Quantitative Studies of \textit{in Vitro} Morphological Transformation of Syrian Hamster Cells by Inorganic Metal Salts

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\textbf{ABSTRACT}

Morphological transformation of Syrian hamster embryo cells was induced by direct exposure to some inorganic salts of nickel, cadmium, chromium, beryllium, and arsenic but not salts of iron, titanium, tungstate, zinc, aluminum, and nickel sulfide amorphous. Furthermore, no transformation was observed in untreated controls. Lethality, indicated by a reduction in cloning efficiency, occurred with carcinogenic and noncarcinogenic metal salts. In addition, transformation of Syrian hamster cells was observed also after transplacental exposure to inorganic salts of nickel, beryllium, chromate, and cadmium which had been injected into pregnant hamsters. The transformed colonies had a variety of morphologies reflecting the cell types present in early passages of cells and were identical to those observed after organic carcinogens. Animal experimental data exist for the carcinogenicity of all positive inorganic metals except arsenic. Epidemiological evidence indicates an association between exposures to metals and cancer. Although in terms of dose-response relationships cadmium and chromium were the most potent transforming agents, the comparative potency of the various metals cannot be determined since it would depend on the ionization potential of the metal, the inorganic salt form, and possible biointeractions of the various metals.

\textbf{INTRODUCTION}

A number of inorganic substances are considered carcinogenic. Various essential elements may influence the growth of autotransplanted tumors. Others are considered to be environmental contaminants that initiate cancer in humans and/or in experimental animals. A number of reviews and meetings have focused on metal carcinogenesis (11, 13, 19, 37, 42) and the problems arising from the fact that some metals can induce cancer. Some inorganic metals are often associated with a variety of environmental contaminants, and much of the experimental data consist of the induction of tumors at the site of injection. Recently, quantitative \textit{in vitro} transformation was induced with lead acetate using fibroblasts derived from Syrian hamster embryo cells (8). A dose-response relationship for transformation was obtained, and the relevance of the transformed cells to neoplasia was demonstrated by the ability of cells derived from the transformed colonies to produce progressively growing fibrosarcomas when injected into nude mice.

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In the present report, 12 metal compounds were tested by direct application to cells that had been seeded for colony formation; 4 of these were tested also by a transplacental \textit{in vivo-in vitro} approach (9). Interestingly, of the 12 metal salts examined, acceptable evidence for human carcinogenicity is available for only arsenic, beryllium, chromium, and nickel; these, plus cadmium, were identified as transforming agents.

\textbf{MATERIALS AND METHODS}

\textbf{Cell Cultures.} Primary HEC\textsuperscript{2} were derived from Syrian embryos 13 to 14 days in gestation. Methods for cultivation have been published in detail (7). Essentially, after trypsinization of the whole embryos, primary cultures were obtained by seeding 1 \times 10\textsuperscript{5} cells/100-mm plastic Petri dish. Secondary cultures were prepared from the primary cultures 3 to 4 days later. After medium was removed, the cells were trypsinized, centrifuged, suspended in complete medium, and seeded at 5 \times 10\textsuperscript{3} to 10\textsuperscript{4} cells/100-mm plastic Petri dishes. Mass cultures were grown in Dulbecco's modification of Eagle's minimal essential medium, supplemented with 10% fetal bovine serum, in incubators at 37\textdegree C in a 10% CO\textsubscript{2} atmosphere. Total cell count for the secondary cultures after 2 days ranged from 10\textsuperscript{7} to 1.4 \times 10\textsuperscript{8} cells/dish.

\textbf{Test Materials.} The inorganic metal salts were purchased from Chem Services, Inc., Westchester, Pa., except for nickel subsulfide and NiS amorphous which were gifts from Dr. F. W. Sunderman and ferric oxide and titanium dioxide which were obtained from National Cancer Institute Chemical Repository. The BP which was used as a positive control was purchased from Eastman Kodak. All chemicals were prepared in a darkened room. Ten mg of each chemical were dissolved in 1 to 2 ml of acetone, added to 100 ml of warm complete medium, and mixed. The NiS amorphous was insoluble and was used in suspension. These stock solutions were stored at 4\textdegree C until used. The desired concentrations were prepared by diluting stock solutions with complete medium. The final concentration of acetone was always less than 0.02%.

