Effect of microcystin on leukocyte viability and function

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Abstract

Microcystin (MC) has been found in several areas of the world. In addition to its hepatotoxicity, microcystin may have an immunomodulatory effect.

Considering that patients receiving hemodialysis may be chronically exposed to variable concentrations of MC, and that they present important changes in this immune response, we have assessed the effect of MC on the function of leukocytes.

Polymorphonuclear leukocytes isolated from healthy volunteers (HV) and patients receiving hemodialysis (HD) were incubated with microcystin (10 µg/L) for 24 h and evaluated for reactive oxygen species production (ROS), phagocytosis and apoptosis. Monocytes incubated with and without LPS (100 ng/mL) and microcystin for 24 h were assessed for TNFα and IL-10 production.

Leukocytes of HV presented an increase in apoptosis rates and leukocytes from HD exhibited a lower production of oxygen-reactive species, both spontaneously and after stimulus with S. aureus, when compared with leukocytes incubated without toxin.

Monocytes presented an increase in cytokine production after stimulation by LPS in both groups, but there was no difference between the groups with and without MC that were incubated with or without LPS.

Low concentrations of microcystin can induce mild changes in leukocyte function of HV and HDP, particularly in the ability to produce ROS.

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1. Introduction

Cyanobacteria are found in several areas of the world and have shown increased incidences of blooms as a result of changes in the level of nutrients in the water and in the weather conditions (Kuiper-Goodman et al., 1999).

Several species of cyanobacteria produce toxins (cyanotoxins), which are released after cell death. Some of these toxins may cause skin irritation, whereas others are neurotoxic. However, the most common intoxications are caused by hepatotoxic peptides (Kuiper-Goodman et al., 1999).

The most common hepatotoxin is microcystin (MC), produced by Microcystis, Anabaena, Nostoc, and Oscillatoria species. This toxin inhibits protein phosphatase 1 and 2A, and acute microcystin poisoning in mammals is
characterized by disruption of hepatic architecture leading to massive intrahepatic hemorrhage, hepatic failure and hypovolemic shock (Kuiper-Goodman et al., 1999).

In vitro studies have shown that hepatocytes exposed to MC immediately develop apoptosis. Other cell types also evolve with apoptosis, however, higher concentrations and longer exposure to the toxin are needed for this response (McDermott et al., 1998). Additionally, the generation of oxygen-reactive species has also been involved in the pathophysiology of hepatic lesions induced by MC (Ding et al., 2001).

Some authors have suggested a possible immunomodulatory effect of MC. Hernández et al. (2000) showed that microcystin and nodularin cyanotoxins increase the spontaneous adhesion of human peripheral polymorphonuclear leukocytes. There is also an evidence that they can suppress the lymphocytic function by decreasing the lymphoproliferation induced by lypopolysaccharide and dose-dependent in vitro inhibition of the production of polyclonal antibodies (Shen et al., 2003). The modulation of cytokine production by microcystin has also been demonstrated (Nakano et al., 1989, 1991).

Intoxications of human populations by oral consumption of water contaminated with toxic strains of cyanobacteria have been described in Australia, England and China (Kuiper-Goodman et al., 1999).

In 1996, in Caruaru (Brazil), 126 patients from a hemodialysis clinic (HD) presented visual complaints, nausea and vomiting after a hemodialysis session. Later on, 100 patients developed acute liver failure, with a total of 52 deaths. The water analysis identified two groups of cyanotoxins: microcystin and cylindrospermopsin. Comparing the victims’ symptoms and the pathological findings with the findings in animal studies, the investigators concluded that the main toxin to which the patients were exposed was MC (Jochimsen et al., 1998). Since then, a lot has been discussed about the presence of cyanotoxins in the water reservoirs all over the country, and in the water offered to dialysis centers. In Brazil alone, around 60,000 patients are in chronic hemodialysis therapy (http://www.datasus.gov.br), and they are in contact with more than 300 L of water per week.

