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CADMIUM RESISTANCE AND CONTENT OF CADMIUM-BINDING PROTEIN IN TWO ENZYME-DEFICIENT MUTANTS OF MOUSE FIBROBLASTS (L-CELLS)

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Abstract—The toxic Cd^{2+} ion accumulates in mammalian organisms, the main storage organs are apparently the liver and the kidney. In these organs Cd^{2+} is bound to low molecular weight proteins (thioneins) as metallothionein. We describe here the development of resistance to otherwise lethal concentrations of Cd^{2+} by two non-epithelial cell lines, both derived from mouse fibroblasts (L-cells). One of the cell lines (clone 1D) is deficient in thymidine kinase and resistant to 5-bromodeoxyuridine, the other (A9) deficient in hypoxanthine-guanine phosphoribosyl transferase and resistant to 8-azaguanine. After stepwise increase in Cd^{2+} concentration, clone 1D cells had apparently normal growth rate in the presence of 100 micromolar Cd^{2+} after 6 months of Cd treatment. The A9 cells were apparently more sensitive to Cd^{2+}, after about one year's Cd treatment they had apparently normal growth in the presence of 100 micromolar Cd^{2+}. This concentration of Cd^{2+} would kill cells of both cell lines not previously exposed to Cd. In the resistant A9 cells about 40 per cent of the cadmium were bound to a cadmiumbinding protein (Cd-BP) of molecular weight of about 12,000, most probably metallothionein, in the resistant clone 1D cells the corresponding figure was 60 per cent. The non-resistant cell lines had apparently no metallothionein. We have thus found that also non-epithelial cells can synthesize low molecular weight Cd-BP and that there apparently is a good correlation between cadmium resistance and content of Cd-BP.

The toxic Cd^{2+} ion accumulates in mammalian organisms. The main storage organs are apparently the liver and the kidney [1]. Cd^{2+} is bound to low molecular weight proteins (thioneins) as metallothioneins in these organs [2]. In the liver of rats [3–5] and rabbits [6], metallothionein is inducible. Pretreatment of rats with low doses of cadmium induce the synthesis of metallothionein and also protects against subsequent exposure to an otherwise lethal dose [7]. The role of metallothionein in cadmium resistance is not clear. Restriction of food intake in the rat increases the level of metallothionein [8], but does not alter the LD_{50} of cadmium [9]. Also, the synthesis of metallothionein seems to continue for a longer time than the protection against cadmium in both rats and mice.

Lucis *et al.* [10], found uptake of Cd in cultured human embryonic fibroblasts, HeLa cells and monkey kidney epithelial cells after 8 days in culture with Cd. At that time the cells also contained a Cd-binding protein similar in its gel-filtration properties to metallothionein from liver. The authors did not, however, describe growth of the cells in the presence of Cd, or the development of cell strains resistant to Cd. Moreover, the levels of Cd-uptake were very low, and it is uncertain whether incorporation occurred through replacement of other cations (e.g. zinc) and thus unaccompanied by metallothionein biosynthesis. Webb and Daniel [11], more recently described the synthesis of a Cd-binding protein in cultures of cells derived from the cortex of the adult pig kidney. We have previously described the development of Cd resistance and a relationship between cadmium resistance and cadmium-binding protein (Cd-BP) in cultured human skin epithelial cells [12]. We describe here the development of resistance to Cd by two non-epithelial cell lines, and the relation between Cd-resistance and development of Cd-BP in these cell lines.

The two mouse fibroblast cell lines used were derived from L-cells, they are designated Clone 1D (Cl 1D) and A9. Clone 1D cells are deficient in thymidine kinase (TK) (ATP: thymidine-5′-phosphotransferase, EC 2.7.1.21) and they are resistant to 30 μg/ml of 5-bromodeoxyuridine (BUDR) [13]. The A9 cells are deficient in hypoxanthine-guanine phosphoribosyl transferase (HGPRT, EC 2.4.4.8) and resistant to 3 μg/ml of 8-azaguanine (8-AG) [14]. The purpose was to see if other eucaryotic cell lines than the human epithelial cell line described previously could be adapted to otherwise lethal concentrations of Cd, if resistant cells contained metallothionein, and further to develop Cd resistant cell lines which are suitable for cell hybridization in selective media [15]. Hybrids of Cd resistant and non-resistant parent cells might be useful in studies of the mechanism of Cd resistance and biosynthesis of metallothionein.

