Biochemical and Biophysical Research Communications 382 (2009) 9–14

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Microcystins -LA, -YR, and -LR action on neutrophil migration

Paula Kujbida [a], Elaine Hatanaka [b,c], Marco Aurélio Ramirez Vinolo [c], Kaline Waismam [a], Danielle Maia de Holanda Cavalcanti [a], Rui Curi [c], Sandra Helena Poliselli Farsky [a,*], Ernani Pinto [a,*]

[a] Department of Clinical and Toxicological Analyses, Faculty of Pharmaceutical Sciences, University of São Paulo, Avenida Prof. Lineu Prestes, 580 bloco 13 B, 05508-900 Butanta, São Paulo, SP, Brazil
[b] Post-Graduate Program in Human Movement Science, Biological Sciences and Health Center, Cruzeiro do Sul University, São Paulo, SP, Brazil
[c] Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP, Brazil

ARTICLE INFO

Article history:
Received 26 January 2009
Available online 10 February 2009

Keywords:
Microcystin
Neutrophils
Migration
Rolling
Adherence
Adhesion molecules
Calcium influx

ABSTRACT

Microcystins (MCs) produced by some freshwater cyanobacterial species possess potent liver toxicity as evidenced by acute neutrophil infiltration. Here, we investigate the ability of three structurally distinct toxins (MC-LA, MC-LR, and MC-YR) to evoke neutrophil recruitment *per se* and their effects on migration pathways. Intravital microscopic studies showed that topical application of only MC-LR enhanced the numbers of rolling and adhered leukocytes in the endothelium of postcapillary mesenteric venules. The latter effects may be dependent upon induction of the synthesis and expression of L-selectin and β2-integrin in neutrophils, as assessed by flow cytometry and RT-PCR, respectively. Conversely, the three toxins promoted direct locomotion of neutrophils and enhanced their migration in response to fMLP, as measured by Boyden chamber assays, and increased intracellular calcium, a messenger in the chemotaxic process. In conclusion, our results show that MCs act on specific pathways of neutrophil recruitment, indicating their potential effect on neutrophils activation.

Introduction

Toxic cyanobacterial blooms in freshwater supplies pose a risk to the environment and human health [1]. Microcystins (MCs), known potent hepatotoxins, are produced by various species of cyanobacteria [2], which can form water blooms under eutrophic conditions in lakes, ponds, and water reservoirs [3].

A severe accident in Brazil during 1996, called "Caruaru Syndrome," illustrates the high human toxicity of MCs. In this fatal accident, untreated water from a reservoir with a cyanobacterial bloom was used at a hemodialysis center. Levels of MC-LR, MC-YR, and MC-AR were found in the tissues and blood of these patients and were related to nausea, vomiting, acute liver damage, and, in some cases, death. Biopsy and autopsy of these livers demonstrated substantial neutrophil infiltration [2,4–6]. It has not yet been elucidated as to whether neutrophils are recruited by the activity of the toxin or after hepatic damage, playing a role in liver failure. Although leukocyte recruitment to the focus of a lesion provides the first line of host defense, exacerbated activity of these cells can induce tissue injury and microvascular dysfunction. The deleterious and undesirable effects in the host are due to the ability of neutrophils to release proteolytic enzymes and reactive oxygen and nitrogen metabolites that cause tissue damage [7]. We have previously shown that MCs activate human and rat neutrophils *in vitro*, generating ROS and cytokine release [8,9].

During the process of migration, circulating leukocytes initially roll and adhere to the endothelium of microcirculatory vessel walls, and subsequently transmigrate into the extravascular space, moving directly to site of injury. A coordinated interplay involving a spectrum of adhesion molecules successively expressed on cellular membranes mediates the interactions in the microvasculature and the extravascular tissue [10].

Neutrophils move by projecting pseudopodia directly into the chemoattractant gradient. The intracellular pathway of chemotaxis is not completely understood, and varies according to the chemoattractant [11]. In general, chemoattractant receptor activation has been shown to lead to the generation of Ca^{2+} signaling via a series of events, including G-protein interactions, inositol 1,4,5-triphosphate production, and Ca^{2+}-channel opening and release of intracellular Ca^{+2} stores to the plasma membrane. The increased Ca_i^{2+} concentration mediates the expression of adhesion molecules responsible for cell to cell contact in the extravascular matrix and regulates myosin function via Ca^{2+}/calmodulin-dependent myosin light chain kinases [10].

Taking into account that MC toxicity is characterized by neutrophil influx into target tissues, we herein investigated the ability of three structurally distinct toxins, MC-LA, MC-YR, and MC-LR, to induce leukocyte recruitment *in vivo* and their specific effects on

* Corresponding authors. Fax: +55 11 3091 9055 (E. Pinto), +55 11 3815 6593 (S.H.P. Farsky).
E-mail addresses: sfarsky@usp.br (S.H.P. Farsky), ernani@usp.br (E. Pinto).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.02.009

pathways of cellular movement. The results clearly demonstrate the *in vivo* inflammatory properties of MC-LA, MC-YR and MC-LR, quantified by their individual actions on leukocyte–endothelial interactions, leukocyte adhesion molecule expression, neutrophil chemotaxis, and Ca^{2+} mobilization.

