Arh Hig Rada Toksikol 2001;52:333-354

REVIEW

IN VITRO TOXICOLOGY OF HEAVY METALS USING MAMMALIAN CELLS: AN OVERVIEW OF COLLABORATIVE RESEARCH DATA

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Received July 2001

The uptake and distribution of selected heavy metals were followed and related to cytotoxicity using various parameters of proliferation and viability of cultured cells. The effects of short-term lead exposure on DNA synthesis were reversible, indicating that lead does not significantly influence genetic cellular function. In contrast, nickel effects persisted, indicating that DNA is one of the main nickel targets. Heavy metals affected all cycle phases, but those related to preparation and commencement of DNA synthesis were the most susceptible. Tolerance appeared in chronic exposure to lead and cadmium. Lead combined with X-rays had additive effect, while manganese acted synergistically and appeared to inhibit the DNA repair processes. Zinc and manganese showed a protective effect against the toxic effects of cadmium. Similar antagonistic interaction was seen for nickel v. manganese cytotoxicity. This model system makes it possible to compare heavy metal effects at the cellular level and to identify cellular targets and metabolic processes.

Key words:
cadmium, cell culture, lead, manganese, metal cytotoxicity, nickel

Metals are indispensable for life. Beside sodium, potassium, calcium, and magnesium, which sustain the internal physiological balance, there are approximately a dozen other trace metals which have proven essential. However, in higher dosage, these can also be

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This overview continues the series dedicated to the activities of the Institute for Medical Research and Occupational Health performed since its foundation either alone or in collaboration with other institutions. For details, please refer to *Archives of Industrial Hygiene and Toxicology* year 1999, vol. 50, pp. 211-222; 309-326; and 405-421.

toxic, including iron, copper, zinc, iodine, fluorine, manganese, selenium, chromium, cobalt, molybdenum, nickel, zinc, and vanadium. For other metals and metalloids including lead, cadmium, mercury and arsenic, a biological role has not yet been established. The functional, morphological and biochemical effects of these elements manifest themselves at different levels: the organism, organs and tissues, the cell, and even at the sub-cellular level. Before organ functions are affected, cellular processes must be altered. The mammalian cell is the smallest self-sustaining functional unit and a complex homoeostatic system. Metals influence cells in several ways; they react with membranes, organelles, and metabolic and mitotic systems.

Cell culture techniques have allowed the propagation of cells *in vitro*, that is, outside the organism, allowing the study of biological processes at the cellular level.

The cell culture system has certain limitations:

- there is a lack of humoral, nervous and other integration mechanisms common for all cells in the organism;
- toxic substances are usually not metabolised. This can sometimes be compensated for by special methods such as co-cultivation with metabolically competent cells or by the addition of liver microsomal extracts, which is not necessary in the case of metals;
- secondary effects resulting from the accumulation *in vivo* are absent.

On the other hand, this test system has its strong advantages:

- it makes it possible to study cellular processes directly;
- experimental conditions are well-defined and reproducible;
- homogeneous cell populations are used;
- no secondary effects resulting from primary alterations at other locations of the whole organism need to be considered (1, 2).

This paper gives an overview of *in vitro* studies with cultured mammalian cells which were carried out singly or in collaboration between the laboratories of the two authors. It does not include very recent work, but exemplifies what has been achieved and what can be done with this methodology. It also acknowledges good scientific and personal relations resulting from this collaboration. This paper reports only the results for heavy metals lead, cadmium, manganese, and nickel, since these studies were either done in co-operation or complement each other.

MATERIALS AND METHODS USED

The methods used have been presented in detail in earlier publications. Here only a brief account is given.

Cell lines

- HeLa, a human epithelial-like portio-carcinoma cell line (3, 4),
- human embryonic fibroblasts FH (4),

- L-A cells, a substrain of the mouse fibroblast line L929, growing in suspension (5),
- the quasi-diploid cells $C_{14}F_{28}$ and V69 derived from peritoneum and lung of the Chinese hamster, respectively (4, 6).

Parameters of cytotoxicity

- proliferation determined by electronic cell counting (Coulter Counter Model FN) or microscopic evaluation (5, 7),
- plating efficiency, i.e. the colony-forming ability of cells plated in small numbers of 100-500 per culture vessel, as another indicator of cellular replication and viability (3, 6),
- viability determined by the trypan blue exclusion test indicating membrane damage due to cell death (5),
- release of lactic dehydrogenase (LDH) indicating membrane damage (5),
- lactic acid production indicating alterations in carbohydrate and energy metabolism (5),
- ^{3}H-cysteine or ^{14}C-phenylalanine incorporation to study effects on protein synthesis (4, 8),
- ^{3}H-uridine incorporation to study effects on RNA synthesis (4),
- ^{3}H- or ^{14}C-thymidine incorporation to determine effects on DNA synthesis and cell replication (4),
- light microscopic examinations of cell morphology including mitotic index, chromosome morphology, and sister chromatid exchanges (6),
- determinations of cell size by Coulter Counter measurements (9),
- determinations of DNA content by flow photocytometry in order to elucidate effects on the progression of the cells through the cell cycle (6),
- determination of metal uptake under different experimental conditions by use of ^{115m}Cd isotope and by chemical analysis (10, 11),
- histochemical staining of intracellular lead by the adapted method of Brunk and Brun (4, 12).

THE RESULTS OF THE STUDIES

ACUTE TOXICITY

The purpose of short-term experiments was to investigate the acute cytotoxicity of trace metals lead, cadmium, manganese, and nickel. Here are reported some of the employed parameters and results.

