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Effects of microcystins on human polymorphonuclear leukocytes

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Abstract

Microcystins (MCs) are cyclic heptapeptides produced by cyanobacteria present in water contaminated reservoirs. Reported toxic effects for microcystins are liver injury and tumour promotion. In this study, we evaluated the effects of two MCs, MC-LR and [Asp^3]-MC-LR, on human neutrophil (PMN). We observed that even at concentrations lower than that recommended by World Health Organization for chronic exposure (0.1 nM), MCs affect human PMN. Both MCs have chemotactic activity, induce the production of reactive oxygen species, and increase phagocytosis of *Candida albicans*. MC-LR also increased *C. albicans* killing. The effect of MCs on PMN provides support for a damage process mediated by PMN and oxidative stress, and may explain liver injury and tumour promotion associated to long-term MCs exposures.

Keywords: Microcystin; Cyanobacteria; Neutrophil; Reactive oxygen species; Polymorphonuclear leukocytes; Phagocytosis

Cyanobacterial blooms of the genus *Microcystis* in water reservoirs have caused serious ecological and public health concern due to their ability to produce cyanotoxins named microcystins (MCs, Fig. 1) [1–3]. MCs are cyclic heptapeptides, which mainly cause morphological and functional changes in hepatocytes [4] inhibiting the activity of phosphatases, especially types 1 and 2A (PP1 and PP2A, respectively), both in vivo [5] and in vitro [6], resulting in cell proliferation and cancer or an apoptotic process and cell death [7].

The first described epidemic of fatal MCs toxicity in a haemodialysis population was called ''Caruaru Syndrome'' [8]. In this dramatic acute exposure event, the liver-biopsy specimens and necropsy revealed a very uniform pathological picture, with disruption of liver plates and cell deformity, extensive necrosis, apoptosis, apart from a dense mixed inflammatory infiltrate consisting predominantly of polymorphonuclear leukocytes (PMN) in the portal tracts [9,10].

We hypothesized here that, MCs affect PMN functions. The migration of PMN to the liver may represent a mechanism to increment MCs toxicity. PMN and other phagocytic cells are able to release high amounts of superoxide anion ($O_2^{\cdot -}$) through the enzymatic complex NADPH oxidase in a process known as oxidative burst. Superoxide anion originates secondary high reactive oxygen species (ROS), such as hydrogen peroxide, hypochlorous acid, and singlet oxygen [11]. Although the oxidative burst represents a host defence response to pathogens, acute or prolonged inflammatory processes may increase host tissue destruction [12]. The effects of two microcystin on human PMN recruitment, ROS production, phagocytosis, and killing activities were investigated herein.

Materials and methods

Cell culture. The cyanobacterium *Microcystis panniformis* strain BCCUSP 100 was grown in laboratory as described in [13].

Microcystin isolation and identification. The toxins MC-LR and [Asp^3]-MC-LR were isolated and identified following the modified method from Bittencourt-Oliveira et al. [14]. Briefly, MCs were extracted with 200 mL MeOH/H_2O (73:27, v/v) from filtered cells (50 g) and submitted to a sonic disruption for 10 min. The extract was centrifuged (10,000 rpm, 15 min)

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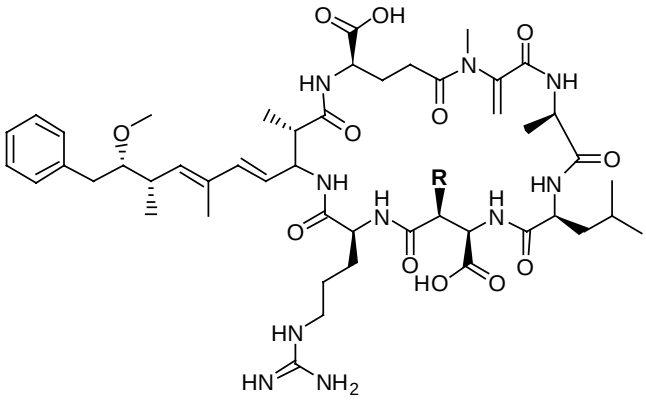

