Toxicity of cadmium and zinc to encystment and *in vitro* excystment of *Parorchis acanthus* (Digenea: Philophthalmidae)

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(*Received 20 March 2000; revised 12 July 2000; accepted 19 July 2000*)

SUMMARY

The toxicity of cadmium, zinc and cadmium/zinc mixtures at concentrations ranging from 1000 to 50000 μg/l were investigated against cercariae and metacercariae of *Parorchis acanthus* obtained from the dog whelk *Nucella lapillus*. Cercarial encystment at concentrations of 25000 μg/l or higher was significantly impaired by all test metals; however, at lower concentrations only zinc demonstrated toxicity. Mixtures of cadmium and zinc had a synergistic effect compared with single metal toxicity but only at 50000 μg/l. Excystment *in vitro* was only significantly affected by cercariae exposed to cadmium/zinc mixtures whilst encysting. Twenty-four h exposures of fully formed cysts had no effect on excystment *in vitro*. Effects on *in vitro* excystment rates over a 2 h period demonstrated widespread effects for cercariae-exposed *P. acanthus*. No effects were evident on excystment rates of cyst-exposed parasites.

Key words: cadmium, zinc, encystment, cercariae, excystment.

INTRODUCTION

Digeneans are appropriate models for the demonstration of pollutant effects on parasites because of their extensive free-living stages. However, the majority of studies have utilized species whose free-living stages actively penetrate their target host and may be mobile in a polluted water body for several hours (e.g. Abd Allah, Wanas & Thompson, 1996). Nevertheless, some species with a more passive transmission phase possess cercariae which only have a short free-living phase before they form a protective cyst in or on a substrate which may form the diet of its target host. One of the more extensively studied of these species is the marine digenean *Parorchis acanthus*, which occurs throughout the coastal fringe of much of Europe and North America (Williams, 1969). In Europe the larval stages occur in the marine snail *Nucella lapillus* with the adult stage establishing in a number of bird species especially gulls.

The effect of pollutants on the digenean cyst has only previously been studied for the freshwater species *Notocotylus attenuatus* (Evans, 1982), where the effects of copper and zinc on cyst formation and the establishment of adult worms in the definitive bird host were investigated.

The present study examines the effects of cadmium and zinc on the cyst biology of *P. acanthus*. Cadmium and zinc are related heavy metals, almost always occurring together in pollution incidents, which have been used widely in industry and are released into the environment as a by-product of ore smelting (Hellawell, 1986). Although toxicological studies have been reported for a number of freshwater digenean species, especially *Schistosoma mansoni* (Abd Allah *et al*. 1996), little is known about the metal toxicity induced in marine digeneans. Indeed only Siddall & des Clers (1994) have studied the toxicity of sewage sludge, containing a number of heavy metals, to the free-living stages of *Zoogonoides viviparus*.

The specific objectives of this study were to elucidate the toxicity of cadmium and zinc singly and in combination, utilizing high metal concentrations which, although rarely encountered in chronically polluted environments, allow an examination of toxic effects on the encystment and *in vitro* excystment of cercariae exposed and cyst exposed *P. acanthus* which may occur in an acute short duration pollution event.

MATERIALS AND METHODS

Test solutions

Stock solutions of 100 mg/l cadmium and zinc were prepared by dissolving either cadmium chloride ($CdCl_2.5/2H_2O$) or zinc chloride ($ZnCl_2$) (Sigma Chemicals) in distilled water. Test solutions (cadmium, zinc, or equal mixtures of cadmium and zinc)