MATERIALS AND METHODS

Methods of cell culture. Both clone 1D cells and A9 cells were grown in Falcon tissue culture flasks (75 cm^2) in Dulbecco's modified Eagle's medium supplemented with 2.5% foetal calf serum and 15% horse serum (Gibco). Penicillin 100 U/ml, streptomycin 0.1

g/ml and nystatin 60 U/ml were added. For subculture the cells were incubated for 10 min at 37° with 0.25% trypsin in a buffered EDTA solution. After gentle shaking the cell suspension was centrifuged at 1000 *g* for 5 min and resuspended in fresh medium. Subcultures were usually made with a 1:4 or 1:6 split. The A9 cells were grown in the presence of 3 μg/ml of 8-azaguanine (8-AG) and 6 μg/ml of thioguanine (TG) (final concentrations). Clone 1D cells were grown in the presence of 30 μg/ml of 5-bromodeoxyuridine (BUDR).

Materials. $CdSO_4$ was purchased from BDH chemicals, BUDR, azaguanine and thioguanine from Sigma.

Gel filtration was performed in a 1.6 cm × 79 cm column (K16/100) (Pharmacia) with Sephadex G75 in 0.01 M Tris–HCl buffer pH 8.6. Radioactively labelled Cd as the dichloride was purchased from NEN. Cold cadmium was added to a specific activity which gave suitable counting statistics with the amounts of cadmium used in the experiments.

Radioactivity. This was counted in Packard autogamma Spectrometer 578.

Protein. Protein in cell cultures was determined by Oyama and Eagle's [16] modification of the method of Lowry *et al.* [17] for protein determination. For other protein samples the method of Lowry *et al.* [17] was used.

Determination of low molecular weight Cd-BP. Growth medium and cell content were tested for low molecular weight Cd-BP by addition of ^{109}Cd, a gamma emitter to cell cultures. To get a high uptake of labelled Cd, cultures of cells were grown for 3 days in Cd-free medium before addition of ^{109}Cd. Labelled Cd (final concentration of 1.3 μm/l (L) was then added to the medium. After growth for 24 hr in medium containing ^{109}Cd, the medium was collected, the cells washed twice with 5 ml prewarmed (37°) medium without Cd, lysed in 10 ml distilled water and frozen and thawed twice. The cell lysate was centrifuged at 2000 *g* for 10 min and the supernatant chromatographed on a Sephadex G-75 column calibrated with Blue Dextran, bovine serum albumin, trypsin inhibitor soybean protein and cytochrome *c*. The elution volume for cytochrome *c* was the same as peak 2 of ^{109}Cd (Figs 3 and 4) and corresponds to a molecular weight of about 12,000.