Materials and methods

Reagents. MC-LA, MC-YR, MC-LR, bovine fetal serum (BSA), penicillin, RPMI-1640 medium supplemented with l-glutamine, *N*-formyl-methionyl-leucyl-phenilalanine (fMLP), May Grumwald–Giemsa, ethylenediaminetetraacetic acid (EDTA), ionomycin A23187, Fluo-3 AM, and oyster glycogen were obtained from Sigma Chemical Co. (St. Louis, MO, USA). L-Selectin monoclonal antibodies conjugated to FITC (anti-rat CD62L) and β2-integrin monoclonal antibodies conjugated to FITC (anti-rat CD18) were purchased from BD PharMingen Technical (San Diego, CA, USA). Sodium pentobarbital was purchased from Cristália (São Paulo, SP, Brazil). Trizol reagent was purchased from Invitrogen (Grand Island, NY, USA). Oligo(dT)15 primer, ribonuclease inhibitor, reverse transcriptase, Taq DNA polymerase, and dNTP mix were purchased from Promega (Madison, WI, USA).

Animals. Male Wistar rats weighing 180 ± 20 g were obtained from the Department of Clinical Chemistry of the Faculty of Pharmaceutical Sciences, University of São Paulo, Brazil. The rats were maintained at 23 °C under a light:dark cycle of 12:12 h. Food and water were provided *ad libitum*. The Animal Care Committee of the Faculty of Pharmaceutical Sciences approved of the experimental procedures employed in this study (Protocol No. 275).

Intravital microscopic assay. Rats were anesthetized by i.p. injection of pentobarbital sodium (65 mg/kg) and the mesentery was exteriorized. After surgery, the animals were maintained on a special board that was thermostatically controlled at 37 °C. This system included a transparent platform on which the tissue to be transilluminated was posicioned. The preparation was kept moist and warm by irrigating the tissue with a warmed Ringer-Locke solution (pH 7.2–7.4; 154 mM NaCl; 5.6 mM KCl; 2 mM $CaCl_2{\cdot}2H_2O$; 6 mM $NaHCO_3$, and 5 mM glucose) containing 1% gelatin. The rate of solution outflow onto the exposed tissue was controlled to keep the preparation in continuous contact with a film of the liquid. Transilluminated images were obtained by optical microscopy. The images were captured with a video camera (ZVS, 3C75DE, Carl–Zeiss) and simultaneously transmitted to a TV monitor and a computer. Digitized images were subsequently analyzed using image-analyzing software (KS 300, Kontron).

The interaction between leukocytes and vessel walls was evaluated by determining the number of rolling and adhered leukocytes on the postcapillary venule wall (20–30 μm diameter, 200 μm length) of the mesentery at 10-min intervals, as previously described by Dahlen et al. [12]. Three fields were evaluated per animal after application of 10 μL of MC-LA, MC-YR, or MC-LR (1 nM). Leukocytes moving peripherally to the axial stream and in contact with the endothelium were considered to be 'rollers'. These leukocytes moved sufficiently slow to be individually visible and were counted as they rolled past a designated point on one side of the vessel for a duration of 10 min after MC-LA, MC-YR, or MC-LR treatment (1 nM). The number of leukocytes adhered to the endothelium (stopped at the vessel wall) was determined in the same vascular segment after 10 min of MC-LA, MC-YR, or MC-LR application.

Blood flow cytometry. In order to quantify l-selectin or β2-integrin expression, leukocytes were isolated from abdominal aorta blood collected in EDTA (100 mg/mL). Erythrocyte lysis was performed using an ammonium chloride solution (0.13 M), and leukocytes were recovered after washing the preparation with PBS. The cells (1.0×10^6) were incubated with MC-LA, MC-YR, MC-LR (1000 nM), or fMLP (10 nM) for 4 h. After washing, the leukocytes were further incubated for 30 min at 4 °C in the dark with 10 μL of monoclonal antibody against l-selectin or β2-integrin. Immediately after incubation, the cells were analyzed using a FACSCalibur flow cytometer (Becton & Dickinson, San Jose, CA, USA). Data were obtained from 10,000 cells and only morphologically viable neutrophils were considered for analysis. Leukocytes were separated based on size and granularity. Fluorescence was determined and the results are expressed as the mean fluorescence of four assays performed in duplicate.

RT-PCR assays. Neutrophils were prepared as described above. Cells (2.5×10^7) were incubated with MC-LA, MC-YR, MC-LR (1000 nM), or fMLP (10 nM) for 4 h. Total RNA was extracted from cultured neutrophils using Trizol reagent according to the manufacturer's instructions. RNA extraction was carried out in an RNAse-free environment, and the isolated RNA was quantified by reading the absorbance of the RNA solution at 260 nm.