Fig. 1. Molecular structure of microcystin-LR (where $R = CH_3$) or [Asp^3]-microcystin-LR (where R = H) used in this study, isolated from *M. panniformis* strain BCCUSP 100.

and the supernatant was collected as dried in a rotoevaporator (bath at 40 °C) and dissolved in 3 mL of ethyl acetate. Samples were submitted to pre-equilibrated ethyl acetate silica column (20 × 5 cm, Silica Gel Keiselgel 60, Merck) and eluted as follows: 30 mL ethyl acetate, 30 mL ethyl acetate:MeOH (1:1, v/v), 60 mL MeOH, and 120 mL MeOH:H_2O (1:1, v/v). The fraction containing MCs (last elution step) was dried and suspended with 1 mL MeOH:H_2O (1:1, v/v) and then repurified in a HPLC system equipped with a pump LC-10AD, a PDA detector (SPD10AV), and a SCL-10Avp System Controller (Shimadzu, Kyoto, Japan). Samples were injected in the system and chromatographed in a semi-preparative HPLC column (Phenomenex, Luna C18, 5 μm, 250 × 10 mm) eluted with a mixture of acetonitrile (ACN) and 20 mM $NH_4CH_3COO^-$ (27:73), pH 5, at a flow rate of 4.7 mL min^{-1}(detected at 238 nm). The two peaks collected were analysed in a Quattro Micro tandem mass spectrometer (Waters Micromass, Manchester, UK). The identification of MC-LR and [Asp^3]-MC-LR was carried out according to Bittencourt-Oliveira et al. [14].

PMN preparation. PMN were obtained from the peripheral blood of healthy donors under endotoxin-free conditions and isolated as previously described by Boyum [15], using a commercial gradient of Ficoll–Hypaque (Histopaque). After purification, PMN were suspended in phosphate-buffered saline (PBS) buffer, pH 7.4, enriched with 1 mg mL^{-1} glucose, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$, counted in a Neubauer chamber and purity was estimated to be higher than 98%.

Cell viability assay. The MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) colorimetric assay (reduction of tetrazolium salt to formazan) was applied to evaluate the PMN viability in the presence of the microcystins (0.01–1000 nM) [16]. Cells (1×10^6 PMN mL^{-1}) were pre-incubated with MC-LR or [Asp^3]-MC-LR in PBS buffer (37 °C, 5% CO_2). After 60 min, (0.5 mg mL^{-1}) MTT was added and plates were incubated for 60 min. Supernatant was discarded and 50 μL of DMSO was added each to all well microplates to dissolve the dark blue crystals. The plates were read on a MicroElisa reader (Quantikine; R&D Systems, Minneapolis, MN) at 540 nm. PMN viability was not significantly affected by MCs. After an incubation time up to 60 min, there was only a reduction of 15% in cell viability, even at concentrations of MCs as high as 1 μM.

Migration assay. PMN migration was determined using the 96-well chemotaxis chamber (Neuro Probe). Both MCs (range from 0.01 to 1000 nM) and 57 nM fMLP were added to the bottom wells of the well plate. PMN (2.5×10^6 cells mL^{-1}) were added to the top wells, incubated for 60 min (37 °C, 5% CO_2) and the non-migrating on the origin side (top) of the filter were removed by gentle scraping. After incubation, the cells that migrated were counted in a Neubauer chamber.

Oxidative burst. The effect of MC-LR and [Asp^3]-MC-LR (10 and 100 nM) on the PMN oxidative burst was evaluated by the luminol-enhanced chemiluminescence assay. PMN (1×10^6 cells mL^{-1}) were pre-incubated (in PBS buffer) with MCs for 5 min in the presence of luminol (1 nM). The reaction was run in PBS buffer at 37 °C in a final volume of 0.3 mL. Chemiluminescence was measured in an EG&G Berthold LB96V microplate luminometer.