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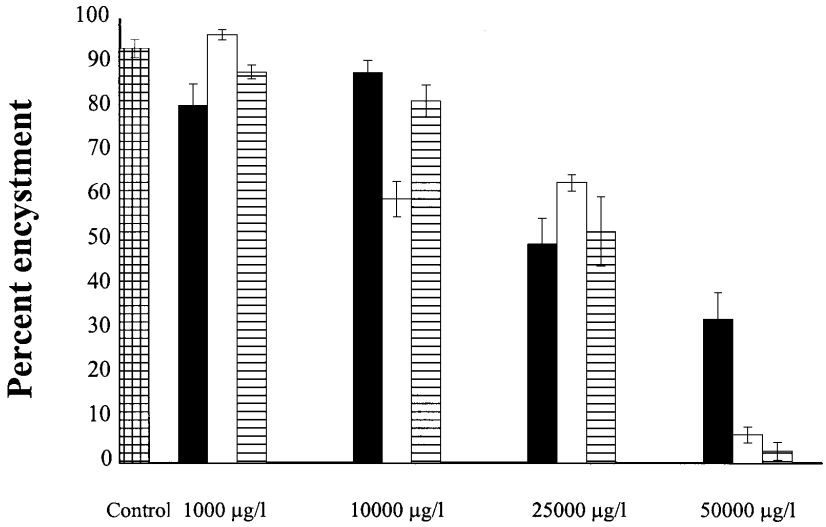

Fig. 1. Encystment of *Parorchis acanthus* in control (▦), cadmium (■), zinc (□), and Cd/Zn mixture (▤) in concentrations ranging from 1000 to 50000 μg/l. Error bars are standard error.

of 1000, 10000, 25000 and 50000 μg/l were obtained by diluting stock solutions in distilled water. Mixtures of cadmium and zinc were added to give concentrations of 50000 μg/l Cd + 50000 μg/l Zn, 25000 μg/l Cd + 25000 μg/l Zn, 10000 μg/l Cd + 10000 μg/l Zn, 1000 μg/l Cd + 1000 μg/l Zn. Salt water was prepared using artificial sea salts (Tropic Marin; Aquarientechnik, Germany).

Source of parasites

Samples of the dog whelk, *Nucella lapillus*, naturally infected with *P. acanthus* were collected on the rocky shore of Tan-y-barlch near Aberystwyth, Wales, and maintained in 8 l perspex tanks in a circulated gravity-fed marine aquarium for the duration of the experiment.

Water analysis

Samples of all test solutions were analysed for decay over 24 h. Solutions were analysed on a Perkin–Elmer Optima 3300 Inductively Coupled Plasma-Atomic Emission Spectrometer which was calibrated with a 1 % nitric acid blank and a standard which consisted of 1000 μg/l of cadmium and zinc in 1 % nitric acid.

Cercarial encystment

Recently shed cercariae, in batches of 20, were placed in 35 mm plastic Petri dishes in 20 ml of sea water containing selected heavy metal concentrations of 1000, 10000, 25000 or 50000 μg/l, or in distilled water as a control. Six replicates of each test solution were used, all tests were conducted at the same time. These were then incubated at 20 °C for 48 h to ensure that a fully formed cyst, normally found after 24 h, had been properly established. At the end of this period the numbers of fully formed cysts were counted and the data analysed using one-way ANOVA (SPSS statistical computer package).

Excystment of metacercariae in vitro

Excystment was investigated by exposing metacercarial cysts to the heavy metals in one of two ways. (i) The parasites were placed in 35 mm plastic Petri dishes containing 20 ml of sea water and the appropriate test solution and freshly shed cercariae were exposed to test solutions in the concentration range 1000–25000 μg/l metal concentration or distilled water added as control whilst encysting. (ii) Fully formed cysts (72 h post-emergence) were exposed to test solutions ranging from 1000 to 50000 μg/l or distilled water added as control for 24 h. In both exposures *in vitro* excystment was observed for metacercariae (96 h post-emergence) in 3 batches of 20 cysts, all tests were conducted at the same time. Cysts were scraped from the exposure Petri dishes and briefly rinsed in Lockes balanced salt solution (BSS). They were then incubated for 15 min in 1 % acid pepsin (pH 2) (Sigma Chemicals) at 42 °C, rinsed in Lockes BSS and incubated in a Fried and Roth medium (Fried & Roth, 1974) for 2 h at 42 °C. The cysts were examined every 15 min and excysted metacercariae counted and removed. Data were analysed using a repeated measure ANOVA (SPSS statistical computer package).