Reverse transcriptase-PCR. cDNAs were synthesized from 1 mg of total RNA using an oligo(dT)15 primer (20 mg/mL) after incubation at 70 °C for 15 min in the presence of 2 mM dNTP mix, ribonuclease inhibitor (20 U), and reverse transcriptase (200 U) in a final volume of 25 μL in reverse transcriptase buffer. The reverse transcription reaction was performed by incubation at 42 °C for 60 min. For PCR, cDNA was incubated with 2.5 U of Taq DNA polymerase, 0.4 mM 3- and 5-specific primers, and 200 mM dNTP mix with buffer-thermophilic DNA polymerase in 1.5 mM $MgCl_2$. The primer sequences used were GAPDH, 5′-TATGAT GACATCAAGA AGGTGG-3′ (forward) and 5′-CACCACCCTGTTGCTGTA-3′ (reverse); l-selectin, 5'-AACGAGACTCTGGGAAGT-3′ (forward) and 5′-CAAAGGCTCACATTGGAT-3′ (reverse); β2-integrin, 5′-TGCGCCCC TCACTGCTGCTTG-3′ (forward) and 5′-GAGATCCATGAGGTAGTACA GATC-3′ (reverse).

In vitro neutrophil migration: Boyden chamber assays. Neutrophils were obtained from adult rats 4 h after intraperitoneal (ip) injection of 10 mL of 1% sterile oyster glycogen solution in phosphate-buffered saline (PBS). The animals were anesthetized with 200 μL of ketamine:xylazine (2:1) and the cells were collected by rinsing the abdominal cavity with 40 mL of sterile PBS. The number of viable cells (98%) was counted in a Neubauer chamber using a light microscope (Nikkon, Japan). The migratory assay was performed using a multiwell chemotaxis chamber, as previously described by Boyden [13] and Zigmond and Hirsch [14].

To evaluate the capacity of MC-LA, MC-YR, or MC-LR to induce neutrophil migration, aliquots of cell suspensions containing 1.5×10^6 neutrophils (in Hanks' balanced salt solution (HBSS) containing 0.01% albumin) were added to the upper compartment of the chamber onto a cellulose filter with an average pore size of 8 μm (Millipore). The lower compartments were prepared as follows: HBSS containing the chemotactic agent fMLP (10 nM) in positive control wells, HBSS only in negative control wells, and solutions of MC-LA, MC-YR, or MC-LR (1, 100, and 1000 nM in HBSS) in test wells. The cells were incubated for 60 min (humidified air, 37 °C, 5% CO_2), followed by removal of the filters for fixation and staining of the cells. Neutrophil migration within the filter was determined under light microscopy, as previously described by Zigmound and Hirsch [14]. The distance was measured from the top of the filter to the farthest plane still containing two cells with an ×40 objective. Duplicate wells were tested for each variable, and five fields were counted and averaged per filter.

To investigate the influence of MC-LA, MC-YR, or MC-LR on neutrophil migration induced by fMLP, aliquots of cell suspensions containing 1.5×10^6 neutrophils were added to the upper compartment of the chamber after incubation for 60 min (37 °C, 5% CO_2) with MC-LA, MC-YR, or MC-LR (1, 100, and 1000 nM). After

incubation, the cells were washed and resuspended in HBSS containing 0.01% albumin for use in the chemotaxis assay. The chemotactic agent (10 nM fMLP) was added to the lower compartment of all wells, excluding negative control wells (HBSS only). The cells were incubated for 60 min (humidified air, 37 °C, 5% CO_2), followed by removal of the filters and evaluation of cell migration using the same methods for fixation and staining described above.

Confocal microscopy. Neutrophils were obtained from adult rats 4 h after i.p. injection of 10 mL of 1% oyster glycogen solution in PBS. The animals were anesthetized with 200 μL of ketamine:xylazine (2:1) and the cells were collected by rinsing the abdominal cavity with 40 mL of sterile Krebs–HEPES buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), and 0.1% bovine serum albumin (BSA), pH 7.3). The number of viable cells (98%) was counted in a Neubauer chamber using a light microscope (Nikkon, Japan). The intracellular Ca^{2+} level ($[Ca^{2+}]_i$) was analyzed using a laser scanning confocal microscope (LSM 410, Carl Zeiss, Jena, Germany). Isolated neutrophils were incubated with 1 mM Fluo-3AM for 45 min at room temperature. Neutrophils were washed and suspended at 4×10^6 cells/mL in Krebs–HEPES buffer. For each experiment, 35 μL of the cell suspension was added to glass-bottomed culture dishes and allowed to settle. The cells were imaged with an inverted confocal microscope (MRC-Bio-Rad 600) using a 63× objective. After 10 min of cell addition, at which time the cells were adherent, the first images were acquired and MC-LA, MC-YR, or MC-LR (1000 nM) was added to the dishes. Immediately after MC stimulation, $[Ca^{2+}]_i$ was analyzed using a laser scanning confocal microscope with excitation and emission wavelengths of 506/526 nm, respectively. Samples were scanned once per second for 200 s. The $[Ca^{2+}]_i$ at each scanning point was analyzed and integrated for 95 s. Maximal fluorescence was obtained by addition of ionomycin (A23187, 100 μM), and minimal fluorescence was obtained by addition of EDTA (4 mM). The integrated values of five cells per experimental group were obtained and the means ± SE of the fluorescence intensity of these cells was then calculated.

