TOXIC ACTION OF SALTS OF HEAVY METALS ON INTACT AND IRRADIATED MAMMALIAN CELLS IN CULTURE

Yu. A. Ershov, L. N. Kublik, T. V. Pleteneva, and L. Kh. Eidus

UDC 615.916.015.4

KEY WORDS: cell culture; ionizing radiation; toxic salts of heavy metals

Among factors polluting the environment an essential role is played by salts of heavy metals [3, 4]. There is an abundant literature on their effect on different organisms [1-4, 7]. Maximal allowable concentrations (MAC) of different salts of heavy metals in bodies of water and in atmospheric air have been established [4], and also in the atmosphere of work places [1]. Mechanisms of the toxic action of these factors have received rather less study, and information on their effects on mammalian cells is absolutely inadequate [2, 7]. With the development of methods of culture of animal cells it has now become possible to remedy this defect by the use of cell models. The problem of the combined action of several different salts of heavy metals, and also their combined action with ionizing radiation, has assumed special importance at the present time.

In this investigation the toxicity of salts of two heavy metals (nickel and chromium) was investigated separately and in combination with radiation, using different criteria of damage to cells in culture.

EXPERIMENTAL METHOD

The principal test object consisted of cells of a transplantable culture of Chinese hamster fibroblasts from lung tissue, of the FAF-D-II-11 strain. These cells are a convenient object in toxicologic research because of the ability of the cells to adhere to the glass surface at the bottom of a Carrel flask, with the formation of a cell monolayer, which enables various test preparations to act on the cells. These preparations can be added in appropriate concentrations to the growth medium, with repeated washing, additions, or changes of solutions, if necessary. Cells of a suspension culture of mouse lymphoma Sp-2 constituted the other test object. The Chinese hamster cells were seeded in Carrel flasks in concentration of 80,000-100,000/ml in nutrient medium 1:1 (Eagle's + medium 199) with the addition of penicillin 100 U/ml, streptomycin 50 U/ml, and 10% bovine serum. After incubation for 24 h at 37°C, when a cell monolayer had formed on the bottom of the flask, the cells were exposed either to a solution of metal salts or to irradiation, or to both these factors in succession. The salts $K_2Cr_2O_7$ and $NiSO_4$ were dissolved in nutrient medium for preparing solutions of the corresponding concentration with pH 7.0-7.2. The cells were treated with the solutions for a certain time, which differed in different series of experiments. In experiments to study the combined action of salts and radiation, after treatment of the cells with a solution of the salt for 1 h at room temperature the solution was poured off and the cells rinsed twice with fresh nutrient medium, after which the medium was replaced by new, containing 10% serum. The cells were irradiated in this medium. Flasks with cells were irradiated at room temperature on a ^{60}Co gamma-ray source (GUBE apparatus) in doses of 2 and 4 Gy for 4 and 8 min respectively. In order to select an effective criterion for assessing the toxicity of the heavy metals and their combined action with small doses of radiation, we used three tests: 1) the direct mortality of the cells treated with solutions of a salt (based on staining with a 0.04% solution of Trypan blue); 2) slowing of cell proliferation (by counting the increase in the number of cells with time during the first two days of incubation in the presence of the salt); 3) weakening of the colony-forming ability of the cells dying reproductively after exposure to the salt (based on counting the

I. M. Sechenov Moscow Medical Academy. Institute of Biological Physics, Academy of Sciences of the USSR. (Presented by Academician of the Academy of Medical Sciences of the USSR G. I. Rumyantsev.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 113, No. 3, pp. 280-283, March, 1992. Original article submitted July 25, 1991.