The World Health Organization (WHO) recommends water for oral consumption to have MC levels of less than 1 µg/L (Kuiper-Goodman et al., 1999), and although there is no specific recommendation about the levels in the water for hemodialysis, it is expected that this toxin is not present. Water treatments recommended for hemodialysis have been relatively efficient in purging this toxin. However, these and other processes considered to be more efficient in the removal of MC, such as chlorination and ozonation, may not be sufficient during periods of blooming or when a large organic load is present; in these settings, the monitoring of toxin levels must be intensified during the water treatment process (Hitzfeld et al., 2000).

It is well known that acute exposure of patients receiving HD to MC leads to hepatic toxicity with hemorrhage and death (Jochimsen et al., 1998), but little has been studied about the risks of chronic exposure of these patients to small amounts of MC. Actually, there are many factors that could lead to the possibility of chronic exposure to MC during dialysis treatment. These factors include the frequent presence of small amounts of MC in the water supply at hemodialysis centers, the non-guaranteed complete removal of MC by activated charcoal and by reverse osmosis, and the sub-optimal control of water quality at many dialysis centers.

Based on the possibility that patients with chronic renal failure (CRF) receiving hemodialysis may be subject to chronic exposure of low concentrations of MC, we investigated the possible effect of MC in the leukocyte function in individuals receiving hemodialysis and in healthy individuals.

2. Patients and methods

A total of 17 mL heparinized blood was collected from healthy volunteers (n = 10), and 10 mL from patients with chronic renal failure (CRF) receiving hemodialysis for at least 3 months (n = 10).

Microcystin was supplied by Prof. W.W. Carmichael (Wright State University, EUA) in a 15 µg/mL solution (ethanol) which was re-diluted in PBS to a concentration of 100 µg/L to be used in cells.

In the first phase, a dose-response study was performed to determine the concentration for the next stages. To this end, polymorphonuclear (PMN) leukocytes from six healthy individuals were incubated with three different concentrations of MC (0.1, 1, and 10 µg/L) 24 h, and evaluated for viability, apoptosis, oxidative metabolism and phagocytosis.

In a second phase, PMN from 10 patients receiving HD (HD) and from 10 healthy volunteers (HV) incubated with microcystin (10 µg/L) 24 h were analyzed and evaluated similarly to the first phase, and compared to the control group without the toxin in each group of individuals.

In the third phase, mononuclear cells (PBMC) from both groups were incubated with 10 µg/L MC 24 h and evaluated for the production of cytokines with and without lipopolysaccharide (LPS) stimulation.

2.1. Laboratory methods

2.1.1. PMN isolation

In brief, leukocyte preparations containing 95–98% PMN were isolated by Ficoll-Hypaque (Sigma Chemical Co., St Louis, MO, NY, USA) density gradient centrifugation and dextran sedimentation (Metcalf et al., 1986). Residual erythrocytes were lysed with hypotonic saline and the cells (1 × 10⁹/mL) were suspended in phosphate buffered saline (PBS, Sigma, St Louis, MO, USA).
2.1.2. Incubation of PMN with microcystin

A 2 mL suspension of PMN (5 x 10^6 cells/mL) was aliquoted into 5 mL test-tubes with culture medium RPMI supplemented with 50% autologous plasma with or without MC (0.1, 1 or 10 μg/L). The suspension was then incubated for 24 h at 37 °C in 5% CO₂ and analyzed for apoptosis. The same process was performed with PMN in the second phase and with PBMC in the third phase.

2.1.3. Cell apoptosis

Cell apoptosis was measured by Annexin V staining. One of the cell membrane changes during the early and intermediate stages of cell apoptosis is the translocation of phosphatidylserine (PS) from the inner to the outer layer of the cell membrane. Annexin V binds to the PS residue. To evaluate apoptosis, cells were washed in phosphate-buffered saline (PBS-Sigma, Co., USA) and density was adjusted to 1 x 10^7/mL. One hundred microlitres of the solution (1 x 10^7 cells) was transferred to a 5 mL culture tube; 5 μL annexin V-FITC (Pharmingen, BD, USA) and 2 μL of propidium iodide (PI) were added and the cells were incubated for 15 min at room temperature in the dark; 400 μL of binding buffer was added to each tube. The following controls were used: unstained cells, cells stained with Annexin V-FITC (no PI) and cells stained with PI (no Annexin-FITC). The degree of apoptosis was assessed by flow cytometry (FACScalibur analyzer; Becton Dickinson Immunocytometry Systems, CA, USA) within 1 h (Omerod, 1998). Cells staining positive for PI were considered as dead cells (necrosis or late apoptosis), cells staining positive only for annexin V were considered as apoptotic, and cells negative for both were considered as viable.

