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First report of cylindrospermopsin effect on human peripheral blood lymphocytes proliferation *in vitro*

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Abstract

Cylindrospermopsin (CYN) is a natural toxin synthesized by several freshwater cyanobacteria (blue-green algae) species. Their expansion in Central European surface waters has been observed in last decades. Potential identified effects of CYN on human health exposed mainly via contaminated drinking water or consuming contaminated food includes hepato-, geno-, cyto- and fetal toxicity. So far there is very little known about CYN effects on immune system. We investigated the effect of different concentrations of this toxin upon the human peripheral blood lymphocytes proliferation and observed significant inhibition induced after 24 h by 1 μg ml^{-1} CYN. We believe this is the first report of CYN effect on this process in human lymphocytes. Authors will continue to study immunotoxicity of this chemical compound.

Key words: *cylindrospermopsin, lymphocytes, proliferation, immunotoxicity.*

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Introduction

Cylindrospermopsin (CYN) is polyketide-derived alkaloid with a central functional guanidino moiety combined with hydroxymethyluracil attached to its tricyclic carbon skeleton. It is highly soluble in water and has a relatively low molecular weight of 415 Da. Cylindrospermopsin which was identified for the first time in 1992 is synthesized as a secondary metabolite by eleven filamentous freshwater and bloom-forming cyanobacteria (blue-green algae) species [1, 2]. Expansion of these prokaryotic, autotrophic and photosynthetic organisms in Central European water bodies has been recently observed and raised serious health concern due to several identifications of CYN occurrence [3-6]. Concentrations of CYN in surface water can widely vary, highest levels are usually observed during bloom phenomenon (massive and rapid increase in cyanobacteria population) and in extreme cases can exceed the concentration of 800 μg l^{-1} [7]. Routes of potential human exposure to CYN can include drinking contaminated water, consuming contaminated food (due to bioaccumulation in freshwater organisms) and recreational activities (swimming, boating, water skiing) during cyanobacterial bloom [8]. However, many countries lack the official regulations concerning guideline safety values of CYN in drinking water, some authors suggested it should not exceed the concentration of 1 μg l^{-1} [9].

Cylindrospermopsin was believed to be primary hepatotoxic chemical compound [10]. Such properties has been observed in rodent model experiments and studies involving human cell lines [11-13]. There has been also two confirmed epidemic cases of CYN poisoning (Palm Island, Australia, 1979 and Caruaru, Brazil, 1996) resulting among many in: painful hepatomegaly, bloody diarrhea, vomiting, anorexia and dehydration. However, concentrations of CYN to which the individuals were exposed remains unknown due to lack of conducted studies in this area [14, 15]. In addition, numerous cases of animal poisoning, including lethal cases (e.g. cattle) after drinking water from dam contaminated with CYN has been recorded [16]. Apart from liver injuries, other potential effects of CYN on human health has been investigated and include geno- [17], cyto- [18] and fetal toxicity [19]. Cancerogenous properties of CYN are still a subject of study. In 2006 International Agency for Research on Cancer (IARC) concluded that there is no sufficient available data to resolve the question

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whether CYN can be involved in carcinogenesis processes [20]. So far there is only one report of experimentally observed of CYN-initiated tumor in mice [21]. A follow-up review of medical records from the children poisoned from the Australian outbreak in 1979 found an increased rate of gastrointestinal cancers in the period of 1982-1999 compared to the unexposed population; however no significance was found probably due to the low number of individuals in the exposed population [22].

Effect of CYN on immune response is not well studied, potential immunotoxicity of this naturally occurring poison was so far indirectly suggested in a few publications. Therefore, we aimed to investigate an effect of CYN on proliferation rate of human blood lymphocytes *in vitro*. We believe this is the first report of CYN effect on this process.

Material and methods

Heparinized samples of blood (8 ml) were collected from healthy donors at Regional Center of Blood and Blood Treatment in Poznań, Poland. Lymphocytes were isolated under sterile conditions by centrifugation (30 minutes, 1750 rpm, g = 569,4) on Gradisol-L (Aqua-Med, Poland) and washed twice in Eagle's medium (Biomed, Poland). The isolated lymphocyte suspension (1×10^6 cells per ml^{-1}) in Eagle's medium was supplemented with 10% fetal bovine serum (Sigma Chemicals, USA) and antibiotic (gentamycine at concentration of 50 µg ml^{-1}, Sigma Chemicals, USA). Lymphocytes cultures were established in a 96-well microplate (200 µl aliquots per well) and were incubated with CO_2 incubator under controlled conditions (5% CO_2, temp. 37°C, humidity 95%). Each culture were done in triplicate.

