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Resistance Mechanisms in Three Human Small Cell Lung Cancer Cell Lines Established from One Patient during Clinical Follow-up[1]

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ABSTRACT

Mechanisms for resistance were studied in three classic type, human small cell lung cancer cell lines, GLC_{14}, GLC_{16}, and GLC_{19}, that were established from one patient during clinical follow-up. Clinically the tumor changed from sensitive (GLC_{14}) to completely resistant to (chemo)therapy (GLC_{19}) during this period. The stain with JSB-1 antibody, detecting the M_r 170,000 multidrug resistance associated glycoprotein, was most pronounced in GLC_{16} and absent in GLC_{19}. Intracellular Adriamycin (Adr) concentrations were decreased in GLC_{16} and GLC_{19} *versus* GLC_{14}. Glutathione levels were 12.9, 15.5, and 16.6 μg/mg protein; total sulfhydryl groups were 36.5, 45.7, and 48.8 μg/mg protein; and glutathione *S*-transferase activity was 13, 29, and 43 nmol 1-chloro-2,4-dinitrobenzene/min/mg protein for GLC_{14}, GLC_{16}, and GLC_{19}, respectively. Incubation with DL-buthionine-*S,R*-sulfoximine increased Adr and cisplatin induced cytotoxicity, whereas X-ray induced cytotoxicity remained the same. Catalase activity increased from 0.88 to 1.73 to 3.83 μmol H_2O_2/min/mg protein in, respectively, GLC_{14}, GLC_{16}, and GLC_{19}. Compared to GLC_{14} and GLC_{16}, Adr induced a higher amount of DNA strand breaks in GLC_{19}. In none of the three cell lines could Adr induced DNA strand breaks be repaired. X-ray induced a comparable amount of DNA strand breaks in all three cell lines but all cell lines were capable of repairing the X-ray induced DNA strand breaks within 90 min.

It is concluded that a number of different mechanisms are operative and that some but not all of the observed changes in mechanisms for drug resistance in these lines correlate with the clinical data.

INTRODUCTION

The development of acquired resistance to antineoplastic drugs represents a significant problem in the treatment of patients with cancer. Elucidation of the mechanisms involved in drug resistance could indicate ways to circumvent such a development. Several studies concerning *in vitro* developed resistance in human tumor cell lines have been reported (1–6). These studies deal especially with *in vitro* acquired resistance for Adr,[3] in which the resistance factor is often very high. Little is known, however, about the correlation between the mechanisms found for *in vitro* acquired resistance and the mechanisms responsible in the clinic. Therefore the study of cell lines derived from tumors with acquired resistance *in vivo* might broaden our understanding of the mechanisms involved. Thus far only few cell lines derived from one patient before as well as after therapy have been characterized (7–9), but no studies on mechanisms of *in vivo* acquired resistance have been published. Mechanistic studies in lines obtained from tumors with *in vivo* acquired resistance most probably will better reflect the *in vivo* situation in the patient.

In the present study, a number of possible mechanisms for *in vivo* acquired resistance were evaluated in three cell lines (GLC_{14}, GLC_{16}, and GLC_{19}). The lines were derived from one patient with small cell lung cancer during clinical follow-up and treatment. During this period the tumor changed from sensitive to completely resistant to (chemo)therapy.

MATERIALS AND METHODS

Chemicals. Adr was provided by Farmitalia Carlo Erba, Milan, Italy. cDDP was obtained from Bristol Myers S.A.E., Madrid, Spain. RPMI was obtained from Gibco, Paisley, Great Britain. Fetal calf serum was from Flow Laboratories, Irvine, Great Britain. GSH, 1-chloro-2,4-dinitrobenzene, catalase, 5,5-dithiobis(2-nitrobenzoic acid), and MTT were obtained from Sigma, St. Louis, MO. H_2O_2 and dimethyl sulfoxide were from Merck, Darmstadt, West Germany. BSO was obtained from Chemalog, South Plainfield, NJ. Ethidium bromide was from Serva, Heidelberg, West Germany.

