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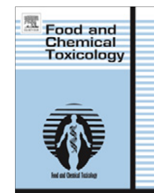

Chlorella vulgaris restores bone marrow cellularity and cytokine production in lead-exposed mice

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ABSTRACT

Chlorella vulgaris (CV) was examined for its modulating effects on the reduction induced by lead (Pb) on the numbers of marrow hematopoietic stem cells (HSCs) (c-Kit^{+}Lin^{-}), granulocyte–macrophage progenitors (Gr1^{+}Mac1^{+}) and total bone marrow cellularity. In mice gavage-treated daily with 50 mg/kg dose of CV for 10 days, concomitant to a continuous offering of 1300 ppm lead acetate in drinking water, the treatment with the algae recovered the significantly reduced numbers of these cell populations to control values. As CV may have a myelostimulating effect through the induction of cytokines, we evaluated its modulating effects on the production of IL-1α, TNF-α, IFN-γ, IL-10 and IL-6. Our results demonstrated that lead significantly impairs the production of IFN-γ, IL-1α and TNF-α and increases the production of IL-10 and IL-6 and that these effects are successfully modulated by the CV treatment. The activity of NK cells, reduced in Pb-exposed animals, was raised to levels higher than those of controls in the exposed group treated with CV. Treatment with the algae also stimulated the production of IFN-γ, IL-1α, TNF-α and NK cells activity in normal mice. In addition, zinc bone concentrations, reduced in lead-exposed mice, were partially, but significantly, reversed by the treatment with CV.

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1. Introduction

Lead (Pb) is known for millennia as a ubiquitous, insidious, and devastating toxin that affects human health and the environment in both industrialized and developing countries. Lead exposure has shown to downregulate various parameters of the immune response. Humoral and cellular immunosuppression appears to be a more subtle effect of exposure to low levels of Pb (Blakley and Archer, 1982; Chen et al., 1999; Faith et al., 1979; Heo et al., 1998; Kowolenko et al., 1991; McCabe et al., 1999).

Chlorella vulgaris (CV) is a microscopic single celled freshwater green algae, which is considered to be a biological response modifier (Noda et al., 1998), as demonstrated by its protective activities against viral and bacterial infections in normal and immunosuppressed mice (Dantas and Queiroz, 1999; Hasegawa et al., 1994, 1997; Queiroz et al., 2003; Tanaka et al., 1986) and against tumors (Justo et al., 2001; Konishi et al., 1985; Tanaka et al., 1984, 1998; Ramos et al., 2010). It is reported to be a rich source of antioxidants such as lutein, α- and β-carotene, ascorbic acid and tocopherol, and supplies large quantities of vitamins, minerals and dietary fibers (Gurer and Ercal, 2000; Rodriguez-Garcia and Guil-Guerrero, 2008; Vijayavel et al., 2007). Likewise plants, *Chlorella* and other algae contain high concentrations of phytochelatins, which are glutathione oligopeptides capable of strongly binding heavy metal ions (Cobbett and Goldsbrough, 2002; Pawlik-Skowronska et al., 2007).

Recently, the chelating ability of CV to reduce Pb toxicity in blood and target organs such as bone, kidney and liver has been demonstrated by our group. In addition, the chelators contained in CV greatly restored the normal levels of ALA, particularly in the liver, and the biosynthesis rate of heme and its precursors, suggesting very high affinity of CV for Pb (Queiroz et al., 2008). This metal binding property seems to be related to the presence of chloroplast in the cellular wall, an organelle rich in sulfur, potassium,

Abbreviations: APC, antigen presenting cells; CFU-GM, Granulocyte–macrophage colony-forming-unit; CV, *Chlorella vulgaris*; FBS, fetal bovine serum; HSCs, hematopoietic stem cells; IFN, interferon; IL, interleukin; NK, natural killer; Pb, lead acetate; TNF, tumor necrosis factor.

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calcium and phosphorus (López et al., 1998; Traviesso et al., 1999; Wong et al., 1997), The ability of sulphydryl-containing compounds to chelate metals is well established in the literature and this could be the main factor involved in the in vitro removal by CV of heavy contaminants (Apasheva et al., 1972; Sinicropi et al., 2010).

A suppressive dose-dependent effect of Pb on bone marrow granulocyte–macrophage progenitors has also been demonstrated by our group and others (Bincoletto and Queiroz, 1996; Queiroz et al., 2003, 2008; Van Den Heuvel et al., 1999). The accumulation of this metal in the bone marrow could result in deleterious effects to the progenitor cells and consequently lower the resistance to a variety of pathogens (Barry, 1975; Westerman et al., 1965). Notably, the stimulation of the pool of hematopoietic stem cells and the activation of mature leukocytes are important aspects of CV effects on the immune system of immunosuppressed hosts (Hasegawa et al., 1990; Konishi et al., 1990, 1996). In this respect, studies from our laboratory have demonstrated that CV induces a significant recovery in the reduced number of myeloid progenitor cells (CFU-GM) found in tumor-bearing, stressed and infected mice (Dantas and Queiroz, 1999; Justo et al., 2001; Queiroz et al., 2003; Souza-Queiroz et al., 2004, 2008). Relevant to our present findings, we recently demonstrated that CV treatment of lead-exposed mice up-modulates the reduced ability of bone marrow stromal cells to display myeloid progenitors in vitro and restores both the reduced numbers of CFU-GM and non-adherent cells in long-term cultures (Queiroz et al., 2008).

