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Effects of Pb^{2+}, Ni^{2+}, Hg^{2+} and Se^{4+} on cultured cells. Analysis of uptake, toxicity and influence on radiosensitivity

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Effects of Pb^{2+}, Ni^{2+}, Hg^{2+} and Se^{4+} on cultured human glioma U-343MG cells were investigated considering uptake, toxicity and, in combination with radiation, clonogenic cell survival. The cells were exposed to 0–100 μM of the metals for a week before the evaluation. The tests showed a tendency to toxicity with 10 μM nickel although not significant ($P > 0.05$). Selenium, lead and mercury exerted a significant toxicity ($P < 0.05$) at 2.5 μM, 10 μM and 1 μM, respectively. To challenge the clonogenic cell survival capacity, the cells were irradiated with ^{60}Co photons after being exposed to the highest nontoxic concentration of the different metals. The clonogenic cell survival tests, after irradiation, showed no significant change if the cells were exposed to 5 μM nickel, 0.5 μM selenium or 5 μM lead compared with those not exposed. Mercury, 0.1 μM, gave a relative reduction in survival compared with only irradiated cells of 58 ± 17%. Thus, only mercury affected the radiation-induced damage and/or repair. When exposed to the highest nontoxic concentrations of the different metals, the cultures did not display a significant uptake ratio (metal concentration ratio of exposed cells to control cells) of nickel (3.1 ± 3.3), only a small uptake ratio of selenium (4.0 ± 0.4), while there was a large uptake ratio of both lead $(2.6 \pm 1.7) \times 10^2$ and mercury $(1.5 \pm 0.2) \times 10^1$. The results indicated that nickel was neither especially toxic nor influenced the clonogenic cell survival after irradiation. Mercury was more toxic and also influenced the radiation sensitivity. Lead was taken up strongly but did not influence the radiation sensitivity. Selenium accumulated but gave no detectable effect on the radiation sensitivity.

Keywords: lead, mercury, metals, nickel, radiation effects, selenium

Introduction

Metals might interfere with cellular metabolism and give toxic effects such as growth disturbances and deficient DNA repair. In this study we investigated to what degree lead, nickel, mercury or selenium influenced the growth of cultured human glioma cells and if such effects were correlated with high uptake of the metals. The clonogenic cell survival was challenged by the exposure to radiation. It is well known that clonogenic cell survival after radiation is strongly dependent on the capacity to repair DNA (Tubiana *et al.* 1990a). The human glioma cells U-343MG were chosen as a biological model because these cells have recently been characterized in some detail regarding their radiation sensitivity and capacity to repair DNA damage and they are also easy to handle in toxicity tests (Lindström *et al.* 1994, Stenerlöw *et al.* 1994).

There is a general agreement that metals may react directly with DNA. The large number of nucleophilic centres in nucleic acids implies a high affinity for metals. The most serious interaction between metals and DNA is, probably, crosslinks between the DNA strands as was noticed after exposure to Cu^{2+}, Zn^{2+}, Co^{2+} and Mn^{2+} (Eichhorn 1979).

Ions such as Ni^{2+} and Cd^{2+} can react with the phosphate groups as well as with the bases of DNA (Jacobson & Turner 1980). Nickel has been reported to induce a variety of DNA lesions. These include

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both DNA strand damage, as well as DNA protein crosslinks (Ciccarelli *et al*. 1981, Ciccarelli & Wetterhahn 1982). Nickel has been shown to inhibit DNA synthesis following either *in vitro* or *in vivo* exposure (Basrur & Gilman 1967, Costa *et al*. 1982, Kasprzak *et al*. 1987).

Different results have been shown as to whether or not mercury affects the activity of glutathione peroxidase, an enzyme necessary for the elimination of lipid hydroperoxides and hydrogen peroxide (Yonaha *et al*. 1980, Barregard *et al*. 1990). Mercury also strongly binds to SH-groups (Vallee & Ulmer 1972) with possible inhibition of repair enzymes or depletion of free SH-groups that could protect cellular compounds from attack by radiation-induced radicals.