Statistical analyzes. Statistical analyzes were carried out by comparing the average ± standard error (SE) of the groups by ANOVA, with the Student–Newman–Keuls multiple comparison tests.

Results

Rolling and adherence of leukocytes is only induced by MC-LR in vivo

Topical application of MC-LR increased both events using intravital microscopy, as evidenced by increased numbers of rolling ($p < 0.001$) and adhered ($p < 0.05$) leukocytes in comparison to topical application of PBS solution, MC-LA, or MC-YR (Fig. 1A and B).

Synthesis and expression of L-selectin and β2-integrin on circulating leukocytes is only enhanced by MC-LR

The expression of both molecules on *in vitro* MC-LR-exposed neutrophils was higher than that observed on MC-LA-, MC-YR-, and vehicle-exposed cells ($p < 0.05$) (Fig. 2A and B). Similar L-selectin and β2-integrin expression levels were observed in MC-LA-, MC-YR-, and vehicle-exposed cells. fMLP-stimulated cells were used as positive controls in the assays, and the data depicted herein show that these cells presented elevated expression of the two molecules tested (Fig. 2A and B).

To evaluate if the elevated adhesion molecule expression reflected their increased synthesis, mRNA was isolated from the cells and RT-PCR assays were performed. The mRNA levels of L-selectin and β2-integrin were significantly increased in MC-LR-treated neutrophils compared to MC-LA-, MC-YR-, or vehicle-exposed cells (Fig. 2C and D). Gene expression was also increased in fMLP-stimulated cells (Fig. 2C and D).

MC-LA, MC-YR, and MC-LR induce neutrophil migration in vitro

MC-LA, MC-YR, and MC-LR induced chemotaxis *per se* in Boyden chambers, as neutrophil migration was enhanced in response to these toxins. Values obtained were higher than those observed in response to vehicle (HBSS) and equivalent to migration induced by fMLP, which was used as a positive control (Fig. 3A, B and C). However, chemotactic potency differed between the toxins. Elevated responses were detected with 1 and 100 nM MC-LA ($p < 0.001$; Fig. 3A), 1 nM MC-YR ($p < 0.01$; Fig. 3B), and 1, 100 and 1000 nM MC-LR ($p < 0.001$; Fig. 3C).

The second assay was carried out to evaluate the ability of the toxins to alter chemotaxis in response to fMLP. To assess this, the migration of cells previously incubated with different concentrations of MC-LA, MC-YR, MC-LR, or HBSS onto the filter was assessed in response to fMLP. Cells incubated with all concentrations of the three toxins employed presented higher migration in response to fMLP than cells incubated with HBSS ($p < 0.01$ or $p < 0.001$, Table 1).

MC-LA, MC-YR, and MC-LR induce intracellular calcium mobilization in neutrophils

$[Ca^{2+}]_i$ was determined by analyzing cells for 0–95 s after MC additions. Means ± SE of the integrated values of five cells per

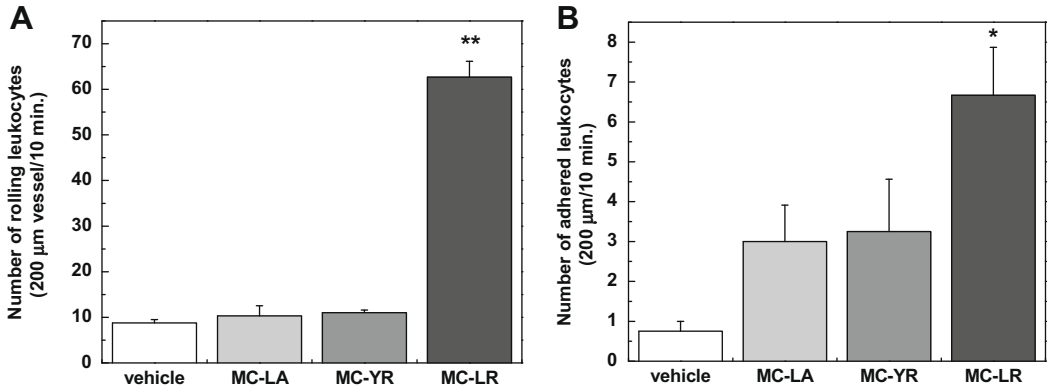

Fig. 1. Number of rolling (A) and adhered (B) leukocytes in postcapillary venules (20–30 μm diameter) of the mesentery of rats exposed to vehicle (PBS), MC-LA, MC-YR, or MC-LR (1000 nM). Results were obtained by intravital microscopy 10 min after topical application of MC-LA, MC-YR, or MC-LR or vehicle. Values are presented as the means ± SE of five animals per group. $^{*}P < 0.05$, $^{**}P < 0.001$ between the vehicle and MC-treatments as indicated by ANOVA, with the Student–Newman–Keuls multiple comparison test.

