Cadmium accumulation and interactions with zinc, copper, and manganese, analysed by ICP-MS in a long-term Caco-2 TC7 cell model

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Abstract

The influence of long-term exposure to cadmium (Cd) on essential minerals was investigated using a Caco-2 TC7 cells and a multi-analytical tool: microwave digestion and inductively coupled plasma mass spectrometry. Intracellular levels, effects on cadmium accumulation, distribution, and reference concentration ranges of the following elements were determined: Na, Mg, Ca, Cr, Fe, Mn, Co, Ni, Cu, Zn, Mo, and Cd. Results showed that Caco-2 TC7 cells incubated long-term with cadmium concentrations ranging from 0 to 10 μmol Cd/l for 5 weeks exhibited a significant increase in cadmium accumulation. Furthermore, this accumulation was more marked in cells exposed long-term to cadmium compared with controls, and that this exposure resulted in a significant accumulation of copper and zinc but not of the other elements measured. Interactions of Cd with three elements: zinc, copper, and manganese were particularly studied. Exposed to 30 μmol/l of the element, manganese showed the highest inhibition and copper the lowest on cadmium intracellular accumulation but Zn, Cu, and Mn behave differently in terms of their mutual competition with Cd. Indeed, increasing cadmium in the culture medium resulted in a gradual and significant increase in the accumulation of zinc. There was a significant decrease in manganese from 5 μmol Cd/l exposure, and no variation was observed with copper.

Abbreviation: AAS – Atomic absorption spectrometry; CRM – Certified reference material; PBS – Phosphate buffered saline without calcium and magnesium; DMEM – Dubelcco's modified Eagle's medium.

Introduction

During the last few years, increasing consideration has been given to interactions between the nutritional status of the organism and the toxicity of heavy metals. Heavy metals contaminating food pose a long-term health risk, because they bioaccumulate and cause toxicity. The heavy metal environmental pollutant cadmium (Cd), widely disseminated in the biosphere, enters the food chain via polluted soils via Cd-contaminated rice, vegetable, and shellfish. Following intestinal absorption estimated to be about 5% (Friberg *et al.* 1986), Cd accumulates mainly in kidneys, showing a biological half-life of 25 to 30 years in humans. Exposure to Cd is associated with renal and skeletal damage, and also some cancers. Cd has recently been recognised as a risk factor for

osteoporosis after long-term exposure (Staessen *et al*. 1999; Alfven *et al*. 2002). The mechanisms behind Cd-induced bone damage are not clear, possibly implicating interactions with minerals. These interactions are complex and involve biometals such as zinc (Zn), copper (Cu), iron (Fe), chromium (Cr), magnesium (Mg), and calcium (Ca). They have been described in various models, *in vivo* and *in vitro*, but comparing the results and the effects of low-dose long-term exposure to Cd on the mineral elements needs simplified models allowing chronic testing, i.e. ''long-term testing'' models as defined by ECVAM (Pfaller *et al*. 2001) and highly sensitive techniques.

The intestinal epithelium is the main route of entrance for nutrients and at the same time the first barrier to be crossed, following oral ingestion of dietary contaminants such as Cd. Studying accumulation and mineral interactions at this step of metabolism is of prime interest. Over the years the Caco-2 cell line has become the best established model of the intestinal absorptive epithelium and has been extensively used to study the transport and toxicity of nutrients and xenobiotics (Delie & Rubas 1997). Originally derived from a moderately well-differentiated human colon adenocarcinoma (Fogh *et al*. 1977), Caco-2 cells exhibit spontaneous morphological and biochemical enterocytic differentiation at confluence in culture (Pinto *et al*. 1983; Hidalgo *et al*. 1989; Zweibaum & Chantret 1989). This differentiation process is growth-dependent, and after confluency, brush border hydrolase activities are similar to those reported for normal villous enterocytes (Zweibaum *et al*. 1991).

We previously studied the effects of long-term exposure to Cd on metal uptake and transepithelial transport (Blais & Lecoeur 1999) on a Caco-2 cell model. However, parental Caco-2 cells have been reported to show heterogeneity, and several clonal cell lines have been developed to increase the homogeneity and stability of the cell population (Ranaldi *et al*. 2003). In the present study, we improved our long-term testing model by using the Caco-2 TC7 clonal cell line, derived from the parental line at late passage (Chantret *et al*. 1994).

