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DNA damage and repair in normal and neoplastic cells treated with adriamycin

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Adriamycin (ADR), a common antineoplastic drug, was used to study DNA repair synthesis, cell cytotoxicity and DNA single strand breaks in normal human fibroblasts — CLV98 and human melanoma cells — ME18. No repair synthesis was observed in ME18 and CLV98 cells exposed to adriamycin in concentrations up to 10^{-5} M. ME18 cells were less sensitive to ADR treatment than CLV98 cells. Adriamycin-induced DNA single strand breaks (at ADR concentration: 1 μg/ml) were incompletely repaired in ME18 cells and unrepaired in CLV98 cells within 24 h after drug removal. Within 48 h strand breaks were completely repaired in both kinds of cells. No repair of single strand breaks was observed in ME18 and CLV98 cells after drug treatment in the concentration of 5 μg/ml.

Adriamycin (doxorubicin HCl, ADR) is a common antineoplastic drug used in the treatment of many forms of cancer including breast cancer, acute leukemia, sarcomas and lymphomas [1, 2]. Its mechanism of action is probably complex. Adriamycin inhibits synthesis of DNA and RNA in living cells, gives a rise in protein-associated breaks and cross-links in DNA, induces mutation [3], sister-chromatid exchange [3, 4] and chromosome aberrations [5]. Like other anthracyclines, adriamycin intercalates in DNA and this is likely to be an important factor for its genotoxic activity [6–8]. A number of investigators studied the repair activity of adriamycin-induced DNA damage but usually in malignant cells.

In our current work, we have examined the repair of single strand breaks observed in normal human fibroblasts (CLV98) and human melanoma cells (ME18) following exposure to adriamycin in various concentrations, 24 and 48 h after treatment. Moreover, DNA repair synthesis and cytotoxicity of ADR in both kinds of cells were studied.

MATERIALS AND METHODS

Chemicals. Adriamycin (ADR) was obtained from Farmitalia, thiazolyl blue (MTT) from Sigma, [^{3}H]dThd (25 Ci/mmol) and [^{14}C]dThd (61 mCi/mmol) from Amersham, Akwascynt from BioCare, minimum essential medium (MEM) and phosphate buffered saline (PBS) from W.S.S. Lublin (Poland), foetal calf serum from Bioproduct.

Cells. Human embryonic cell line CLV98 and human melanoma cell line ME18 were used. Cells were grown in MEM supplemented with 10% foetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin.

Cell viability assay (MTT assay). MTT assay, originally described by Mosmann [9], is based on the ability of viable cells to reduce the soluble yellow tetrazolium salt (MTT) to an insoluble blue-black formazan precipitate. The suspension of cells was diluted, usually to 5×10^5 cells/ml, in MEM and 100 μl of this suspen-

sion was placed into individual wells on a 96-well multiplate. ADR dissolved in water was then added in a volume of 100 µl at double strength drug dilution. The wells, containing MEM without ADR, were used for the control of cell viability. The plate was then incubated in a humidified atmosphere for 24 h at 37°C in 5% CO_2. Cells were continuously exposed to ADR at the concentration ranging from 10^{-9} to 10^{-5} M throughout this period. After the ADR exposure the plate was inverted to remove the medium, then 100 µl of a 5 mg/ml MTT-solution in PBS was added to each well and the plate was incubated for another 4 h. Then the plate was inverted again to remove the unconverted MTT, and the formazan crystals were left at the bottom of the wells. These crystals were dissolved in 100 µl of dimethylsulphoxide by agitating on a plate shaker for 5 min, and absorbance at 500 nm was measured. The effects of ADR treatment were determined by calculating the absorbance of the test wells as a percentage of that of the control wells. In all experiments eight replicate wells were used at each point. The results represent the mean of three independent determinations.