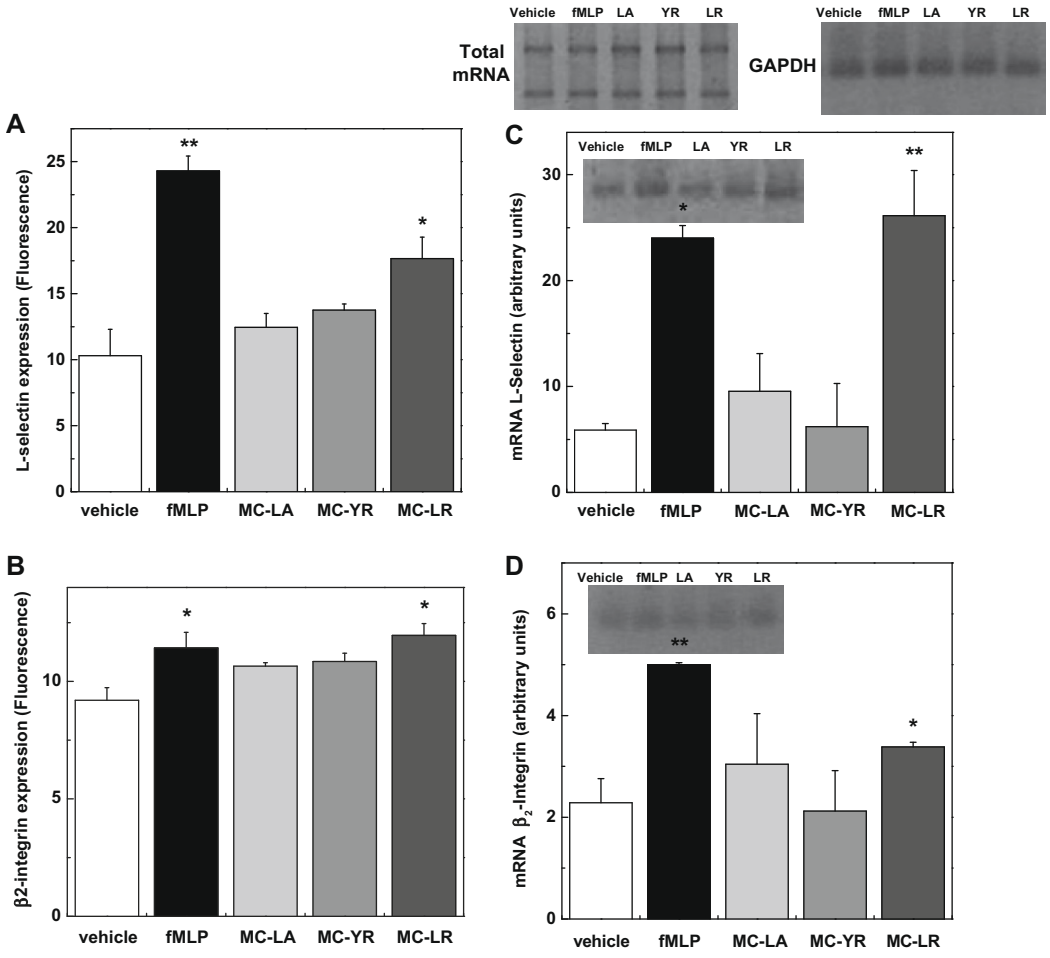

Fig. 2. Effect of vehicle (PBS)-, fMLP-, MC-LA-, MC-YR-, or MC-LR-treatments on the membrane and gene expression of adhesion molecules in neutrophils. Expression of L-selectin (A) and β2-integrin (B) were analyzed by flow cytometry. The assays were carried out in neutrophils isolated from the abdominal aorta blood of adult rats. Cells (1.0×10^6) were incubated with MC-LA, MC-YR, MC-LR (1000 nM), or fMLP (10 nM) for 4 h. Images represent agarose gel electrophoresis of total mRNA, GAPDH; L-selectina (C), and β2-integrin (D). Values are presented as the means ± SE of four animals per group. $^{*}P < 0.05$, $^{**}P < 0.001$ between the vehicle and fMLP-, MC-LA-, MC-YR- or MC-LR-treatments, as indicated by ANOVA, with the Student–Newman–Keuls multiple comparison test.

