Review paper

Immunotoxic, genotoxic and carcinogenic effects of cyanotoxins

ANNA SIEROSŁAWSKA

Department of Physiology and Ecotoxicology, The John Paul II Catholic University in Lublin, Poland

Abstract

Released during water blooms cyanotoxins may evoke different negative effects on exposed organisms. Except well studied hepatotoxicity, neurotoxicity or dermatotoxicity, which became the basis of the toxin classification, some other effects, such as immunotoxicity, genotoxicity and tumor promoting potency are also suggested.

Key words: *water blooms, cyanotoxins, immunotoxicity, genotoxicity, carcinogenesis.*

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Introduction

Cyanotoxins, produced and released into the water during and after water blooms, pose a growing problem for human and animal health. Exposure to cyanotoxins may occur via several routes, e.g. by consuming contaminated water, food or dietary supplements, during recreational activities or by inhaling toxin-containing aerosols [1, 2]. Their toxic effects on vertebrates include different symptoms and are based on various mechanisms.

According to the target organ, cyanotoxins are divided into hepatotoxins (microcystins, nodularins, cylindrospermopsins), neurotoxins (anatoxin-a, anatoxin-a(s), saxitoxins), cytotoxins and irritating toxins, also influencing the gastrointestinal system (lyngbiatoxins, aplysiatoxins, lipopolysaccharides) [3]. The above mentioned directions of toxic impact are clearly seen after acute exposure. However, the chronic exposure to low cyanotoxin levels brings also other effects, of not fully understood mechanisms and sometimes difficult to predict. That possibility should be taken into consideration in toxicity assessment, especially in case of the toxins of long persistence in water, such as microcystins (MCs).

This review attempts to sum up information concerning conditions and mechanisms of immunotoxic, genotoxic and carcinogenic activity of cyanotoxins. While MCs are the most studied cyanobacterial toxins, presented herein data in a large extend concerns that group of substances.

Immunotoxic effects of cyanotoxins

There is a growing evidence, that cyanotoxins may evoke immunotoxic effects. This is of great concern taking into consideration, that the disorders of immune functions may induce many negative changes in organism functioning, including carcinogenesis. Immunomodulatory potency of cyanotoxins seems to be of dualistic way, inducing both immunostimulatory and immunosuppressive responses. Comprehensive reviews of the results of studies on the cyanotoxin immunomodulatory potency are given in [4-6]. In this paper only selected effects, connected with non-specific immunity will be discussed, in the respect to their hypothetical mechanisms.

Phagocytosis, being the fundamental immune response, is of special interest in immunotoxicity assessment. The process involves cytoskeleton rearrangements, followed by secondary activities, such as superoxide production and inflammatory cytokine release, controlled by different signaling pathways [7]. The main mechanism of toxic activity of such cyanotoxins, as MCs or nodularins, is the inhibition of serine/threonine protein phosphatase 1 and 2A (PP1 and PP2A), leading to the hyperphosphorylation of cytosolic and cytoskeletal proteins [8, 9]. MCs are supposed to interfere with all of three main cytoskeleton protein components [9, 10].

As phagocytosis is an actin-dependent process [7], microcystin-induced collapse of microfilaments in the

Correspondence: Anna Sierosławska, Department of Physiology and Ecotoxicology, The John Paul II Catholic University of Lublin, Norwida 4, 20-061 Lublin, Poland, phone number/fax: +48 81 536 70 89, e-mail: ansie@kul.lublin.pl

primary human and rat hepatocyte cultures [11, 12] and in other, non-hepatic cells [9], suggests that disruption of subtle remodeling of actin cytoskeleton in professional phagocytes exposed to the toxin may also be expected. Additionally, increased phosphorylation of the actin-associated proteins, such as vinculin or talin, may result in alternations in microfilaments binding to a cell membrane [citations in 9]. Actin cytoskeleton changes induced by MC-LR in all studied cell types are preceded by reorganization of intermediate filaments and microtubules, which suggests that these two latter ones are at least partially the cause of the observed microfilament collapse [9]. Toivola *et al.* [13] found, that MC-induced hyperphosphorylation of cytokeratin 18 and 8, the basic proteins of intermediate filaments, leads to their disruption. It is also suggested, that MCs may disrupt the microtubule cytoskeletal structures by direct binding to the tubulin cysteine residues [8]. The tubulin synthesis is dependent on the feedback regulated by the protein monomer pool. MC-mediated microtubule polymer collapse increases the free tubulin pool and inhibits new tubulin generation, which in the authors opinion, in some extend explains the severe loss of microtubules, observed in the cells treated with microcystic cyanobacteria extract [8]. MC-LR, the most toxic isoform of MCs, has been also found to cause increased phosphorylation of cytoplasmic dynein [14], which may be reflected in impaired microtubule motor functions. Dynein, microtubule-associated protein, is engaged in retrograde phagosome transport towards lysosomes allowing their fusion and degradation of the internalized particles [15, 16]. Indeed, reduction in the recovery of endosomal/lysosomal membranes by MCs in hepatocytes exposed to MC-LR has been reported [14], but no data from studies on phagocytic cells is available.