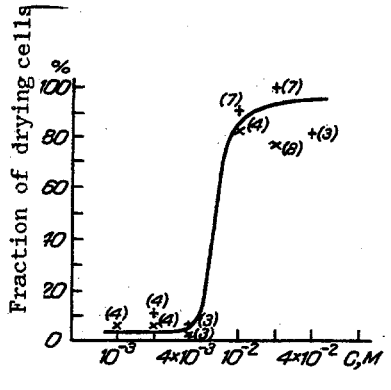


Fig. 1

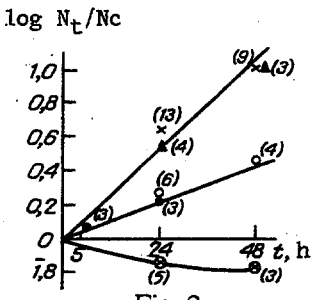


Fig. 2

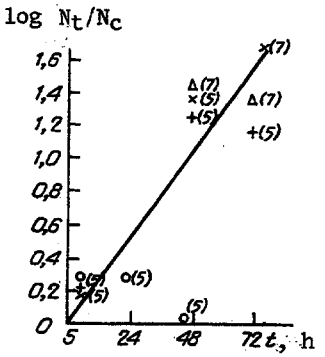
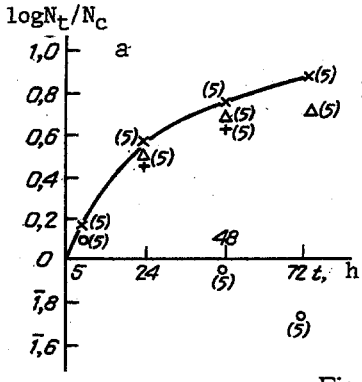


Fig. 3



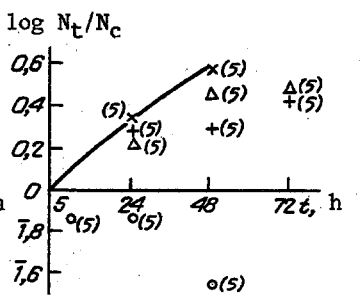


Fig. 4

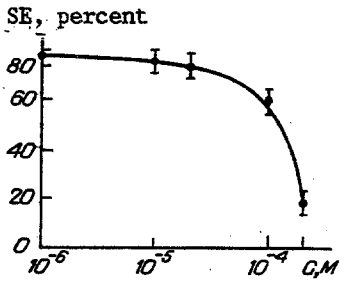


Fig. 5

Fig. 1. Death of Chinese hamster fibroblasts treated with $K_2Cr_2O_7$ solution in different concentrations for 2 h (×) and 4 h (+) (mean of number of experiments in parentheses). Abscissa, concentration (C) of salt (in M); ordinate, fraction of dying cells (in per cent).

Fig. 2. Effect of $K_2Cr_2O_7$ solutions on kinetics of growth of Chinese hamster fibroblast population. Abscissa, duration of incubation (t); ordinate, log of ratio of number of cells (N_t) to initial number (N_0). Multiplication sign – control, △ – 10^{-4} M, empty circle – $2 \cdot 10^{-4}$ M, filled circle – $3 \cdot 10^{-3}$ M, cross inside circle – $6 \cdot 10^{-3}$ M.

Fig. 3. Effect of $NiSO_4$ on kinetics of growth of Chinese hamster fibroblast population. Abscissa, duration of incubation (t), ordinate, log of ratio of number of cells (N_t) to initial number (N_0). Multiplication sign – control, △ – 10^{-3} M, + – $2 \cdot 10^{-3}$ M, empty circle – 10^{-2} M.

Fig. 4. Effect of $NiSO_4$ on kinetics of growth of suspension culture of mouse lymphoma Sp-2, differing in initial cell density: 1) 100,000/ml, 2) 400,000/ml. Abscissa, duration of incubation (t); ordinate, log of ratio of number of cells (N_t) to initial number (N_0). Multiplication sign control, △ – 10^{-3} M, + – $2 \cdot 10^{-3}$ M, empty circle – 10^{-3} M.

Fig. 5. Effect of $K_2Cr_2O_7$ on seeding efficiency (SE) of Chinese hamster fibroblasts. Abscissa, concentration (C) of salt (in M); ordinate, SE – ratio of number of growing colonies to number of cells seeded.