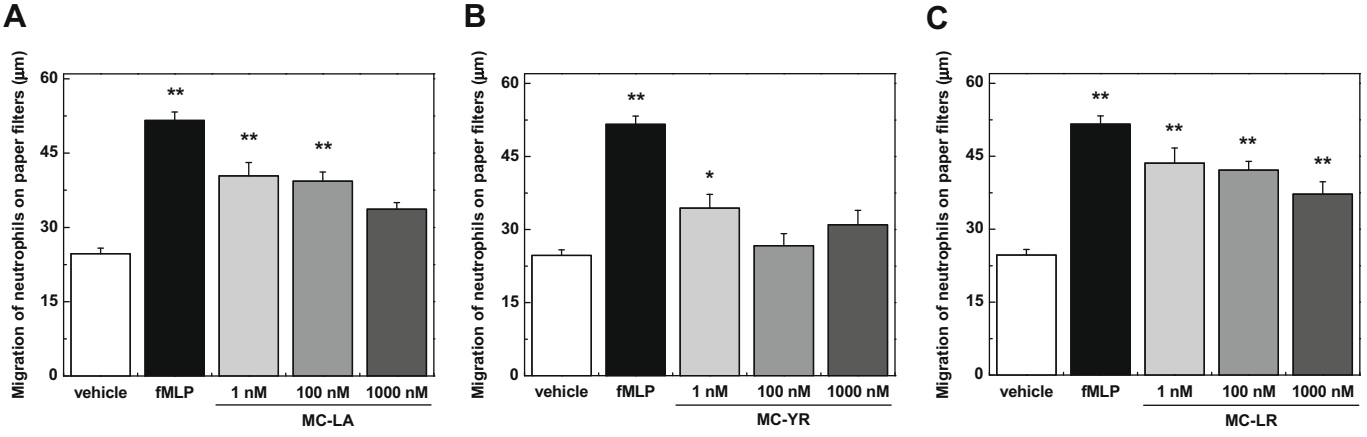

Fig. 3. Chemotactic responses (micropore filter system) of neutrophils to vehicle (HBSS), fMLP (10 nM), MC-LA, MC-YR, or MC-LR (1, 100 and 1000 nM). Neutrophils (1.5×10^6 cells) were placed in the top compartment of the chamber and allowed to migrate for 60 min. Values are presented as the means ± SE of four animals per group. $^{*}P < 0.01$, $^{**}P < 0.001$ between fMLP, (A) MC-LA, (B) MC-YR, or (C) MC-LR and vehicle as indicated by ANOVA, with the Student–Newman–Keuls multiple comparison test.

group (MC-LA, MC-YR, or MC-LR-stimulated neutrophils) demonstrated that toxins increased the $[Ca^{2+}]_i$ (Fig. 4). $[Ca^{2+}]_i$ was increased immediately after additions of MC-LA, -YR, and -LR and attained a maximum level within 20, 27, and 40 s, respectively. In spite of the fact that all MCs were able to trigger calcium influx in neutrophils, MC-LR incubation maintained the elevated $[Ca^{2+}]_i$ until to the end of the investigation period (Fig. 4). Integrated values for the area, as shown in the minor insert of the figure,

Table 1
Chemotactic responses (micropore filter system) of neutrophils to fMLP (10 nM).

Cells pre-incubated with	Concentration of MC (nM)	Migration of neutrophil on paper filter (μm) in response fMLP (mean ± SE)
HBSS	0	51.60 ± 1.705**
MC-LA	1	64.25 ± 4.505*
	100	77.50 ± 4.667**
	1000	68.25 ± 4.092*
MC-YR	1	68.00 ± 3.022*
	100	80.25 ± 4.666**
	1000	74.00 ± 6.341**
MC-LR	1	76.75 ± 4.549**
	100	84.75 ± 4.552**
	1000	79.75 ± 2.844**

The values are presented as means ± SE of four animals per group $^{*}P < 0.01$, $^{**}P < 0.001$ for comparison between vehicle (HBSS)- and MC-LA-, MC-YR- or MC-LR-treatments in response fMLP, as indicated by ANOVA with Student–Newman–Keuls multiple comparison test.

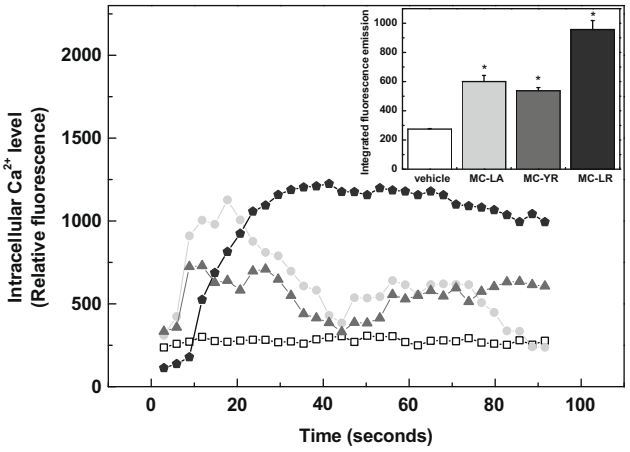

Fig. 4. Vehicle- (—□—), MC-LA- (—●—), MC-YR- (—▲—) or MC-LR- (—⬟—) induced increases in intracellular Ca^{2+} levels. Neutrophils were incubated with 1 mM Fluo-3 AM for 45 min at room temperature. Cells were washed and suspended at 4×10^6 cells/mL in Krebs–HEPES buffer. The cell suspension (35 μL) was plated in glass microwell dishes, and the intracellular Ca^{2+} level was analyzed using a laser scanning confocal microscope. The change in 506/536 nm was monitored. The inserts represent the means ± SE of the integrated fluorescence emission of neutrophils in the presence of MC-LA, MC-YR, or MC-LR. $^{*}P < 0.001$, between the vehicle and MC-LA-, MC-YR- or MC-LR-treatments, as indicated by ANOVA, with the Student–Newman–Keuls multiple comparison test.

represent significantly increased $[Ca^{2+}]_i$ in neutrophils incubated with MC-LA MC-YR and MC-LR ($p < 0.001$) in comparison to that in cells incubated with vehicle.