2.1.4. Phagocytosis and ROS production

Phagocytosis was evaluated using heat-killed S. aureus, strain ATCC 25923 (Difco, Detroit, MI, USA), labeled with propidium iodide (PI) at 5%, 2.4 x 10^7 colony-forming units/mL. The PMN ROS production was assessed by examining the ability of PMN to respond to an appropriate stimulus. To do this, cells were incubated with PI-labeled S. aureus, 4-ß phorbol 12-ß-myristate 13-acetate (PMA, 100 ng/mL, Sigma, St Louis, MO, USA). A mixture of 100 μL of heparinized whole blood, 100 μL of PI-labeled S. aureus and 900 μL of PBS were prepared in a plastic tube in order to evaluate phagocytosis. Results were presented as percentage of cells ingesting S. aureus (Hasui et al., 1989).

To study unstimulated and stimulated ROS production, a mixture of 100 mL of heparinized whole blood, 200 mL of 0.3 mM 2’’7’dichlorofluorescein diacetate (DCFH-DA, Sigma, St Louis, MO, USA) in PBSg, 700 μL of PBS, and 100 μL of each stimulus (S. aureus, PMA, and FMLP) were prepared in separate plastic tubes. To evaluate unstimulated ROS production, one of the tubes received no stimulus, and 800 μL of PBS was used. The tubes were incubated with agitation for 30 min at 37 °C in a shaking water bath, and then 2 mL of ethylene diamine tetraacetic acid (EDTA, Sigma, St Louis, MO, USA) were added in order to terminate phagocytosis. After centrifugation, the erythrocytes were removed by hypotonic lysis as described above, and the leukocyte pellet was resuspended in 1.0 mL of 3 mM EDTA in PBSg. Intracellular DCFH and PI-labeled S. aureus fluorescence of PMN were studied by flow cytometry (Hasui et al., 1989).

Histograms of the fluorescence intensity were constructed for each tube and the geometric mean of the fluorescence intensity (mean fluorescence intensity—MFI) of DCFH in that population of cells was determined. Phagocytosis was determined by assessing the percentage of cells staining positive for PI.

2.1.5. Flow cytometry analysis

Intracellular DCFH fluorescence of PMN was determined by flow cytometry (FACScalibur analyzer; Becton Dickinson Immunocytometry Systems, CA, USA). In the final suspension, monocytes, lymphocytes, a few contaminating erythrocytes, aggregated cells and debris were excluded from analysis using a gate analysis method based on forward light scatter and side scatter (linear 90° light scatter). Histograms of the fluorescence intensity were constructed for each tube and the geometric mean of the fluorescence intensity (mean fluorescence intensity—MFI) of that population of cells was determined. For each experimental condition, we subtracted the MFI of the control tube from the MFI of the test tube. For the unstimulated cells and cells exposed to PMA, the control tube contained cells and buffer, without DCFH-DA. For the S. aureus-stimulated cells, control tubes contained PI-stained S. aureus.

2.1.6. Assessment of cytokine production in monocytes of peripheral blood

After the isolation described above, four aliquots of PBMC of each subject (n=4 in each group) with 1 x 10^6 cells/mL each were incubated with RPMI, RPMI + LPS (100 ng/mL), RPMI + microcystin (10 μg/L) and RPMI + LPS + microcystin (10 μg/L) for 24 h in CO₂ at 37 °C. After incubation, the cells were kept at −70 °C until analysis. Immunoenzymatic assays were performed to assess the production of tumor necrosis factor-alpha (OPTEIA Human TNF-α Elisa BD, USA) and interleukin 10 (OPTEIA Human IL-10 Elisa BD, USA).

