Lysosomal changes in the hemocytes of the freshwater mussel *Dreissena polymorpha* experimentally exposed to lead and zinc

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ABSTRACT: This study examines the structural changes of the lysosomal system of the hemocytes of the zebra mussel *Dreissena polymorpha* experimentally exposed to lead and zinc. A cytochemical technique which demonstrated acid phosphatase activity as a lysosomal marker was used on blood cell monolayers. The results indicate that the effects of both metals on hemocytic lysosomes were variable and that no marked linear relationship between lysosomal changes and metal concentration and exposure time was observed. Hemocytes of exposed zebra mussels exhibited enlarged and/or more numerous lysosomes. The use of the lysosomal changes in the hemocytes as a biomarker of contaminant exposure is discussed.

KEY WORDS: Freshwater mussel · Heavy metals · Hemocytes · Lysosomal changes · Biomarker

INTRODUCTION

Aquatic organisms are able to concentrate in their soft tissues a wide range of contaminants occurring in their environment. Therefore, they have been used as sentinel organisms in the monitoring of environmental pollution. In these studies, particular attention has been given to marine filter-feeding bivalves (Phillips & Rainbow 1988, Browler 1989, Cossa 1989) and, most often, only the xenobiotic body burdens have been taken into account. During the last decade, several authors have shown the importance of assessing the biological effects of pollutants in biomonitoring programs (Bayne 1989, Livingstone et al. 1989, Widdows & Donkin 1989, Narbonne et al. 1991, Veldhuizen-Tsoerkan et al. 1991, Depledge et al. 1995). In this context, numerous markers of pollution effects and/or exposure have been developed at different levels of the biological organization (Bayne 1985, Galgani et al. 1992, Regoli 1992, Cajaraville et al. 1995a, Regoli & Principato 1995, Sole et al. 1995). Cellular markers can offer rapid and sensitive indicators of adaptive

responses to environmental contamination, before pollutant-induced injury manifests at the level of the whole organism (Moore 1985, Cajaraville et al. 1995b). In many cases, the earliest detectable changes are associated with subcellular organelles such as lysosomes (Moore 1985). Lysosomes are involved in a broad spectrum of both physiological and pathological functions (Holtzman 1976, Moore 1982, Cheng 1983). Lysosomes are also involved in accumulation and detoxication of both metallic and organic xenobiotics (George 1980, George & Viarengo 1985, Viarengo et al. 1985, 1988). Many microanalytical investigations have demonstrated the occurrence of trace metals in the lysosomal-vacuolar system of different cell types in bivalve molluscs (Schulz-Baldez 1977, George et al. 1978, Lowe et al. 1979, Ballan-Dufrançais et al. 1985, Nigro et al. 1992). In molluscs, the digestive gland represents one of the main target organs of pollutant accumulation. Therefore, using various technical approaches, the responses of the digestive cell lysosomal system have received the principal attention in pollution assessment in marine ecosystems. Numerous field and experimental studies have shown that various environmental stressors (metallic and organic xenobiotics, thermal, hypoxia and salinity stress, oxygen-

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free radicals) caused 2 main types of general stress responses in mollusc digestive gland cells: reduction in lysosomal membrane stability and enlargement of lysosomes (Moore 1976, Lowe et al. 1981, Patel & Patel 1985, Viarengo et al. 1987, Lowe 1988, Moore 1988, Cajaraville et al. 1989, Lowe & Clarke 1989, Mari-gomez et al. 1989, Winston et al. 1991, Regoli 1992, Etzeberria et al. 1994, Krishnakumar et al. 1994, Lowe & Pipe 1994, Svenden & Weeks 1995).

On the other hand, the biological tools to assess the health condition of organisms are lacking for the monitoring of freshwater ecosystems. Previous investigations have reported the use of the freshwater mussel *Dreissena polymorpha* as a bioindicator of water pollution (Kraak et al. 1991, Mersch & Pihan 1993). In zebra mussel, as in other bivalve species, the hemocytes are rich in lysosomal hydrolases and are involved in metal transport and detoxication (Giamberini 1993). In this context, the purpose of the present study was to evaluate cytochemically the lysosomal responses in the hemocytes of *D. polymorpha* experimentally exposed to high concentrations of an essential metal (Zn) or of a non-essential metal (Pb).