Measurement of DNA repair synthesis. A sensitive method to measure DNA repair synthesis, described by Trosko & Yager [10] and modified by Anuszewska & Koziorowska [11] was used. Cells, 2×10^5/ml, were seeded into 35 mm plastic Petri dishes (2 ml per dish) and grown to confluence at 37°C in a 5% CO_2 humidified atmosphere for 96 h in MEM supplemented with 10% foetal calf serum and antibiotics. After a further 1 h-incubation with hydroxyurea (10 mM), ADR dissolved in water and [^{3}H]dThd (1 µCi/ml) were added and the cultures were incubated for 3 h. Incorporation of [^{3}H]dThd into DNA was stopped by addition of cold thymidine at the concentration of 50 µg/ml, then the cells were washed several times with cold PBS and dried in the air. The cell fractions insoluble in trichloroacetic acid were collected onto millipore filters, dried and placed in vials, containing 8.0 ml of a mixture of toluene with POPOP and PPO for subsequent liquid scintillation counting. Controls contained all additions except ADR. DNA repair synthesis was expressed as percentage of [^{3}H]dThd incorporation in relation to controls.

Alkaline elution assay. The technique of alkaline elution, as described by Kohn [12, 13] and modified by Brunborg *et al.* [14, 15] was used for detection of single-strand DNA breaks after ADR treatment. The rate of DNA elution from the filter at high pH is inversely related to strand size. After 24 h of cell growth, DNA was labelled by adding [^{14}C]thymidine (0.01 µCi/ml). Radioactive medium was removed after 24 h and the cells were resuspended in fresh medium for not longer than 20 h. After that, they were exposed to ADR at the concentration of 1 or 5 µg/ml for 1 h at 37°C. Then, one set of cultures was washed with PBS, scraped and resuspended in cold PBS to prevent repair of ADR-induced damage of DNA. Other sets of cultures were refed with MEM and incubated for 24 h (ADR_{24}) or 48 h (ADR_{48}). After that time, the cells were rinsed with PBS and scraped. Cell suspensions were layered onto membrane filter, lysed (sodium dodecyl sulfate: Na_2EDTA: proteinase K) and then single-stranded DNA was eluted from the filter in the dark by passing 10 mM Na_2EDTA (pH = 12.1) through the filter with the use of a peristaltic pump (0.3 ml/min).

Fractions of eluted DNA (2.0 ml) were collected and mixed with 7.0 ml of Akwascynt for scintillation counting. The fractions, washes and the filter were counted using a scintillation counter and the percentage of DNA retained on the filter during the process of elution, was calculated. Three replicates were done for each point.

Effects observed in ADR-treated cultures were compared with untreated control cultures.

RESULTS

To estimate the cytotoxic effect of adriamycin on CLV98 and ME18 cells the MTT method was used. This method, a semi-automated colorimetric assay, is based on the premise that the mitochondria of living cells reduce tetrazolium salt to formazan. A modified form of this assay is currently being successfully applied by the National Cancer Institute U.S.A. for chemosensitivity testing of drugs [16–18].

Survival of the cells was measured after 24 h of continuous exposure to various concentrations of ADR from 10^{-9} M to 10^{-5} M. The dose-response curves for both cell lines, CLV98 and ME18, are shown in Fig. 1. ME18 cells appeared