The possibilities of biological monitoring have been restricted by the limited performance of previous analytical techniques. To study metal bioavailability as well as metal toxicity, we developed a new technique allowing rapid, sensitive, and highly accurate measurements of Caco-2 TC7 intracellular metal levels (Noël *et al*. 2003a). This procedure is based on closed vessel microwave digestion and inductively coupled plasma mass spectrometry (ICP-MS) for studying intracellular accumulation of individual minerals and essential trace elements. The ICP-MS technique, which allows the analysis of small samples with low analyte concentrations and the simultaneous determination of many elements, combined with a single sample preparation, such as closed vessel microwave digestion, and allows rapid semi-quantitative and quantitative analysis with high accuracy (Barnes 1993; White *et al*. 1998).

The purpose of the present work was to study the influence of long-term exposure to cadmium on intracellular accumulation of essential minerals using a multi-analytical tool: microwave digestion followed by ICP-MS determination. The emphasis will be put on three elements – zinc, copper, and manganese – known to play a major role in the absorption and toxicity of cadmium (Yanagiya *et al*. 2000; Brzoska & Moniuszko-Jakoniuk 2001).

Material and methods

Cell culture

The Caco-2 TC7 clone, selected from a late passage (P-198) of the Caco-2 cell line was kindly given by Dr M. Rousset (INSERM U505, Pierre and Marie Curie University, Paris, France). Cells were seeded in 25 cm^2 or 75 cm^2 plastic flasks (Corning Costar, Cambridge, USA) at a density of 10^4 cells/cm^2 and cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, 862 mg/l glutamax-I (L-alanyl-L-glutamin), supplemented with 20% heat-inactivated (56 °C, 30 min) foetal calf serum, 1% non-essential amino acids, 50 IU/ml penicillin and 50 μg/ml streptomycin in a 10% CO_2/90% air atmosphere. The medium was changed 60 h after seeding, and daily thereafter. Under these culture conditions, cells became confluent 5–6 days after seeding. Cells were detached using trypsin-EDTA in phosphate-buffered saline for 10 min at 37 °C. All tissue culture media were obtained from Gibco (Glasgow, Scotland, UK).

Caco-2 TC7 cells exposed long-term to cadmium

A 1000 mg/l standard stock solution of cadmium chloride was sterilised through a 0.22 μm filter (Millipore S.A., St Quentin en Yvelines, France) and kept at 4 °C for use in all the assays. In order to obtain cells exposed long-term to Cd, $CdCl_2$ was added to the culture medium at the concentrations of 1, 5, or 10 μmol Cd/l, a concentration previously shown not to be cytotoxic (Huynh-Delerme *et al.* 2005), for 4 weeks (Figure 1), corresponding to 4 passages. To obtain a sufficient amount of cells for mineral analysis, cells were grown in 75 cm^2 plastic flasks for 7 days and collected in the first and fifth weeks. On day 7 of those weeks, as indicated in the Figure 1, the medium was removed and collected, then cells were washed with PBS solution, and separated by trypsinisation. Complete medium was then added to inhibit trypsine activity. The cell suspension was centrifuged at 100 g for 5 min, the medium was discarded and the cells were weighed (0.2 to 0.3 mg). In order to determine the metal content in the media, the latter were collected before the beginning of the study and on days 3, 4, 5, 6 and 7. For that purpose, media were centrifuged at 1000 *g* for 5 min to remove cell debris and kept at −20 °C until analysis.

Copper, zinc, and manganese treatment in Caco-2 TC7 cells exposed long-term to cadmium

Stock solutions of cadmium, zinc, copper, and manganese were sterilised through a 0.22 μm filter and kept at 4 °C for use in all the assays. The cells exposed long-term to cadmium were cultured in 75 cm^2 plastic flasks in culture media containing 1, 5, 10 and μmol Cd/l, with or without 30 μmol/l of copper, zinc, or manganese. Cells exposed long-term were tested under the same conditions as for the first week, and cadmium, copper, zinc and manganese were analysed in the media as shown in Figure 1.