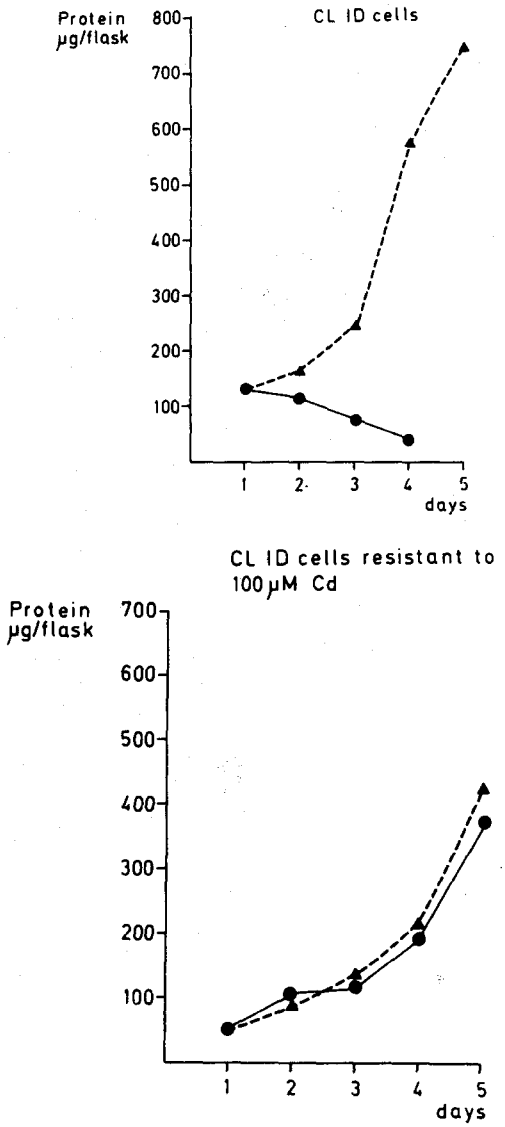

Fig. 1. Growth of clone ID cells not made resistant to cadmium (a) and clone (D cells made resistant to 100 μmoles/l Cd in the growth medium (b). Both cell lines were grown in medium without Cd added (▲----▲) and with 100 μmoles/l Cd added (●——●).

RESULTS

Development of Cd-*resistant cell strains.* $CdSO_4$ was added (5, 10 or 20 μmoles/l (final concentration)) to the culture medium. For both cell lines all the cells died within 2–3 days at 10 or 20 μmoles/l concentration. With 5 μmoles/l concentration most of the cells died, the remaining ceased to grow for 2–3 weeks. After that time the cells regained their growth. After additional 2–3 weeks the growth rate in 5 μmoles/l was apparently normal and the concentration was increased to 10 μmoles/l. Again most of the cells died, the remaining ceased to grow for weeks. The A9 cells seemed to be more sensitive to Cd; for each increase in concentration they required longer time to regain their growth compared to the clone 1D cells. The $CdSO_4$ concentration was stepwide increased by 10 μmoles/l when the apparent growth rate had become normal after the last increase. For every increase the cells altered shape, most of them died, and it took weeks for those remaining to regain normal growth. After approximately 6 months the clone 1D cells had an apparently normal growth in 100 μmoles/l $CdSO_4$, whereas it took almost a year to have an apparently normal growth rate at 100 μmoles/l $CdSO_4$ for the A9 cells.

Growth rate estimated by measuring cellular protein as described was determined in resistant and non-resistant A9 cells and CLID cells grown in the presence of 100 μmoles Cd/l and without any Cd added. The results are shown in Figs 1 and 2.

Cells resistant to Cd, but grown for 4 weeks without Cd still were able to grow in medium containing 100 μmoles Cd/l. Cd content in the cells were not