3. Statistical analysis

The results were presented as median and extremes. ANOVA was used for the analysis of results with different concentrations of microcystin. The non-parametric Wilcoxon Signed Rank Test was used to compare the results obtained with toxin and each corresponding control group. The rejection level of the null hypothesis was set at 5%.
Table 1
Viability, apoptosis, necrosis, ROS production and phagocytosis in PMN isolated from patients with CRF receiving HD (HD) and from healthy volunteers (HV), incubated with or without 10 µg/L microcystin (MC) 24 h (n = 10)

<table>
<thead>
<tr>
<th></th>
<th>HD</th>
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<tbody>
<tr>
<td>Viability (%)</td>
<td>59 (19–70)</td>
</tr>
<tr>
<td>Apoptosis (%)</td>
<td>15 (8–51)</td>
</tr>
<tr>
<td>Necrosis (%)</td>
<td>7 (2–176)</td>
</tr>
<tr>
<td>Spontaneous ROS (MFI)</td>
<td>90 (14–225)</td>
</tr>
<tr>
<td>S. aureus stimulated ROS (MFI)</td>
<td>325# (76–786)</td>
</tr>
<tr>
<td>PMA stimulated ROS (MFI)</td>
<td>478# (172–1645)</td>
</tr>
<tr>
<td>Phagocytosis (%)</td>
<td>84 (64–94)</td>
</tr>
<tr>
<td>HD + MC</td>
<td>62 (21–72)</td>
</tr>
<tr>
<td></td>
<td>14 (5–54)</td>
</tr>
<tr>
<td></td>
<td>5 (1–17)</td>
</tr>
<tr>
<td></td>
<td>65* (14–175)</td>
</tr>
<tr>
<td></td>
<td>271#* (53–415)</td>
</tr>
<tr>
<td></td>
<td>284# (19–1385)</td>
</tr>
<tr>
<td></td>
<td>75 (32–92)</td>
</tr>
<tr>
<td>HV</td>
<td>70 (45–86)</td>
</tr>
<tr>
<td></td>
<td>10 (0.3–25)</td>
</tr>
<tr>
<td></td>
<td>8 (2–24)</td>
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<tr>
<td></td>
<td>27 (10–75)</td>
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<tr>
<td></td>
<td>125# (19–244)</td>
</tr>
<tr>
<td></td>
<td>331# (29–627)</td>
</tr>
<tr>
<td></td>
<td>78 (55–89)</td>
</tr>
<tr>
<td>HV + MC</td>
<td>64 (48–89)</td>
</tr>
<tr>
<td></td>
<td>13* (1–29)</td>
</tr>
<tr>
<td></td>
<td>9 (2–26)</td>
</tr>
<tr>
<td></td>
<td>24 (8–65)</td>
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<tr>
<td></td>
<td>104# (19–258)</td>
</tr>
<tr>
<td></td>
<td>244# (36–653)</td>
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</tbody>
</table>

ROS, in vitro production of peroxide per cell (PMN). S. aureus, after stimulation with S. aureus. PMA, after stimulation with phorbol myristate acetate. *P < 0.05 vs. compared to controls without the toxin. #P < 0.05 vs. spontaneous ROS. MFI, mean fluorescence intensity.

(P < 0.05). True Epistat program was used to perform the statistical analysis.

4. Results

4.1. Determination of dose-response of microcystin in PMN of healthy volunteers

There was no statistically significant difference in viability or necrosis of PMN leukocytes of healthy volunteers with MC in concentrations of 0.1, 1 or 10 µg/L and the control group. However, PMN incubated with MC 10 µg/L presented increased apoptosis rates compared to controls without toxin (control = 3.9; 0.3–21.2 vs. MC = 5.0; 1.1–25.4, P < 0.05, n = 6).

No significant differences were found either in spontaneous or stimulated production of ROS (PMA or S. aureus) or in phagocytosis of S. aureus in PMN from healthy volunteers with microcystin in the three concentrations and the control group.

4.2. Analysis of microcystin toxicity in PMN of patients with CRF receiving hemodialysis

Polymorphonuclear leukocytes from patients receiving HD incubated with MC did not show statistically significant differences in viability, apoptosis or necrosis when compared to controls without the toxin (Table 1).

There was increased production of ROS by PMN when stimulated with PMA or S. aureus compared to baseline (P < 0.05). When incubated with MC, PMN of patients receiving HD presented a significant reduction in both spontaneous and S. aureus-stimulated production of ROS when compared with the same group without the toxin (P < 0.05). PMA-stimulated ROS production was also mildly reduced in the presence of microcystin (P = 0.06) (Table 1). There was no significant difference in phagocytosis with and without MC.