Multi-element analysis

Multi-element concentrations were determined in control cells, cadmium-exposed cells, and media, in the first and fifth weeks of culture, by ICP-MS (VG PlasmaQuad ExCell, TJA solutions, Montigny-le-bretonneux, France), after digestion using a closed vessel microwave procedure (Anton-Paar, Courtaboeuf, France). The following elements were determined: sodium, magnesium, calcium, manganese, copper, zinc, and cadmium. Note that the analysis of iron is more difficult by ICP-MS because of the poor sensitivity of ^{54}Fe in plasma standard mode and the potential interference of $^{40}Ar^{14}N$ on the iron signal. Therefore, iron was analysed in this study using an in-house AAS technique. Other elements (i.e. cobalt, nickel) could not be measured due to their low cellular incorporation. The performance characteristics of the analytical system, evaluated by calibration and linearity, the limits of detection and quantification, the accuracy with the use of spiking, and the trueness and the repeatability with the use of available CRMs, have been previously reported (Noël *et al.* 2003a). Details of the instruments, operating conditions and internal quality controls were slightly adapted from a previous study (Noël *et al.*, 2003b). Briefly, homogenised samples were treated with 3 mL HNO_3 65% (v/v) in the microwave acid digestion vessels and the resulting digests, after treatment for 50 min (30 min heating + 20 min cooling), were diluted with deionised water to a

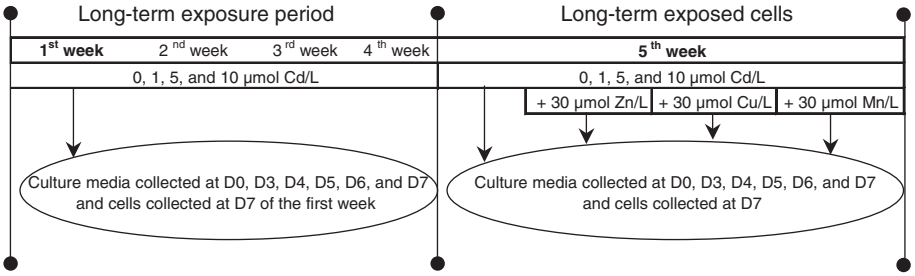

Figure 1. Treatment chronology of the Caco-2 TC7 cells. Cells are considered exposed long-term to cadmium after 4 passages (corresponding to 5 weeks) in contact with cadmium.

final volume of 50 ml and stored in acid-cleaned polyethylene tubes until analysis under the same conditions as for calibration standards.

Statistical methods

In all the experiments, measurements were made in four flasks, and the experiments were repeated at least twice. Concentrations are expressed in mg of contaminants per kg of fresh material. Results are presented as mean ± SD. To determine significant differences between treatments for a particular experiment, data were analysed by ANOVA, followed by Pearson's correlation procedure. A p value of less than 0.05 was considered significant. All analyses were performed using the SAS System (SAS Institute, Cary, NC).

Results

Multi-element analyses in Caco-2 TC7 cells exposed long-term to cadmium

As shown in Figure 2, cells incubated with cadmium at concentrations ranging from 0 to 10 μmol Cd/l exhibited an increase in cadmium accumulation as a direct linear function of intracellular cadmium concentrations. The cadmium intracellular accumulation was significantly greater in cells exposed long-term (fifth week) than in control cells (first week). Measurements made in the culture medium indicated that there was less Cd (whatever the Cd concentration considered) in the culture medium of the cells exposed long-term than in that of control cells (results not shown). The intracellular accumulation of sodium, magnesium, calcium, manganese, iron, copper, zinc, and cadmium in media containing 0, 1, 5, and 10 μmol Cd/l is presented in Table 1. Copper and zinc accumulation increased significantly from 1 to 10 and from 5 to 10 μmol Cd/l, respectively. Pearson's correlation showed that there was a positive correlation between cadmium and copper content ($r = 0.95$; $p < 0.0001$ instead of $r = 0.69$; $p < 0.01$ in control cells) and cadmium and zinc content ($r = 0.93$; $p < 0.0001$) in Caco-2 TC7 cells exposed long-term to cadmium. There was no significant effect of varying amounts of cadmium on sodium, magnesium, calcium, manganese and iron accumulation in cells exposed long-term to cadmium.