RESULTS

Water analysis

Loss of dissolved metals over 24 h occurred in all test solutions. The greatest losses occurred at the higher metal concentration (50000 μg/l–47·6 % for cad-

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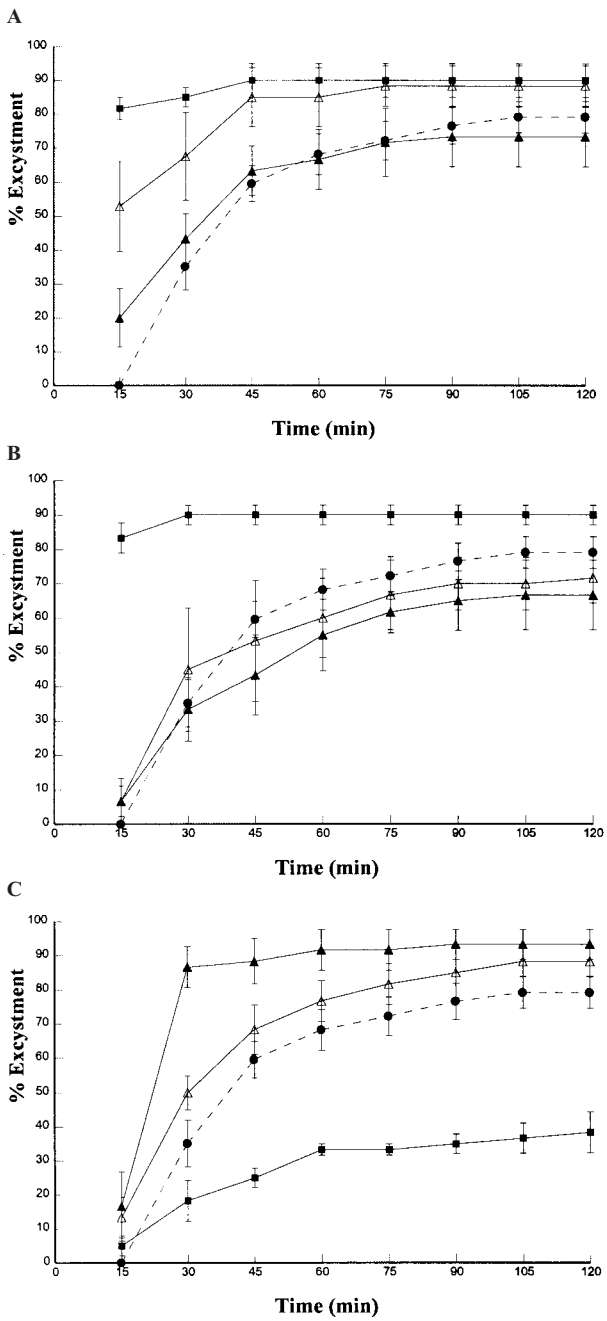

Fig. 2. Excystment of *Parorchis acanthus* cysts *in vitro* from encysting cercariae exposed in (A) cadmium, (B) zinc, (C) Cd/Zn mixtures in concentrations ranging from 1000 to 25000 μg/l. Error bars are standard error. (●) Control; (▲) 1000 μg/l; (△) 10000 μg/l; (■) 25000 μg/l.

