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Testing of chelating agents and vitamins against lead toxicity using mammalian cell cultures†

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Mammalian cell cultures were used to determine the capacity of antidotes to modify (a) lead uptake, (b) lead toxicity and (c) lead release from cells. The following chelating agents were tested: Na, Ca-ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), nitriloacetic acid, ethylene glycol-bis(aminoethyl)tetraacetic acid (EGTA), D,L-mercaptosuccinic acid (MSA), *meso*-2,3-dimercaptopropanesuccinic acid (DMSA), D,L-2,3-dimercaptopropane-1-sulfonic acid (DMPS), penicillamine (PA), *N*-acetylpenicillamine (NAPA), and diethylcarbodithioate (DDTC). The following vitamins were tested: thiamine (B_1), riboflavine (B_2), pyridoxine (B_6), cobalamin (B_{12}) and ascorbic acid (C). Inhibition of lead uptake was produced by EDTA, EGTA, DMSA, DMPS, MSA, PA, NAPA and vitamins B_1, B_6 and C, vitamins B_2 and B_{12} being ineffective. The same compounds reduced lead cytotoxicity. Interestingly DDTC and DTPA increased lead uptake, but did not exacerbate lead toxicity. Significant release of lead from preloaded cells was caused by DTPA, NAPA, DMPS and PA, while the other chelators were ineffective.

Keywords: *Lead; chelating agents; B vitamins; thiamine; riboflavine; pyridoxine, cobalamin; cellular metal uptake; cellular metal release; cellular metal toxicity*

Mammalian cell cultures are established tools in many medical and biological disciplines. Several working groups have realised the usefulness of this experimental system for toxicological studies into the effects of chelating agents.[1–4] On the one hand cell cultures can be used for the screening of antidotes to identify possible candidates against toxic metals before they are tested in experimental animals; on the other hand events at the cellular level can be elucidated. Thus the use of cell cultures may also help to spare or reduce animal experiments, a goal that is desirable for financial as well as ethical reasons.

In previous studies an experimental approach for the assessment of the antidotal efficacy of chelating agents directed against cadmium-inducd cytotoxicity was presented and the salient methodological points for the determination of the toxicity of the antidotes and their modifying effects on metal uptake and cytotoxicity as well as metal mobilization were discussed.[5,6] In the present work, tests with chelating agents of potential and proven therapeutic efficacy against lead are communicated.

About a decade ago the B vitamin complex were found to prevent lead poisoning in rats.[7] We were intrigued to find out if these antidotes were also effective in our experimental system.[8] These studies, which identified thiamine and pyridoxine as inhibitors of lead intoxication, have been extended and are reported here.

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Experimental

Chemicals

The chelating agents, vitamins and their sources were as follows: Na, Ca-ethylenediaminetetraacetic acid (EDTA) from Serva, Heidelberg, Germany; diethylenetriaminepentaacetic acid (DTPA), D,L-mercaptosuccinic acid (MSA), *meso*-2,3-dimercaptopropanesuccinic acid (DMSA), D,L-2,3-dimercaptopropane-1-sulfonic acid (DMPS), penicillamine (PA), *N*-acetylpenicillamine (NAPA) from EGA-Chemie, Steinheim, Germany; ethylene glycol-bis(aminoethyl)tetraacetic acid (EGTA), nitriloacetic acid (NTA) and diethylcarbodithioate (DDTC) from Merck, Darmstadt; the vitamins thiamine (B_1), riboflavine (B_2), pyridoxine (B_6), cobalamine (B_{12}) and ascorbic acid (C) from Merck, Darmstadt, Germany and cell culture media and reagents from Seromed, Berlin, Germany.