Primitive hematopoietic stem cells (HSCs) in the bone marrow are rare pluripotent cells with the capacity to give rise to all lineages of blood cells. They are operationally defined as cells that can completely reconstitute a recipient following bone marrow ablation. Similarly, they must have the capacity of self-renewal, giving rise to other stem cells (Domen and Weissman, 1999). HSCs are highly enriched in a population that is negative for lineage markers (Lin^-) and positive for Sca-1 and c-Kit (Spangrude et al., 1988). c-Kit, which is the receptor for stem cell factor, has a wider expression pattern than Sca-1, marking most multipotent progenitors. Sca-1 and c-Kit are often used together for positive selection of HSCs from Lin^- cells. Surface antigens lineage-specific as Gr-1 and Mac-1 (Lin^+) characterize compromised cells of the myeloid lineage (Larsson and Karlsson, 2005; Lyman and Jacobsen, 1998).

The production of hematopoietic cells is under the tight control of a group of cytokines. Th1/Th2 balance is widely recognized for its role in the regulation of homeostasis and the function of mature lymphoid/myeloid lineage cells, and it also plays a role in maintaining the homeostasis of hematopoietic stem cells and progenitor cells. Recent study has shown that Th1 cell products, but not those of Th2 cells, caused a rapid expansion of lineage $Sca\text{-}1^+c\text{-}Kit^+$ cells in vivo and in vitro. Among Th1 cytokines, interferon (IFN)-γ was found to play a major role in this expansion by converting the lineage $Sca\text{-}1^-c\text{-}Kit^+$ cells to lineage $Sca\text{-}1^+c\text{-}Kit^+$ cells (Zhao et al., 2010).

Natural killer (NK) cells act as regulatory keys during inflammation and influence subsequent adaptative immune responses (Orange and Ballas, 2006; Vivier et al., 2008), due to their capacity to produce cytokines, particularly IFN-γ. Relative to IFNs, it is well demonstrated that IFN or IFN-inducers potentiate NK cell reactivity (Justo et al., 2003; Queiroz et al., 2002; Zwirner and Domaica, 2010), thus favoring Th1-type responses. In this regard, previous studies from our laboratory and others demonstrated that CV enhances the levels of Th1 cytokines with no effects on Th2-type response (Hasegawa et al., 1997; Queiroz et al., 2002).

Another important aspect in the development and maintenance of immune competence consist in the adequate concentrations of zinc. This mineral is one of the most important trace elements in the body for many biological functions, as cell growth and division, and it is required for the activities of a variety of enzymatic systems in the body (Muzzioli et al., 2007). In addition, zinc has been shown to minimize lead poisoning in children exposed to this metal (Winneke, 1996).

In order to better characterize the immunoprotective effects of CV in lead-exposed mice, flow cytometric quantifications of hematopoietic stem cells – $c\text{-}Kit^+Lin^-$ and granulocyte–macrophage progenitors – $Gr1^+Mac^+$, as well as total cellularity, were performed in bone marrow. In addition, cytokines production (IL-1α, TNF-α, IFN-γ, IL-10 and IL-6), NK cell activity and zinc concentrations were also evaluated.

2. Material and methods

2.1. Mice

Male BALB/c, 6–8 weeks old, were bred at the University Central Animal Facilities (Centro de Bioterismo, Universidade Estadual de Campinas, Campinas, SP), raised under specific pathogen-free conditions, and matched for body weight before use. Standard chow and water were freely available. Animal experiments were done in accordance with institutional protocols and the guidelines of the Institutional Animal Care and Use Committee (Olfert et al., 1993).

2.2. Treatment regimens

The dried *C. vulgaris*, a strain of unicellular green algae, was kindly provided by Dr. Hasegawa (Research Laboratories, Chlorella Industry Co. Ltd., Fukuoka, Japan). Chemical analysis, performed by Hasegawa et al. (1990) revealed that CV contained 44.4 g of protein, 39.5 g of carbohydrates and 15.4 g of nucleic acids in 100 g (dry weight) of whole material. No lipids were detected. CV was prepared in distilled water and doses of 50 mg/kg were given orally by gavage in a 0.2 ml volume/mouse for 10 consecutive days, concomitantly to continuous offering of 1300 ppm lead acetate in drinking water. The selection of doses for CV and lead was based on previous studies performed in our laboratory (Bincoletto and Queiroz, 1996; Dantas and Queiroz, 1999; Queiroz et al., 2003). In all groups, femoral marrow was collected 24 h after the last administration of CV.