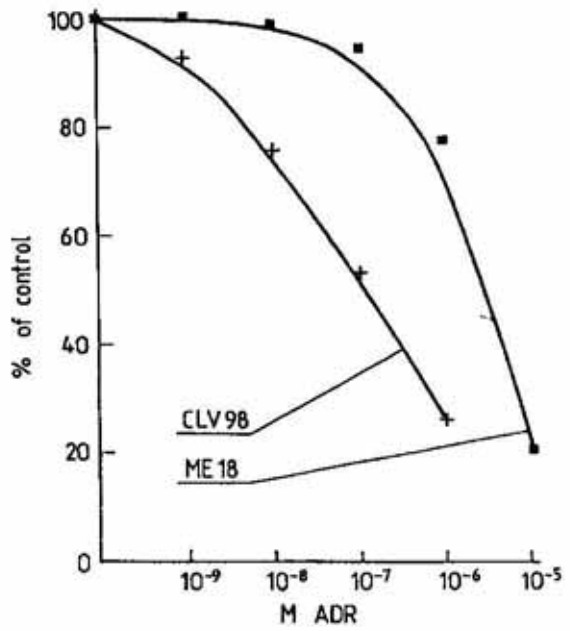

Fig. 1. Response to adriamycin of CLV98 and ME18 cells in the MTT assay.
The data represent the percentage of viable cells compared to control cells, incubated in the absence of adriamycin.

to be less sensitive to ADR treatment than CLV98 cells.

DNA repair synthesis was measured as the increase of [^{3}H]dThd incorporation into CLV98 and ME18 cells in the presence of 10 mM hydroxyurea, that is known to inhibit preferentially replicative DNA synthesis [11]. As the results in Table 1 show, there was no increase of [^{3}H]thymidine incorporation after ADR treatment in either cell line, whereas CLV98 cells treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) were able to carry out the DNA repair synthesis (Table 1). A significant decrease in the level of [^{3}H]dThd incorporation after treatment with ADR at the concentration of 10^{-5} M may result from inhibition of residual DNA synthesis caused by ADR itself.

To measure the rate and the amount of DNA single strand breaks released in alkali that were able to pass through polycarbonate membrane filter, alkaline elution was used.

A comparison of kinetics of DNA elution from cells lysed in the presence or absence of proteinase K showed that, as expected, enzymatic deproteinization was necessary to detect the DNA breaks associated with covalently bound protein [6].

A 1 h exposure to ADR at the concentration of 5 μg/ml, caused an increase in the rates of ^{14}C-labelled DNA elution from the filters observed at 24 and 48 h after treatment in CLV98 and ME18 cells. The results of this study shown in Figs. 2a and 2b indicate that ADR produced single strand breaks and that incubation of CLV98 and ME18 cells for 24 or 48 h after drug removal did not influence elution patterns. This indicates that there was no repair of ADR-induced single strand breaks up to 48 h. The differences between the curves obtained at various times of incubation after ADR treatment, were not significant.

The exposure to ADR at the concentration of 1 μg/ml of both kinds of cells caused changes in the elution patterns. As shown in Figs. 3a and 3b, the fraction of single-stranded DNA amounted to about 50% in cells treated with ADR at the concentration of 5 μg/ml. ADR-induced single strand breaks in ME18 cells were almost completely repaired within 24 h after drug removal and completely repaired within

Table 1

Effects of adriamycin on [^{3}H]dThd incorporation into DNA (repair synthesis) of CLV98 and ME18 cells expressed as a percentage of tritiated thymidine incorporation (c.p.m./culture) as compared with control (samples containing 10 mM hydroxyurea in the absence of adriamycin).

		CLV98 Mean ± S.D.	ME18 Mean ± S.D.
ADR	10^{-8} M	105.0 ± 12.6	99.5 ± 10.9
	10^{-7} M	98.9 ± 8.9	104.0 ± 15.6
	10^{-6} M	81.0 ± 12.0	95.0 ± 9.1
	10^{-5} M	66.8 ± 7.5	72.3 ± 7.9
MNNG	15 μg/ml	492.0 ± 74.0	not tested