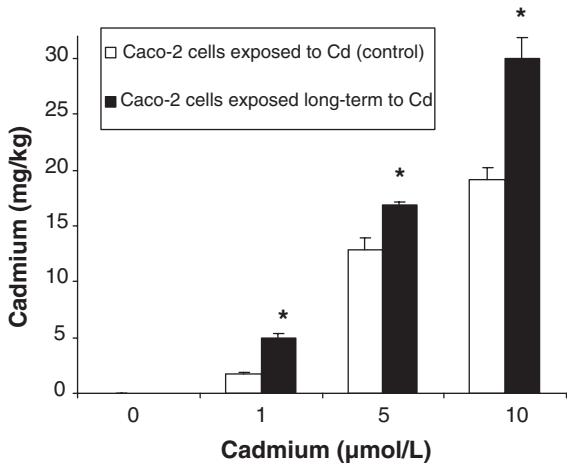

Figure 2. Accumulation of cadmium by control and Caco-2 TC7 cells exposed long-term to cadmium. Values are mean ± SD of four cultures. Cells were incubated with cadmium concentrations ranging from 0 to 10 μmol Cd/l. Main effects of cadmium (control cells vs. Caco-2 TC7 cells exposed long-term to cadmium). (*) Values significantly different from controls (ANOVA, Tukey's Studentised range test, $p < 0.05$).

Effect of zinc, copper, and manganese on cadmium accumulation in Caco-2 TC7 cells

In cells exposed long-term to cadmium, a concentration of 30 μmol/l of zinc, copper, or manganese was added to the media during the fifth week of treatment in addition to the cadmium. Results are shown in Figure 3. Cadmium accumulation was significantly inhibited by each of these three elements. Compared to controls, the higher the cadmium concentration in the medium, the lower the inhibition potential (ratio between element and cadmium concentrations) in the case of zinc and manganese. Manganese showed the highest inhibition potential and copper the lowest. The concentration of 30 μmol Mn/L decreased cadmium accumulation by 86, 77, and 58% for 1, 5, and 10 μmol Cd/l, respectively. Concentrations of 30 μmol Zn/l and 30 μmol Cu/l decreased cadmium accumulation by 59, 50, and 41% and by 2, 21, and 20%, respectively. Measurements showed that more Cd remained (whatever the Cd concentration) in the culture medium in the presence of zinc, copper, or manganese than when these elements were not added, which was in agreement with the intracellular dosages (results not shown).

Table 1. Effect of cadmium exposure on metals accumulation in cadmium long-term exposed Caco-2 TC7 cells (mg/kg).

Cd exposure (μmol/l)	Cd	Na	Mg	Ca	Mn	Fe	Cu	Zn
0	<0.002	2145 ± 281	94 ± 10	17 ± 5	0.24 ± 0.02	1.7 ± 0.3	1.2 ± 0.3	7.8 ± 0.3
1	4.9 ± 0.4*	2215 ± 234	95 ± 17	22 ± 13	0.20 ± 0.02	2.1 ± 0.4	2.0 ± 0.1*	8.8 ± 0.7
5	16.8 ± 0.4*	1832 ± 174	105 ± 2	21 ± 7	0.23 ± 0.02	2.3 ± 0.3	2.5 ± 0.1*	10.6 ± 0.4*
10	30.0 ± 1.8*	1961 ± 302	102 ± 7	19 ± 8	0.23 ± 0.03	2.0 ± 0.6	2.7 ± 0.2*	11.9 ± 0.9*

Values are mean ± SD of four cultures.*Values significantly different from control (ANOVA, Tukey's Studentised range test, $p < 0.05$).

Effect of cadmium present in the media on zinc, copper, and manganese accumulation in Caco-2 TC7 cells

The accumulation of zinc, copper and manganese in cells exposed long-term to 0, 1, 5, and 10 μmol Cd/l in the presence of 30 μmol/l of those respective elements is shown in Figure 4. Comparison of those intracellular accumulations between cell cultures incubated with the different elements (Zn, Cu, or Mn) and with the buffer accumulation (blank), gives an estimation of the cellular accumulation of these mineral elements. In our assays, the values for zinc, copper, and manganese corresponded to the amount of element accumulated by the cells. As cadmium in the culture medium increased, there was a gradual and significant increase in the accumulation of zinc, a significant decrease in manganese at 5 and 10 μmol Cd/l, and no significant variation in copper accumulation.