2.3. Total bone marrow cellularity

Femoral marrow cells were aseptically collected by flushing. Total cell counts of marrow cell suspensions were then performed, 24 h after the last administration of CV in Neubauer chamber by Trypan-blue exclusion method.

2.4. Flow cytometric analysis

To determine hematopoietic cells populations, bone marrow cells were collected by flushing (1×10^6 cells), fixed with 2% *p*-formaldehyde for 30 min, labeled with anti-Gr-1 fluorescein isothiocyanate (1.0 μg/ml), anti-Mac-1-phycoerythrin (1.0 μg/ml) in 1% bovine serum albumin and dissolved in PBS for 30 min on ice. To identify murine progenitor cells, a biotin-conjugated lineage (Lin) antibody cocktail was used (anti-Gr-1, Mac-1, CD3ε, TER119, and B220) for 20 min, followed by labeling with anti-c-Kit-allophycocyanin (APC, 1.5 μg/ml) in 1% bovine serum albumin and dissolved in phosphate buffered saline for 30 min on ice. The cells were collected on a FASCalibur (Becton Dickinson, San Jose, CA, USA). Data analyses were performed using CELLQuest 3.4 software (Becton Dickinson, San Jose, CA, USA). The antibodies were purchased from BD Biosciences (San Diego, CA, USA).

2.5. Quantification of TNF-, IFN-γ, IL-1, IL-10 and IL-6 levels

Cytokines IL-1α, TNF-α, IFN-γ, IL-10, and IL-6 were quantified by sandwich ELISA using purified anti-mouse IL-1α (Cat. 550347), TNF-α (Cat. 555268), IFN-γ (Cat. 555138), IL-10 (Cat. 555252) and IL-6 (Cat. 555240), purchased from BD Pharmingen, USA. Cytokine determinations were done according to BD OptEIA™ ELISA Set protocol. Cytokine titers were expressed as picograms/ml, calculated by reference to standard curves constructed with known amounts of recombinant cytokines.

2.6. Natural killer cell activity

2.6.1. Preparation of effectors cells for the NK cell assay

Suspensions of spleen cells from all mice were prepared by gently pressing aseptically removed spleen through a stainless steel mesh net into RPMI-1640 (Cultilab) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Cultilab). The resultant mononuclear cells were isolated from the cell suspension by Ficoll–Hypaque gradient separation (density 1.077 g/ml; Pharmacia, Piscataway, USA), washed three times and resuspended in RPMI-1640 supplemented with 10% heat-inactivated FBS. Cell suspensions were placed in 150 mm tissue culture dishes

and incubated at 37 °C, in 5% CO_2, for 90 min to remove adherent cells. Non-adherent cells were then harvested by gently pipetting. The cells were washed three times and the concentration adjusted to 5×10^6 cells/ml.

2.6.2. Preparation of target cells for the NK cell assay

YAC-1, a Moloney virus-induced mouse T-cell lymphoma of A/SN origin, was used as target cell in the 4 h ^{51}Cr-release assay. Briefly, 5×10^6 pelleted YAC-1 cells were resuspended to 0.2 ml of FBS and labeled with 100 mCi of sodium chromate (^{51}Cr) (IPEN, Brazil) for 90 min at 37 °C in a shaking water bath. After labeling, the cells were washed twice with RPMI 1640 culture medium and resuspended at a concentration of 1×10^5 cells/ml in enriched medium supplemented with 10% FBS.

2.6.3. NK cell cytolytic assay

NK activity of effector cells was measured with a 4 h ^{51}Cr-release assay using YAC-1 target cells. Effector cells and targets were dispensed in triplicates into round bottom microtiter plate wells (Corning) producing effector to target ratios of 50:1. Plates were centrifuged at 800 rpm for 5 min and incubated 4 h at 37 °C in a humidified CO_2 incubator. After the incubation period, the plates were centrifuged again at 1200 rpm for 10 min and 0.1 ml of the supernatants were collected for radioactivity counts in a Beckman Biograma Counting System (Beckman 5500 B, Irvine, USA). Spontaneous release was determined by adding 100 labeled target cells to 0.1 ml of medium in the absence of effector cells and were always less than 10% of the maximum release, which was determined by exposure of labeled target cells to 0.05% Tween-20. Percentage of cytotoxicity, as measured by specific ^{51}Cr release, was calculated by using the formula: (cpm experimental − cpm spontaneous)/(cpm maximal − cpm spontaneous) × 100.

2.7. Determination of zinc concentrations

Zinc concentrations in bone, as well as in CV, were measured by inductively coupled plasma-optical emission spectrometry (ICP OES); model VISTA-MPX CCD Simultaneous from Varian Inc. (Mulgrave Victoria, Australia) and equipped with axial vision, a radio frequency (RF) source of 40 MHz, a CCD (Charge Coupled Device)-type simultaneous multi-elementary solid-state detector, a peristaltic pump, a nebulization chamber, and a sea spray nebulizer. The system was controlled by ICP Expert software using liquid argon as plasma gas with 99.996% purity (Air Liquid, SP, Brazil). The wet-digestion using HNO_3 (65%) and H_2O_2 (37%) in a microwave-assisted digester oven (Milestone, Italy) were used for all samples. The detection and quantification limits were 1.4 µg/kg and 9 µg/kg, respectively, and the recovery was 95% for zinc. The analytical curve was linear in the range 0.01–1.00 mg/L (r = 0.9998). Analytical quality was maintained by repeated analysis of the reference standards (Horwitz, 2005).