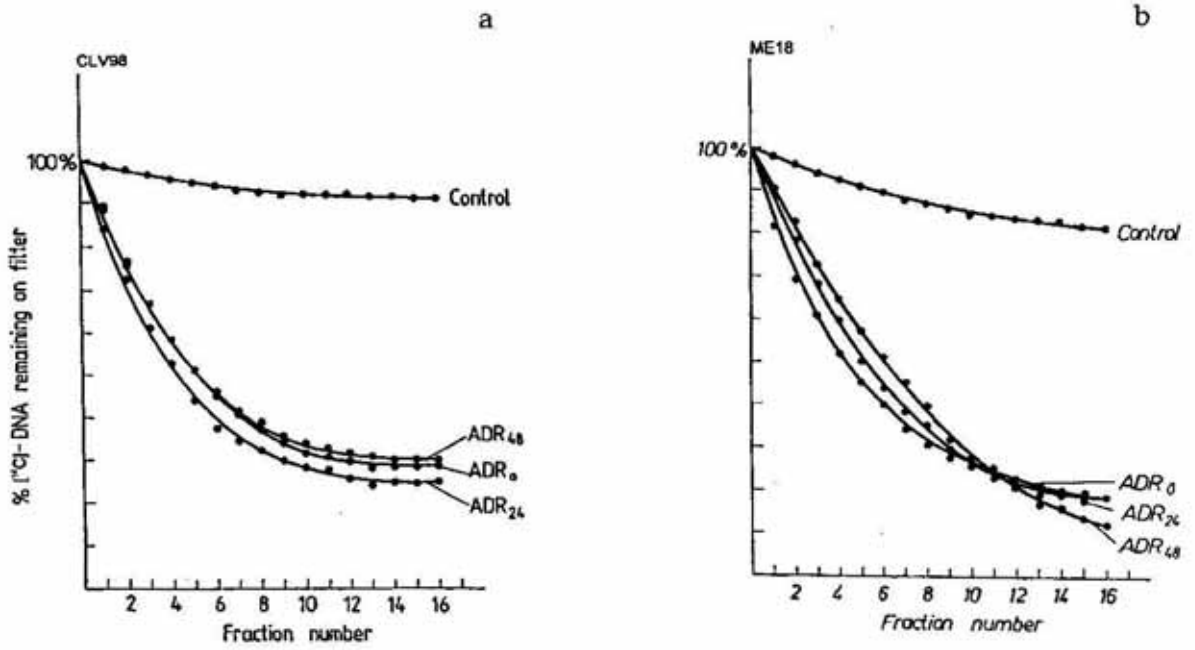

Fig. 2. Representative alkaline elution profiles of CLV98 and ME18 cells at various times after adriamycin treatment (0, 24, 48 h) at the concentration of 5 μg/ml (10^{-5} M) for 30 min at 37°C.
Data is expressed as a percentage of the total radioactivity recovered. Three replicates were done for each incubation time.

48 h. Repair of single strand breaks in CLV98 cells after treatment with ADR at the concentration of 1 μg/ml was not observed during the first 24 h. However, ADR-induced single strand breaks were completely repaired during the following 24 h.