Discussion

Exacerbated influx and activation of neutrophils contribute to tissue injury in a number of disease states, including certain xenobiotic-induced liver injuries [15]. In this context, neutrophil infiltration was observed around necrotic foci in human and laboratory animals after MC-induced intoxications [5,16]. Nevertheless, the role of neutrophils in the pathogenesis of MC-induced organ toxicity has not been established. In this study, we demonstrated the ability of three structurally distinct and potentially toxic MCs to promote activation and migration of neutrophils into the focus of MC activity.

Neutrophil recruitment into inflamed tissues is initially dependent upon its interaction with the vessel endothelium of the microcirculatory network, subsequent transmigration into the extravascular space, and direct locomotion to the site of inflammation [17]. Herein, topical application of MC-LR induced rolling of neutrophils to postcapillary venules of the mesentery, and these cells were able to adhere to the vessel wall. Hernández et al. [18] previously suggested that MC-LR activates and induces the adherence of human neutrophils to an inert membrane. Together, these results suggest that MC-LR induces neutrophil-endothelial interactions by promoting, at least in part, expression of neutrophil adhesion molecules. In fact, incubation of MC-LR with neutrophils evoked the synthesis and expression of both L-selectin and β2-integrin. Interestingly, these effects were only induced by MC-LR toxin. The ability of MCs to evoke secretion of chemokines [9] and MC-LR to induce synthesis of adhesion molecules, suggest potential effects of MCs on mRNA transcription proteins related to inflammatory events. The effect of MCs on activation of NF-kappaB and activator protein 1 (AP-1) may be important areas for further investigation, as these transcriptional factor mediates the synthesis of chemokines and adhesion molecules [19,20]. Actually, Wei et al. [21] have recently showed that MC-LR induces a time dependent increase in AP-1 activity in liver of mice, via c-Jun-N-terminal protein kinase, which in turn induces mitochondrial-dependent apoptosis.

Using the Boyden chamber model, we demonstrated that toxins are chemoattractants for neutrophils and previous incubation of neutrophils with MCs enhanced their direct migration in response to an inflammatory agent. Therefore, it is clear that MCs *per se* activate intracellular mechanisms responsible for oriented locomotion and prime neutrophils to respond to another chemoattractant.

In accordance with other data presented in this paper, actions on neutrophil chemotaxis suggests that MCs interact with membrane components or penetrate into a cell, activating, in both cases, intracellular routes responsible for pro-inflammatory actions. How MCs enter the cell or trigger intracellular activation pathways is not well-established. MC-LR was shown to enter hepatocytes and cross the blood brain barrier through organic anion transporting polypeptides (OATPs) [22,23], that comprise a family of transporters exhibiting broad distribution and a range of substrates and display an important role in drug disposition and toxicity [24], but the expression of OATPs by neutrophils has not been demonstrated.

Here, we show that Ca_i^{2+} may be a mechanism by which MCs modulate neutrophil activity. The three toxins studied increased intracellular Ca^{2+} levels with different kinetic profiles, indicating that MCs acts via distinct pathways of neutrophil activation. The response evoked by MC-LR was delayed, but was more intense and sustained in comparison to that induced by MC-YR and MC-LA. In accordance with these data, the role of Ca_i^{2+} in MC toxicity has already been shown. Ca_i^{2+} released from mitochondria after oxidative stress induced by MC-LR and activation of Ca^{2+}/calmodulin-dependent protein kinase has been implicated in MC-induced hepatocyte apoptosis [25,26]. The source of Ca_i^{2+} and its real participation in the neutrophil migration pathways requires further investigation.

Together, the results described herein show that the three MCs studied induce inflammation and act as chemotactic agents for neutrophils, an important mechanism associated with toxicity. Therefore, a better understanding of the mechanisms by which MCs affect neutrophils may provide novel approaches for therapeutic intervention in response to severe MC-induced acute toxicity.

Acknowledgment

This work was supported by the Brazilian research funding agency Conselho Nacional de Desenvolvimento e Tecnologia (CNPq), Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

References

[1] M.E. Van Apeldoorn, H.P. Van Egmond, G.J.A. Speijers, G.J.I. Bakker, Toxins of cyanobacteria, Mol. Nutr. Food Res. 51 (2007) 7–60.

[2] W.W. Carmichael, S.M.F.O. Azevedo, J.S. An, R.J.R. Molica, E.M. Jochimsen, S. Lau, K.L. Rinehart, G.R. Shaw, G.K. Eaglesham, Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins, Environ. Health Perspect. 109 (2001) 663–681.

[3] I. Chorus, J. Bartram, Toxic Cyanobacteria in Water — A Guide to their Public Health Consequences, Monitoring and Management, Spon Press, London, 2003.

[4] E.M. Jochimsen, W.W. Carmichael, J.S. An, D.M. Cardo, S.T. Cookson, C.E.M. Holmes, M.B.C. Antunes, D.A. Melo-Filho, T.M. Lyra, V.S.T. Barreto, S.M.F.O. Azevedo, W.R. Jarvis, Liver failure and death after exposure to microcystins at a haemodialysis center in Brazil, N. Engl. J. Med. 33 (1998) 873–878.

[5] S. Pouria, A. De Andrade, J. Barbosa, R.L. Cavalcanti, V.T.S. Barreto, C.J. Ward, W. Preiser, G.K. Poon, G.H. Neild, G.A. Codd, Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil, Lancet 352 (1998) 21–26.