Cell cultures

Chinese hamster peritoneal cells, line B14F28, were serially cultivated in minimal essential medium (MEM) supplemented with 5% new-born calf serum (NCS), non-essential amino acids, glutamine and penicillin/streptomycin. The pH of the medium was stabilised to 7.0 ± 0.1 with 20 mmol l^{-1} HEPES buffer.[9]

Procedure

Cytotoxicity experiments

Nearly confluent Chinese hamster cell cultures (inoculum of 30 000 cells per 25 cm^2 flask, 3 d old; four replicates per experimental group) were exposed to 0.6 μmol l^{-1} $PbCl_2$ and different concentrations of the chelating agents or vitamins in serum-free medium. Cell numbers were determined 24 h later by detaching the cells from the flasks with trypsin (0.5% in PBS) and counting aliquots in an electronic counter (Coulter Counter).[10] The results are expressed as the percentage of the attained cell increase of the cultures treated only with lead; the proliferation of these lead-treated cultures was usually reduced by 20–30% compared with untreated controls. The standard deviation of the replicate cell counts within experimental groups was generally $< 4\%$.

Lead uptake experiments

Semi-confluent cultures (four replicates per experimental group) were exposed to 0.6 μmol l^{-1} $PbCl_2$ and different concentrations of the chelating agents or vitamins in serum-free medium. After 24 h incubation at 37 °C the cells were washed four times with PBS, detached by trypsinization and aliquots counted. Cells were processed and their lead contents analysed by atomic absorption spectrometry (AAS). The lead content of the samples was related to cell numbers.

Lead release experiments

Semi-confluent cultures (5 000 000 cells per 75 cm^2 flask, 3 d old; four replicates per experimental group) were exposed to

75 μmol l^{-1} $PbCl_2$ for 24–27 h at 37 °C in medium containing 5% serum. After washing the cells four times with PBS they were incubated for 24 h in serum-free medium without addition of test substances followed by 24 h incubation with two concentrations of the chelating agents in serum-free medium. This protocol was chosen because *ca*. 30–40% of the incorporated lead was released from the cells even in the absence of chelating agents during the first 24 h, while spontaneous release during the next 24 h amounted to *ca*. 20%. Cell numbers were determined, the lead contents of the cells were analysed and related to cell numbers.

Lead analyses

The preparation of the samples and lead analysis were performed, with modifications due to the fact that cell cultures are an unusual matrix, according to methods used for biological material in our laboratory.[8,11] Cells, including untreated controls, were processed by wet ashing in HNO_3 (Baker-Instra-Analized), samples were slowly heated to 180 °C and the residues dissolved in HNO_3. Standard lead solutions were used for calibration. In addition to the original samples standard dosages were added to aliquots of the samples, which were analysed in parallel. The lead contents were determined by AAS (PU 92100, TJA-Unicam, VG Elemental, Offenbach, Germany) with an electrothermal atomiser. The retrieval rate obtained in round robins was > 97%.

Statistics

Student's *t*-test was used for the evaluation of cytotoxicity and lead uptake. For the lead release experiments one-way analysis of variance was combined with Scheffe's test.

Results

Chelating agents

In previous experiments it was shown that the majority of the employed chelating agents were well tolerated by the cells; a 50% reduction of cell proliferation (IC_{50}, 2 d exposure) occurred at > 1 mmol l^{-1}. The exceptions were MSA, with a slightly elevated IC_{50} of 0.1–0.2 mmol l^{-1}, and DTPA and DDTC with a substantially higher ID_{50} of 10–20 μmol l^{-1}.[11] Therefore DTPA and DDTC were applied at one tenth the strength of the other chelating agents in the experiments reported here. The results of the simultaneous exposure of B14F28 cells to 0.6 μmol l^{-1} lead and the chelating agents on cellular lead uptake is shown in Fig. 1. The substances DMSA, PA, DMPS, EDTA, NTA, MSA, EGTA and NAPA caused a dose-dependent decrease of lead incorporation and correspondingly the cytotoxicity of lead was also diminished by these chelating agents (Fig. 2). A different result was observed in the case of DTPA and DDTC, which caused a clear dose-dependent increase of cell-bound lead. However, contrary to an expected increase in cytotoxicity, the proliferation of the cells exposed to lead and these two agents was unchanged compared with the cells treated with lead alone (Figs. 1 and 2).