4.3. Analysis of microcystin toxicity in PMN of HV

PMN from HV incubated with MC presented increased apoptosis rates compared to controls without toxin, but there were no differences in viability and necrosis rates. There were also no significant differences in the spontaneous or stimulated production of ROS, or in the percentage of phagocytosis with and without MC (Table 1).

4.4. Analysis of the cytokine production in monocytes

Monocytes presented a higher production of IL-10 and TNF after stimulation by LPS both in HD and in HV. This response was maintained in monocytes incubated with MC at baseline and after LPS stimulation in both groups. (Tables 2 and 3).

5. Discussion

MC is primarily hepatotoxic, but damage to other organs such as kidneys, lungs and intestines have also been reported (Kuiper-Goodman et al., 1999).

Several authors have recently discussed the possible role of this toxin as an immune modulator; some authors have described the effects on the humoral immunity with

Table 2
Cytokine production (TNF-α and IL-10) by monocytes of patients with CRF receiving HD (HD) incubated with RPMI, or after stimulation with LPS (100 ng/mL) and/or microcystin (10 µg/L) for 24 h (n = 4)

<table>
<thead>
<tr>
<th></th>
<th>HD</th>
<th>HD + LPS</th>
<th>HD + MC</th>
<th>HD + LPS + MC</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (pg/ml)</td>
<td>5*</td>
<td>1633 (1617–1732)</td>
<td>5*</td>
<td>1613 (1535–1700)</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>4 (4–28)</td>
<td>1237 (826–1664)</td>
<td>4*</td>
<td>915 (564–1586)</td>
</tr>
</tbody>
</table>

* Values found are below the detection level of the test.
lymphoproliferation modulation and in vitro production of polyclonal antibodies (Shen et al., 2003), but few studies have investigated the effect of this toxin on human leukocytes (Hernandez et al., 2000). The current study analyzed, for the first time, the effect of MC on the viability, production of oxygen-reactive species and phagocytosis of leukocytes in healthy individuals and in patients with chronic renal failure receiving hemodialysis, as well as the production of cytokines by monocytes of these individuals. The concentrations of MC used were based on values close to those found occasionally in the water supplied to the general population and to the dialysis centers. A differentiated response between the two assessed populations was evidenced, with higher rates of apoptosis in PMN of healthy individuals and a reduction in the production of ROS at baseline and after S. aureus-stimulation in PMN of patients receiving HD exposed to MC.

HD patients usually present PMN dysfunction in addition to increased levels of pro-inflammatory cytokines; these factors are related with higher morbidity and mortality rates. The use of bioincompatible membranes, non-sterile dialysate and back leak of dialysate through the dialysis membrane has been associated with an inflammatory reaction in patients receiving HD (Stenvinkel and Alvestrand, 2002).

On the other hand, studies with MC have evidenced increased spontaneous adhesion of human peripheral PMN (Hernandez et al., 2000), and up-regulation in the production of cytokines in macrophages (Nakano et al., 1989, 1991), suggesting that this toxin may participate in the inflammatory response. The hypothesis that this toxin might also be involved in leukocyte dysfunction in patients receiving HD had not been investigated until the present moment.

In this study, only PMN of healthy volunteers presented more apoptosis after exposure to MC 24 h. Other authors (McDermott et al., 1998) observed apoptosis in hepatocytes exposed to MC (0.8 μM) after 30 min. However, other cell types (human endothelial cells, human fibroblasts, human epithelial cells and rat promyelocytes) showed apoptosis with much higher concentrations of MC (100 μM) and needed longer periods of treatment. Botha et al. (2004) identified apoptosis and loss of viability in human colon carcinoma cells (CaCo2) only 48 h after exposure to MC (50 μM). Hence, the response to MC can vary according to the cell type, toxin concentration and duration of exposure.

It may also depend on the previous level of cell dysfunction, as in the case of patients receiving HD, who are constantly exposed to uremic toxins and present high rates of apoptosis even before being exposed to microcystin.