The presence of selenium increases the activity of glutathione peroxidase and enhances the destruction of peroxide (Sandström *et al*. 1987, 1989). Selenium confers protection in part by inducing or activating cellular free-radical scavenging systems and also by enhancing peroxide breakdown (Borek *et al*. 1985).

Lead has been reported to be capable of forming complexes with the phosphate groups of nucleotides and nucleic acids, and of catalyzing the non-enzymatic hydrolysis of nucleoside triphosphates (Martell 1981).

Clonogenic cell survival (*vide infra*) can be used to assess how cells cultured in metal supplemented medium react on irradiation, thereby indicating a direct interaction between metals and DNA damage and repair. The objective of the present study was to examine how the elements Pb^{2+}, Ni^{2+}, Hg^{2+} and Se^{4+} affect the radiation sensitivity, i.e. if they act as protective agents or sensitizers. Selenium was chosen as a possible protective agent because recent studies showed selenium protection against mercury and cadmium toxicity (Lindh *et al*. 1996). Lead, mercury and nickel were chosen because they might replace zinc in zinc finger proteins and thus cause genotoxicity (Barber & Sunderman 1988). The strategy was to expose the cells to the highest nontoxic concentration of the metals and then analyze possible modifying effects on the radiosensitivity.

Materials and methods

Cell culture

Human glioma cells U-343MGaC12:6 (Nistér *et al*. 1987), called U-343MG in this study, were grown and maintained in complete growth medium (Ham's F-10 medium supplemented with 10% fetal calf serum, 2 mmol l^{-1} L-glutamine, 20 μg ml^{-1} streptomycin, 20 U ml^{-1} penicillin and 2.5 μg ml^{-1} amphotericin B, all from Biological Industries, Israel). The last three days prior to some of the experiments the cells were grown in serum-free medium (*vide infra*). The serum-free medium was prepared as the complete medium but without 10% fetal calf serum. The U-343MG cells were grown as monolayers in 25 cm^2 plastic flasks (Bibby, UK) containing 10 ml of medium at 37°C (humified air containing 5% CO_2). The U-343MG cells were seeded at a density of $(2–3) \times 10^6$ cells per flask and their doubling time was about 36 h.

Trypsinization and counting of cells

The cells were trypsinized using a solution containing 0.25% trypsin and 0.02% EDTA (Biological Industries, Israel). First, the medium in each flask was removed and the cells were washed briefly in 0.5 ml trypsin solution, which was then removed. Thereafter, another 0.5 ml trypsin solution was added and the cells were incubated at 37°C for 10–15 min. The trypsinization period was interrupted by adding 10 ml complete growth medium. The cells were well mixed to obtain a cell suspension without any cell aggregates. Half a ml of the suspension was used for counting in a cell counter (Model ZM, Coulter Counter, UK).

Metal solutions

The following compounds were used for preparation of solutions: $NiCl_2 \cdot 6H_2O$, $PbCl_2$, $HgCl_2$ and $Na_2SeO_3 \cdot 5H_2O$. All chemicals were obtained from Merck, Germany. Each metal was dissolved and diluted with distilled water to an original stock solution with a concentration at least 100-fold the concentration added to the cells. The original stock solutions were, before used, sterile filtered with a 0.2 μm filter (Gelman Sciences, USA). The final concentration was obtained by dilution in culture medium.

Schedule of exposure to metals

The following schedule of exposure was used to see how the cells were affected when exposed during at least two doubling times ($t_D = 36$ h for U-343 cells). On day zero, the cells were plated at a density of $(2–3) \times 10^6$ cells per 25 cm^2 flask. The metal was then added to the cell cultures. On the second day, the medium was changed for 10 ml of fresh medium containing the metal. On the fourth day, the medium was changed again. This time the metal was diluted with serum-free medium. The cells were then allowed to grow until the seventh day, to trap the cells in a non-proliferative state (G_1 or G_0 phase), before irradiation and the clonogenic cell survival test. The control cells were also growing according to this schedule but without any metal addition to the medium.