To stimulate lymphocytes proliferation phytohaemagglutinin-L (PHA-L, Roche Diagnostics, Sweden) was used in a concentration of 2.5 µg ml^{-1}.

100 µg of purified (> 95%) CYN (Alexic Chemicals, USA) isolated from *Cylindrospermosis raciborskii* was first dissolved in 1 ml of 50% methanol and stored in –20°C. After 48 h of lymphocytes incubation CYN was added to the culture in three different concentrations: 0.01 µg ml^{-1}, 0.1 µg ml^{-1} and 1 µg ml^{-1}. Final concentration of methanol in the investigated samples was 0.5%. To exclude potential effect of methanol on lymphocytes proliferation, two types of negative control (non-treated cells) for each experiment were included – with and without 0.5% methanol. Simultaneously with CYN [3H]-thymidine (Amersham, UK) was added in 1 µCi per well concentration. All samples were incubated for next 24 h. Ten repetitions of experiment for each CYN concentrations were conducted.

In order to measure lymphocytes proliferation, cultures were transferred by the harvester (SKATRON Instruments, Norway) on glass fiber filters (Perkin Elmer, USA), later placed in a scintillation cocktail (Perkin Elmer, USA). Measurement of thymidine incorporation was determined using scintillation counter (Perkin Elmer, USA). Results were expressed in counts per minute (CPM).

Data were analyzed by Wilcoxon signed rank test. Statistical significance was accepted at $p < 0.05$.

Results

Only the highest assayed concentration of CYN (1 µg ml^{-1}) had an adverse impact on human peripheral blood lymphocytes proliferation after 24 h of incubation. An effect was observed when compared with both control trials – with PHA-L and with PHA-L + alcohol. Statistically significant differences were noted ($p < 0.01$ in both comparisons). Rate of thymidine incorporation decreased averagely by 27.4% (compared to PHA-L control) and 23.9% (compared to PHA-L + alcohol control). Decrease was observed in every investigated sample with maximum 43.9% inhibition (compared to PHA-L control). No significant inhibition of thymidyne incorporation was reported for 0.01 µg ml^{-1} and 0.1 µg ml^{-1} CYN concentrations (Fig. 1). However, slight decrease of thymidine incorporation ratio was noted when compared with PHA-L control samples (4.3% for 0.01 µg ml^{-1} and 5.4% for 0.1 µg ml^{-1}, respectively). Comparison with PHA-L + alcohol control samples revealed lower differences (0.2% for 0.01 µg ml^{-1} and 0.9% for 0.1 µg ml^{-1}, respectively). There was no statistical difference between control trials (PHA-L vs. PHA-L + alcohol, $p > 0.05$) although average thymidine incorporation was 4% lower in PHA-L + alcohol samples.

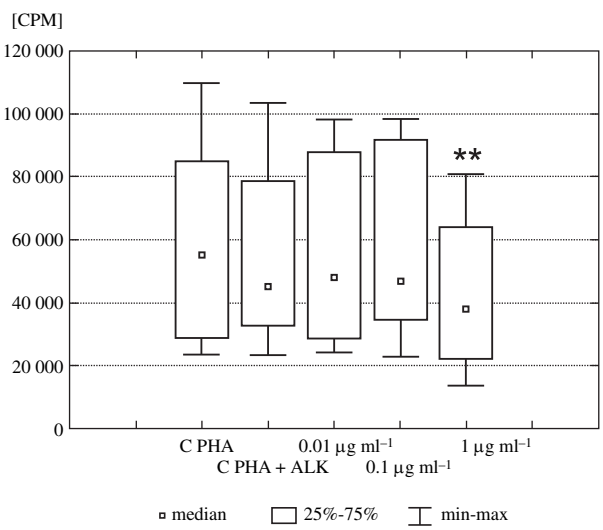

Fig. 1. Effect of different CYN concentrations on lymphocytes proliferation (CPM – counts per minute; C PHA – control with PHA; C PHA + ALK – control with PHA and methanol). Asterisks denotes a response that is significantly different from the both controls ($p < 0.01$)

Discussion

We have shown that the highest investigated concentration of CYN (1 μg ml^{-1}) had an effect upon the proliferation of human peripheral blood lymphocytes and resulted in inhibition of thymidyne incorporation. Use of two control trials (PHA-L and PHA-L with 0.5% methanol) allowed to exclude potential effect of an alcohol on lymphocytes proliferation.