Phagocytosis and killing assays. These assays were performed using a method modified from Hampton [17]. *Candida albicans* strain ATCC 5374 was plated on Sabouraud's dextrose agar (Oxoid, Wesel, Germany) and incubated at 37 °C for 18 h. Colonies were added in human serum (0.3 mL) and incubated for 30 min (37 °C). After opsonization, 2.7 mL of PBS buffer was added. PMN (1×10^6 cells mL^{-1}) were incubated with *C. albicans* suspension (5×10^6 cells mL^{-1}) and MC-LR or [Asp^3]-MC-LR (1 and 1000 nM) for 10 and 15 min. The samples (100 μL) were concentrated in a cytocentrifuge and fixed with Rosenfeld stain. This dye does not stain dead fungi. The percentage of neutrophils that have phagocytized *C. albicans* and the percentage of killing [(number of died *C. albicans* ingested/number of total *C. albicans* ingested) × 100] were calculated.

Statistical analysis. The statistical analysis was carried out comparing the average ± SD of the group samples. It consisted of the one-way analysis of variance (ANOVA) with the Student–Newman–Keuls multiple comparisons test.

Results and discussion

Effect of MCs on PMN migration

Both MCs substantially enhanced neutrophil migration (Figs. 2A and B). For [Asp^3]-MC-LR a dose-dependent

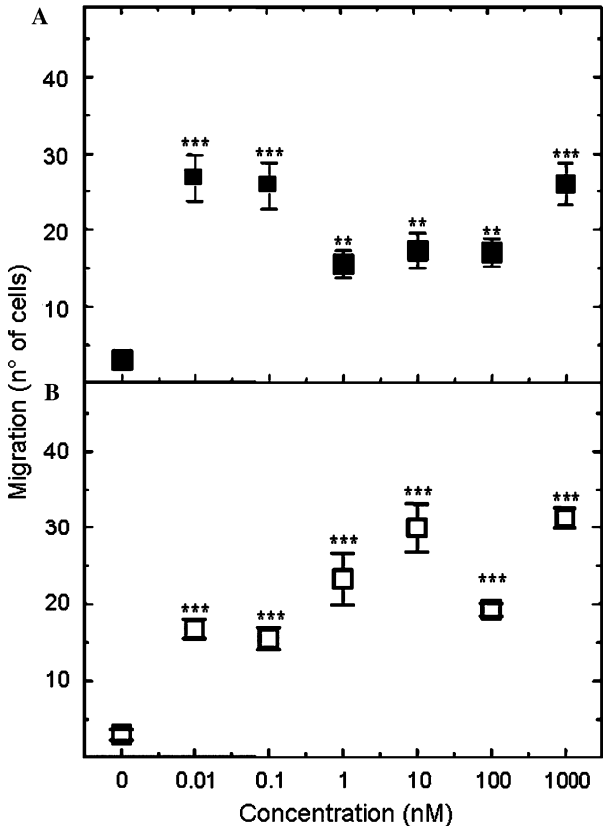

Fig. 2. Effect of MC-LR (A) and [Asp^3]-MC-LR (B) concentration on PMN migration. Migration was measured in a 96-well chemotaxis plate. The number of migrated cells was quantified in a Neubauer chamber. Cells (2.5×10^6 cells mL^{-1}) were incubated with MC-LR, [Asp^3]-MC-LR (0.01–1000 nM) for 60 min (37 °C and 5% CO_2). Results for each procedure are means from triplicate determinations from three experiments. Asterisk denotes a response that is significantly different from the positive control (**$P < 0.01$, ***$P < 0.001$).

effect was more evident. The increased migration observed here complements the previously reported enhancement of PMN spontaneous adherence in the presence of MC-LR [18] once these processes, adherence and migration, are important steps in inflammation [19].