[6] S.M.F.O. Azevedo, W.W. Carmichael, E.M. Jochimsen, K.L. Rinehart, S. Lau, G.R. Shaw, G.K. Eaglesham, Human intoxication by microcystins during renal dialysis treatment in Caruaru-Brasil, Toxicology 181 (2002) 441–446.

[7] B.M. Babior, Phagocytes and oxidative stress, Am. J. Med. 109 (2000) 33–44.

[8] P. Kujbida, E. Hatanaka, A. Campa, P. Colepicolo, E. Pinto, Effects of microcystins on human polymorphonuclear leukocytes, Biochem. Biophys. Res. Commun. 341 (2006) 273–277.

[9] P. Kujbida, E. Hatanaka, A. Campa, R. Curi, S.H.P. Farsky, E. Pinto, Analysis of chemokines and reactive oxygen species formation by rat and human neutrophils induced by microcystin-LA, -YR and -LR, Toxicon 51 (2008) 1274–1280.

[10] V. Witko-Sarsat, P. Rieu, B. Descamps-Latscha, P. Lesavre, L. Halbwachs-Mecarelli, Neutrophils: molecules, functions and pathophysiological aspects, Lab. Invest. 80 (2000) 617–653.

[11] V. Niggli, Signaling to migration in neutrophils: importance of localized pathways, Int. J. Biochem. Cell Biol. 35 (2003) 1619–1638.

[12] S.E. Dahlen, J. Bjork, P. Hedqvist, K.E. Arfors, S. Hammarstrom, J.A. Lindgren, B. Samuelsson, Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules: in vivo effects with relevance to the acute inflammatory response, Proc. Natl. Acad. Sci. USA 78 (1981) 3887–3891.

[13] S. Boyden, The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leukocytes, J. Exp. Med. 15 (1962) 453–466.

[14] S.H. Zigmond, J.G. Hirsch, Leukocyte locomotion and chemotaxis: new method for evaluation and demonstration of cell-derived chemotactic factor, J. Exp. Med. 137 (1973) 387–410.

[15] L.J. Dahm, A.E. Schultze, R.A. Roth, An antibody to neutrophils attenuates alpha-naphthylisothiocyanate-induced liver injury, J. Pharmacol. Exp. Ther. 256 (1991) 412–420.

[16] J.M. Benson, J.A. Hutt, K. Rein, S.E. Boggs, E.B. Barr, L.E. Fleming, The toxicity of microcystin LR in mice following 7 days of inhalation exposure, Toxicon 45 (2005) 691–698.

[17] M. Kelly, J.M. Hwang, P. Kubes, Modulating leukocyte recruitment in inflammation, J. Allergy Clin. Immunol. 120 (2007) 3–10.

[18] M. Hernández, M. Macia, C. Padilha, F.F.D. Campo, Modulation of human polymorphonuclear leukocyte adherence by cyanopeptide toxins, Environ. Res. 84 (2000) 64–68.

[19] A.A. Nanji, K. Jokelainen, A. Rahemtulla, L. Miao, F. Fogt, H. Matsumoto, S.R. Tahan, G.L. Su, Activation of nuclear factor kappa B and cytokine imbalance in experimental alcoholic liver disease in the rat, Hepatology 30 (1999) 934–943.

[20] V. Tergaonkar, NFkappaB pathway: a good signaling paradigm and therapeutic target, Int. J. Biochem. Cell Biol. 38 (2006) 1647–1653.

[21] Y. Wei, D. Weng, F. Li, X. Zou, D.O. Young, J. Ji, P. Shen, Involvement of JNK regulation in oxidative stress-mediated murine liver injury by microcystin-LR, Apoptosis 13 (2008) 1031–1042.

[22] W.J. Fischer, S. Altheimer, V. Cattori, P.J. Meier, D.R. Dietrich, B. Hagenbuch, Organic anion transporting polypeptides expressed in liver and brain mediate uptake of microcystin, Toxicol. Appl. Pharmacol. 203 (2005) 257–263.

[23] H. Lu, S. Choudhuri, K. Ogura, I.L. Csanaky, X. Lei, X. Cheng, P. Song, C.D. Klaassen, Characterization of organic anion transporting polypeptide 1b2-null mice: essential role in hepatic uptake/toxicity of phalloidin and microcystin-LR, Toxicol. Sci. 103 (2008) 35–45.

[24] R. Evers, X. Chu, Role of the murine organic anion-transporting polypeptide 1b2 (Oatp1b2) in drug disposition and hepatotoxicity, Mol. Pharmacol. 74 (2008) 309–311.

[25] W.X. Ding, H.M. Shen, C.N. Ong, Pivotal role of mitochondrial Ca^{2+} in microcystin-induced mitochondrial permeability transition in rat hepatocytes, Biochem. Biophys. Res. Commun. 285 (2001) 1155–1161.

[26] W.X. Ding, H.M. Shen, C.N. Ong, Calpain activation after mitochondrial permeability transition in microcystin-induced cell death in rat hepatocytes, Biochem. Biophys. Res. Commun. 291 (2002) 321–331.