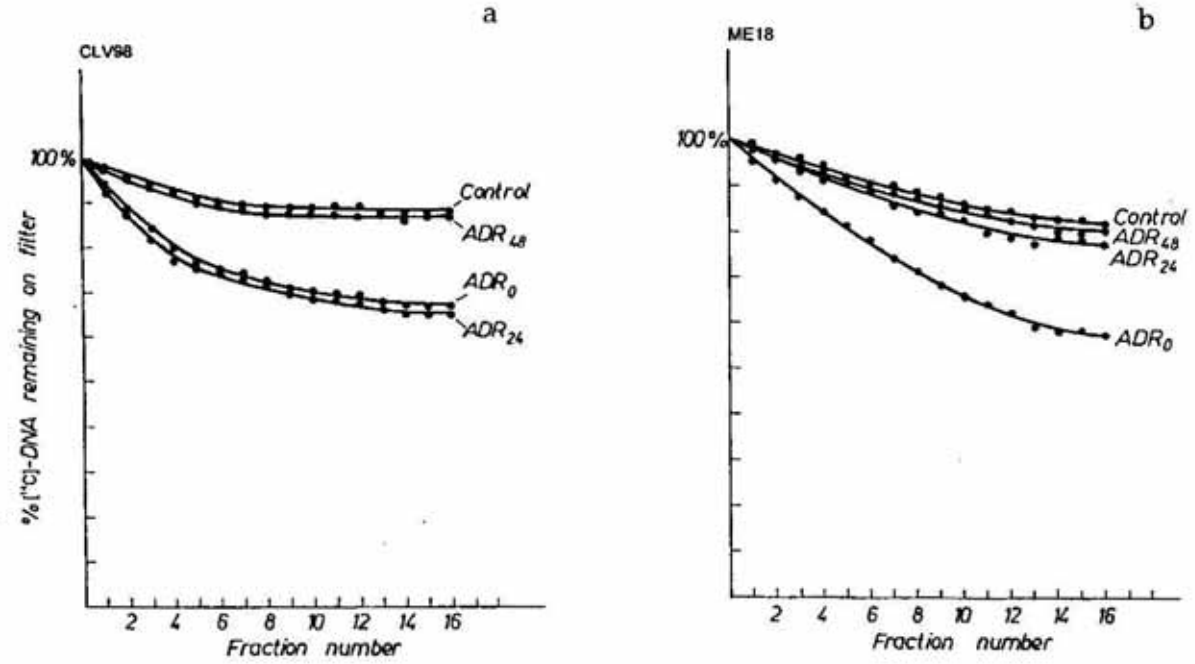

Fig. 3. Representative alkaline elution profiles of CLV98 and ME18 cells at various times after adriamycin treatment (0, 24, 48 h) at the concentration of 1 μg/ml (2×10^{-6} M) for 30 min at 37°C.
Data is expressed as a percentage of the total radioactivity recovered. Three replicates were done for each incubation time.

DISCUSSION

The mechanism underlying the biological activity of ADR is not yet completely understood, although a number of injurious effects of the drug have been described. It is likely that the cytotoxic and genotoxic activities of ADR are due to a complex interaction between several types of cellular damage. We have postulated that the expression of these activities may depend on the kind of cells.

On the basis of the MTT assay it is possible to conclude that ME18 cells appear to be less sensitive to ADR treatment than CLV98 cells. Neither kind of the cells used seemed to evoke the DNA repair replication after ADR treatment. Under analogous experimental conditions, CLV98 cells treated with MNNG were able to carry out the DNA repair synthesis. These results indicate that the inability to evoke repair synthesis depends on the kind of the DNA damage and does not depend on the kind of cells.

Lambert *et al.* [4] studied the effects of ADR on DNA repair synthesis in human lymphocytes *in vitro*. No repair replication was observed in lymphocytes exposed to ADR at the concentration up to 10^{-3} M. In our study, as indicated by the shape of the elution pattern at pH = 12.1, ADR induced single strand breaks in both kinds of cells. The number of single strand breaks induced in CLV98 and ME18 cells, was dose dependent (Figs. 2 and 3). These breaks were not repaired till 48 h after treatment with ADR at the concentration of 5 μg/ml (Fig. 2). However, in both kinds of used cells, single strand breaks disappeared till 48 h after treatment with ADR at the concentration of 1 μg/ml. It is interesting that CLV98 cells did not repair the breaks within 24 h after drug removal. These breaks disappeared between 24 and 48 h of incubation in the medium without ADR (Fig. 3a). Breaks induced by ADR at the same concentration in ME18 cells were nearly repaired within 24 h after drug removal (Fig. 3b).

Ross & Smith [8] have presented evidence that ADR-induced single strand breaks in DNA of mouse leukemia L1210 cells, were slowly and incompletely repaired over 24 h after drug removal. Also Zwelling *et al.* [7], who studied the effect of ADR on L1210 cells, noted slow repair of ADR-induced strand breaks. On the basis of their data, Ross & Smith [8] suppose that the persistence of DNA lesions is largely dependent on the continued presence of the drug in the cells. Further studies are therefore needed to explore the possibility that the drug is removed from CLV98 cells slower than from ME18 cells. In our studies in progress we try to elucidate also the possibility that the difference between the two kinds of cells under study depends on the level of detoxifying enzymes, induced in the cells after ADR treatment.

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