Discussion

The present study using ICP-MS in a long-term Caco-2 TC7 model showed that increased cadmium accumulation was correlated with intracellular essential minerals copper and zinc, and confirmed the inhibitory potential of copper, zinc and manganese on cadmium accumulation. To our knowledge, this is the first study using the ICP-MS technique to analyse the cellular content of minerals in cells exposed long-term to cadmium.

We characterised the effects of chronic exposure to cadmium on the mineral status of intestinal epithelium cells. The long-term Caco-2 TC7 model can be considered to simulate repeated low dose exposure to dietary cadmium in humans, leading to accumulation of cadmium. Caco-2 cells, a cell line with enterocytic-like differentiation, have been shown to be suitable for evaluating uptake of various trace metal such as cadmium (Mata *et al.* 1996; Rossi *et al.* 1996; Jumarie *et al.* 1997;

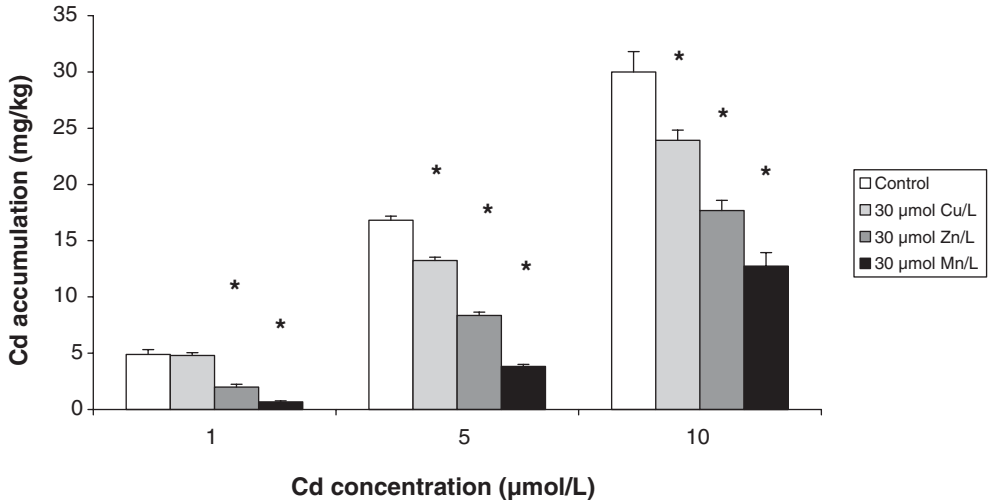

Figure 3. Effect of a fixed metal concentration (30 μmol/l of copper, zinc, or manganese) on cadmium accumulation. Values are mean ± SD of four cultures. (*) Values significantly different from control (ANOVA, Tukey's Studentised range test, $p < 0.05$).