2.8. Statistical analysis

Data were analyzed for statistically significant experimental differences using analysis of variance (ANOVA) and the Tukey post test. Probability values greater than 0.05 were considered non-significant. In all cases, at least three independent experiments were conducted to warrant that the results were representative.

3. Results

3.1. Total bone marrow cellularity

The effects of CV treatment on total bone marrow cellularity of Pb-exposed mice are presented in Table 1. As can be seen from these results, exposure to Pb significantly ($P < 0.05$) reduced cellularity, reaching levels 1.6-fold lower than those from controls. Treatment with CV restored these values to control levels. Treatment of non-exposed mice with CV produced no changes in total bone marrow cellularity.

3.2. Flow cytometric analysis

The numbers of c-Kit$^+$Lin$^-$ and Gr1$^+$Mac$^+$ cells in the bone marrow were significantly reduced in Pb-exposed mice ($P < 0.05$) (Figs. 1A and 1B), reaching, respectively, levels 50% and 40% lower than those observed in the control group. As can be seen in both figures, the simultaneous daily administration of 50 mg/kg of CV for 10 consecutive days restored the numbers of these two populations to control values. As before, CV treatment did not affect the number of both populations in non-exposed mice.

Table 1
Total bone marrow cellularity.

Groups	Total bone marrow cellularity ($\times 10^6$)
Control	26.2 ± 1.6
CV	26.3 ± 1.5
Pb	16.7 ± 1.2[a]
CV + Pb	27.5 ± 1.3

Mice were gavage treated orally with 50 mg/kg/daily of CV for 10 consecutive days, simultaneously to the administration of 1300 ppm of lead acetate in the drinking water. Bone marrow was removed 24 h after the end of lead exposure. Control mice received diluent only. Results are the means ± SD of six animals/group. [a]$P < 0.001$ compared to control and CV + Pb.

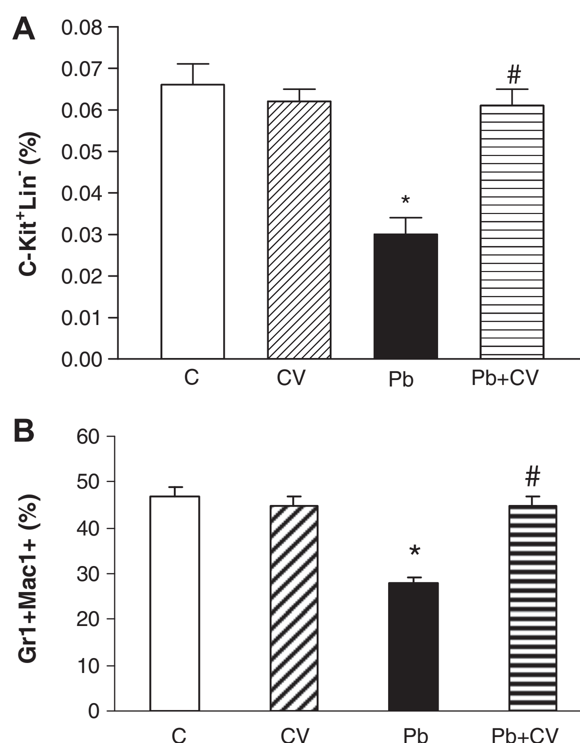

Fig. 1. Protective effects of *Chlorella vulgaris* (CV) on the percentage of primitive hematopoietic progenitor cells (c-Kit$^+$Lin$^-$) (A) and granulocytes/macrophages progenitor cells (Gr1$^+$Mac1$^+$) (B) in the bone marrow of mice gavage treated daily with a single 50 mg/kg dose of CV for 10 consecutive days, concomitantly to continuous offering of 1300 ppm lead acetate in drinking water. Bone marrow cells were removed and fixed 24 h after the end of the 10-day study. Control mice (C) received vehicle only. The results are represented as the mean ± SD of six mice/group. *$P < 0.05$ in relation to control and to CV-treated groups, #$P < 0.05$ in relation to Pb group.

3.3. Quantification of cytokine levels

The effects of the treatment with CV on the production of IL-1α, TNF-α, IFN-γ, IL-10 and IL-6 by splenocytes of Pb-exposed mice are presented in Figs. 2 and 3, respectively. Notably, in non-exposed mice, CV treatment significantly increased ($P < 0.05$) the production of IL-1α, TNF-α and IFN-γ, whereas no significant changes were observed in the levels of IL-10 and IL-6. Exposure to Pb significantly reduced ($P < 0.05$) IL-1α, TNF-α and IFN-γ production to levels 3, 2 and 4-fold, respectively, lower than controls, and significantly increased ($P < 0.05$) the production of IL-6 and IL-10, at levels 2 and 1.5-fold higher than controls. As previously, CV treatment of these animals recovered the levels of all cytokines studied to values similar to those of controls ($P < 0.05$).