Clonogenic cell survival and ^{60}Co irradiations

Plating efficiency (PE) is defined as the relative number of cells having the capacity to form colonies containing at

least 50 cells. The clonogenic cell survival (S_c) after irradiation is defined as the ratio between the plating efficiency for the given radiation dose (PE_D) and the plating efficiency for the control (PE_0) and thus can be expressed as $S_c = PE_D/PE_0$ (Tubiana *et al.* 1990b).

Cells are plated in conventional 8.5 cm culture dishes (Bibby, UK) and complete medium is added. Thereafter, the cultures are incubated at 37°C during fourteen days, with a change of the medium after seven days, before fixation in formalin and staining with haematoxylin. Colonies with more than 50 cells are counted with a 10× enlargement lens. Higher radiation doses tend to lower the number of colonies. This fact is compensated by plating more cells. For experiments within the low-dose range, 200–2000 cells were plated, whereas for the high-dose range 10 000–20 000 cells were plated (Tubiana *et al.* 1990b). This plating procedure has also been used when exposing the cells to different amounts of a metal.

The plastic flasks, containing the monolayers of cells, were placed in a ^{60}Co irradiation apparatus (Scanditronix AB, Sweden). The irradiation procedure was similar to that described previously (Stenerlöw *et al.* 1994). The flasks were irradiated at 20°C with doses in the interval 0–6 Gy. These doses provide a relevant clonogenic cell survival for this study. Thereafter, the cells were transferred to the 37°C incubator. The photon dose rate was 0.93 Gy min^{-1}.

Six hours after irradiation, the cells were trypsinized, resuspended, counted (only 0.5 ml) and plated for analysis of clonogenic cell survival. The plating efficiency of cultures from these U-343MG cells was about 0.4.

Toxicity test

For each metal a toxicity test was made to determine at what concentration the cell growth was disturbed. The cells were grown, according to the schedule described above, in three or four flasks for each concentration. Thereafter, the cells were cloned without irradiation into five dishes for each concentration. After fourteen days the cultures were fixed, stained and counted. The following concentrations of metals were used:

Ni and Pb at 1, 5, 10, 25, 50, 100 μM.
Hg at 0.01, 0.1, 1 and 10 μM.
Se at 0.05, 0.5, 2.5 and 10 μM.

Metal exposure and radiosensitivity

The U-343MG cells were grown with the highest nontoxic metal concentrations (5 μM Ni, 5 μM Pb, 0.1 μM Hg and 0.5 μM Se) for seven days as described earlier. The cells were then irradiated with the ^{60}Co source at doses of 0, 2, 4 and 6 Gy, and thereafter cloned.

Binding test

This test was made to assess the uptake of Ni, Pb, Hg or Se in the cells. The cells were exposed to the concentrations 5 μM Ni, 5 μM Pb, 0.1 μM Hg and 0.5 μM Se according to our specific schedule. After one week the medium was removed from the flasks and the cells were washed six times with serum-free medium.

Two ml trypsin were added to the cells exposed to mercury and the solution was then incubated at 37°C for 10–15 min. One ml of the solution was taken for ICP–MS analysis (Houk 1994). To obtain a measure of the uptake, the results were normalized by comparison with one ml trypsinized unexposed cells (control).

Due to circumstances beyond our control, the mass spectrometer was not available for the analysis of nickel, lead and selenium. The following protocol was therefore adopted for these elements. Four ml trypsin were added to the exposed cells. The solution was incubated at 37°C for 10–15 min. Five ml of complete medium were added to the cell dish. The solution was suspended and transferred to a 15 ml tube. A further 5 ml medium was added to the dish and transferred to the tube. The tubes were then centrifuged for 5 min at 470 *g*. The supernatant was removed and two pellets, which had been exposed to the same element, were pooled and transferred to one Eppendorf tube which was centrifuged for 2 min at 9.5×10^3 *g*. When the supernatant had been removed, the cell pellet was analyzed for the metal content and normalized with a cell pellet of unexposed cells (control). Nickel was examined by graphite furnace atomic absorption spectrometry, GF–AAS (Dürnberger *et al.* 1987, Lindberg *et al.* 1988). Lead was investigated by inductively coupled plasma atomic emission spectrometry, ICP–AES (Thompson 1987). Selenium was studied by hydride generation atomic absorption spectrometry, HG–AAS (Narasaki 1985, Yamamoto *et al.* 1985).