\textbf{Transformation.} For the transformation assay in which chemicals were applied directly to cell cultures, 300 cells from secondary cultures of HEC were plated in complete medium with 20% serum in 50-mm plastic Petri dishes along with 6 \times 10\textsuperscript{4} HEC cells which had been irradiated as confluent monolayer cultures. The feeder cells were produced using a Picker portable industrial X-ray apparatus (T55-433) at 100 kV and 5 ma, 3000/min adjusted to deliver...
Transformation by Inorganic Metals

a total of 4400 R in air at a distance of 22.2 cm. The HEC were exposed to the metal salts 1 day after cells had been seeded for colony formation. Chemicals as 2× concentrations were added in 4 ml of complete medium so that each dish had a total of 8 ml of medium. Experiments were terminated 7 to 8 days after the addition of the test metal; the medium was removed, and the cells were washed with Dulbecco’s phosphate-buffered saline, fixed, and stained with Giemsa for examination. Colonies (a minimum of 2 mm diameter) were counted using a stereoscopic microscope at a relatively low magnification. The CE was determined by dividing the average number of colonies per dish by the number of cells seeded per dish multiplied by 100. The frequency of morphological transformation was expressed as the average number of transformed colonies per dish relative to total number of colonies per dish and as the average number of transformations per dish. Morphological transformation is defined as an altered colony morphology consisting of criss-crossing and piling up of cells not observed in the controls. Each experimental point was repeated 3 to 7 times with cells that originated from different animals; but since the results were consistent, only one set of data is presented for each chemical.

Additional experiments were done with some metals using a host-mediated in vivo-in vitro assay. This system differs from the established quantitative in vitro assay by including in vivo transplacental exposure of the fetuses to the chemical or to its metabolites. Pregnant Syrian golden hamsters at 11 days of gestation were given i.p. injections of the chemical in alcohol using 2.5 or 5 mg/100 g maternal weight. The injected animals were fed water and standard laboratory chow, and the embryos were excised on Day 13 of gestation, 48 to 60 hr after maternal injection. The preparation and culture of cells from these embryos and the identification for transformation were as described above.

RESULTS

The colonies formed by untreated HEC were either light or dense as previously described and were made up of various cell types reflecting the diversity of cells found in the embryo. Morphology of control colonies was constant with regular cell arrangements and easily distinguished from those classified as having been transformed. BP treatment produced, in addition to colonies that appeared similar to those in control, some colonies with a random or criss-cross orientation of cells. When 2.5 μg BP per ml of medium were used, the transformation frequency, on a colony basis, was 4 to 6% (data not shown). The relevance of the transformed phenotype as an indicator of neoplastic properties is that cells derived from these transformed colonies repeatedly have been shown to demonstrate the ability to produce progressively growing tumors when inoculated into appropriate hosts. Typical colonies scored as transformed after direct treatment with metal carcinogen were identical to those obtained with other chemical carcinogens. These were classified as either light (Figs. 1, 3, and 4) or dense (Figs. 2, 5, and 6).

Of the 12 metals studied, 6 produced transformation; 4 of the 6 positives were also tested and were found to be positive in the transplacental assay. The nontransforming metal salts, ferric oxide, titanium dioxide, sodium tungstate, zinc chloride, aluminum chloride, and NiS amorphous, were used at concentrations as high as 20 μg/ml of medium. At the highest concentrations used, the nontransforming metal salts reduced the CE by 20 to 25% relative to controls even though transformation was not observed; however, with NiS amorphous (5 μg/ml in suspension), toxicity was even less (~5%).

The positive results with the transforming metal salts are summarized in Table 1. All metals caused transformation at more than one concentration which was accompanied by toxicity, as indicated by a reduction in CE of approximately 25 to 50%. The carcinogenic metals were as potent as BP which was also used with 20% fetal bovine serum. The data indicate, on the basis of the absolute number of transformation, that the transformation frequency was dose dependent. Concentrations of metals higher than those given in Table 1 resulted in further lethality and in some cases essentially eliminated all colonies.

The largest absolute number of transformation observed, disregarding concentration, was obtained with nickel sulfate at 10 μg/ml medium. The more potent metal carcinogens on the basis of dose-response were sodium chromate and cadmium acetate, which induced an average of 1.75 transformations/dish at a concentration of 0.5 μg/ml of medium. When the concentration of cadmium acetate was doubled to 1 μg/ml medium, there was a reduction in both transformed colonies and the average number of colonies per dish. Results obtained with sodium chromate indicate that a transformation frequency in the same range was observed with cadmium acetate but with less lethality, as indicated by the higher CE. Nickel subsulfide (1 to 5 μg/ml medium) also showed a trend of increased number of transformations with increased concentration. The percentage of transformations obtained with nickel subsulfide was the highest observed, regardless of the metal carcinogen used. The average number of transformations ranged from 0.66 to 1.75/dish or 1.5 to 11% on the basis of transformed colonies relative to total colonies scored. The absolute numbers of transformations per dish after beryllium sulfate or sodium arsenate were approximately 1 and 2/dish at 2.5 and 5 μg/ml medium, respectively; but on a relative colony basis, in the experiments with 5 μg/ml, beryllium produced a higher transformation frequency (6.4 versus 4.13) than arsenic.