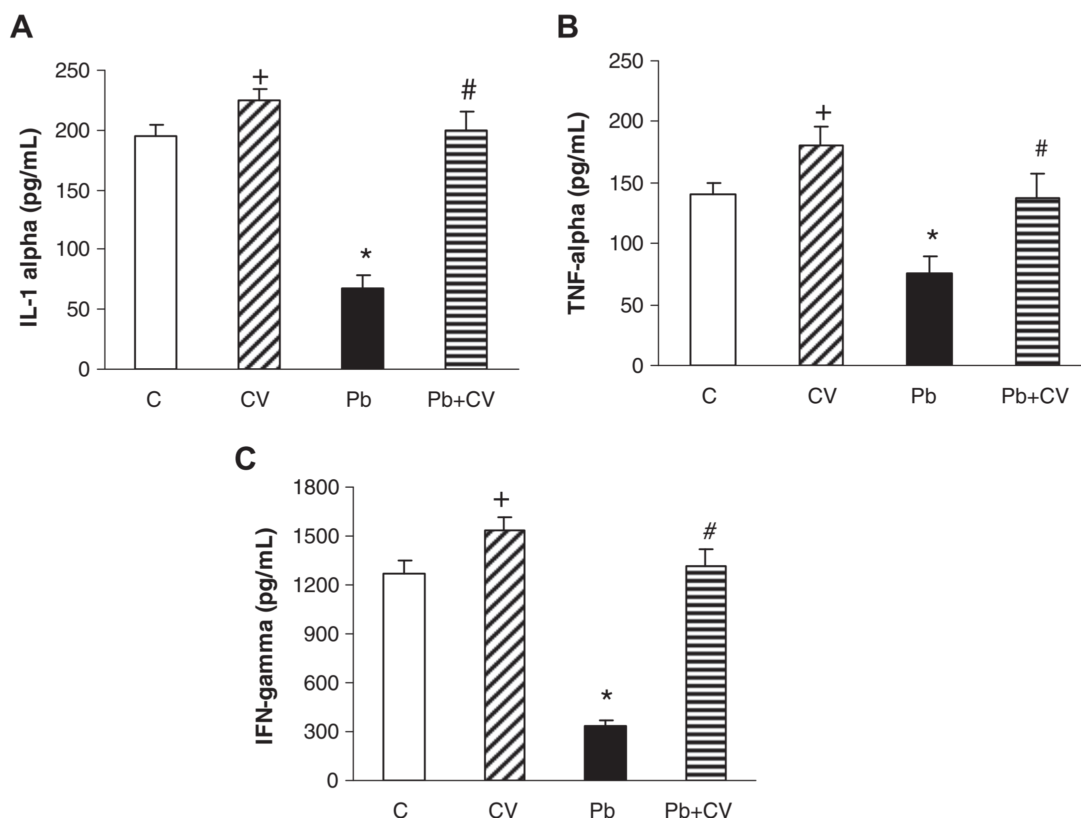

Fig. 2. Levels of IL-1α (A) TNF-α (B) and IFN-γ (C) in the supernatant of spleen cells culture from mice gavage treated daily with a single 50 mg/kg dose of CV for 10 consecutive days, concomitantly to continuous offering of 1300 ppm lead acetate in drinking water. Mice were sacrificed 24 h after the end of the 10-day study. Control mice (C) received vehicle only. Results are the means ± SD of six mice/group. $^{+}P < 0.05$ in relation to control group, $^{*}P < 0.05$ in relation to control and to CV-treated groups, $^{\#}P < 0.05$ in relation to Pb group.

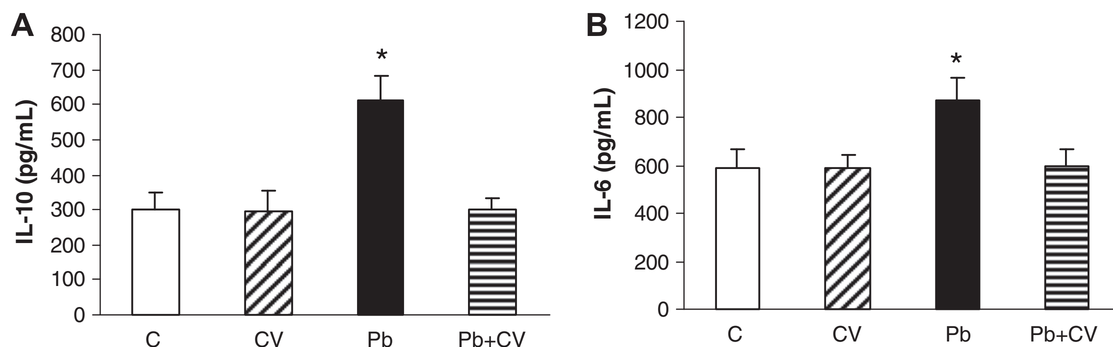

Fig. 3. Levels of IL-10 (A) and IL-6 (B) in the supernatant of spleen cell cultures from mice gavage treated daily with a single 50 mg/kg dose of CV for 10 consecutive days, concomitantly to continuous offering of 1300 ppm lead acetate in drinking water. Mice were sacrificed 24 h after the end of the 10-day study. Control mice (C) received vehicle only. Results are the means ± SD of six mice/group. $^{*}P < 0.05$ in relation to control, to CV-treated and to Pb + CV-treated groups.