The following chelating agents were able to mobilise lead from preloaded cells: PA, NAPA, DTPA, DMPS and MSA, while DDTC, EDTA, NTA, DMSA and EGTA were ineffective under the given experimental conditions (Fig. 3).

Vitamins

Studies of the effect of the vitamins on cellular lead uptake showed that thiamine and pyridoxine as well as vitamin C depressed lead incorporation markedly. A small, but statistically insignificant effect was noted in the case of riboflavine and cobalamin (Fig. 4). The substances depressing lead incorporation also had a favourable effect on lead toxicity (Fig. 5).

Discussion

In earlier studies we demonstrated the reduction of lead-induced cytotoxicity in the mouse fibroblast line L-A by chelating agents. The most effective substances were DMSA, EDTA, NTA, and PA, while NAPA, DMPS, and EGTA had no significant effect and MSA even increased the cytotoxic effects of lead.[12] These present results with the Chinese hamster cell line are partly in agreement, partly contradictory; a greater number of chelating agents was found to be effective under the new, apparently more sensitive experimental conditions. This can be either due to specific characteristics of the employed cell cultures or to other experimental variables. Therefore, it would be desirable to use different cell cultures for the screening of metal antidotes.

In the present investigations we found that the reduction of lead toxicity correlated with a reduced metal uptake. An interesting deviation from this rule was observed with DTPA and DDTC, which did not exacerbate lead toxicity in spite of markedly increased lead uptake. A similar finding was obtained when the combination of cadmium and DDTC was tested.[5] We

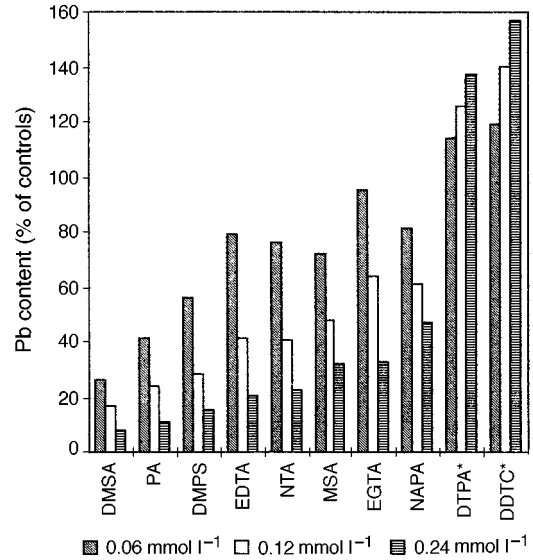

Fig. 1 Effect of chelating agents on lead uptake. Semi-confluent cultures of B14F28 cells were simultaneously exposed for 24 h to 0.6 μmol l^{-1} $PbCl_2$ and 60–240 μmol l^{-1} of the chelating agents. DDTC and DTPA were applied at 6–24 μmol l^{-1}. Lead was determined by AAS.

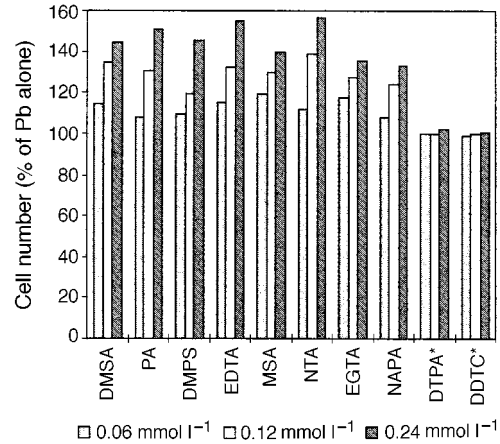

Fig. 2 Effect of chelating agents on lead toxicity. Semi-confluent cultures of B14F28 cells were simultaneously exposed to 0.6 μmol l^{-1} $PbCl_2$ and 60–240 μmol l^{-1} of the chelating agents and cell numbers were determined 24 h later. DDTC and DTPA were applied at 6–24 μmol l^{-1}.

interpret this to mean that the cells incorporate and store the complex formed between the metal and the chelating agent, which is less toxic than the free metal ions since these are no longer available to react with cellular ligands.