MATERIALS AND METHODS

Zebra mussel exposure. Mature specimens of *Dreissena polymorpha* (18 to 22 mm in length) were collected at a reference location and acclimatized to the experimental conditions for 1 wk. The intoxication experiment was conducted at $14 \pm 1^\circ\text{C}$ on a 16 h light: 8 h dark cycle. Fifty zebra mussels were placed on tiles to which they could become attached, kept in 9 l tanks, and exposed to lead chloride (PbCl_2) at concentrations of 400 and 800 $\mu\text{g l}^{-1}$ or to zinc chloride (ZnCl_2) at concentrations of 500 and 1000 $\mu\text{g l}^{-1}$ for 5 wk. Lethal concentrations have been established for *D. polymorpha* populations in The Netherlands (Kraak et al. 1994). These are for lead: LC_{50} (8 wk) = 2330 $\mu\text{g l}^{-1}$, LC_{50} (10 wk) = 497 $\mu\text{g l}^{-1}$; and for zinc: LC_{50} (3 wk) = 4293 $\mu\text{g l}^{-1}$, LC_{50} (10 wk) = 1065 $\mu\text{g l}^{-1}$. The control group received only dechlorinated tap water. Turnover of the tank volume was achieved by means of a peristaltic pump. PbCl_2 and ZnCl_2 stock solutions were continuously introduced into the tanks by a second pump to yield the desired concentration of metals. Food was continuously added in the form of a monospecific suspension of the algae *Chlamydomonas variabilis*, which is readily assimilated by *D. polymorpha* (Ten Winkel & Davids 1982). Four zebra mussels were sampled after 7, 21 and 35 treatment days from each experimental group.

Hemolymph collection and hemocyte monolayers. Hemolymph was obtained by making a notch near the

posterior margin of the mussel shell in the dorsal portion. A needle was inserted between the valves, and the hemolymph was withdrawn from the posterior adductor muscle sinus. Portions of hemolymph samples were placed onto a cleaned glass slide, in a moist chamber, and the hemocytes were allowed to settle for 15 min at room temperature. Subsequently, the hemocytes were fixed for 5 min with 1.25% glutaraldehyde (grade I; Sigma Chemical Co., St. Louis, MO, USA) in cacodylate buffer solution (0.025 M, pH 7.4) and rinsed with buffer solution.

Enzyme cytochemistry. Acid phosphatases (ACP) (E.C.3.1.3.2) were demonstrated by the azo-dye coupling method (Diagnostic kit 386, Sigma Chemical Co.) using naphthol AS-BI phosphate as substrate and Fast Garnet GBC as coupler (Goldberg & Barka 1962). The change in the presence and relative amounts of ACP following the exposure to metals was assessed according to the method of Moore & Gelder (1985) modified for the present study. To indicate the relative degree of reactivity, 200 hemocytes were examined on each slide and assigned to 1 of 4 categories: class 1 (0 to 25% of the endoplasm filled with positive granules), class 2 (25 to 50% of the endoplasm filled with positive granules), class 3 (50 to 75% of the endoplasm filled with positive granules) and class 4 (75 to 100% of the endoplasm filled with positive granules). The degree of reactivity reflects the relative number and/or size of positively staining granules in hemocytes and not the amount of final reaction per granule. The data were then expressed as percentages.

Statistical analysis. Data of the control and experimental groups were compared using the Mann-Whitney *U*-test.