The induction of the production of ROS has also been investigated as a possible pathophysiologic mechanism of the lesions induced by MC, especially in hepatocytes. Ding et al., (2001) observed that the production of ROS induced by MC caused rupture of the cytoskeleton of cultured hepatocytes even before significant changes in viability. The generation of ROS by other cell types was also demonstrated by Botha et al. (2004), who observed the production of H2O2 by CaCo2 cells and MCF-7 in the first 30 min of exposure and returning to baseline production after 120 min.

When incubated with MC, PMN of patients receiving HD showed a reduction in the production of baseline and S. aureus-stimulated ROS compared with the control group that, in spite of being small, was statistically significant. However, there was no change regarding the production stimulated by PMA. In this specific setting, MC seems not to directly interfere with the production of ROS as in hepatocytes. Considering that S. aureus stimulates the production of ROS in PMN through the stimulus of a specific receptor of cellular membrane, and that PMA acts by stimulating protein kinase C (PKC) in the distal portion of the signaling pathway for the production of ROS, the data found in this study suggest that microcystin in human PMN interferes with the mechanisms of intracellular signaling pre-PKC, probably due to protein hyperphosphorylation. This effect was not found in PMN of healthy volunteers, which suggests that this response is facilitated by the uremic environment (Vanholder et al., 1995).

Cytokine production was also assessed, since there are studies showing increased levels of cytokines in patients receiving HD (Kaysen, 2001) and MC has also been related to the modulation of cytokine production in some studies (Nakano et al., 1989, 1991). We noticed that the production of IL-10 and TNF-α increased in both groups after stimulation with LPS. However, the baseline and stimulated production were not modified by the presence of MC. Previous studies had demonstrated that cell extracts of M. aeruginosa stimulated the in vitro production of interleukin 1 (Nakano et al., 1989) and TNF–α (Nakano et al., 1991) by peritoneal macrophages in mice. However, the MC concentrations (10–500 μg/mL) used in these studies were much higher than the one used in our study, and do not

**Table 3**

<table>
<thead>
<tr>
<th>Cytokine production (TNF-α and IL-10) by monocytes of healthy volunteers (HV) incubated with RPMI, or after stimulation with LPS (100 ng/mL) and/or microcystin (10 μg/L) for 24 h (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HV + LPS</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
</tr>
</tbody>
</table>

*a Values found are below the detection level of the test.*
reflect the values that can really be found in the water to which healthy individuals and HD patients are exposed.

Concentrations of microcystin between 1 and 10 μg/L have been reported in the water supply in Sao Paulo. Activated charcoal and reverse osmosis units of dialysis centers should be able to remove these amounts of MC; however, activated charcoal filters have removal rates between 60 and 98% (Hrudey et al., 1999), and there is only one study assessing the effectiveness of reverse osmosis membranes that showed retention levels between 96.7 and 99.9% (Neumann and Weckesser, 1998). Additionally, it is possible that the inadequate maintenance of these systems leads to ineffective removal of MC.

For this reason, in addition to the usual water treatments for dialysis, the use of filters of ultra-pure water has spread in hemodialysis centers with some advantages, such as a reduced risk of exposure to endotoxins. We evaluated this type of filter in our laboratory with a hemodialysis system similar to the one used in clinical practice. MC was added to the dialysis bath (10 μg/L) after the treatment with reverse osmosis and a simulation of a hemodialysis session in laboratory was performed with a hemodialysis machine and an ultra-pure water filter (PEPA Model EF-01, Nikkiso, Japan).

In this system, we identified retention levels around 75% after 60 min of hemodialysis and 55% retention after 240 min. We also observed that after cleaning the system and restarting the procedure, the toxin retained in the filter was released into the dialysate. More detailed studies should be carried out with this type of filter in order to define its real importance in the water treatment in terms of cyanotoxins retention.

In summary, this study shows that human leukocytes exposed to low concentrations of MC occasionally found in water, present mild changes in function that have been differentiated in healthy volunteers and in patients with chronic renal failure receiving hemodialysis. It is possible that with longer periods of exposure (chronic exposure) this response may be intensified; moreover, other functions in these cells may be impaired. Further studies are necessary to assess this possibility.

References


