeruginosa* after the concentration process.

In the present study, these toxins and/or other cyanobacterial compounds may have caused the effects described above by reach-

---

**Figure 2.** Photomicrographs of lung parenchyma stained with hematoxylin-eosin. A, Control group; B, C, D, E, F, and G, adult mice sacrificed at 2 and 8 h and at 1, 2, 3, and 4 days after the injection of cyanobacteria extract, respectively. Photographs were taken at an original magnification of 200X. As shown in the insets (1000X) polymorphonuclear cells were the predominant cell type in this process.
ing the lung through the blood stream after ip injection. Therefore, intravenous exposure to microcystins can represent a risk to the lungs in addition to the known targets, i.e., liver and kidney. Thus, whenever human health depends on the quality of water for direct consumption and recreational or medical use, such as dialysis treatment, the increase of cyanobacterial blooms producing microcystins in the water supplies ought to be carefully considered.

References