These findings support the idea that, in cases of MCs intoxication, there is a movement of PMN from the bloodstream toward areas that concentrate the toxin, for instance the liver [20]. This chemotactic effect of MCs may cause an increment in the inflammatory cell influx that is a condition that already accompanies MCs intoxication. The inflammatory cell influx occurs in response to the direct effect of MCs on hepatocytes causing cytoplasm damage, necrosis, and liver haemorrhage and fibrosis [8,20,21]. Thus, liver damage, by itself, is a factor that promotes leukocyte migration and this event may be amplified by the chemotactic activity of the MCs. The extravasation of PMN is a pre-state for the production of reactive oxygen species (ROS), followed by the establishment of an oxidative stress condition that culminates with the development of tissue injury [22].

Effect of MCs on the production of ROS by PMN

Under appropriated stimuli PMN generate ROS for a large period of time [11]. PMN activation mediated by membrane receptors occurs, for instance, for bacterial products and particulate material, and soluble substances able to directly activate protein kinase C. The process of ROS production by PMN is known as oxidative burst and depends on a complex signalling involving a fine balance of protein phosphorylation and dephosphorylation. Besides ROS production, PMN activation also includes cytoskeletum organization and degranulation processes. For PMN, a cell rich in azurophil granules, the production of ROS is accompanied by degranulation of the enzyme myeloperoxidase. Although the luminol-enhanced chemiluminescence assay is not selective, it is safely used as a tool to evaluate the resultant of the events of production of ROS and degranulation [23]. The simultaneous presence of ROS and MPO activity are able to catalyse oxidative reactions in lipids and proteins, and are associated with cellular damage and disease.

MC-LR or [Asp^3]-MC-LR was able to trigger the luminol-enhanced chemiluminescence by human PMN, in a dose–response manner and over to 20 min (Fig. 3). The kinetics of light emission is very similar to those triggered by recognized activators, such as phorbol esters. Thus, it is reasonable to conclude that a direct action of PMN might be involved in the toxicity of MCs. In hepatocytes, it is known that ROS generated by PMN induce an oxidative stress able to cause a necrotic injury in less than one hour. In this case, the mechanism of injury does not involve gross lipid peroxidation [24] but may be caused by the opening of the membrane permeability transition pore and the collapse of the mitochondrial membrane potential [25]. Some recent studies strongly suggest that the

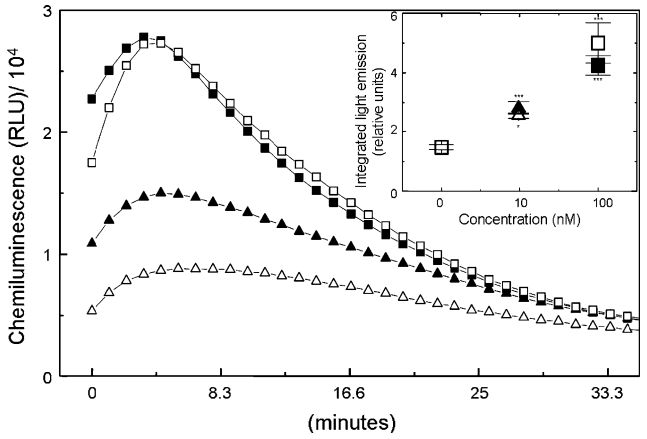

Fig. 3. Effect of MC-LR and [Asp^3]-MC-LR concentration on ROS production by human PMN. Luminol (1 nM)-enhanced chemiluminescence induced in PMN (1×10^6 cells mL^{-1}) by (▲) 10 nM, (■) 100 nM MC-LR and (Δ) 10 nM, (□) 100 nM [Asp^3]-MC-LR. The inset represents the integrated light emission (relative light units) in the presence and absence of these MCs. Asterisk denotes a response that is significantly different from the control (*$P < 0.05$, ***$P < 0.001$).

induction of ROS formation and mitochondrial alterations are the two major events found in MC-treated culture of rat hepatocytes [26]. In addition to cell injury, ROS promote inflammation by the activation of the transcription factor NF-κB (nuclear factor-κB), which controls the formation of cytokines, chemokines, and adhesion molecules [22]. Additionally, increased lipid peroxidation and oxidative stress conditions were shown in studies with cells and animals exposed to MCs [25–30].