Results

Toxicity

Figure 1a shows the effect on the U-343MG cells when exposed to different concentrations of nickel and selenium; Figure 1b shows the effect of lead and mercury. The test was performed by clonogenic assay without irradiation. The possible significant difference in plating efficiency (PE) between cells exposed to a metal and cells without exposure (control) was evaluated by Student's two-sample *t*-test. The *t*-test showed that cells exposed to 0.05 μM Se and 1 μM Ni had a significantly higher PE than the control cells. The following concentrations of the trace elements did not give a significant toxic effect for the U-343MG cells and were therefore chosen for the survival test after irradiation: 5 μM Ni, 5 μM Pb, 0.1 μM Hg and 0.5 μM Se.

Survival after irradiation

Figure 2a–d shows the dose-response, measured with clonogenic assay, for the U-343MG cells after

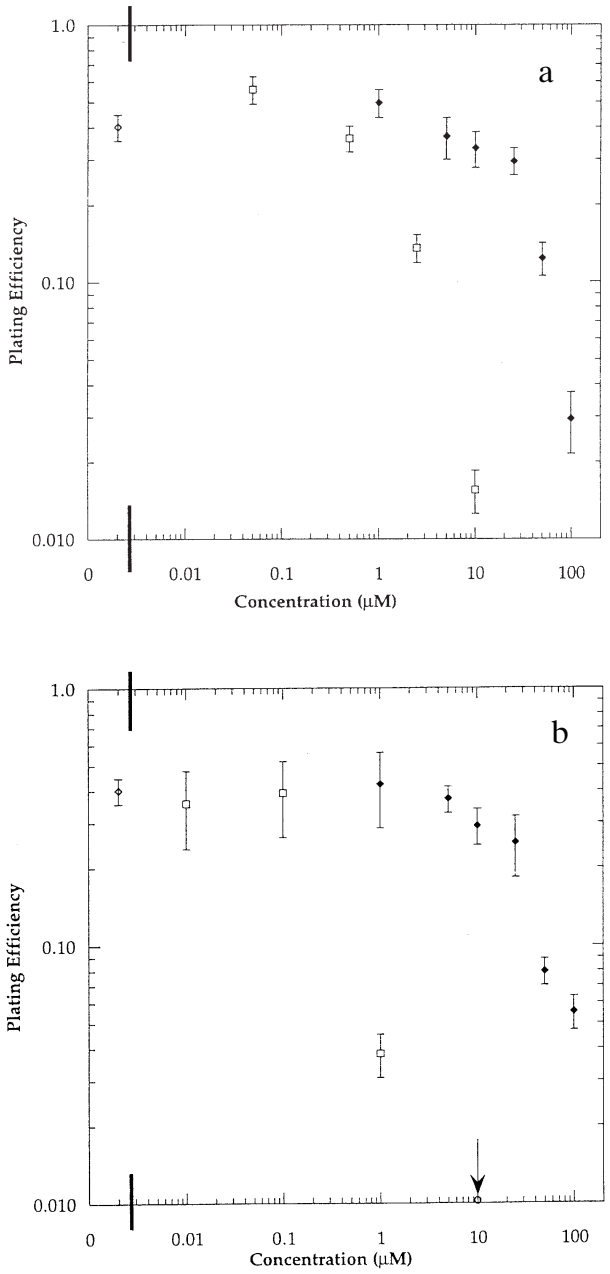

Figure 1. Plating efficiency for the U-343MG cell line grown with different concentrations of nickel, selenium, lead and mercury. Mean ± SD values based on five samples are shown. The control value (◇), cells grown without metals, is shown to the left of the transversal bars in each diagram. (a) Plating efficiency for selenium (□) and nickel (◆); (b) plating efficiency for mercury (□) and lead (◆). The arrow indicates no measured surviving cells.