3.4. Natural killer cell activity

The effects of the treatment with CV on NK cells activity from Pb-exposed mice are demonstrated in Fig. 4. Exposure to Pb significantly ($P < 0.05$) decreased NK cells activity, reaching values 1.5-fold lower than those of controls. CV treatment increased the activity of these cells, reaching levels 1.4-fold higher than those observed in control group ($P < 0.05$). Notably, a significant ($P < 0.05$) enhancement in NK cells activity was also observed after treatment of normal mice with CV.

3.5. Zinc concentrations

As seen in Fig. 5, a significant ($P < 0.05$) reduction in the levels of femoral zinc was observed in Pb-exposed mice, reaching levels 1.2-fold lower than control values. This effect was partly, but significantly, reversed by the treatment of these animals with CV ($P < 0.05$), resulting in bone zinc levels 1.1-fold lower than those observed in controls. Determination of zinc content in CV showed that the algae presents 10.6 mg/kg of this mineral.

4. Discussion

Despite increasingly successful efforts to reduce the rate of deposition of lead in the environment worldwide, the metal remains a crucial contaminant of humans and wildlife (Bishayi and Sengupta, 2006; Choi and Kim, 2003; Jarup, 2003). Recently, the recognition that lead exposure has the capacity to alter neurological and immunological functions in children, even at low exposure

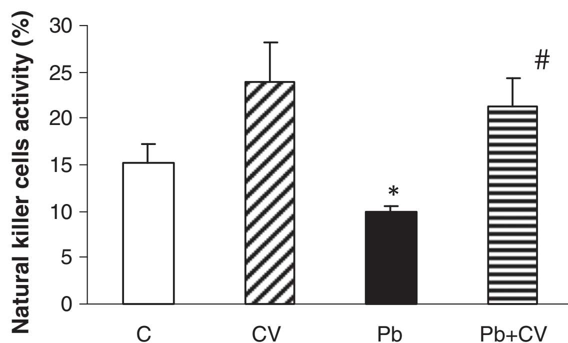

Fig. 4. Protective effects of *Chlorella vulgaris* (CV) on the natural killer (NK) cells activity of mice gavage treated daily with a single 50 mg/kg dose of CV for 10 consecutive days, concomitantly to continuous offering of 1300 ppm lead acetate in drinking water. Mice were sacrificed 24 h after the end of the 10-day study. Control mice (C) received vehicle only. The results are represented as the mean ± SD of six mice/group. $^{+}P < 0.05$ in relation to control group, $^{*}P < 0.05$ in relation to control and to CV-treated, $^{\#}P < 0.05$ in relation to control and Pb groups.

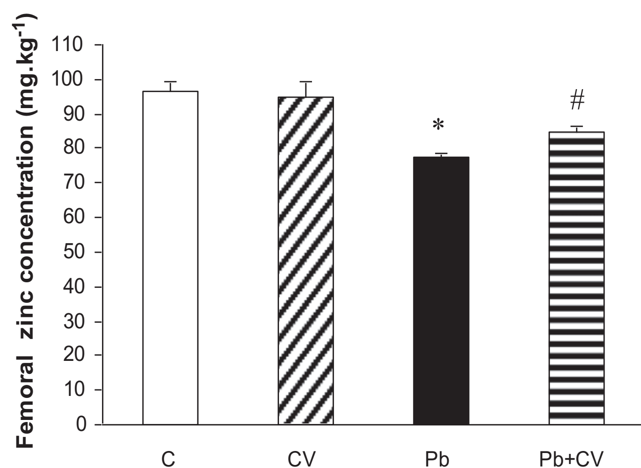

Fig. 5. Zinc concentrations in bone of mice gavage treated daily with a single 50 mg/kg dose of CV for 10 consecutive days, concomitantly to continuous offering of 1300 ppm lead acetate in drinking water. Mice were sacrificed 24 h after the end of the 10-day study. Control mice (C) received vehicle only. The results are represented as the mean ± SD of six mice/group. $^{*}P < 0.05$ in relation to control and to CV-treated groups, $^{\#}P < 0.05$ in relation to Pb group.

levels, led to increasing concern over the long-term effects of early-life low level exposure to this metal (Bellinger, 2004; Dietert and Piepenbrink, 2006). In this respect, the lack of safety and efficacy demonstrated by conventional chelating agents (Gurer and Ercal, 2000) has encouraged the search for new ways to remove heavy metals from the body. New technologies involving the removal of toxic metals from waste waters has directed attention to biosorption, based on metal-binding capabilities of algae and other aquatic plants. In this context, the use of *C. vulgaris*, due to its metal absorption properties, has been reported by several authors (López et al., 1998; Slaveykova and Wilkinson, 2002; Veglio and Beolchini, 1997; Wilde and Benemann, 1993; Wong et al., 1997).