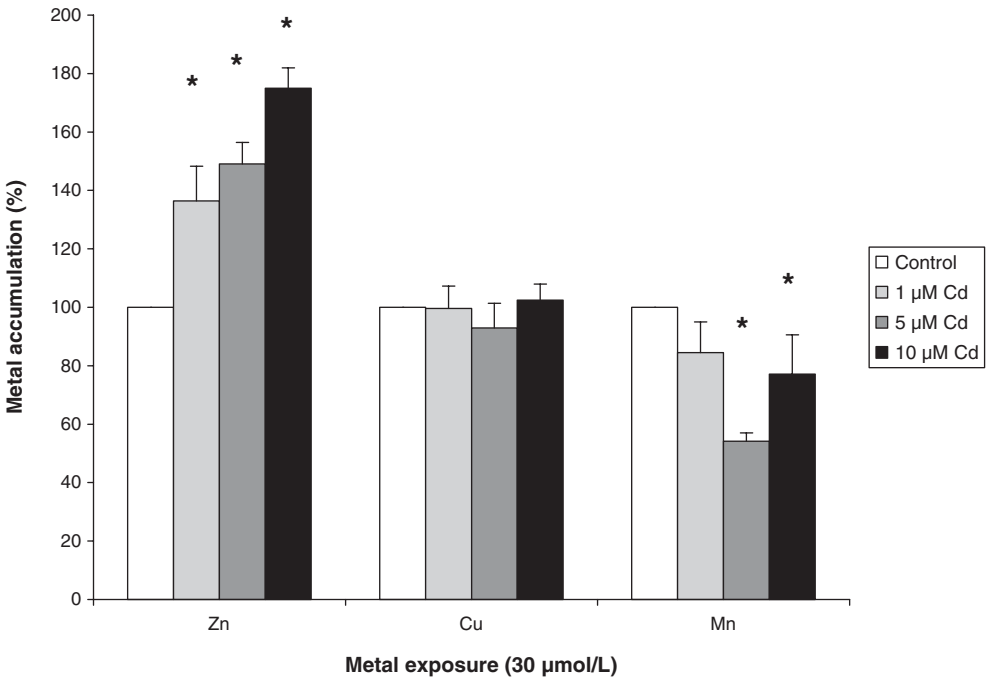

Figure 4. Effect of cadmium concentration on zinc, copper and manganese accumulation. Values are expressed as % of controls (mean of four cultures). (*) Values significantly different from controls (ANOVA, Tukey's Studentised range test, $p < 0.05$).

Pigman *et al*. 1997; Jumarie *et al*. 1999; Tallkvist *et al*. 2001; Eklund *et al*. 2003), copper (Rossi *et al*. 1996; Ferruzza *et al* 2000), manganese (Yanagiya *et al*. 2000), and zinc (Jovani *et al*. 2001) among another. Furthermore, the TC7 clone offers noticeable advantages over parental Caco-2 cells and represents a good alternative to the parental line for drug transport studies (Le Ferrec et al. 2001). Most studies using this model exposed cells acutely before determination of time-course and transport across the monolayer, which presupposes seeding cells on filters. We preferred to measure accumulation after long-term exposure. We purposely seeded our cells on flasks and not filters, which allowed us to harvest a sufficient quantity of cells to perform ICP-MS analysis. Closed vessels microwave digestion is then performed followed by inductively coupled plasma mass spectrometry (ICP-MS). The ICP-MS technique, which permits analysis of small samples containing a low analyte concentration and concurrent determination of many elements, is a fast semi-quantitative and quantitative method of analysis with high precision (Barnes 1993; White *et al*. 1998). This technique has been used to measure various materials but we were the first to develop it for studying intracellular accumulation of individual minerals and essential trace elements (Noël *et al*. 2003a).

In this study, we used long-term testing up to 10 μmol/l cadmium, a concentration previously shown not to be cytotoxic (Boveri *et al*. 2004; Huynh-Delerme *et al*. 2005). Some authors have studied the influence of a serum-free medium or a serum-supplemented medium (Finley *et al*. 1995; Reeves *et al*. 2001; Ranaldi *et al*. 2003) on the cells. However, in most studies, the culture medium used during uptake-transport experiments was different from the culture medium used during cell growth and differentiation. We designed our culture conditions to simulate the normal status of the intestinal cells. We used the same serum-supplemented culture medium throughout the experiments since the accumulation of elements depends on the various chelators usually present in the complete culture medium as found under *in vivo* conditions.

Our results showed that Caco-2 TC7 cells incubated with cadmium concentrations ranging from 0 to 10 μmol Cd/l exhibited an increase in cadmium accumulation as a direct linear function of intracellular cadmium concentrations. The

cadmium accumulation was significantly higher in cells exposed long-term to cadmium (five weeks) than in cells exposed 1 week. These results are in agreement with a previous study on parental Caco-2 cells (Blais *et al.* 1999). Moreover, compared to control cells, only intracellular concentrations of copper and zinc increased in Caco-2 cells exposed long-term to cadmium but not the other minerals measured (Table 1). It is well known that zinc interacts with cadmium at the level of absorption and tissue binding (Orlowski & Piotrowski 2003). Exposure to cadmium disturbs zinc levels in the organism, while dietary zinc intake has a marked effect on cadmium absorption, accumulation, and toxicity (Brzoska & Moniuszko-Jakoniuk 2001). Both absorption and metabolism of copper has been shown to be affected by cadmium (Davies & Campbell 1977; Reeves *et al.* 1996). Moreover, it is generally believed that cadmium decreases copper uptake and/or transport in the gut (Davies & Campbell 1977; Reeves et al. 1996). However, in the present study, there was a significant increase in copper accumulation. Contrary to copper and zinc, increased cadmium accumulation was not correlated with the manganese intracellular concentration (Table 1). The initial manganese concentration in the culture medium was low, thus its absorption could not be influenced by the cadmium accumulation in the medium. These results could by explained by the fact that cadmium, copper, and zinc are well known to combine with metallothionein, an intracellular protein that binds metals, whereas this is not the case with manganese (Watanabe *et al.* 1991).