RESULTS

The morphology of the hemocytes belonging to the 4 classes defined in the present study is illustrated in Fig. 1. Granules reacting for ACP activity occur in the endoplasm. The percentage of the hemocytes in each class, indicating the amount of positively staining granules in cells, are reported for the zebra mussels non-exposed (Fig. 2A) and exposed to 400 $\mu\text{g l}^{-1}$ (Fig. 2B) and 800 $\mu\text{g l}^{-1}$ (Fig. 2C) of Pb for 35 d. In general terms and for the whole exposure time, the number of hemocytes with <<50% of the endoplasm containing positive granules (classes 1 and 2) tends to decrease, whereas the number of hemocytes with >>50% of the endoplasm occupied by lysosomes (classes 3 and 4) tends to increase in exposed mussels. The differences between the control and exposed mussels are significant for the hemocytes in classes 1 and 3 (Fig. 2B, C). In these classes, the lyso-

Fig. 1. *Dreissena polymorpha*. Acid phosphatase reactivity (small curved arrows) in the endoplasmic granules in the 4 classes of hemocytes. (A) Hemocytes of class 1 (B) Hemocytes of class 2 (double arrowheads) and of class 3 (simple arrowheads). (C) Hemocytes of class 4. Abbreviations: e = ectoplasm; g = endoplasmic granules; n = nucleus; p = pseudopod

Fig. 2. *Dreissena polymorpha*. Percentages of hemocytes in 4 classes (mean $\pm$ SD). (A) Non-exposed, exposed to (B) 400 $\mu\text{g l}^{-1}$ or to (C) 800 $\mu\text{g l}^{-1}$ of Pb, and exposed to (D) 500 $\mu\text{g l}^{-1}$ or to (E) 1000 $\mu\text{g l}^{-1}$ of Zn. * $p < 0.05$; ** $p < 0.02$; ° $p < 0.01$; °° $p < 0.001$

somal changes are more marked at short times (7 d) for mussels exposed to the lower lead concentration (Fig. 2B). Nevertheless, the response seems to be modified with time, and the differences between control and exposed groups are not significant at the end of the experiment (35 d). The proportion of the hemocytes in class 4 (Fig. 2B, C) remains low, but tends to increase with lead concentration. The large increase of hemocyte percentage in class 4 for mussels exposed to 400 $\mu\text{g l}^{-1}$ of Pb for 7 d (Fig. 2B) is difficult to explain since this response is observed neither for the other exposure times nor for the

higher lead concentration. However, in class 4, the response of the lysosomal system is more evident at short exposure time for the zebra mussels exposed to both concentrations of lead. The experimental exposure of zebra mussels to zinc induces qualitatively the same lysosomal changes in the 4 classes of hemocytes (Fig. 2D, E).

The results indicate that experimental exposure to Pb or Zn provokes an increase in lysosomal number and/or size in the hemocytes of zebra mussels which does not always show a linear dependence with the time or with the dose of exposure.

DISCUSSION

This preliminary experimental and cytochemical study shows that sublethal concentrations of lead and zinc induce lysosomal changes in the hemocytes of *Dreissena polymorpha*. Zebra mussels exposed to both metals exhibit enlarged and/or more numerous lysosomes. Enlargement of lysosomes, whether or not this is associated with an increase in lysosome number, seems to be a non-specific stress biomarker (Cajaraville et al. 1995a, b, Ettxeberria et al. 1995). We report here, for the first time, this type of alteration in blood cells, but enlarged lysosomes have been observed in the digestive cells of both bivalve and gastropod molluscs exposed to a wide variety of environmental stress. It is difficult to compare our results since the effects of lead on biochemical functions have rarely been studied (Gnassia-Barelli & Romeo 1993), particularly in the case of freshwater invertebrates (Rainbow & Dallinger 1992). The exposure to sublethal concentration of lead (500 ppm) induced in the earthworm *Eisenia foetida* a destabilisation of chloragosomal membranes leading to the partial release of their acid phosphatases into the cytosol (Cancio et al. 1995). Bolognani-Fantin & Franchini (1991) reported an increase of both lysosome number and enzyme activity in the neurons of *Viviparus ater* experimentally exposed to acute concentrations of lead. Under laboratory conditions, metals such as Cu and Cd caused enlargement of lysosomes in the digestive gland of *Mytilus edulis* (Lowe & Clarke 1989) and *Littorina littorea* (Marigomez et al. 1989). A mixture of metals, such as Fe, Mn, Cu, Zn and particularly those rich in Pb, led to the same results in natural populations of the marine mussels *Mytilus galloprovincialis* (Regoli 1992) and *M. edulis* (Lowe 1988). In these studies, an increase in lysosome size was concomitant with a decrease in their numbers. This suggests a process of smaller secondary lysosomes fusing to form large autolysosomes (Lowe et al. 1981) which are associated with membrane destabilization or increased permeability resulting in reduced latency of lysosomal hydrolases (Moore 1985, Regoli 1992). Our results are similar to those of Ettxeberria et al. (1994), who report an increase in both size and number of the lysosomes in the digestive cells of *M. galloprovincialis* exposed to Zn, Cu or Cd under field and experimental conditions. The lysosomal alterations they observed did not always show a linear dependence with metal concentrations, and they did not report different impacts of metals which do or do not have a biological function. The heterogeneity of the lysosomal responses when exposed to both organic (Moore 1988, Cajaraville et al. 1995a) and metallic (Suresh & Mohandas 1990) xenobiotics has often been stressed. The enlargement of lysosomes