Comparative metal cytotoxicity and cell susceptibility

Comparative studies investigated lethal and proliferation-inhibiting effects of lead, cadmium, manganese, and nickel. In L-A mouse fibroblasts the viability as determined by the trypan

blue exclusion test as well as cell proliferation following 7-day exposure decreased in the following manner: $CdCl_2$>$NiCl_2$>$MnCl_2$>$PbCl_2$. Table 1 shows the concentration ranges leading to a minimal and 50% growth inhibition (IC_m and IC_{50}) or to 50% lethality (LC_{50}) (2). Comparative cytotoxicity tests of these heavy metals in the human lines HeLa and FH exposed for two days yielded, however, a different rank order: $CdCl_2$>$MnCl_2$>$NiCl_2$≈$PbCl_2$ (3, 13, 14). These differences may be due to two causes. The first concerns the differing susceptibility of various cell lines, depending on parameters studied, which can be related to differences in susceptibility observed in different species and also a varying sensitivity of the organs in the whole organism (15). The second concerns the experimental protocol; for HeLa and FH an exposure time of seven days is too long and therefore an exposure period of two days was chosen. In further experiments concerning the plating efficiency it was seen that the effects of nickel were delayed and appeared only after a 2-day exposure, which could mean that the toxicity of Ni may have been underestimated in the 2-day test (see cell cycle effects below).

Table 1 *The effect of different heavy metals on the proliferation and viability of* L-A mouse fibroblasts. 120,000 suspension cells were inoculated into 100 ml Breed-Demeter flasks (4 flasks per experimental group) and exposed to metal chlorides in Medium 199 supplemented with 10% horse serum for 7 days (9)

Metal	Minimal concentration (IC_m)	Inhibitory 50% concentration (IC_{50})	Inhibitory 50% lethal concentration (LC_{50})
Cd	2 μM	3 μM	4 μM
Ni	2 μM	3-5 μM	10-30 μM
Mn	3 μM	8-12 μM	100-200 μM
Pb	30 μM	30-50 μM	ca. 300 μM

All tested heavy metals inhibited cell proliferation, which was manifest through the decrease in the cell number, plating efficiency and DNA synthesis, and through altered mitotic rates before the viability was affected and LDH leaked through the damaged membranes. Another index of cellular stress is the elevated production of lactic acid which accompanies the growth inhibition. An example is given in Table 2 (13).

REVERSIBILITY OF EFFECTS OF HEAVY METALS

Lead

Experiments investigating whether the effects of lead were reversible paid particular attention to the effects preceding toxic manifestations. HeLa cells incubated with $2.5x10^{-4}$ M lead chloride showed a time-dependent decrease in the uptake of ^{3}H-thymidine, ^{3}H-uridine, and ^{14}C-phenylalanine. In 30 hours, the incorporation of DNA, RNA, and protein precursors dropped to 40%, 37%, and 27% of the control incorporation, respectively. Under these

Table 2 *Cytotoxicity of $NiCl_2$ for human diploid fibroblasts; 0.9 million FH cells* were inoculated into 25 cm^2 flasks and 24 h later exposed to 0-4x10^{-4} M $NiCl_2$ in MEM supplemented with 10% newborn calf serum. 49 h later, the cultures were examined for cell number (trypsinisation and Coulter counting), viability (dye exclusion test), release of intracellular lactic dehydrogenase (LDH) into the supernatant and lactic acid production (test-sets Boehringer Mannheim), as well as for the mitotic rate (Giemsa stain, 300 cells counted) (13, 14).

$NiCl_2$	Cell number (10^6/culture)	Viability (%)	LHD (mU/10^6 cells)	Lactic acid (mg/10^6 cells)	Mitotic index (%)
0	3.079	98.5	6.6	0.9	2.37
10^{-4} M	2.828	98.6	6.8	1.37	1.77
2x10^{-4} M	1.542	93.7	13.2	2.22	1.48
4x10^{-4} M	0.702	58.1	18.5	n.d.	n.d.

n.d. = not determined

conditions the generation time was prolonged from ca. 20 h (control value) to 55 h after 72 h. If the cells treated with 2.5x10^{-4} M lead chloride were then cultured in lead-free growth medium and pulse-labelled with the radioactive precursors, DNA, RNA, and protein syntheses returned to control values within approximately 10 h. This shows that the measured early lead effects are reversible and that lead does not significantly influence genetic cellular function (16). During the inhibition of macromolecular syntheses, the histochemical sulphide-silver method showed that lead was present in the cells in granules assembling in the cytoplasm around the nucleus. These granules slowly disappeared, after the cells had been returned into the normal medium (4, 17).

Nickel

The reversibility of nickel effects was investigated using the Painter test (16) which measures the recovery of DNA synthesis following the depression by a toxic agent. The uptake of ^{14}C-thymidine did not normalise immediately after the cessation of treatment with $NiCl_2$, contrary to the control substance DMSO which is non-mutagenic (Figure 1). Our results confirm those of Painter, indicating that DNA is one of the main nickel targets. Other known effects are the induction of DNA strand breaks, DNA-protein cross-links, inhibition of DNA polymerase, genetic miscoding (18-20), abnormal mitotic figures, chromosome breaks, sister chromatid exchanges (8, 21-23), and cell transformation (24, 25).

Cell cycle effects

As demonstrated above, a dose-dependent decrease in cell proliferation was seen upon heavy metal exposure. Since proliferation is a sensitive indicator of cytotoxicity, it was of particular interest to see which part of the cell cycle might be the most susceptible to metals and whether cells reacted in a different way between single phases of the cell cycle.

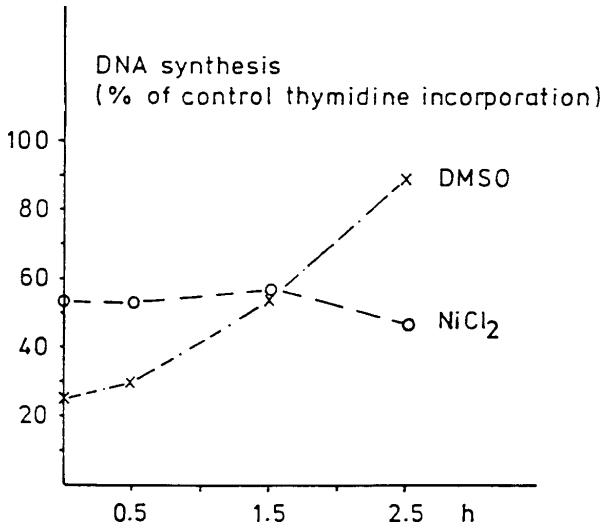

Figure 1 *The effect of nickel on DNA synthesis. HeLa cells were treated for 24 h with 20 mM $NiCl_2$ in serum-free medium. After washing, the cultures were incubated for 15 min with ^{3}H-thymidine at different times after cessation of exposure and the thymidine incorporation was determined by liquid scintillation counting (13).*