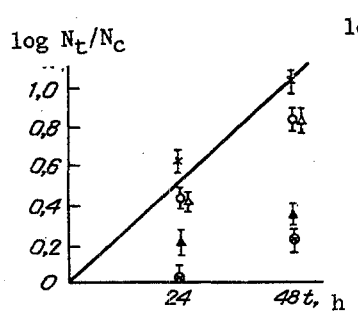


Fig. 6

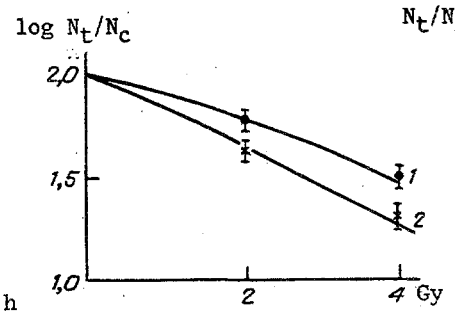


Fig. 7

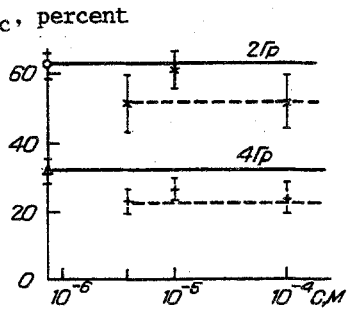


Fig. 8

Fig. 6. Effect of combined action of radiation and toxic action of $K_2Cr_2O_7$ in a concentration of $2 \cdot 10^{-4}$ M (Fig. 1) on kinetics of growth of Chinese hamster fibroblast population. Abscissa, duration of incubation (t); ordinate, log of ratio of number of cells (N_t) to initial number (N_0). Multiplication sign – control, △ – irradiation 2 Gy, empty circle – irradiation 4 Gy, combined effect of salt and irradiation: 2 Gy (△), 4 Gy – cross inside circle.

Fig. 7. Dose curves of reproductive death of Chinese hamster fibroblasts. 1) Irradiation of intact cells, 2) irradiation of cells treated beforehand with $K_2Cr_2O_7$ solution. Abscissa, dose of irradiation; ordinate, log of fraction (in per cent) of irradiated cells, forming colonies (N_t) as a ratio of number of colonies from intact cells (N_c).

Fig. 8. Combined action of radiation and various concentrations of $K_2Cr_2O_7$. Abscissa, concentration (C) of salt (in M); ordinate, fraction (in per cent) of irradiated cells forming colonies (N_t) as a ratio of number of colonies from intact cells (N_c); ○, △ – continuous horizontal line – after irradiation in doses of 2 and 4 Gy respectively, without treatment with salt solutions; × and + – during combined fraction.

number of macrocolonies after 7 days' growth of the culture). The threshold of toxicity was established by changing the concentration of the salt in solution. In the last case, to assess the toxic effect we determined the seeding efficiency of the control of untreated cells and cells exposed to the action of solutions containing different concentrations of the salt. The ratio between them gave a measure of the toxic effect. The results of the combined action of salt and radiation were judged from the change in the effect of irradiation within the range of nontoxic concentrations of the salts. The results of each series were averaged over all the repeated experiments, the number of which is indicated on the figures.

EXPERIMENTAL RESULTS

In experiments to determine the toxicity of the $K_2Cr_2O_7$ salt for Chinese hamster fibroblasts, using death of the cells immediately after exposure, treatment with solutions of the salt was given for 2 or 4 h at 37°C. Fractions of "living" and "dead" cells, in the presence of different concentrations of the agent, were determined in at least three wells of plastic cultural planchets (from "Filtertek"). The total number of cells analyzed was 250-300 in each well. Death of the cells was expressed as the ratio of the number of "dead" cells to the total number of cells counted, in per cent. The results are given in Fig. 1 and they show that a solution of potassium dichromate is absolutely toxic in a concentration of 10^{-2} M, but dependence on concentration is very strong. A fall of only 2.5 times in the concentration ($4 \cdot 10^{-3}$ M) virtually abolishes toxicity when determined by this test.