Four metal salts also produced transformation in the host-mediated in vivo-in vitro assay. When the salts of nickel (sulfate), beryllium, chromate, and cadmium were injected into pregnant Syrian hamsters, transformation was observed in cells cloned from the third subpassage. Beryllium, chromate, and cadmium were used at 2.5 mg/100 g body weight, and nickel was used at 5 mg/100 g body weight. Doubling the concentrations proved lethal to the pregnant hamsters.

DISCUSSION

Inorganic metals may cause cancer in animals and may have mutagenic properties in microbial systems. Some metals may also be human carcinogens. Of the 6 metals reported to be negative for transformation, only zinc salts...
Transformation and cloning efficiency of Syrian hamster embryo cells treated with diverse inorganic metal salts

Petri dishes (50 mm) were seeded with 300 cells from a secondary culture with an irradiated hamster feeder layer (6 \times 10^6 cells). Chemicals were added 24 hr later. Dishes were fixed and stained 9 days after seeding. Colonies were scored blind by 2 observers.

<table>
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<th>Chemical</th>
<th>µg/ml</th>
<th>No. of transformed colonies</th>
<th>Transformed colonies/dish</th>
<th>Av. no. of colonies/dish</th>
<th>CEa (%)</th>
<th>Total colonies</th>
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<td>28.1</td>
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<td>12</td>
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<td>5.45</td>
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<td>25</td>
<td>51</td>
<td>17.5</td>
<td>4.13</td>
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Table 1

- Determined by dividing the average number of colonies per dish by the number of cells seeded per plate multiplied by 100.

are reported to induce tumors. Tumors were obtained by injection of zinc salts into the testes of fowl (2, 27) or hamsters (16) but not by s.c. or i.m. routes (43). Zinc does not affect the fidelity of DNA synthesis in vitro (35), but zinc chloride or sulfate are weak enhancing agents for SA7 transformation of HEC (5).

A positive correlation exists between the capacity of metals to cause transformation in vitro and tumor formation in animals with all metals tested except arsenic. Although most human carcinogens produce some type of cancer in animals, arsenic is unique because attempts to produce arsenical cancer have failed. Skin cancer in humans results from arsenic in drugs, drinking water, and occupational environment (3, 30, 35, 46). Although the incidence of deaths from lung cancer among metallurgical workers incriminates arsenic (40), the role of sulfur dioxide must be considered (24); the lung cancer mortality data reflect an increasing average dose of arsenic in terms of time and concentration (28).

Epidemiological evidence for the carcinogenicity of nickel compounds is convincing. Cancer of the lung and nasal cavity occurs in nickel refinery workers (19, 42). The increased incidence of respiratory tract cancer suggests that metallic nickel, nickel subsulfide, nickel carbonyl, and nickel oxide may be responsible (45). A variety of inorganic nickels produced fibrosarcomas and/or rhabdomyosarcomas when injected i.m. into mice or rats (19, 42, 44). Inhalation of nickel sulfide induced malignant lung tumors in rats (32), and nickel subsulfide caused a carcinogenic synergism when given with nickel oxide or carcinogenic polycyclic hydrocarbons (20, 26). Transformation of HEC by nickel subsulfide was found by Costa but not with amorphous nickel. No tumors occurred in rats after amorphous nickel (44).

Occupational exposure to cadmium may be relevant to carcinoma of the prostate, kidney, and of respiratory cancer (21, 43) and provides an example of the apparent increase of risk in men who smoke and work in high-risk occupations compared to men who do neither (21). Rats have developed sarcomas after s.c. injection of the chloride, sulfate, sulfide, and dioxide of cadmium and with cadmium powder (43). When soluble cadmium salts (sulfate and chloride) were injected s.c. into rats or mice, interstitial testicular tumors associated with testicular atrophy occurred (19). However, lung adenoma incidence did not increase in A/St mice treated i.p. (41) or in Swiss mice (38) when cadmium acetate was administered in the drinking water. Dose-dependent necrotic events were caused by cadmium in confluent human cultures (WI-38) (17). Maintaining the culture in the log growth phase prevented such morphological changes. The ability of cadmium salts to produce tumors systemically in animals and the presumptive data in humans are further...
reinforced by the transformation of HEC after either direct application or i.p. injection of cadmium acetate into pregnant hamster.