exposure to different metals and irradiation with ^{60}Co photons. Repeated data sets of each metal were least-square fitted to the linear-quadratic (LQ) and single hit, multi-target (SHMT) models (Stenerlöw *et al.* 1994). The SHMT model is described by $S = 1\text{-}(1 - \exp(-D/D_0))^n$ where D is the dose (Gy) and D_0 is a constant (Gy) giving the slope of the curve in the high-dose region. The extrapolation number n gives the radiosensitivity in the low-dose region. The LQ model is described by $S = \exp - (\alpha D + \beta D^2)$ where α is the slope of the curve near the dose zero and β is the constant describing the shape of the curve at high doses (Tubiana *et al.* 1990b). The survival parameters α, β, D_0 and n are listed in Table 1. Repeated experiments with mercury produced somewhat varying survival levels and only one data set is shown as an example (Figure 2d). The mean relative reduction in mercury-dependent survival for U-343MG cells from all experiments was 58 ± 17% and is presented in Table 2. These tests did not show, by Student's *t*-test, any significant differences between irradiated cells exposed to nickel, selenium or lead and only irradiated cells. Significant effects in survival were seen after exposure to mercury.

Binding

The calculated element uptake values for cells grown with exposure to metals are shown in Table 3. The uptake is calculated as the ratio between the concentration of a specific metal in a sample exposed to this element and an unexposed sample. The measurements are based on four samples for nickel and two samples for the rest of the elements.

Discussion

The toxicity, as estimated by a significantly decreased plating efficiency compared with the control plating efficiency, for the U-343MG cell line revealed that 0.05–0.5 μM selenium was not toxic. However, 2.5 and 10 μM decreased the plating efficiency significantly. A clear toxicity was observed with lead and nickel ($P < 0.05$) beginning at 10 μM and 25 μM, respectively. Mercury was toxic at 1 μM and seemed to be the most toxic of the considered metals.

The survival after irradiation of the U-343MG cells in combination with exposure to selenium, nickel and lead resulted in survival curves that were similar to the control survival curves. They all had shoulders in the low-dose region after irradiation with photons and were very similar to the curve for U-343MG cells obtained after gamma photon irradiation in a previous study on survival and DNA repair (Stenerlöw *et al.* 1994). It has been reported that selenium does not change the radiosensitivity