For a possible therapeutic use chelating agents should, in addition to being non-toxic, be able to mobilise metals from cells. This was clearly demonstrated for NAPA, DTPA, MSA, and PA. The ability of NAPA to mobilise intracellular lead was also observed in mice exposed to lead acetate; although survival was not improved after intraperitoneal treatment with NAPA, lead concentrations in liver, kidney and brain were significantly reduced.[13] Penicillamine (PA) has been clinically used to treat lead poisoning and it has also been shown in experimental animals that tissue lead is reduced and renal lead elimination increased by PA.[13–15] Our finding that DMPS reaches the intracellular space is also supported by investigations which found that DMPS is taken up into erythrocytes by an anion transport protein and can even be concentrated there.[16,17] In rats 40% of the parenterally applied DMPS was found in the bile which indicates that a cellular passage occurs in the liver.[18] The low cytotoxicity of this substance and its protective effect which were observed in our *in vitro* studies has been confirmed *in vivo*. DMPS increased the survival of lead-poisoned mice clearly[19] and in rats it caused a substantial elevation of renal lead elimination as well as a reduction of the lead contents of blood, liver, kidney and bones.[3,20,21] In clinical studies with lead-intoxicated children lead concentrations decreased also.[22]

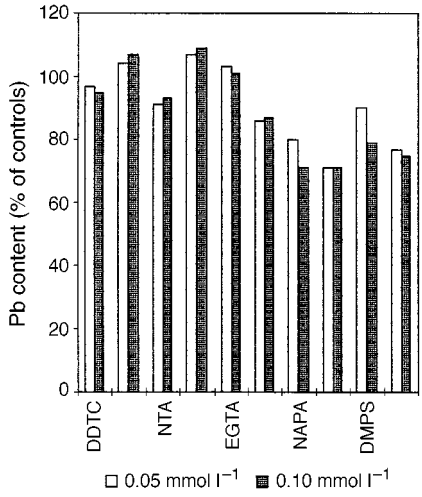

Fig. 3 Effect of chelating agents on lead release. Semi-confluent cultures of B14F28 cells were exposed for 24 h to 75 μmol l^{-1} $PbCl_2$, washed and incubated for another 24 h in serum-free medium followed by 24 h with 50 and 100 μmol l^{-1} of the chelating agents. Cell numbers were determined and lead was analysed by AAS and related to cell counts.

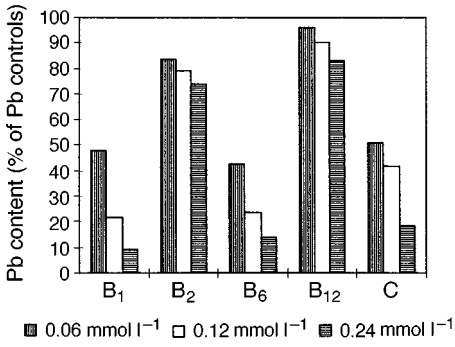

Fig. 4 Effect of vitamins on lead uptake. Semi-confluent cultures of B14F28 cells were simultaneously exposed for 24 h to 0.6 μmol l^{-1} $PbCl_2$ and 60–240 μmol l^{-1} of the vitamins. Lead was determined by AAS and related to cell counts.