A high lung cancer risk is associated with occupational exposure to chromium compounds in chrome-producing plants (1) and in the chrome pigment industry but not in chrome ore mines (23). The evidence points toward an exposure-cancer relationship for hexavalent chromium compounds (18). Since hexavalent chromium can be reduced to the trivalent state, such compounds may also be carcinogenic in humans. Both metallic chromium and its hexavalent compounds produce sarcomas by a variety of routes in rats, mice, and rabbits (43). Carcinomas and adenocarcinomas in lungs of rats occur after intratracheal implantation of pellets of calcium chromate (22) and in mice after chronic inhalation of calcium chromate (29). Calcium chromate putatively transformed BHK 21 cells, but the possibility of spontaneous transformations could not be excluded (12). It is not surprising that transformation followed sodium chromate treatment of HEC or in cells derived from fetuses following transplacental exposure.

Until recently, epidemiological data on the incidence of respiratory cancer among beryllium workers was considered equivocal. Questions existed: could the methodology of analysis be responsible for the excess cancer incidence found in patients with berylliosis; and might the lung cancer mortality among beryllium workers be explained by correcting for cigarette smoking (18)? Recent data demonstrated that a higher percentage of noncigarette smoking workers exposed to beryllium developed cancer than did members of the United States white population (49). Beryllium is the lightest element known to cause cancer. Inhalation of various beryllium salts caused cancer in rats (33, 36, 48). Injections of beryllium salts i.v. into rabbits and mice induced osteogenic sarcomas (14). Thus, the positive results with HEC are consistent with human and animal data that beryllium is a carcinogen.

The underlying mechanism by which metals cause carcinogenicity is not understood. Some are lipid soluble and would be expected to enter the cell without metabolic alterations. Other would require organic compounds or bridging anions that bind in coordination covalent complexes to nucleic acids in DNA and/or RNA (15). The comparative potency of the carcinogenic metals is obviously dependent upon the physicochemical state of the metal, and the multitude of biological interactions depend upon whether the metal is elemental or ionized. Similar to organic carcinogens, metals can complex with proteins or nucleic acids and induce and inhibit enzymes. Metals have a striking influence on in vivo metabolism or have their effect profoundly altered or abolished by other metals. For example, cadmium affects calcium metabolism and vitamin D synthesis, but its effect can be averted or abolished by other metals such as zinc and selenium (10).

A number of metals are mutagenic in bacteria or phage, but not all carcinogenic metals are mutagenic. Both sodium and calcium salts of chromate were mutagenic for Escherichia coli in a spot test that demonstrated reversion from try+ to prototrophy (47). Chromate and arsenite increased the frequency to try+ revertants of E. coli; but when tested on another derivative of E. coli that lacks the functional rec (a DNA repair) gene, neither was mutagenic (31). Similarly, negative reports were obtained with E. coli phage T4 with chromate and lead (6). The latter produced neoplastic transformation of HEC (8). A number of metals were tested for potential mutagenicity using recombinant-deficient strains of B. subtilis rec (31). Arsenic, cadmium, and chromate were more inhibitory for rec+ cells. Among the metal compounds that showed no mutagenicity were the chlorides of beryllium and nickel. Fidelity of DNA synthesis decreased with the salts of most metal carcinogens, including beryllium and nickel (39). With the Salmonella reversion assay, which depends on histidine revertants, iron (4) and chromate but not arsenic (25) were mutagenic, but iron neither induced transformation nor affected the fidelity of DNA synthesis (39). Sodium arsenite added to E. coli after UV radiation caused decreased survival attributable to the inability to correct excision repair. The decreased mutation frequency by arsenite after UV in bacteria containing rec+ and lex+ genes indicates inhibition of mRNA (34). In mammalian cells, however, alkaline sucrose gradient analysis indicates that not all metals induce breakage of DNA (8). Whereas nonlethal concentrations of arsenic and cadmium caused breaks, beryllium or nickel produced no breaks even at concentrations that caused 100% cell death (6, 8). Therefore, it is probable that metals are very broad in their activity and that the resulting carcinogenicity is due to a number of different mechanisms.

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