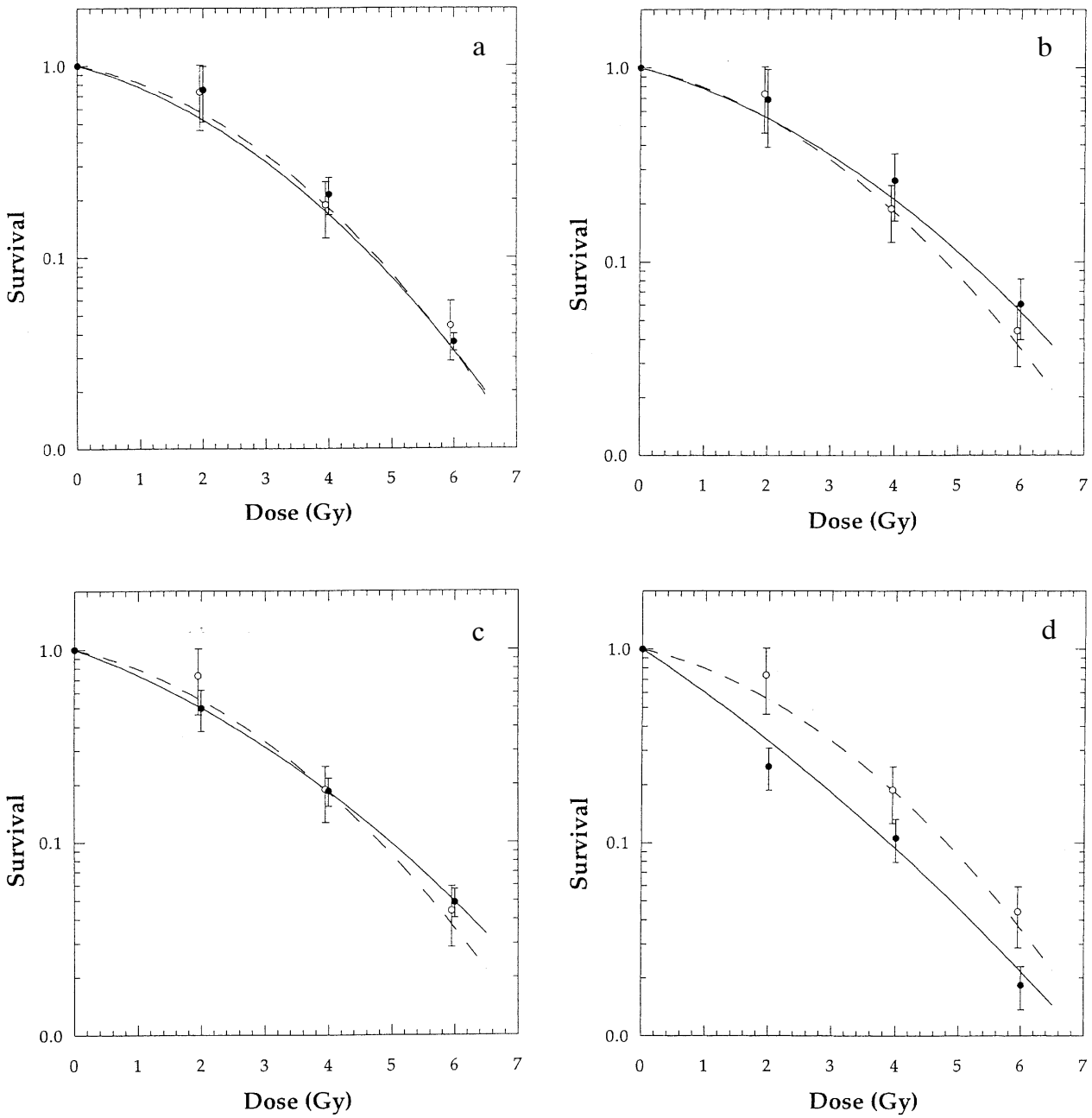

Figure 2. Clonogenic cell survival for the U-343MG cell line with (●) and without (○) exposure to different metals. Each experiment was based on three samples with one replicate. Mean ± SD values from the experiments are shown. The curves were fitted using the least-squares method for the linear-quadratic clonogenic cell survival model. (a–d) exposed to Ni, Se, Pb and Hg respectively (solid lines). The control curve (dashed lines) from (a) was superimposed on the data in (b), (c) and (d).

of cultured cells, although it can increase the activity of glutathione peroxidase (Sandström *et al.* 1987, 1989). It is possible that the level of glutathione peroxidase activity was increased in the present experiments although this was not analyzed. There does not seem to be any simple relation between the relative reduction in mercury-dependent survival as a function of radiation dose (Table 2). The mean relative reduction in mercury-dependent survival was 58 ± 17%.

Table 1. Survival parameters[a] for the U-343MG cell line after irradiation with ^{60}Co photons and exposure to different elements. Each experiment was based on three samples with one replication

	Control	Ni	Pb	Hg[b]	Se
D_0(Gy)	1.29 ± 0.08	1.19 ± 0.10	1.51 ± 0.05	1.53 ± 0.42	1.58 ± 0.11
n	4.00 ± 1.20	4.86 ± 2.00	3.10 ± 0.41	1.15 ± 1.10	2.31 ± 0.58
α (Gy^{-1})	0.173 ± 0.120	0.171 ± 0.120	0.207 ± 0.049	0.500 ± 0.260	0.270 ± 0.053
β (Gy^{-2})	0.0638 ± 0.0190	0.0689 ± 0.0190	0.0461 ± 0.0077	0.0233 ± 0.0440	0.0388 ± 0.0088

[a]Mean and standard deviations.
[b]Survival parameters only for the given example.