Lead

In L-A cells different parameters of proliferation were studied following exposure to $PbCl_2$. The differences between controls and cells treated with $2x10^{-5}$ M for 2-3 days were in no instance significant ("no-effect level"). The cultures exposed to 8 and $32x10^{-5}$ M $PbCl_2$ showed a clear reduction in cell numbers and ^{3}H-thymidine labelling, whereas viability dropped moderately (to ca. 80%) only at the highest concentration (Table 3) (5). It can be

Table 3 *Cytotoxicity of $PbCl_2$ for mouse fibroblasts. 136,000 L-A suspension cells were inoculated into 100 ml Breed-Demeter flasks (4 flasks per experimental group) and exposed to 0-$4x10^{-4}$ M $PbCl_2$ in Medium 199 supplemented with 10% horse serum. Two days later, the cultures were examined for cell number (trypsinisation and Coulter counting), viability (dye exclusion test), ^{3}H-thymidine-labelling (500 cells microscopically counted per sample), and for the mitotic rate (5000 cells counted per experimental group) (5).*

$PbCl_2$	Cell number * d 2	Viability * d 2/d 3 (%)	% labelled cells (d 2-d 3)	Mitotic index (%)
0	264,778	92.7/95.5	94.2	1.3
$2x10^{-5}$ M	253,212	91.8/96.5	93.7	1.7
$8x10^{-5}$ M	161,048	91.7/93.7	83.3	0.7
$32x10^{-5}$ M	141,348	82.0/78/0	39.9	n.d.

* The standard deviation was <2.5%

n.d. = not determined

concluded that there is a sizable proportion of cell population which does not take part in DNA synthesis or enters DNA synthesis after longer intervals. This was confirmed by cytophotometric measurements. In controls, 76.7% of the cells were in G1, 14.8% in S, and 6.3% in G2. In cells treated with 50 and 100 μM $PbCl_2$ for 24 h, G1 increased to 89.1% and 91.8%, respectively, S decreased to 7.5% and 5.1%, respectively, and G2 nearly halved to 3.4% and 3.1%, respectively (27). The cells are arrested before DNA synthesis, that is, in G1. Cell size measurements showed a clear increase in the number of enlarged lead-treated cells (Figure 2) (5). Control cells, which were predominantly in G1, that is, smaller when inoculated, grew in size during the lag phase one day after inoculation and then gradually returned to their original size until the stationary culture phase. In contrast, the lead-exposed cells grew and remained bigger throughout the observation period of 13 days. It is conceivable that a substantial fraction of cells treated with lead which are arrested in G1 continues to synthesise proteins without starting DNA synthesis. This enlargement could precede vaculoations seen at still higher lead doses. The mitotic rate is stimulated by the lowest lead concentration and decreases with the rising exposure to lead (Table 3). This can be explained by the influence of lead on the spindle. Like colcemide, heavy metals induce "C-mitoses" resulting in reduced chromosome length (28). In addition, microscopy of mitotic cells shows that some mitoses change morphologically and that the index of pro- plus metaphases: ana- plus telophases is increased (27). Thus the progression through mitosis is inhibited in the early division stages and a failure of chromosome distribution to daughter cells must be expected. Increasing lead concentrations impair cell division to the point that the mitotic rate diminishes probably due to the effects of lead on the genetic material. It was further seen that $PbCl_2$ caused a moderate, but dose-

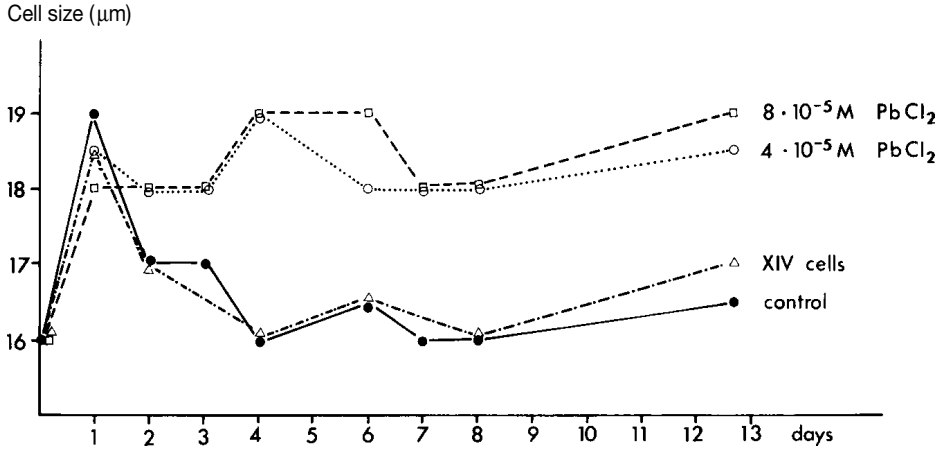

Figure 2 *The effect of lead on the cell size of L-A cells during a full growth cycle. Cultures were started and exposed to 4 and 8x10⁻⁵ M $PbCl_2$ (as described in Table 3) and the cell sizes of cell suspensions measured with the Coulter Counter Model FN. Only the most frequent size classes are shown while the fractions containing smaller and larger cells are not presented. The measurements of cells adapted to and continuously cultured with 14x10⁻⁵ M $PbCl_2$ (XIV cells, cf. Figure 5) are included (5).*

dependent increase in sister chromatid exchanges (8, 29). It can be concluded that probably all cell-cycle phases (G1, S, G2 and *M*), are affected by lead in a concentration-related fashion.

Cadmium

Cadmium caused effects on cell proliferation similar to lead, but at substantially lower concentrations. Another notable feature is the close proximity of growth-inhibiting and lethal concentrations (Table 1). We investigated the cell-cycle factors determining the expression of cadmium cytotoxicity in detail.