and/or the increase of their numbers are better illustrated by classes 1 and 3. However, the variability observed here in the hemocytic lysosomal responses still remains difficult to explain and further studies are necessary to fully understand these structural changes. The effects of both metals, nonessential (Pb) and essential (Zn), follow the same general pattern. Zebra mussels can regulate Zn up to $191 \mu\text{g l}^{-1}$, but capacity decreases with exposure time. On the contrary, lead cannot be regulated and is accumulated (Kraak et al. 1994). The short-term responses observed under lead exposure could correspond to an adaptive response. This may be related to the detoxication function of the lysosomes. However, the total number of hemocytes of animals exposed both to Pb or Zn did not show significant changes during this contamination experiment (Giambérini 1993). It is generally accepted that bivalve hemocytes are involved in metal transport from uptake to excretion organs. In cells, metals (and especially lead) are sequestered and immobilized in inert form in lysosomes before excretion (Viarengo 1989, Nott 1993).

Lysosomal enlargement in the mollusc digestive gland could be induced not only by metals but also by various organic xenobiotics such as PAH or PCB (Lowe 1988, Moore 1988, Lowe & Clarke 1989), oil-derived hydrocarbons (Lowe et al. 1981, Cajaraville et al. 1995a) and 1-naphthol (Cajaraville et al. 1989). These studies concluded that lysosomal enlargement represented a good indicator of general environmental stress.

Various techniques, such as microdensitometry or stereology, principally in the digestive gland cells, have been used to assess lysosomal changes (membrane destabilization, number and size variations). Other cell types, such as blood cells, rich in lysosomes (Cheng 1981) and involved in internal defense mechanisms and metal accumulation have been only rarely investigated. Using biochemical techniques, Pickwell & Steinert (1984) and Suresh & Mohandas (1990) concluded that copper caused destabilization of lysosomal membrane or hypersynthesis of acid hydrolases in bivalve hemocytes before their release into the hemolymph. Very recently, lysosomal responses have been determined in blood cells of several species of molluscs by *in vitro* tests using neutral red retention (Lowe et al. 1995, Svedsen & Weeks 1995). Due to several characteristics of bivalve hemocytes (richness in lysosomes, the presence of several acid hydrolases which are cytochemically demonstrable and an isolated stage), measurements of the lysosomal structure in this cell type have an enormous potential for the assessment of pollutant effects (Cajaraville et al. 1995b).

In conclusion, these results indicate that the hemocytic lysosomes of zebra mussels exposed to lead and

zinc exhibit structural changes. Thus, we could consider the response of the vacuolar-lysosomal system in the hemocytes of *Dreissena polymorpha* as a potential biomarker for exposure to freshwater pollution. However, several further investigations are necessary before validation of this biomarker. First, an automated image analysis will improve the objectivity and the precision of the method. Moreover, parameters such as size and number can be identified separately and more rapidly. In addition, experimental approaches with different and more realistic metal concentrations will be useful to precisely measure the sensitivity of the response. The sensitivity of this potential biological marker must be tested in natural conditions where mixtures of contaminants and various environmental stresses occur. Finally, to obtain an integrated evaluation of organism health, the lysosomal changes will be extremely useful in concert with other biomarkers at different levels of the biological organization, such as the gametogenetic index, and the scope-for-growth and body condition index.

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