Despite the evident potency of MCs to induce changes in cytoskeleton, the results from the studies on the toxin effects on the process of phagocytosis are divergent. After 24 h incubation of human neutrophils with MC-LR concentrations up to 10 ng/ml, no effects on cell viability or phagocytosis have been found [17]. On the contrary, concentration-dependent suppression of phagocytic ability was seen in mice peritoneal macrophages after cell triggering with MC-LR at 1 – 1000 nmol/l for 4 h [18]. In the same experiment no effects were seen when mouse macrophage-like RAW264.7 cells were used. Chronic exposure of the early life stages of common carp on the cyanobacterial extract containing MCs by bath, as well as oral administration of MC-containing biomass to common and silver carp caused significant decrease of phagocytic activity in blood leucocytes [19, 20]. Decreased phagocytic index was also reported after injection of cyanobacterial extract containing 9.94 and 19.88 μg of MCs equivalents/kg mouse b.w. for 14 days, without any influence on percentage phagocytosis [21]. However, observed in the *in vivo* experiments changes in phagocytic ability may not be the results of direct toxin influence on phagocyte physiology, some indirect effects should also be considered.

Oppositely, there are reports suggesting toxin-induced non-specific activation of immune cells [22]. Stimulation of human and rat neutrophils after cell exposure on MC isoforms LA, YR and in the highest degree MC-LR, manifested as increased cell rolling and adherence, accompanied by higher L-selectin and b2-integrin expression and stimulated chemotaxis, was observed by Kujbida *et al*. [23, 24]. Toxins concentrations used were 1 – 1000 nM, exposure time not exceeded 60 min. Likewise, nodularin and in smaller extend MC-LR, occurred to stimulate the early spontaneous polymorphonuclear cell adhesion at the toxin concentrations up to 1 nM [25]. In our *in vitro* study rainbow trout phagocytes incubated for 35 min with MC-LR at 5 μg/ml revealed the increase of zymosan particle phagocytosis [26].

Except of the toxin influence on cell adherence and engulfing ability of phagocytes, also other stages of phagocytosis were reported to be interfered by the cyanotoxins. Elimination of invading microorganisms by professional phagocytes depends heavily on the generation of reactive oxygen species (ROS) during the process termed respiratory burst [27]. MCs are known to stimulate reactive oxygen production in different cell types, such as hepatocytes [10, 28] or lymphocytes [29]. In our study dose-dependent, dualistic effects of MC-LR on respiratory burst activity in phorbol myristate acetate triggered rainbow trout neutrophils were recorded [26]. It that study stimulation of reactive radicals production after cell incubation with the toxin at 1 and 5 μg/ml was recorded, while the higher used doses (10 and 20 μg/ml) diminished studied parameter. On the contrary, decreased spontaneous ROS generation in mice peritoneal macrophages after cell exposure on MC-LR was already noticeable at the concentrations starting from 10 nmol/l [18]. In turn, inhibitory effects of MC-LR at 10 μg/l on spontaneous and *Staphylococcus aureus* stimulated ROS production were observed in neutrophils isolated from patients receiving hemodialysis, but not from the healthy donors [17].

Observed increase of intracellular Ca^{2+} (iCa^{2+}) levels in the cells exposed on MCs may be a part of mechanisms on which the toxins influence the phagocyte functions, however the way of affecting the iCa^{2+} homeostasis and its real consequences are not yet fully known [10, 24]. The role of calcium ions in phagocytosis is significant, among others in actin cytoskeleton depolymerization and remodeling, in mediating phagosome-lysosome fusion and also in ROS production [30]. MC-induced elevated iCa^{2+} content was reported in different cell types, including neutrophils and lymphocytes [10, 24, 29]. Toxin effects on ROS production seems to be mediated by surge of calcium ions [8, 29], but also other mechanisms are proposed, such as MC-induced increase of NADPH oxidase activity or up-regulation of pro-apoptotic proteins Bax and Bid [28, 30, 32].