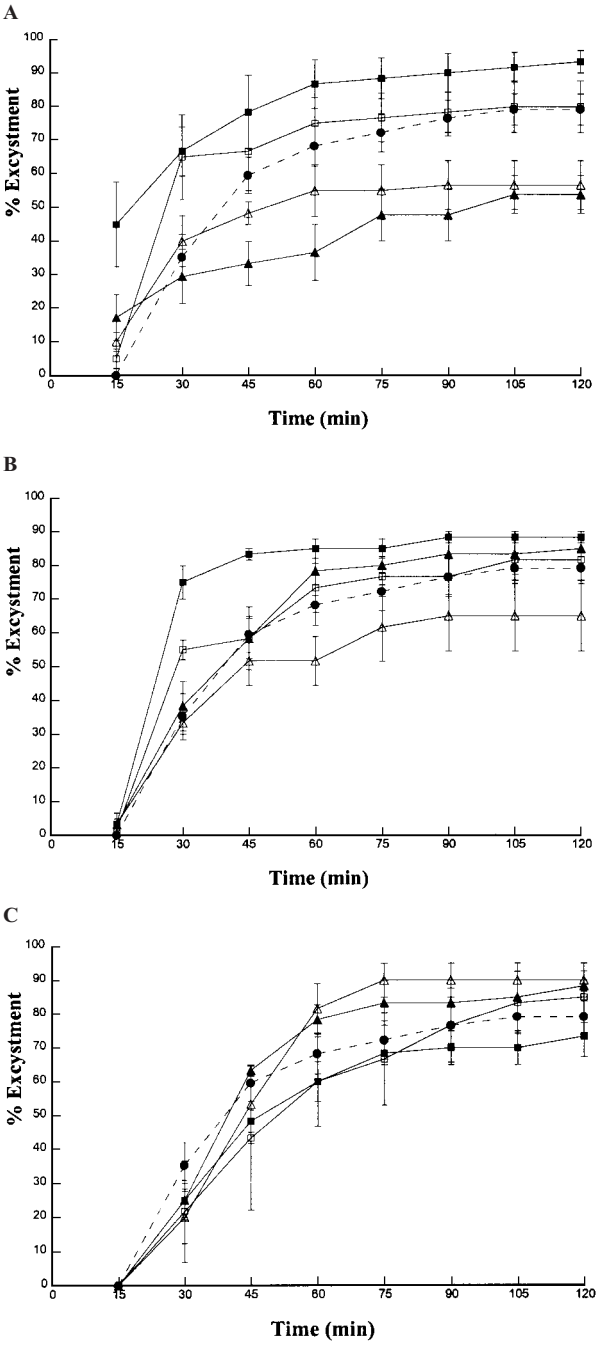

Fig. 3. Excystment of *Parorchis acanthus* cysts *in vitro* from fully formed cysts exposed in (A) cadmium; (B) zinc, (C) Cd/Zn mixtures in concentrations ranging from 1000 to 50000 μg/l. Error bars are standard error. (●) Control; (▲) 1000 μg/l; (△) 10000 μg/l; (□) 25000 μg/l; (■) 50000 μg/l.

mium, 15·6 % for zinc; 25000 μg/l–58·0 % for cadmium, 29·2 % for zinc) where cadmium showed a greater concentration of loss than zinc.

Cercarial encystment

At heavy metal concentrations of 25000 μg/l or higher, encystment was significantly impaired in all toxic media (Fig. 1) ($P < 0{\cdot}001$). However, only zinc showed adverse toxicity at the lower concentration of 10000 μg/l ($P < 0{\cdot}001$). Mixtures of cadmium and zinc caused increased toxicity when compared with individual metal toxicity at the highest metal concentration, but at lower concentrations there was no difference in the degree of toxicity except for the 10000 μg/l concentration where Cd/Zn mixtures caused an inhibition of the toxic effects demonstrated by zinc alone (Fig. 1).

In test solutions causing significant toxicity all unencysted cercariae were dead by 48 h post-exposure and the majority were dead by 24 h if they

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had not begun to encyst. An exception to this occurred at 50000 μg/l Zn. This test solution caused a significant inhibition of encystment, nevertheless many cercariae were still alive after 48 h post-exposure and had secreted much mucous material which, in many cases, had wrapped itself around the cercarial body as well as discharging freely into the test solution.

Excystment in vitro

Only the Cd/Zn mixture at 50000 μg/l had significantly reduced excystment ($P = 0{\cdot}001$) after 2 h, although exposure to cadmium and zinc caused a wide variation in excystment rates (Fig. 2). Cadmium caused an increased rate of excystment compared with controls with increasing concentrations when cercariae of *P. acanthus* were excysting at 25000 μg/l ($P = 0{\cdot}005$) and 10000 μg/l ($P = 0{\cdot}035$) (Fig. 2A). This was only repeated in cercariae exposed to the highest concentration of zinc (25000 μg/l; $P = 0{\cdot}003$) (Fig. 2B). In contrast Cd/Zn mixtures of 25000 μg/l produced a much reduced rate of excystment in cercariae ($P = 0{\cdot}003$) when compared with controls. However, as the concentration of the Cd/Zn mixture decreased, the excystment increased until at 1000 μg/l it was much more rapid than controls ($P = 0{\cdot}018$) (Fig. 2C). Excystment rates of exposed *P. acanthus* metacercariae were, with the exception of 50000 μg/l Cd concentration ($P = 0{\cdot}034$), not significantly different from controls (Fig. 3).