In previous studies we observed that treatment with CV, given simultaneously to continuous offering of 1300 ppm of lead in drinking water, restored to control values the myelosuppression observed in infected/lead-exposed mice and produced significant increases in serum colony-stimulating activity. The efficacy of CV was evident when these mice were challenged with a lethal dose of *Listeria monocytogenes*, reaching survival rates up to 30%. Evidence that these protective effects of CV are partly due to its chelating effect was given by the significant reductions observed in blood lead levels (Queiroz et al., 2003). Monitoring of lead poisoning demonstrated that CV treatment significantly reduced lead levels in blood and tissues, completely restored the normal hepatic ALA levels, decreased the abnormally high plasma ALA and partly recovered the liver capacity to produce porphyrins (Queiroz et al., 2008). The chelating effect of CV was significantly higher when the algae was given simultaneously, rather than following lead exposure, thus indicating that the immunomodulatory effects of CV play an important role in the ability of the algae to reduce lead levels in blood and tissues (Queiroz et al., 2003, 2008).

In the present study, we investigated the effects of CV on both marrow populations c-Kit^{+}Lin^{-} and Gr1^{+}Mac1^{+} and on total bone marrow cellularity of Pb-exposed mice. Using an ex vivo analysis of bone marrow cells, we observed that both the more immature c-Kit^{+}Lin^{-} and the more mature Gr1^{+}Mac1^{+} hematopoietic populations, as well as total bone marrow cellularity, are all reduced in lead-exposed mice, thus indicating a toxic effect of this metal on these cell populations in association to the impaired functional ability and reduced production of regulatory factors by bone marrow cells demonstrated previously (Queiroz et al., 2008). CV treatment of Pb-exposed animals restored to control values the numbers of c-Kit^{+}Lin^{-} and Gr1^{+}Mac1^{+} lineages, as well as total bone marrow cellularity.

Studies from our laboratory and others have demonstrated that CV may have a direct myelostimulating effect through the induction of endogenous cytokine production (Hasegawa et al., 1997; Kumamoto et al., 1999; Queiroz et al., 2002, 2003, 2008; Souza-Queiroz et al., 2008; Ramos et al., 2010). Corroborating previous studies (Queiroz et al., 2002; Ramos et al., 2010), we observed here, in the normal host, the ability of CV to increase the production of IFN-γ, IL-1α and TNF-α, which are mediators of the inflammatory response and which are released from different cell types (Schuerwegh et al., 2003). The reports that CV stimulates macrophages to produce pro-inflammatory cytokines support this contention (Hasegawa et al., 1997, 2000). Similarly, increased production of IL-2 and IL-12 by CV in the normal host has also been demonstrated by other studies (Hasegawa et al., 1997; Queiroz et al., 2002). These findings clearly demonstrate the ability of the algae to promote Th1 response.

Conversely, in relation to lead, it has been previously demonstrated that it significantly impairs the production of IL-1α and TNF-α (Hemdan et al., 2005). Considering the ability of IL-1 and TNF-α to promote Th1 pathway by antigen presenting cells (APC) (Cervi et al., 2004; Hemdan et al., 2005), it seems that lead exerts an inhibitory effect on APC, leading to the inhibition of both cytokines. Our present findings also demonstrate a preferential stimulation of Th2 response by lead through the significantly reduced IL-1α, TNF-α and IFN-γ and increased IL-6 and IL-10 levels in lead-exposed mice. Treatment of these mice with CV restored to control values both the reduced levels of IL-1α, TNF-α and IFN-γ and the increased levels of IL-6 and IL-10. In this respect, previous experiments with gene knockout C57BL/6 mice lacking the IFN-γ gene demonstrated that this cytokine is of paramount importance in the protection afforded by CV (Queiroz et al., 2003). Therefore, it is likely that enhanced Th1 response might be involved in the protective effects of CV in lead-exposed mice, thus making Th1-driven development dominant over Th2-induced response (Heo et al., 1998).