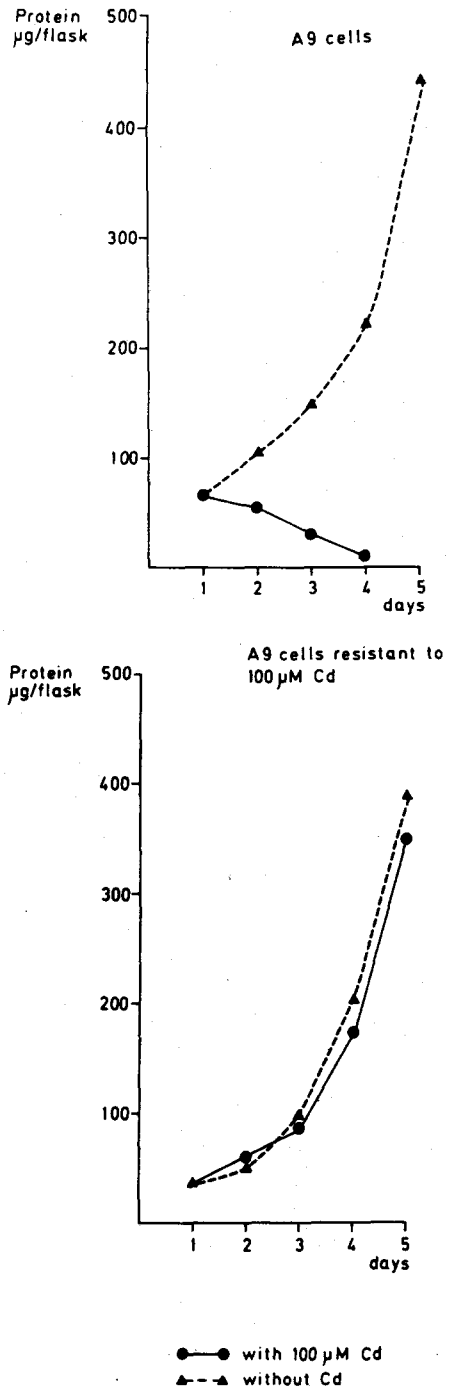

Fig 2. Growth of A9 cells not made resistant to cadmium (a) and A9 cells made resistant to 100 µmoles/l Cd in the growth medium (b). Both cell lines were grown in medium without Cd added (▲----▲) and with 100 µmoles/l Cd added (●——●).

followed, and we do not know if the cells contained minute amounts of Cd at that time.

Cadmium-binding proteins in growth medium and cell lysates. Figures 3 and 4 show the results of gel filtration of cell lysates from ordinary and Cd-resistant A9 cells and C1 1D cells grown for 24 hr in the presence of 1.3 µmoles/l of ^{109}Cd. In the non-resistant cell lines no peaks corresponding to Cd-BP of molecular weight about 12,000 could be detected. In the resistant A9 cells about 40 per cent of the labelled cadmium in the cell lysate were bound to a Cd-BP with an elution volume corresponding to that molecular weight, in the resistant C1 1D cells the corresponding figure was about 60 per cent (Figs 3 and 4). All cell lines had Cd peaks corresponding to a protein eluted with the void volume, which means that its molecular weight is above 70,000. Some cell lysates also had a Cd peak corresponding to the total volume of the column, that is Cd conjugated to some compound with a molecular weight of less than 1000–3000. Growth medium from resistant or non-resistant A9 or C1 1D cells grown in the presence of 1.3 µmoles/l ^{109}Cd never contained any CdBP of molecular weight about 12,000; the labelled Cd was eluted in peaks corresponding to the total volume and the void volume of the column.

After growth for 4 weeks in medium without any Cd added, A9 cells and C1 1D cells previously made resistant to 100 µmoles/l Cd still would grow and divide in culture when 100 µmoles/l Cd were added to the growth medium, and the cell lysates still contained a CdBP of a molecular weight of approximately 12,000. We do not know, however, whether minute amounts of Cd were present in the cells at that time.

In some experiments Cd isotope was added to the medium and the cell lysates after collection of the cells. The results were essentially the same; Cd-BP of molecular weight about 12,000 could be demonstrated in lysates from Cd-resistant cells, but not in growth medium or in cells not previously exposed to Cd.

DISCUSSION

We have previously found that human epithelial skin cells can be made resistant to Cd in the growth medium in concentrations which would kill cells not previously exposed to Cd [12]. The lysates of resistant cells, but not those from non-resistant cells contained large amounts of a cadmium-binding protein of a molecular weight of approximately 12,000.

In the present paper we describe the development of Cd-resistance in two additional cell lines, both derived from mouse fibroblasts. In the resistant cells 40–60 per cent of cellular Cd were bound to a Cd-BP with a molecular weight of about 12,000, whereas the non-resistant cells contained no such Cd-BP. However, the cells were frozen and thawed twice prior to centrifugation and subsequent gel filtration for determination of low molecular weight Cd-BP. It is possible that the freezing and thawing may cause an aggregation of Cd-BP due to which part of the Cd-BP may appear in the fraction of high molecular weight. The true share of low molecular weight of Cd-BP might thus be higher than 40–60 per cent. This shows that also non-epithelial cells may produce Cd-BP in response to Cd, also we have again found a good correlation between cadmium resistance and content of Cd-BP. It seems reasonable to assume that induction of Cd-BP is a general feature of eukaryotic cells and that increased levels of this Cd-BP may play a role in development of resistance towards Cd.