DISCUSSION

Several studies have investigated the effects of stressful environmental parameters on the encystment abilities of *Parorchis acanthus*. Increasing and decreasing salinity levels reduce the number of successful encystments (Stunkard & Shaw, 1932; Rees, 1937) as does lower water temperature (Williams, 1969). In this study we found that heavy metals also decrease encystment in a similar manner to that reported for the freshwater digenean *Notocotylus attenuatus* exposed to copper and zinc (Evans, 1982). However, the concentrations that inhibit *P. acanthus* are much higher than those that inhibit encystment in *N. attenuatus*. This is probably because heavy metal ions form relatively inert complexes with salts present in sea water, thereby reducing metal toxicity (Depledge, Weeks & Bjerregaard, 1994).

The dramatic morphological effects of high zinc concentrations on encystment are possibly due either to inhibition of one or more of the secretions from the gland cells necessary for cyst formation or to inhibition of the 'setting' of the secretions forming the cyst wall. Ultrastructural studies by Evans (1982) on *N. attenuatus* cercariae killed by copper or zinc in the act of cyst formation found that the metals inhibited the complete extrusion and unrolling of the 'keratin-like' granules from the cystogeneous glands necessary for forming the inner cyst wall. 'Keratin-like' granules have also been demonstrated in the formation of *P. acanthus* cysts (Cable & Schutte, 1973). If inhibition of their extrusion occurs in a similar fashion to that demonstrated for *N. attenuatus*, it is possible that the mucous material present in the sea water of cercariae exposed to 50000 μg/l Zn is the remnant of these cyst-forming secretions. These may not be inhibited by heavy metals, but are unable to initiate a proper cyst without the presence of the 'keratin-like' granules.

The toxic effects of cadmium and zinc on *in vitro* excystment are comparable with the *in vivo* study performed on *N. attenuatus* by Evans (1982), where the recovery of excysted metacercariae from cysts exposed to copper or zinc for either 1 or 6 weeks showed no significant difference from controls. In the present study cysts exposed for a much shorter period of 24 h demonstrated no significant effect on excystment. Only when cercariae were exposed to 25000 μg/l Cd/Zn mixture in the process of encystment was *in vitro* excystment subsequently reduced. This suggests that the cyst is a highly efficient protective mechanism against all but the highest concentration of heavy metals in the environment. However, extended periods of exposure to mixtures of heavy metals at lower concentrations may prove more effective at inhibiting excystment, especially if in natural systems the parasite must remain within the cyst for many weeks before transmission to the next host (Williams, 1969) and also considering that an increase in cyst age has been demonstrated to cause a gradual decline in excystment *in vitro* (Asanji & Williams, 1985). The increased rate of excystment *in vitro* of cercariae-exposed *P. acanthus* clearly demonstrated in this study is possibly due to partial inhibition of a properly formed cyst by the suppression of the 'keratin-like' granule secretions previously mentioned. Consequently, the partial inhibition of cyst formation by cadmium or zinc could subsequently allow for much faster excystment rates than the control exposed *P. acanthus* which would already possess fully formed cysts prior to exposure. It is also possible that a heavy metal may be sufficient to weaken the integrity of fully formed cysts at extreme concentrations by toxic actions on the 'keratin-like' granules present in some cyst layers. This would allow cysts exposed to 50000 μg/l Cd to excyst more rapidly than controls *in vitro*.

Although the concentrations of metals used in the present study are higher than those reported for polluted estuarine and coastal waters (10000 μg/l Cd, Knutson, Klerks & Levinton (1987); 3560 μg/l Zn, Stenner & Nickless (1974)), the inhibition of normal cyst formation by cadmium

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and zinc demonstrated by this work may nevertheless have long-term consequences. If the protective effects of a fully formed cyst are undermined allowing an increase in metacercariae permeability and subsequently an increased risk of metal toxicity, this may reduce the long-term survival of metacercariae in cysts and prevent the development of viable and gravid adult worms in the definitive bird host.

We would like to thank John Dingley for collecting the infected snails.

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