Non-specific activation of immune system, observed in cyanotoxin-exposed patients [22] could be explained by stimulation of proinflammatory cytokine release, which in turn is often concomitant with oxidative stress. Indeed, dose-dependent induction of interleukin 1 (IL-1) and TNF-α production was found in mouse macrophages stimulated *in vitro* with MC-LR at up to 1 μg/ml [33]. Incubation of human neutrophils with MC-LA, YR and LR at 1 or 1000 nM for 24 h increased the production of another proinflammatory mediator, IL-8 [34]. Additionally, these toxins stimulated cytokine-induced neutrophil chemoattractant (CINC)-2αβ release from rat neutrophils [34]. On the contrary, the other authors [35, 36] reported decrease of mRNA levels of IL-1β and TNF-α in mice treated with MC-LR-containing cyanobacterial bloom extracts and in mouse macrophages induced with pure MC-LR.

As stated above, there is a lot of discrepancies observed in the toxin effects on phagocytic cells. Some of them may be at least partially explained by different experimental conditions, especially used toxin concentrations and durations of exposure. Cellular uptake of MCs is mediated by multi-specific organic anion transporting peptides (rodent Oatps; human OATPs) [30], of which multiple types are expressed at high level on hepatocytes, enabling relatively quick influx of the toxin and faster effect exerting in the liver cells. The way MCs enters the other cell types, including phagocytes, is not fully clear, probably in some extend also via Oatps/OATPS [37]. Anyway, occurrence of the toxin influence on the non-hepatic cell morphology/functions is prolonged in time, compared to the effects on hepatocytes [9].

As phagocytes play an important role in regulation of cancer development and spontaneous tumorigenesis [38, 39], the immunomodulatory activity of the toxins should also be considered in relation to the observed carcinogenic effects of cyanotoxins.

Carcinogenic and genotoxic effects of cyanotoxins

Public concern about the carcinogenic potency of cyanotoxins arose when the correlation between incidences of primary liver and colorectal cancers and the presence of toxic cyanobacteria or their toxins in drinking sources has been suggested [40-44]. In most cases MC-LR was suspected to be responsible for these effects, however the laboratory studies on the toxin ability to induce cancers give sometimes ambiguous information.

The mechanisms underlying carcinogenic activity of MC-LR are probably divergent and realized on both genotoxic and epigenetic pathways [45]. It has been established, that during chronic exposure, MC-LR may act as the tumor inducer, as was found in the studies on mice given i.p. the toxin at the sublethal dose (20 μg/kg), five times per week over 28 weeks [46]. In that study neoplastic nodules were observed in mice livers without the use of any initiator. Similar findings were also reported in the study by Sano *et al*. [47], where the toxin was i.p. injected to mice for 14 months, causing the dose-dependent increase in hepatic adenomas and adenocarcinomas.

MC-LR is also supposed to be a potent liver tumor promoter. Nishiwaki-Matsushima *et al*. [48] found, that MC-LR administered i.p. at the dose of 10 μg/kg twice per week for 8 weeks to rats previously treated with tumor inducer, diethylnitrosamine, increased the number and percentage area of positive foci for the placental form of glutathione S-transferase, the sensitive marker of cancer initiation, in rat liver. The authors did not record such effects in the non-induced animals, which suggests that the duration of exposure may decide of the way of carcinogenic activity of MC-LR. Agreeable results were achieved by Charbonneau *et al.* [49] with the use of the similar experimental layout. Additionally, in that study carcinogenic effects were found also after oral uptake of the toxin at the dose of 80 μg/kg for 7 weeks. The results obtained by Lian *et al.* [41] confirmed tumor-promoting potency of MC-LR rather than initiating, in the short-term experiments. Mice were initially treated with a single dose of aflatoxin B1 and then were given i.p. MC-LR or nodularin at the dose of 10 μg/kg, once a week for 15 weeks. The other group of mice were treated with the cyanotoxins without previous induction. There was no increase in adenoma or carcinoma appearance in animals without pre-treatment, however a few altered hepatocellular foci were identified. On the contrary, in the aflatoxin-initiated animals both cyanotoxins intensified liver tumors development, nodularin in higher degree than MC-LR.

While the toxin main target in the cell is PP1 and PP2A, enzymes of pleiotropic activity able to directly regulate the activities of multiple protein kinase cascades, their inhibition leads to the hyperphosphorylation of a number of proteins. In the consequence deregulation of cell-cycle control, uncontrolled cell proliferation, as well as the inhibition of proteins involved in DNA repair occurs [45, 50-53]. One of the MC-LR targets undergoing hyperphosphorylation is nuclear protein p53, an important tumor suppressor which contributes to the repair of genotoxic damage, stops proliferation and induces apoptosis of impaired cells [54, 55]. That is of special importance, taking under consideration that in the same time MC-LR is able to cause oxidative stress resulting in DNA damage in the exposed cells [30, 56]. The mechanisms of DNA repair such as the nucleotide excision repair (NER) and the DNA double strand break repair by the nonhomologous end joining, in which p53 is engaged [57, 58], are known to be impaired by MC [30, 45].