Contrary to our present findings of a systemic elevation in IL-6 levels by lead, our previous studies using the supernatant of long-term bone marrow culture of lead-exposed mice demonstrated significantly local reduction in the levels of this cytokine (Queiroz et al., 2008). The apparent controversy in local and systemic production of IL-6 after lead exposure is clarified by Zhou et al. (1993) findings showing that increased systemic IL-6 in

stressed animals may be under the regulation of the neural and endocrine systems and may not come from immune tissues. Specifically, these studies demonstrated that the production of IL-6 by splenic cells and peripheral blood mononuclear cells from stressed animals was increased. Indeed, adrenalectomy substantially attenuated the elevation of plasma IL-6 due to stress. It seems therefore that, besides a direct myelostimulating effect on cytokine release, a possible explanation for lead effects on IL-6 systemic production could be linked to the metal-enhanced stress response, thus compromising the innate and cell-mediated immunity by inhibition of the development and activation of macrophages (Kishikawa et al., 1997; Virgolini et al., 2005). In this respect, Hasegawa et al. (2000) demonstrated that the oral administration of CV significantly inhibited the elevation of serum glucocorticoids levels in mice after stress and concurrently prevented the decrease in the number of immune cells. Corroborating our previous findings showing the ability of CV to restore to control values the local reduced production of IL-6 by stromal cells of lead-exposed mice (Queiroz et al., 2008), we demonstrate here the ability of the algae to down modulate the increased IL-6 production by splenocytes of lead-exposed mice. Therefore, with respect to the variety of IL-6 functions, our results seem to indicate that CV-induced modulation of IL-6 production contributes to the maintenance of homeostasis.

Several studies on the mechanisms by which a stressor modulates immune status demonstrate that the release of catecholamine co-operatively inhibits IFN-γ production by Th1 cells and triggers secretion of substantial amounts of IL-10 by Th2 cells (Woiciechowsky et al., 1998). In this respect, an increasing body of evidence indicate that suppression of cellular immunity through selective inhibition of Th1 functions, in favor of Th2 responses, is an important feature of stress in general (Elenkov et al., 1996, 2000; Zhang et al., 2005; Souza-Queiroz et al., 2008). Relevant to our present findings is the fact that noradrenaline containing nerve fibers entering the hematopoietic tissue of bone marrow terminate with synapses on hematopoietic cells and promote a negative regulation of hematopoietic activity affecting both hematopoiesis and the release of cells from the marrow (Heyworth et al., 1992). These observations assume additional significance in view of the fact that adrenoreceptors are expressed on Th1 cells, but not on Th2 cells (Sarders et al., 1997; Elenkov et al., 2000), thus providing a mechanistic basis for the differential effects on Th1/Th2 functions.

NK cells are thought to be important in innate immunity because of their potent capacity to produce cytokines, particularly IFN-γ (Kim et al., 2000), which increases NK cell reactivity and activates macrophages (Yamamoto et al., 1995; Misawa et al., 2000). Considering the increase of the IFN-γ levels by CV, we investigated its effects towards NK cells activity and observed that the treatment with the algae recovered the decreased NK cells function in Pb-exposed mice, in addition to enhancing this activity in normal mice. These results reinforce literature data (Dantas et al., 1999; Ibusuki and Minamishima, 1990) demonstrating the ability of CV to increase NK cells activity.

With the purpose to investigate some additional mechanism by which CV modulates the immune response, we investigated bone levels of zinc in lead-exposed mice treated with the algae. Zinc is essential for the growth, development, and maintenance of healthy bones (Yamaguchi, 1998), being its deficiency associated with disturbance of hematopoietic functions (King and Fraker, 2002). Data in the literature show that even mild to moderate degrees of zinc deficiency can impair macrophage and neutrophil functions, NK cell activity, and complement activity (Muzzioli et al., 2007). In human studies, the production of Th1 cytokines (IFN-γ and IL-2) is decreased in zinc deficiency (Prasad, 2000). Another important point that must be considered is that zinc supplementation blocks the intestinal absorption of lead, most likely by inducing intestinal metallothionein (Brewer et al., 1985), antagonizes lead-induced enzyme δ-aminolevulinic acid dehydratase (Haeger-Aronsen and Schutz, 1976) and reduces tissue lead deposition in animal models (Jamieson et al., 2006). In this respect, clinical studies emphasize the role of zinc in the treatment or prevention of childhood lead poisoning (Schmitt et al., 1996). Our results corroborate these findings by showing reduced level of zinc in lead-exposed mice. Treatment of these animals with CV produced a partial, but significant, restoration of zinc concentrations in the femur, which might be related to the presence of this mineral in the algae in association to its immunomodulating effect.

Delineating how lead influences the immunohematopoietic function is important for the development of potential pharmacological interventions to lower the incidence of lead-induced immune dysfunction. Irrespective of the mechanisms involved, the immunomodulatory effect of CV in lead-exposed mice may have an important role in the activity suggested to the algae in protecting hosts from immune and non-immune stressful situations, leading to an increase in the ability of the immune system to respond to challenge. Our results may be attributed to integration between CV Th1-stimulating activity, its chelating ability in vivo and its ability to minimize zinc deficiency. We therefore propose that stimulation of hematopoiesis and consequent efflux of new effector cells of the immune system has potential for treatment of lead-induced suppression of host defense mechanisms, including Th1 cytokine production and increased NK cell activity. Although the ultimate mechanisms of this action needs additional studies, our previous and the present findings can provide a venue for promoting the translation of this understanding into effective prevention strategies and new treatment regimens to improve human health.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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