Pilot studies compared the effects of cadmium chloride on the colony-forming ability of $B_{14}F_{28}$ cells in the logarithmical and stationary (plateau) growth phases. The logarithmically growing cultures, which are composed of all cycle phases, were synthesising more DNA than stationary cultures (252 cpm/10^6 cells vs 83 cpm/10^6 cells) which are predominantly arrested in G1. When the cells were exposed to different cadmium concentrations for 1 h, the number of colonies derived from cells in the logarithmic phase was significantly lower; for example, 1 and 2 μM $CdCl_2$ reduced the colonies to 41.5% and 10.5% of control numbers in logarithmic cells, respectively, as opposed to respective 62% and 34.3% in cells derived from the plateau phase (6).

Synchronized Chinese hamster cells, line $B_{14}F_{28}$, were exposed for 1 h to 3.5 μM cadmium chloride for a whole cell cycle and the plating efficiency was determined. Figure 3 shows that the colony number decreased to ca. 35% of controls and that the reduction was the greatest in the period corresponding to the late G1 to middle S-phase. Cells of the G2+*M*-phase are less sensitive than those of the late DNA-synthesising S-phase, but are less resilient than cells in the early and middle G1 (6).

The finding that cells exposed in different growth phases or cell-cycle phases show a different cadmium susceptibility allows two possible explanations: (i) either sensitive cells accumulate more cadmium or (ii) cadmium effects on metabolic processes related to DNA-synthesis are most critical for cell survival. The two hypotheses do not exclude one another. The first agrees with the finding that the uptake of cadmium is markedly higher (per cell number and protein content) in logarithmically growing cultures which contain a higher proportion of DNA synthesizing cells than stationary cells (10). Greater vulnerability of cells in the S-phase is compatible with the demonstration that cadmium is a mutagen. We observed a significant dose-dependent occurrence of sister chromatid aberrations in these cells (6, 8, 29, 30).

In addition, a cell cycle prolongation by cadmium could be determined by means of flow cytophotometry. Mitotically synchronised $B_{14}F_{28}$ cells continuously exposed to 1 μM $CdCl_2$ had a 77% longer cell cycle than controls, while the duration of G1, S, and G2+*M* increased 40%, 6%, and 31%, respectively. This confirms the relative decrease in DNA-synthesis due to cadmium (6). The finding that G2+*M* is prolonged corresponds to that of *Bakka and Digernes* (31) who described a dose-dependent accumulation of cells in the mitotic prophase and early metaphase in a human epithelial cell line. *Harnett and co-workers* (32) reported an S-phase block in Chinese hamster cells, which does not agree with our findings that the duration of S changes little and is relatively shortened. *Bakka*

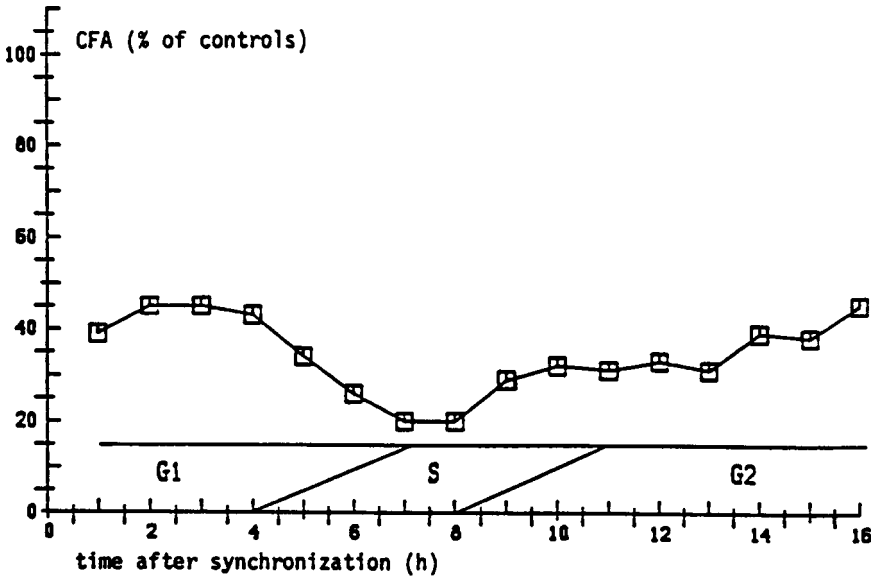

Figure 3 *Cadmium susceptibility of different cell-cycle phases. Five flasks per experimental group were seeded with 100 metaphases of the Chinese hamster cell line $B_{14}F_{28}$ and exposed to 3.5 µM cadmium chloride at 1-hour intervals throughout the cell cycle. The progress of the cell cycle was checked at hourly intervals by cytofluorescence on parallel cover slips (6).*

and Digernes (31) speculated that *Harnett and co-workers* (32) could have made a methodological fault.

Nickel

Similar studies were executed with V79 cells of the Chinese hamster. In order to find out which part of the cell cycle was the most responsive to the effects of nickel, synchronised cells were treated with $NiCl_2$ for 1 and 4 hours at different points of the cell cycle. Figure 4 shows corresponding results for both incubation periods. The late G_1 and early S-phase are the most sensitive, while the late S-phase is the least susceptible (13, 14). It has been reported that CHO cells are blocked by nickel in the S-phase (31). This is compatible with our results, as cells damaged in G1 and early S could be directly held up in S. In addition, the blockage in S should result in a decreased mitotic rate, as was indeed observed (21).

Manganese

With this heavy metal, a comparison of effects on V79 Chinese hamster cells exposed in the plateau phase and in logarithmic growth to 10, 50 and 250 µM $MnCl_2$ showed that