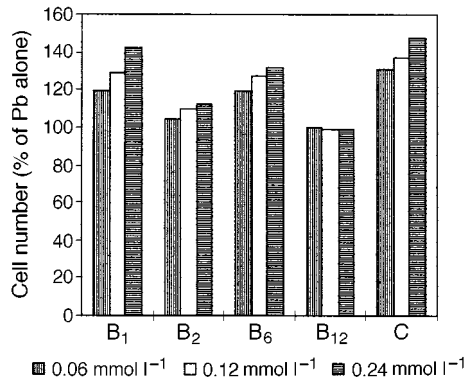

Fig. 5 Effect of vitamins on lead toxicity. Semi-confluent cultures of B14F28 cells were simultaneously exposed to 0.6 μmol l^{-1} $PbCl_2$ and 60–240 μmol l^{-1} of the vitamins and cell numbers were determined 24 h later.

As shown above, DTPA increased lead uptake without an accompanying increase in lead toxicity. This indicates that the substance is transported into the cells, which was also postulated because of the chelating agent's pharmacokinetics.[23,24] Although it has been demonstrated that DTPA is effective against lead in mice[19,24] and that it is well-tolerated,[25] its use has so far been restricted to the treatment of transuranic elements in humans.

In our experiments MSA also showed a good efficiency in mobilising lead and it also decreased lead uptake and cytotoxicity in B14F28 cells. The depression of proliferation caused by MSA in L-A cells indicates that the substance enters the cells and possesses a certain toxic potential, which would make it unsuitable for therapeutic use. A related substance DMSA caused no lead release from the cells under our experimental conditions. *In vivo* studies confirmed that distribution in the organism was restricted to the extracellular compartment.[18] In experimental animals this chelating agent increased lead elimination and lowered tissue lead levels.[13] The therapy of lead-intoxicated persons had similar results. Since DMSA is well tolerated and can be applied by the oral route so that hospitalisation can be avoided, it has several advantages compared with EDTA.[26,27]

In our experimental system no lead release could be induced by EDTA although the chelator showed clear protective effects both in B14F28 and in L-A cells. Our results are in contrast to *in vitro* tests with osteoclasts and hepatocytes where EDTA mobilised lead significantly.[28,29] This difference is probably due to the fact that bone and liver represent typical target cells for lead compared to the fibroblasts which were used in this study. *In vitro* studies have shown that the substance is extracellularly distributed and this has been confirmed by experiments conducted *in vivo*.[24] Although the mechanism of action is still unclear, it has been proved that the renal excretion of lead is stimulated and tissue levels can be decreased by EDTA and it has been regularly used to treat lead-intoxicated patients.[15,27,30] The remaining chelating agents NTA and EGTA, which did not affect cellular lead release, will not be discussed further here, since their therapeutic potential appears to be small.

The tests with the vitamins demonstrate that the B vitamins pyridoxine and thiamine have a protective effect against lead uptake and toxicity. This corresponds to our earlier results with the mouse fibroblasts L-A[11] and to the experiments by Tandon and co-workers. These authors showed in rats that lead intoxication could be prevented by the application of vitamin B complex and different parameters of lead poisoning were aggravated by vitamin B deficiency.[10,31] The authors also treated lead poisoned rats with B vitamins, alone and in

combination with $CaNa_2$-EDTA, and found that folic acid and pyridoxine might be the factors responsible for the favourable effects.[32] In our experiments vitamin C was also highly effective against lead-induced cellular intoxication. This was also observed in rats.[33] To our knowledge the promising approach of employing vitamins for the prevention and therapy of lead intoxication has not yet been introduced into human medicine. Our tissue culture studies confirm that there may be some scope in this strategy.

Our results demonstrate the usefulness of the employed experimental system to identify substances that are capable of modifying the uptake, cytotoxicity and release of lead from mammalian cells. This system can easily be standardised and is comparatively rapid and inexpensive. However, it must be stated that effective substances can be missed (false negatives), a shortcoming that may be reduced by the use of several parallel culture systems. In the case of substances exhibiting antidotal properties *in vitro*, animal tests must confirm the findings, particularly for pharmacokinetic considerations.

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