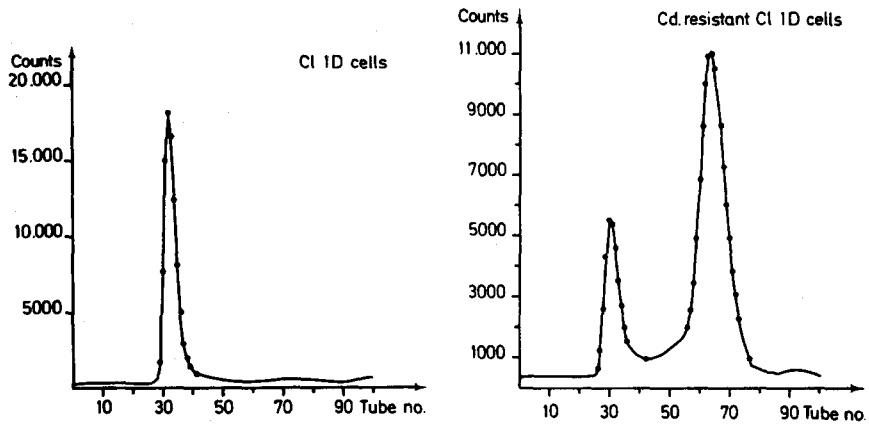

Fig. 3. Gel filtration (Sephadex G-75) of lysate of Clone 1D cells not made resistant to cadmium and clone 1D cells made resistant to 100 μmoles/l Cd in the growth medium. Both cell lines were grown for 24 hr with 1.3 μmoles/l of ^{109}Cd. Tube no. 30 represents the exclusion volume (mol. wt approx. 70,000), tube 65 corresponds to a mol. wt of about 12,000. Ordinate: cpm.

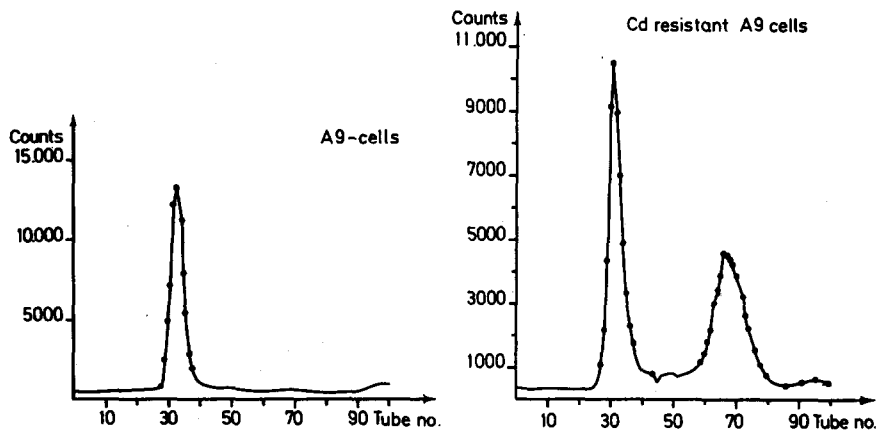

Fig. 4. Gel filtration of lysate of A9 cells not made resistant to Cd and A9 cells made resistant to 100 μm/l Cd in the growth medium. Experimental procedure as given in Fig. 3.

The cells used are particularly well suited for cell hybridization in that their enzyme defects allow the use of selective media [15]. Previous [12] and present work might indicate that Cd resistance is genetically determined. Studies on hybrids of resistant and non-resistant cells might give information on the genetic control of levels of Cd-BP.

The Cd-resistant cells with their high content of low molecular weight Cd-BP may be useful in studies on the biosynthesis, and catabolism of this unique protein. They may also prove useful in experiments on the effects of metal-chelating agents used in metal poisoning.

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