The results of experiments to determine the toxicity of the salts $K_2Cr_2O_7$ and $NiSO_4$, using as criterion delay of cell proliferation, are given in Fig. 2a. Figure 4 illustrates the effect of adding solutions of the salt $K_2Cr_2O_7$ to the incubation medium of the cells on the kinetics of growth of a Chinese hamster fibroblast culture at 37°C (without rinsing the cells to remove the salt). Clearly a concentration of this salt of 10^{-4} M has virtually no effect on the increase in the number of cells, which changes as a logarithmic function of time. With an increase in concentration there is progressive inhibition of growth of the culture. A concentration of $6 \cdot 10^{-3}$ M is highly toxic and not only prevents growth of the culture, but also causes death of some of the cells seeded initially. Toxicity is exhibited within roughly the same concentration range

(10^{-3}-10^{-2} M) according to this criterion of inhibition of growth of the culture for Chinese hamster cells and for the nickel salt (Fig. 3).

Nickel sulfate was used to determine toxicity in relation to inhibition to proliferation of cells cultured in suspension (lymphoid cells). The results of two repeated experiments, differing in the initial density of lymphoid cells in suspension, namely 10^5 (1) and $4 \cdot 10^5$ (2) cells/ml, are given in Fig. 4. In the control, the increase in the number of cells also was a logarithmic function of time, with gradual transition to the steady state taking place the more rapidly, the higher the initial density of the suspension. This rule is evidence of the quality of the particular culture.

It will be clear from Fig. 4 that inhibition of growth begins to be observed with a concentration of about 10^{-3} M. With a concentration of 10^{-2} M marked toxicity is observed. Thus the limits of toxicity (10^{-3}-10^{-2} M) were identical for the two test cultures.

It can be concluded from a comparison of these limits with toxicity using cell death as the criterion (Fig. 1) that with an increase in concentration of the tested salts above 10^{-3} M, proliferation is at first inhibited, but later, with concentrations of $(4\text{-}5) \cdot 10^{-3}$ M, death of the cells begins. With a concentration of about 10^{-2} M all the cells die within a few hours.

It is demonstrative that inhibition of proliferation was observed within the same range of concentration of the salt for completely different mammalian cells.

In experiments to determine toxicity of $K_2Cr_2O_7$ reproductive cell death as the criterion, Chinese hamster fibroblasts were treated with solutions with different concentrations of the salt for 1 h, after which the cells were washed twice and seeded in Carrel's flasks. Seeding efficiency of the cell as a function of salt concentration is shown in Fig. 5. Clearly the toxicity of the salt according to this criterion of injury becomes manifested in concentrations of about 10^{-4} M, and with a further increase in concentration it rises very sharply. The lower threshold of toxicity compared with the two previous criteria of injury can naturally be explained by the integral nature of this parameter. This is connected with the effect both of inhibition of growth and of direct death of the cells, treated with the toxic agent, on this parameter. Meanwhile, for populations of dividing cells, the efficiency of proliferation is the most important indicator of their function.

The combined action of $K_2Cr_2O_7$ and radiation on Chinese hamster fibroblasts was assessed in relation to two criteria of injury: inhibition of proliferation and reproductive cell death. The effect of irradiation itself in doses of 2 and 4 Gy on the kinetics of cell proliferation is illustrated in Fig. 6b. Clearly in these doses the kinetics of proliferation did not undergo any marked changes. Data for the combined action of the salt in a concentration of $2 \cdot 10^{-4}$ M and irradiation in these doses are given in the same figure. Comparison with Fig. 2b shows that irradiation in dose of 2 Gy did not appreciably enhance the toxicity of the salt, but in a dose of 4 Gy, only a certain tendency was observed for its toxicity to increase with respect to this criterion of inhibition of proliferation.

Thus the increase in the total number of cells is not a sensitive test for assessing the combined action of radiation in small doses and potassium dichromate, although the threshold of toxicity with respect to this criterion is sufficiently clearly established.

The combined action of $K_2Cr_2O_7$, using reproductive cell death as the criterion, was studied with two doses of irradiation (2 and 4 Gy). The corresponding dose curve is given in Fig. 7. Surviving cells, forming normal macrocolonies after irradiation in these doses, comprised 62 and 33% of the number of adherent cells.

The effect of combined action of the salt and irradiation, using this criterion of cell viability, is illustrated in Fig. 8. Clearly salt concentrations not affecting the seeding efficiency of intact cells (Fig. 5) potentiate the action of radiation in both doses used.

The experiments thus showed that irradiated cells are more sensitive to the toxic action of this chromium salt than intact cells.