As already mentioned in introduction of this paper, environmental concentrations of CYN widely vary and can greatly exceed the studied levels especially during mass invasion of cyanobacterial species in surface waters [6, 7]. One of potential serious source of human exposure to CYN can include the consumption of contaminated food. Bioaccumulation of CYN in tissues of aquatic organisms including mussels, crayfishes, snails and fishes was observed and varied from 100-1000 μg kg^{-1} depending on investigated species and aqueous CYN level. Reported CYN bioaccumulation factor (BAF) defined as the ratio of the concentration of a chemical accumulated inside an organism (resulting from sorption or/and consumption of organisms lower in the food chain) to the concentration in the surrounding environment varied from 20 to 250. Biomagnification, where toxin concentrations are increased through successive trophic level interactions, may also be possible for CYN and can also put human health at risk [23]. However, no studies involved commercially used species have been conducted so far, this threat cannot be entirely ruled out. Therefore it should be highlighted that water quality and livestock shall be a subject of regular monitoring wherever the risk of CYN-producers development occurs.

So far immunotoxic effects of CYN has not been well studied. First report by Terao *et al.* (1994) described a massive necrosis of lymphocytes in the cortical layer of the thymus of male mice given a single intraperitoneal dose of 0.2 mg kg^{-1} purified CYN [24]. Atrophy in lymphoid tissue of the spleen (follicular lymphocyte loss due to lymphophagocytosis) and thymus (degeneration and necrosis of cortical lymphocytes) has also been observed in orally exposed (4.4-8.3 mg CYN kg^{-1}) mice [25]. In other rodent experimental model induction of lymphophagocytosis in the mouse spleen at dosing with the cell-free extract at 0.05 mg CYN kg^{-1} was shown [26]. Žegura *et al*. (2011) found that CYN can induce oxidative stress in human lymphocytes. This process can eventually lead to adverse immune response. Authors also concluded that human lymphocytes can be a target of CYN induced genotoxicity resulting in the formation of DNA single strand break, increased frequency of micronuclei and nuclear buds, changes in the mRNA expression of P53 and its downstream regulated DNA damage responsive genes MDM2, GADD45α and apoptosis genes, BCL-2 and BAX, as well as oxidative stress responsive genes (GPX1, SOD1, GSR, GCLC) [18].

Immunotoxicity of other secondary metabolite synthesized by cyanobacterial species and commonly detected in European surface water, microcystin (MR), received wider attention so far. Microcystin extracts induced human peripheral blood lymphocytes apoptosis and strong inhibition of proliferation [27]. DNA damage and inhibiting effect on the repair of radiation-induced damage was also reported [28]. Other authors reported mild changes in leukocytes functions when exposed to low doses of MR (10 μg l^{-1}), particularly in the ability to produce reactive oxygen species. Higher rates of apoptosis were also observed [29]. Kujbida *et al*. (2008) investigated MR effects on human neutrophils and found increased interleukin-8, cytokine-induced neutrophil chemoattractant-2ab (CINC-2ab) and extracellular reactive oxygen species levels [30]. However, MR (cyclic peptide) widely differs in chemical structure from CYN (alkaloid), above cited studies indicate that cyanotoxins can have potential to adversely affect human immune system.

We have shown that among previously studied potential health effects, CYN can adversely affect innate human immune response. We believe this is the first report to describe such CYN attribute. Obviously, data obtained from *in vitro* assays cannot be extrapolated directly to the *in vivo* situation, the *in vitro* peripheral blood lymphocytes system used in the present study indicated possible *in vivo* immune responses to CYN in human. In order to decide whether CYN can be classified as a immunotoxicant further and wider studies are necessary. Therefore, authors of this paper will continue investigations of CYN effects on different immune system functions.

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