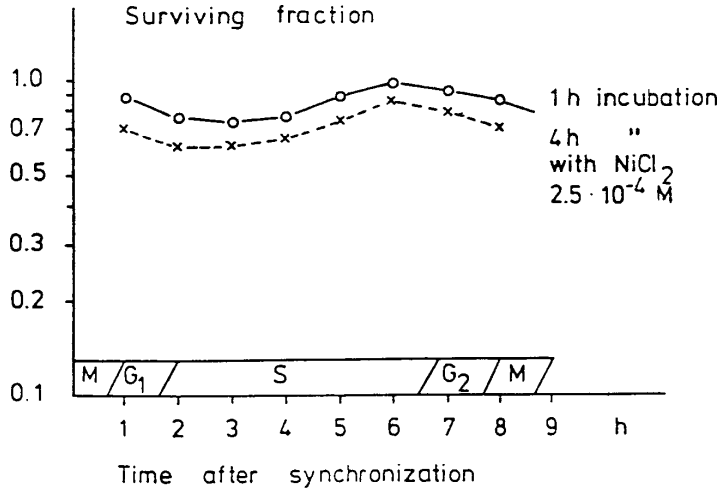

Figure 4 *Cell cycle effects of nickel. Synchronised Chinese hamster V79 cells were obtained by shaking off loosely attached mitotic cells. About 500 cells were plated onto plastic dishes (5 flasks per experimental group) and exposed to $NiCl_2$ at 1 or 4-hour intervals. The cultures were then washed, fed with metal-free medium, and incubated for 8 days, and the resulting colonies were counted (14).*

stationary cells were more sensitive than logarithmically growing cells, especially if they were exposed for up to 6-20 hours. This was determined by the colony formation test. There are two hypotheses to explain this finding; either the quiescent state of V79 cells favours manganese binding to more sites in the cells, slowing down their metabolism more than when the cells are growing exponentially or the stationary cells, which are partly synchronised so that they contain mainly G1 cells, have to prepare themselves for DNA synthesis, and these processes may be especially sensitive, as has already been observed with cadmium. The latter hypothesis is more likely to be true, as the enhanced susceptibility of quiescent cells manifests itself only during longer exposure periods. The progress into S, G2, and M during manganese exposure would then predominantly affect the early stages of the cell cycle in cells derived from the plateau growth phase, while exponentially growing cells contain a mixture of all cell-cycle phases, including the less sensitive ones (33). The increased susceptibility to manganese seems comparable to that observed by other authors for bleomycin and nitrosurea derivatives, but it is not known whether the same mechanisms are responsible (34, 35).

In further experiments where synchronized V79 cells were exposed to the low concentration of 2.5 μM $MnCl_2$ for 1 or 4 h, only a small fluctuation in sensitivity during different cell-cycle phases was noted, but the first part of the cycle corresponding to G1 and early S appeared to be the most susceptible to manganese. This confirms our findings and the explanatory hypotheses at the beginning of this section (36).

To conclude, the investigated heavy metals affect all cell-cycle phases in a complex

manner, including many functions pertaining to the processes related to cell survival, proliferation, and other metabolic processes. The synthesis of DNA, RNA, and protein decreases and a general retardation of cellular proliferation occurs at concentrations which are not yet lethal. However, the processes related to DNA synthesis, that is, late G_1-phase where many preparative events occur and early S-phase are usually most disturbed and should have the greatest consequences. Similar observations have been reported with other heavy metals (31, 32, 37-39).

CHRONIC EFFECTS

Cellular reaction to chronic intoxication with heavy metals was yet another issue; do cells become even more sensitive or can they gradually become more tolerant of these metals?

Lead

Our studies included L-A mouse fibroblasts and HeLa cells. L-A cells were successively cultured with 3, 5, 7, 10.5, and $14x10^{-5}$ M lead chloride, and their proliferation normalised within 1-10 weeks. Lead pre-treated cells tolerated elevated $PbCl_2$ concentrations significantly better than naive cells. Cells that gradually adapted to 10.5 and $14x10^{-5}$ M lead chloride showed markedly better growth rates than naive cells, but they were clearly inferior to those of unexposed controls (Figure 5). When the lead-adapted cells were cultured without lead, the tolerance disappeared within a few weeks (Figure 6) (9).

Comparable effects were seen in HeLa cells. Cultures incubated with increasing $PbCl_2$ concentrations for several weeks showed no effect on DNA synthesis when acutely toxic lead concentrations were added. Lead tolerance disappeared when the adapted cells were returned into lead-free medium (40).

These experiments show that cells chronically exposed to low lead levels are protected during exposure to higher concentrations and that there is a limit to resistance, evidenced by the fact that L-A cells continuously exposed to the two highest concentrations 10.5 and $14x10^{-5}$ lead chloride did not attain the same proliferation rates as controls. The resistance developed so fast that it could not be due to selection, but must have been caused by adaptation. Moreover, lead tolerance was lost within a few cell generations from the cessation of lead exposure, which would not have occurred in a genetically fixed trait. The mechanisms of lead resistance, which can also be demonstrated *in vivo* (41-43), remain unclear. Measurements of lead content in treated cultures showed that the resistant cells did not exclude the heavy metal. This was confirmed in lead-adapted kidney cells which accumulated amounts of lead similar to naive cells (44). It has been suggested that the intranuclear inclusion bodies observed in kidney cells hepatocytes and astrocytes, which are composed of lead-protein complexes, serve to spare toxic injury to cytoorganelles (45, 46). In addition, a low-molecular glycine-rich protein has been discovered in the blood of lead-exposed workers, whose lack correlated with clinical and biochemical signs of lead intoxication in workers with relatively low blood-lead concentrations (47).