One of the consequences of oxidative stress in the cell is the c-Jun *N*-terminal kinases (JNK) pathway activation, which is also attributed to MC-LR activity [59]. It has been found, that elevated activation of JNK plays a major role in promoting tumorigenesis of primary human brain tumors [60].

Genotoxic activity of MC-LR, manifested as increased micronucleus frequency, was determined in human-hamster hybrid AL cell line after 30-day treatment with the toxin at 0.1 µg/ml [61]. Similarly, increase in micronucleus frequency in human lymphoblastoid cell line TK6 after MC-LR intoxication was observed by Zhan *et al*. [62], but the authors used much higher toxin concentration (80 µg/ml) with short duration of exposure (24 h). Moreover, the authors found increase in mutation frequency at the thymidine kinase (TK) locus. Most of the TK mutants induced by MC-LR were the result of loss of heterozygosity, which is known to be an important genetic event in tumorigenesis and has a common occurrence in a variety of human tumors. Confirming results were also obtained when cyanobacterial extract containing MCs was used [63]. In that study genotoxicity was reported in both bacteria model, with the use of SOS chromotest and human lymphocyte model, with the use of comet assay. Moreover, the mutagenic effects of MC-LR, determined as ouabain resistance (Ouar), mutations induced in various mammalian cell types by many known mutagens, were found in cultured human RSa cells, with the highest frequency at 15 µg/ml. Base substitution mutations at K-ras codon 12 in genomic DNA were also found in the cells exposed to 7.5-15 µg/ml of the toxin for 6 days [64].

On the contrary, Lankoff *et al*. [51] reported, that MC-LR had no effect on the frequency of chromosome aberrations in human lymphocytes. Moreover, the authors suggested, that observed in their study microcystin-LR-induced DNA damage might be rather connected with early stages of apoptosis due to toxin cytotoxicity, not genotoxicity. A lack of mutagenic effects of MC-LR containing extract was found with the use of Ames test and SOS umu test [65].

An excellent critical summary of research on MCs genotoxic effects are given in Annex to the SKLM opinion "Microcystins in Algae Products Used as a Food Supplement" [66].

Much fewer data is available for the other cyanotoxin suspected to be carcinogenic, cylindrospermopsin (CYN). The toxin works by inhibition of protein synthesis and its toxicity results from CYN metabolic activation [22, 67]. As the structure of CYN includes sulphate, guanidine and uracil groups, it has been suggested that CYN may act on nucleic acids and exert carcinogenic effects on exposed organisms [68].

Shen *et al*. demonstrated that the toxin given i.p. to mice in a single dose of 0.2 mg/kg caused DNA strand breaks which might be one of the key mechanisms for CYN genotoxicity [69]. Moreover, covalent binding of CYN or its metabolites to DNA in mice, as well as early activation of P53 genes in human cell lines were observed [70, 71]. Cytogenetic damage in WIL2-NS lymphoblastoid cell line was also reported by Humpage *et al*. [72]. The authors suggest two mechanisms of CYN activity, one at the level of the DNA, inducing strand breaks and the other, at the level of kinetochore/spindle function, inducing loss of whole chromosomes. On the contrary, no clastogenic effects were detected after CHO-K1 cells exposure at 0.05-2 µg/ml, with or without metabolic activation of the toxin [73]. No increase in cancer was found in $P53^{def}$ transgenic mice after oral administration of chlorinated purified CYN or toxic cell-free extract for 90 and 170 days [74]. In that study similar effects were also found when MC-LR, and another cyanotoxin, saxitoxin, were used. In conclusion, the information on genotoxic/carcinogenic potency of CYN is incomplete and rather preliminary, so at the current state of knowledge it is difficult to predict such toxin activity.

Conclusions

Cyanotoxins, having diverse chemical properties, constitute a group of highly active compounds, potent to exert varied biological effects, including cancer. However, on the basis of the available literature it can be seen, that only one cyanotoxin, MC-LR, has been more extensively studied on the effects being the main problems of that review. In case of the other ones, data is very scared. Moreover, there is no studies on the possible connection between observed immunomodulating activity of MCs or other cyanotoxins and tumor cases. The need of investigation on that area seems to be obvious.

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