We focused on the interactions of cadmium with zinc, copper, and manganese, which are essential minerals whose adverse effects on human health are related to deficiency as well as excess. They clearly play a major role in the absorption, accumulation, and toxicity of cadmium due to their chemical nature, and their common interactions within living systems with cadmium. After addition of those three elements to cells exposed long-term to cadmium, the greatest reduction in cadmium accumulation was produced by manganese in a dose-dependent manner (Figure 3). The inhibition of cadmium accumulation by these metals has been explained by competition for binding sites at the plasma membrane as well as for transport through the membrane (Blazka & Shaikh 1992). The decrease in Cd toxicity may require the presence of other divalent metallic ions, which are capable of competing with cadmium for binding sites possibly involved in accumulation. Due to the existence of both high- and low-affinity Mn^{2+} transport systems, the total concentration of manganese in cells or tissues may not exhibit a major change when cadmium is added to cells or administered to animals. In this study, we found a mutual inhibition between cadmium and manganese, depending on the concentration of each element in the culture medium. A marked decrease in manganese and cadmium incorporation, but only at low concentrations (less than 1.0 μM) has been shown in a Cd-resistant fibroblastic cell line (Cd-rB5) (Yanagiya *et al.* 2000). Furthermore, the addition of $MnCl_2$ in a range of 1–30 μM reduced the cytotoxicity of Cd in parental cells but not in Cd-rB5 cells. These results suggest that the reduction in Cd toxicity is related to a change in cellular Cd incorporation due to the presence of Mn even at a high concentration. The mutual competition of Mn and Cd in terms of their incorporation into cells is complicated and depends on both low- and high-affinity Mn accumulation systems.

With regards to the inhibition of cadmium accumulation by the essential metals copper, zinc, and manganese, our results are similar to those reported in epithelium cells and in other cell types. For example, cadmium accumulation was reduced in the presence of zinc and copper in a renal proximal epithelial cell line from pig kidney (LLC-PK1), and zinc and copper appeared to competitively inhibit the uptake of cadmium (Templeton 1990; Endo *et al.* 1996). Zinc and copper have also been reported to inhibit cadmium uptake in hepatocytes (Blazka & Shaikh 1992) and intestinal cells (Caco-2) (Jumarie *et al.* 1997). Cadmium uptake is competitively inhibited by zinc in freshly isolated rabbit renal cortical cells (Gachot & Poujeol 1992). These *in vitro* results, confirming negative interactions between accumulated cadmium and exposure to minerals, are in agreement with previous *in vivo* results in ewes (Houpert *et al.* 1997) and other experimental animals (Brzoska & Moniuszko-Jakoniuk 2001; Noël *et al.* 2004). In male rats, the rate of gastrointestinal absorption of Cd is decreased by supplementation of the drinking water with a 'non-toxic' dose of manganese (Sarhan *et al.* 1986). In humans, it has been shown in the liver that zinc results in a decrease in Cd

absorption (Torra *et al*. 1995). The consequence of these results taken together is that a low intake of Zn, Cu, and Mn could have a profound effect on the amount of cadmium entering the body. Thus human populations deficient in these minerals may well more susceptible to cadmium toxicity than those that are adequately nourished (Chaney *et al*. 2001).

In conclusion, the Caco-2 TC7 cells line is a good model for investigating the effect of long-term exposure to environmental pollutants. It allowed us to show that cadmium accumulation was increased in correlation with copper and zinc. We also demonstrated that the extracellular presence of copper, zinc, and manganese resulted in a decrease in intracellular cadmium accumulation, but showed that these elements behave differently in terms of their mutual competition with cadmium. The present study provides a good base for future toxicological studies that will need an improved analytical system to permit the simultaneous analysis of a wider range of elements.

Acknowledgments

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