Table 2. Relative mercury dependent reduction, RR, in survival for U-343MG cells incubated with Hg compared with control cells. Pooled data from three experiments

Dose (Gy)	RR[a] (%)
1	73.0
2	64.8
3	52.0
4	39.8[b]
5	59.0
6	57.0
Mean[c]	58 ± 17

[a]RR = relative reduction in survival of mercury treated and irradiated cells in relation to only irradiated cells.
[b]No significant difference ($P > 0.05$).
[c]Mean value and maximum deviation.

In Table 3 the binding test shows a small uptake of selenium and no specific uptake of nickel compared with the control cultures. There was a large uptake of both lead and mercury. The cells were washed extensively before the analysis so the high uptake of lead and mercury must either be due to a strong binding that resisted washing, or to trapping in a cell compartment that prevented leakage. It is, from the present test, not possible to discriminate between these two possible explanations.

One explanation for the low uptake of nickel could be that the uptake of nickel is inhibited by complexing agents (EDTA, L-histidine, serum albumin, D-penicillamine) that sequester the metal ion (Nieboer *et al.* 1984); the medium used in this study contains serum albumin and L-histidine. A negative influence on uptake of selenium is not expected because it is suggested that selenium is transported in plasma by selenoprotein P (Motsenbocker & Tappel 1982). The complexing agents might also inhibit the uptake of mercury but the observed high uptake indicates that the interaction is not as strong as for nickel. Studies on lead distribution in human blood have shown that 99% of the lead content is in the red cells and only about 1% is in the plasma (De Silva 1981). Therefore, uptake inhibition from serum albumin and L-histidine should be weak.

A high uptake of lead did not, surprisingly enough, cause a significant change in radiosensitivity and we have, at present, no explanation for this behaviour. Maybe, it is reasonable to assume that the accumulated lead did not associate to DNA, at least not in such a way that it influenced DNA synthesis and/or DNA repair.

High concentrations of nickel or lead were neither severely toxic nor influenced clonogenic cell survival after irradiation. Selenium was toxic at high concentrations but did not influence the radiosensitivity when applied at a nontoxic level. Mercury was toxic at high concentrations and, by contrast with selenium, changed the radiation sensitivity when applied at a nontoxic level. Mercuric chloride has been shown to cause DNA strand breaks similar to those caused by X-rays. The breaks may be caused by generated radicals (Cantoni *et al.* 1982). However,

Table 3. Effect of incubation with different elements on the element uptake in U-343MG cells. The uptake values are the amplification in times between sample and control

	Se	Ni	Pb	Hg
Sample (ppb)	$(4.5 \pm 0.3) \times 10^2$	$(1.4 \pm 1.1) \times 10^3$	$(3.1 \pm 1.3) \times 10^5$	$(4.4 \pm 0.3) \times 10^1$
Control (ppb)	$(1.1 \pm 0.1) \times 10^2$	$(0.5 \pm 0.1) \times 10^3$	$(1.2 \pm 0.3) \times 10^3$	2.9 ± 0.1
Uptake	4.0 ± 0.4[a]	3.1 ± 3.3[b]	$(2.6 \pm 1.7) \times 10^2$[a]	$(1.5 \pm 0.2) \times 10^1$[a]

[a]Quotient calculated from means and maximum deviations from two sample runs and two control runs.
[b]Quotient calculated from means and maximum deviations from four sample runs and four control runs.

strand breaks induced by mercuric chloride are not as easily repaired as the breaks caused by X-rays (Cantoni & Costa 1983). Our suggestion is that mercury has a greater affinity for the repair enzymes than does lead and that this may explain the strong toxic effect from mercury and radiation. This is supported by several studies showing cancerogenesis from lead but not from mercury (IARC 1994, Hartwig 1995).

The findings of the present study warrant further experiments to increase the knowledge about uptake, toxicity and interactions between metals and radiation.

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