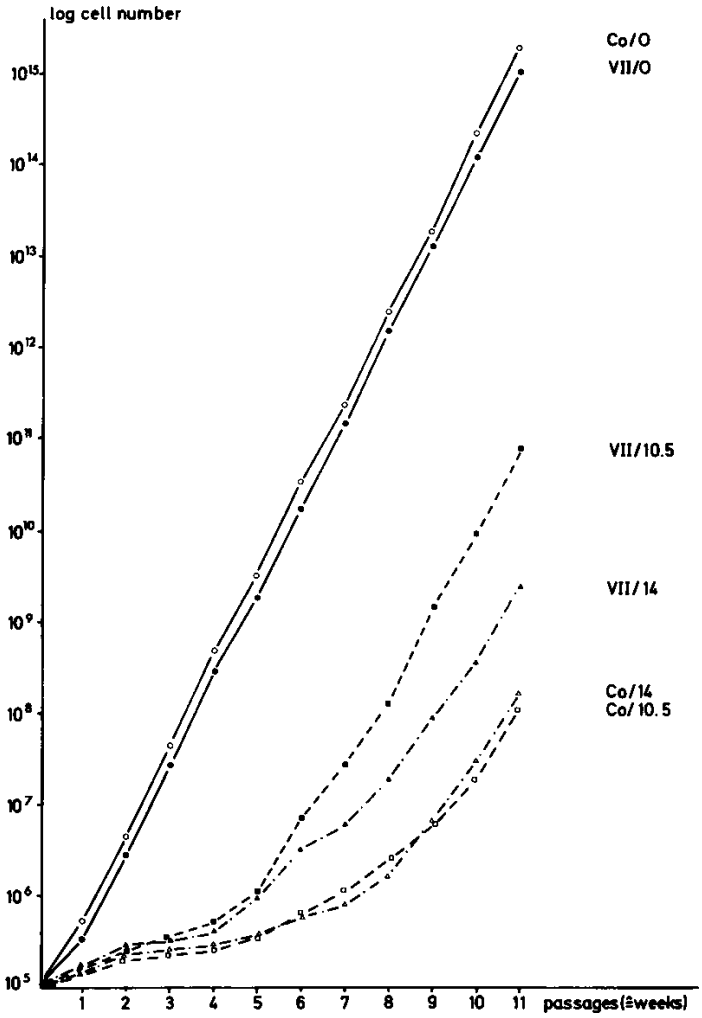

Figure 5 *Long-term effect of lead on L-A cell growth. Control cells (Co) and cells made resistant to 70 μM $PbCl_2$ by gradually increasing exposure to $PbCl_2$ concentrations were continuously cultured in the absence or presence of 10.5 or $14x10^{-6}$ M $PbCl_2$. Cumulated cell numbers are logarithmically plotted on the ordinate and the number of passages (1 passage/week) linearly on the abscissa (9).*

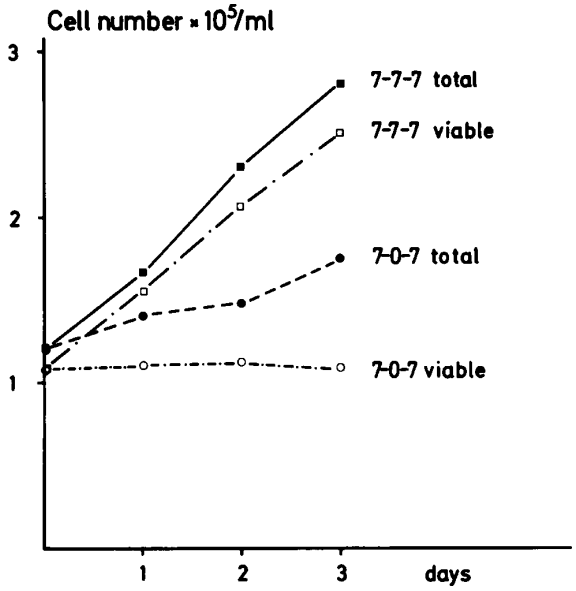

Figure 6 *Comparative effect of 70 μM $PbCl_2$ on the proliferation of L-A cells continuously cultured with this lead concentration (7-7-7-) and cells resistant to lead which were cultured for 7 passages (39 days) without lead. The inoculation involved 120,000 cells/ml and 4 replicates were studied per experimental group (9).*

Cadmium

Studies with experimental animals and cultured mammalian cells show that continuous exposure to cadmium leads to the development of resistance caused by the induction of metallothionein (26, 48-51). We observed adaptation to cadmium in several cell lines including $B_{14}F_{28}$, HeLa, FH, and LA. The cell sensitivity decreased within 1-2 days of pre-treatment (Figure 7) and tolerance increased in long-term exposure. This effect was not produced by the reduced cadmium uptake of the pre-treated cells, but the concentration-related increase in cysteine during cadmium treatment indicated that the cells were synthesising metallothionein (8, 26).

COMBINATION EFFECTS

Metals and X-rays

When Chinese hamster fibroblasts V79 were exposed to the combination of lead and X-rays, additive effects on DNA, RNA and protein synthesis were observed. The effects of

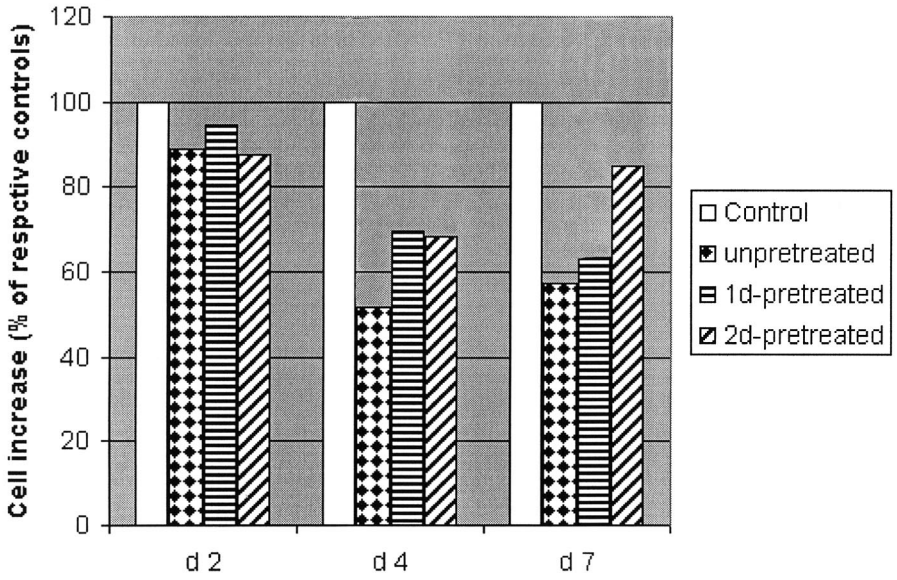

Figure 7 *Comparative effect of 5 μM $CdCl_2$ on the proliferation of L-A cells without pre-treatment (Co) or with 1-2-day pre-treatment with 0.5 μM $CdCl_2$. Cells were cultured and exposed as described in Figure 5 (26).*

irradiation on macromolecular syntheses were reversible; they were blocked by lead, but once lead was removed, the incorporation of the precursors proceeded at the same rate as in cells previously not exposed to lead. It was concluded that lead chloride did not primarily affect the genetic system of HeLa cells and that lead did not inhibit the repair processes (52, 53).