Effect of MCs on phagocytosis and killing

Using the Rosenfeld stain, we measured simultaneously the effect of MCs on the ingestion (phagocytosis) and intracellular killing (killing activity) of *C. albicans*. Both MCs increased phagocytosis (Figs. 4A and B); in the presence of 1 nM of MCs, there was an increment of approximately 70% of phagocytosis. Higher concentration of MCs (1000 nM) did not cause further increment in phagocytosis. In respect to the intracellular microbial activity only the MC-LR affected this activity (Figs. 4C and D). The incubation of PMN with MC-LR at 1000 nM caused an increment in killing activity, especially evident at 10 min.

Although at this moment it is not possible to explain how MCs affect phagocytosis, it is clear that MCs interfere with phosphorylation/dephosphorylation processes. On one hand, independently of the mechanism by which MCs act on phagocytosis and killing, MCs do not compromise the microbicidal activity of PMN. On the other hand, they increase the killing efficiency of PMN.

Conclusion

These findings that MC-LR and [Asp^3]-MC-LR have a direct chemotactic activity towards PMN and are inducers of the oxidative burst in these cells provide support for a

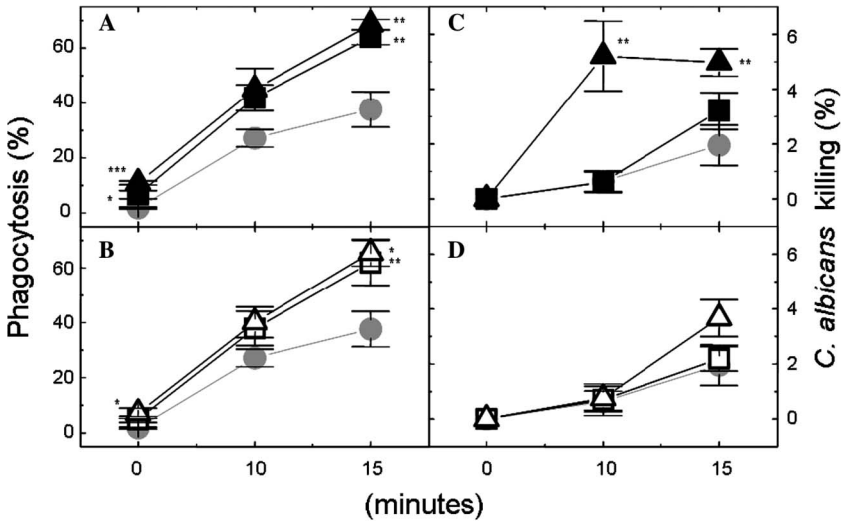

Fig. 4. Effect of MC-LR and [Asp^3]-MC-LR concentration on phagocytosis (A,B) and killing activity (C,D). Cells (1×10^6 cells mL^{-1}) were incubated with (▲) 1 nM, (■) 1000 nM MC-LR and (Δ) 1 nM, (□) 1000 nM [Asp^3]-MC-LR for 10 and 15 min (37 °C). In the control (●), no toxin was added. Asterisk denotes a response that is significantly different from the positive control (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

central role of PMN in the damage process associated to short and long term MC exposure. Despite the fact that the mechanism of MC action on PMN has not yet been demonstrated, MCs may represent an additional class of chemotactic agents and oxidative burst inducers. This could be suggestive for further research about the mechanism operating in the effects triggered by MCs on PMN, especially at chronic exposure to low levels. The recommendations regarding safe algal toxin levels in drinking water as proposed by WHO (0.1 nM) [31] and other countries like Brazil [32] can still offer a significant health risk. Potential tumour promotion is a primary concern. Part of these effects may be associated to the activity of MCs on phosphatases that may cause alterations in signalling processes.

Acknowledgments

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