LITERATURE CITED

1. Harmful Chemicals [in Russian], Leningrad (1989), p. 592.
2. Yu. A. Ershov and T. V. Pleteneva, Mechanisms of the Toxic Action of Inorganic Compounds [in Russian], Moscow (1989), p. 272.
3. A. Kabata-Pendias and H. Pendias, Trace Elements in Soils and Plants [Russian translation], Moscow (1989).
4. J. W. Moore and S. Ramamurty, Heavy Metals in Natural Waters [Russian translation], Moscow (1987), p. 280.

5. Zh. Safiyazov and L. Kh. Eidus, Radiobiologiya, 5, 32 (1965).
6. L. Kh. Éidus, Biofizika, 1, 544 (1956).
7. B. Venugopal and T. D. Luckey, Metal Toxicity in Mammals, Vol. 2, New York (1978), p. 409.

DISTURBANCE OF MEDULLARY HEMATOPOIESIS IN THE LATE STAGES AFTER EXPOSURE TO CYTOSTATICS

I. V. Bogdashin, E. V. Sycheva, V. V. Zhdanov, O. N. Afanas'eva, O. P. Zapuskalova, V. V. Novitskii, and E. D. Gol'dberg

UDC 615.277.3.015.4:612.119].07

KEY WORDS: cytostatic drugs; stem cells; hematopoiesis-inducing microenvironment; bone marrow; late effects

Clinical observations and experimental studies in recent years have fully confirmed the correctness of our theoretical ideas regarding the true nature of late effects of the damaging action of antitumor preparations on healthy (not affected by a tumor) organs and tissues [4]. Under these circumstances disturbances in the nervous, endocrine, and cardiovascular systems are comparatively easily diagnosed, whereas changes in the blood system may be latent in character and may be discovered only as a result of penetrating investigations or after additional hematopoiesis-inducing procedures (blood loss, stress, inflammation, etc.) [1, 2, 7]. It is considered that depression of the reserve capacity for granulocytopoiesis and erythropoiesis after treatment with cytostatics is linked with exhaustion of the pool of hematopoietic stem cells with different degrees of maturity [8]. It has also been suggested that disturbances of hematopoiesis discovered in the late stages after treatment with cytostatic drugs may be linked with changes in the hematopoiesis-inducing microenvironment [9], which plays an important role in the regulation of proliferation and differentiation of hematopoietic stem cells (HSC) [3].

The aim of this investigation was to study the state of bone marrow hematopoiesis and functional activity of adherent bone marrow cells in mice in the late period after administration of cytostatic drugs widely used in clinical practice: doxorubicin, vinblastine, and cyclophosphamide.

EXPERIMENTAL METHOD

Experiments were carried out on CBA mice aged 8-12 weeks (from the "Rassvet" nursery, Tomsk). Standard preparations of doxorubicin (Pharmorubicin, India), vinblastine (Hungary), and cyclophosphamide (USSR) were dissolved immediately before use in isotonic solution and given as a single intraperitoneal injection in the maximally tolerated dose (MTD), based on the results of graphic probit analysis, of 6 mg/kg for doxorubicin, 2.2 mg/kg for vinblastine, and 250 mg/kg for cyclophosphamide. The mice were killed by cervical dislocation 6 months after administration of the cytostatics. The total number of karyocytes (TNK) and the myelogram were counted (on bone marrow films stained by the method of Nocht and Maximow). The number of committed precursor cells of the granulocytic-monocytic series in the hematopoietic tissue was determined by cloning bone-marrow nuclears in methylcellulose in vitro by the method in [6]. A suspension of bone marrow cells in a concentration of $2 \cdot 10^7$ cells/ml was incubated in medium RPMI-1640 with 5% fetal calf serum for 30 min on plastic Petri dishes to separate the adhesive fraction of myelokaryocytes. Nonadherent cells were then collected, their viability estimated, and added in a concentration of $3 \cdot 10^5$ cells/ml to culture medium with the following composition:

Institute of Pharmacology, Tomsk Scientific Center, Academy of Medical Sciences of the USSR. Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 113, No. 3, pp. 283-284, March, 1992. Original article submitted August 7, 1991.

0007-4888/92/0003-0367$12.50