Further experiments investigated the combined effects of manganese and X-rays on the plating efficiency of synchronized V79 cells. Contrary to the results obtained with lead, there was a marked synergism of the two agents when $MnCl_2$ was administered immediately after irradiation, while the effects were only additive, when the metal salt was added 3 hours after irradiation. It was concluded that manganese probably impaired the repair processes occurring immediately after X-irradiation, causing thus the observed synergism (36).

Metal-metal interactions

Simultaneous exposure to several metals is to be expected in the environment, and it is important to know how they interact and which factors modify their toxicity. Our studies focused on the combined effects of cadmium and zinc, cadmium and manganese, and nickel and manganese.

Zinc had a protective effect against cadmium cytotoxicity. When HeLa cells were concomitantly exposed to different concentrations of $CdCl_2$ and $ZnCl_2$, a partial antagonism to adverse cadmium effects was noted already at $ZnCl_2$ concentrations corresponding to 0.25 and 0.5 of the molar $CdCl_2$ concentration applied. The antagonistic efficiency was at its peak with molar ratios of 5:1 and 10:1 (Table 4) (54). The antagonism can be associated either with metallothionein production, which may be accelerated in Zn-treated cells, providing thus more sites for binding cadmium, or with direct competition of the two metals for Zn-containing enzymes. We tested the first hypothesis and found that zinc pre-treatment of L-A cells indeed had a protective effect, but it was inferior to cadmium pre-treatment. This indicates that intracellular events unrelated to metallothionein synthesis may be more relevant. Experiments with radiolabelled cadmium show that $ZnCl_2$ inhibits cadmium uptake in a concentration-related manner (Table 5), which may account for the observed antagonism (2, 55).

Table 4 *Influence of $ZnCl_2$ on the proliferation inhibiting and lethal effects of $CdCl_2$. HeLa cells were inoculated into 25 cm^2 Falcon flasks at a density of 0.5 million per flask and 24 h later simultaneously exposed to 0, 1 and $2x10^{-5}$ M $CdCl_2$. and varying $ZnCl_2$ concentrations. Cell numbers and viability were determined 48 h later. Each pair of horizontal rows depicts an independent experiment (54).*

	No $CdCl_2$		10^{-5} M $CdCl_2$		$2x10^{-5}$ M $CdCl_2$	
$ZnCl_2$	Cell increase (% of respective controls)	Viability (%)	Cell increase (% of respective controls)	Viability (%)	Cell increase (% of respective controls)	Viability (%)
0	100	95.3	80.7	88.9	45.0	79.5
$5x10^{-6}$ M	100	92.8	91.2	90.7	69.1	86.2
0	100	96.5	84.2	89.8	28.8	74.0
10^{-5} M	100	95.7	82.3	92.8	63.5	85.1
0	100	95.4	81.5	91.4	60.6	77.0
$5x10^{-5}$ M	100	94.4	105.9	93.7	90.2	92.1
0	100	93.3	64.7	83.3	-11.2	52.6
10^{-4} M	100	93.1	102.9	91.0	86.3	88.6

The reported effects were not only seen in one cell line, but could be confirmed in all the cells employed (7, 8). A drop in cellular cadmium influx and toxicity was also detected in cultured Chinese hamster cells and erythrocytes in the presence of zinc (56, 57). The interaction betweeen zinc and cadmium is well-documented (58).

Similar effects were seen in combined exposure of cells to $CdCl_2$ and $MnCl_2$ (Table 6) (2, 54, 59). The observed marked decrease in cadmium uptake in the presence of $MnCl_2$, suggests that the protective effect predominantly occurs trough inhibition of cadmium incorporation (Table 5) (55). Literature is scarce about the interaction between cadmium and manganese. In experimental animals, pre-treatment or simultaneous exposure to manganese mitigated the toxicity of cadmium (60, 61). Manganese chloride lessened the toxic effects of $CdCl_2$ in mouse pre-implantation zygotes *in vitro* (62).

Table 5 *The effect of $ZnCl_2$ and $MnCl_2$ on the incorporation of cadmium. Cultures of HeLa and $B_{14}F_{28}$ cells were concomitantly exposed to 1 µM $CdCl_2$ labelled with ^{115m}Cd and varying concentrations of $ZnCl_2$ and $MnCl_2$. The exposure was carried out in serum-free MEM stabilized with HEPES buffer and lasted for 2-3 h. The values are expressed as percent of cpm of the respective controls exposed only to $^{115m}CdCl_2$ (55).*

Metal	Cells	Metal concentrations added				
		2 µM	5 µM	10 µM	20 µM	50 µM
$ZnCl_2$	HeLa	74.7	48.8	34.9	36.5	13.2
	$B_{14}F_{28}$	n.d	74.2	69.3	66.9	n.d.
$MnCl_2$	HeLa	25.6	15.0	12.7	n.d.	n.d.
	$B_{14}F_{28}$	50.8	30.1	n.d.	n.d.	n.d.

n.d.= not determined

Table 6 *Influence of $MnCl_2$ on the proliferation inhibiting and lethal effects of $CdCl_2$. HeLa cells were inoculated as described in Table 4 and 24 h later simultaneously exposed to 0,10 and 20 µM $CdCl_2$ and varying $MnCl_2$ concentrations. Cell numbers and viability were determined 48 h later. Each 2-3 horizontal rows represents an independent experiment (54)*

$MnCl_2$	no $CdCl_2$		10 µM $CdCl_2$		20 µM $CdCl_2$	
	Cell increase (% of respective controls)	Viability (%)	Cell increase (% of respective controls)	Viability (%)	Cell increase (% of respective controls)	Viability (%)
0	100	93.8	35.7	73.3	-5.2	34.9
3 µM	100	93.6	33.1	73.4	3.4	39.4
0	100	93.5	67.4	82.8	-3.0	54.8
7.5 µM	100	91.7	83.1	84.0	12.5	64.0
0	100	92.8	40.6	87.1	27.1	79.9
20 µM	100	92.4	91.4	93.2	73.0	89.5
0	100	96.2	64.6	90.3	-5.9	67.3
30 µM	100	94.8	104.3	95.3	75.7	87.9
0	100	93.0	72.5	88.4	40.5	71.9
40 µM	100	93.9	90.6	90.3	66.7	86.2
0	100	94.3	6.2	72.9	-14.6	56.8
60 µM	100	95.1	95.8	95.4	38.9	82.2
80 µM	100	94.5	98.7	93.2	51.5	85.8
0	100	95.2	37.4	73.6	-19.9	52.0
120 µM	100	94.6	117.0	91.2	97.7	91.5

Rats receiving Cd^{2+} and Mn^{2+} in drinking water had lower cadmium levels in blood, urine, liver, and brain cortex than those receiving only Cd^{2+} (63). Conversely, it was shown that cadmium inhibited intestinal manganese uptake (64). A recent study has shown that cadmium incorporation into mammalian cells is partly mediated by a manganese transport system (65).

Further combination experiments on V79 cells included manganese and nickel. Table 7 shows the proliferation rates of cells treated separately or in combination. Concentrations of 2.5-25 µM $MnCl_2$ showed a dose-related effect on proliferation, whereas 50 and 100 µM killed the cells immediately. $NiCl_2$ retarded proliferation at 100 µM, while 250 µM was lethal. The addition of 0.625-100 µM $NiCl_2$ alleviated the effects of manganese when it was given within the concentration range that inhibits the cell growth. The two metals did not interact at the highest, acutely toxic $NiCl_2$ concentrations (7). The protective mechanism of nickel against adverse manganese effects remains unclear. A similar interaction has previously been observed in rat tracheal rings (66).

Table 7 *Proliferation of V79 cells in media containing varied concentrations of $NiCl_2$ and $MnCl_2$. Cells were seeded into microtiter wells (LINBRO ISF 8.96, ca. 5000 cells per well) and metal solutions added after cell attachment. The cultures were microscopically checked after 48 h. The interaction domain is enclosed in the frame (7)*

$MnCl_2$ (µM/L)	**→ 0**	**0.25**	**0.625**	**1.25**	**2.5**	**6.25**	**12.5**	**25**	**50**	**100**
↓ $NiCl_2$ (µM/L)										
0	3	3	3	3	2	2	1	0	0	0
0.25	3	3	3	3	2	2	1	0	0	0
0.625	3	3	3	3	2	2	2	1	0	0
1.25	3	3	3	3	2	2	2	1	0	0
2.5	3	3	3	3	2	2	2	2	0	0
6.25	3	3	3	3	2	2	2	2	0	0
12.5	3	3	3	3	3	3	3	3	0	0
25	3	3	3	3	3	3	3	3	0	0
50	3	3	3	3	3	3	3	3	0	0
100	2	2	2	2	2	2	2	2	0	0
250	00	0	0	0	0	0	0	0	0	0

0 = no cell division, cell death
1 = slow proliferation arrested after one day
2 = proliferation during 48 h, but slower than controls
3 = proliferation at approximately the same rate as controls

Recent experiments investigated the combined effects of chelating agents and vitamins on the uptake and release of lead and cadmium in cultured mammalian cells and their effects on metal cytotoxicity (8, 67-69). The results of these studies correspond well to the findings obtained in experimental animals (70-73) and demonstrate that this test system is suitable for screening such interactions.

CONCLUSIONS

Cultured cells provide a good model for the study of cellular effects of toxic agents. They make it possible to observe the uptake and the distribution of heavy metals, to identify different cellular targets and metabolic processes, and to investigate cell-cycle effects and interactions with other metals or agents (radiation, detoxifying substances). Depending on the cells used and toxic endpoints studied, even very specific metal effects at the cellular level can be elucidated.

Acknowledgment

The authors wish to thank Dr Maja Blanuša, Dr Martina Piasek and Professor Krista Kostial for their help and valuable advice in preparing the manuscript.

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Sažetak

PROCJENA OTROVNIH UČINAKA TEŠKIH METALA NA STANICE SISAVCA *IN VITRO* - PREGLED REZULTATA SURADNIH ISTRAŽIVANJA

Dan je pregled istraživanja *in vitro* na staničnim kulturama sisavca koja su bila provedena pojedinačno ili u suradnji laboratorija koautorica članka, Laboratorija za celularnu biologiju Instituta za medicinska istraživanja i medicinu rada u Zagrebu i suradnog laboratorija u Institutu za higijenu i zdravstvenu ekologiju Sveučilišta u Giessenu. Namjera rada bila je dati primjere kakvi se rezultati mogu dobiti primijenjenim tehnikama i metodama na staničnim kulturama. Iznijeti su samo najvažniji rezultati na području istraživanja olova, kadmija, mangana i nikla koji su dobiveni ili suradnim istraživanjem ili komplementarnim istraživanjima u oba laboratorija. Ovim su primjerima istaknuti i dobri znanstveni i osobni odnosi koje je polučila znanstvena suradnja dvaju laboratorija. U staničnim kulturama praćeno je unošenje i razdioba metala u stanicama te njihovi citotoksični učinci koji su procjenjivani na temelju različitih pokazatelja stanične proliferacije i vijabilnosti. Učinci kratkotrajne izloženosti olovu na sintezu DNK bili su reverzibilni, što pokazuje da olovo ne utječe značajno na gensku staničnu funkciju. Nasuprot tomu, učinci nikla na DNK bili su trajni, što upućuje na to da je to jedno od glavnih ciljnih djelovanja ovog metala. Ispitivanja djelovanja na stanični ciklus pokazala su da otrovni metali imaju učinke na sve stanične faze, ali da su najosjetljivije faze u kojima se priprema i počinje sinteza DNK. Pri kroničnoj izloženosti olovu ili kadmiju pojavljuje se tolerancija zbog procesa prilagodbe. Pri združenim izloženostima rendgenskim zrakama, olovo ima uvijek aditivni učinak, dok je djelovanje mangana sinergističko i čini se da inhibira procese popravka DNK. U kombiniranim pokusima cink i mangan pokazuju zaštitno djelovanje na učinke kadmija, a slično antagonističko djelovanje nikla opaža se pri citotoksičnim učincima mangana.
Prikazani primjeri pokazuju da se upotrijebljeni pokusni model može primijeniti za usporedbe učinaka otrovnih metala na staničnoj razini, kao i za utvrđivanje ciljnih staničnih dijelova i metaboličkih procesa.

Ključne riječi:
citotoksičnost metala, kadmij, mangan, nikal, olovo, stanična kultura

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