Patient and Cell Lines. Three classic-type, human small cell lung cancer cell lines (GLC_{14}, GLC_{16}, and GLC_{19}) have been established from one patient during longitudinal follow-up. During this period the tumor changed from sensitive to completely resistant to (chemo)-therapy. The characterization of these lines is described by Berendsen *et al.* (7). In short, GLC_{14} was derived before therapy. Treatment consisted of cyclophosphamide 1 g/m^2 every 3 weeks, Adr 45 mg/m^2 every 3 weeks, and etoposide 100 mg/m^2/days 1, 3, and 5 every 3 weeks (cyclophosphamide/Adr/etoposide). After a complete response lasting 7 months, because of tumor recurrence reinduction with cyclophosphamide/Adr/etoposide was started. After 4 cycles a partial response was seen and therapy was discontinued. At that moment GLC_{16} was derived. Radiotherapy (10 × 300 rad) was given to the left hilar region and the mediastinum. After recurrence of disease GLC_{19} was established from a biopsy of the left upper lobe, the area which had been irradiated. Two additional cycles of cyclophosphamide/Adr/etoposide were given, but the tumor progressed.

GLC_{14}, GLC_{16}, and GLC_{19} were kept in continuous culture in RPMI 1640 with 10% heat inactivated fetal calf serum. GLC_{14} grows almost completely attached to the culture flask, whereas GLC_{16} and GLC_{19} grow floating in aggregates. The culture doubling time was 26, 27, and 44 h in, respectively, GLC_{14}, GLC_{16}, and GLC_{19}. For all experiments, cells were incubated 1:1 with 0.02% EDTA solution (0.35 g $NaHCO_3$, 8 g NaCl, 0.4 g KCl, 1 g dextrose, 0.2 g EDTA, and 1.0 liter H_2O) for 5 min to prepare a cell suspension containing small clumps with a viability of 80% for all lines.

Sensitivity Assay. The microculture tetrazolium assay is based on the cellular reduction of MTT by the mitochondrial dehydrogenase of viable cells to a blue formazan product which can be measured spectrophotometrically (10, 11). Before the assays were performed the linear relationship of cell number to MTT formazan crystal formation was checked and cell growth studies were performed. Cell survival studies were performed on day 4 in all three cell lines. Thus, all lines were in an exponential phase of growth and had proceeded two to three cell divisions.

Equal numbers of cells (35,000/well for these cell lines) were incubated in a total volume of 0.1 ml of culture medium with one of the following agents, Adr, cDDP, and H_2O_2, for 1 h, in 96-well culture plates (Nunc; Gibco). For X-ray treatment (Philips X-ray source at 200 kV, 15 mA, 0.5 mm Cu/Al filter), cells were irradiated in tubes in a volume of 0.5 ml and then transferred to 96-well culture plates. For experiments after BSO incubation under conditions as described below, cells in medium without BSO were incubated with Adr or cDDP for 4 h or irradiated. After the incubation, cells, in wells, were washed three

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[3] The abbreviations used are: Adr, Adriamycin; cDDP, cisplatin [*cis*-diamminedichloroplatinum(II)]; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; BSO, DL-buthionine-*S,R*-sulfoximine; PBS, phosphate buffered saline (0.14 M NaCl, 2.7 mM KCl, 6.4 mM $Na_2HPO_4 \cdot 2H_2O$, and 1.5 mM KH_2PO_4, pH 7.4); MDR, multidrug resistance; GSH, glutathione; GST, glutathione *S*-transferase.

times by removing the medium after centrifugation of the microtiter plates (10 min, 180 × *g*) followed by addition of fresh medium. After a culture period of 4 days, 20 μl of MTT solution (5 mg MTT/ml PBS) were added to each well for 3.5 h. Thereafter the plates were centrifuged (30 min, 180 × *g*) and the supernatant was carefully aspirated, in order not to disturb the formazan crystals. Dimethyl sulfoxide (100%; 200 μl) was added to solubilize the formazan crystals and the plate was read immediately at 520 nm using a scanning microtiter well spectrophotometer (Titertek Multiskan, Flow Laboratories). The percentage of cell survival was calculated as the mean of three test samples/mean of three untreated samples. Controls consisted of media without cells (background extinction), and cells were incubated in wells with medium instead of the drug.

P-Glycoprotein Expression. The monoclonal antibody JSB-1 which is directed against the M_r 170,000 MDR associated glycoprotein was tested on cytospins using an indirect immunoperoxidase staining protocol (12).

Intracellular Adr Level. Cellular Adr levels were determined after 1 h incubation with Adr at 0°C and at 37°C. After the incubation cells were washed three times with ice-cold PBS, extracted with 0.3 N HCl-50% ethanol, and centrifuged. Adr fluorescence was measured in the supernatant at excitation and emission wavelengths of 474 and 549 nm, respectively, in a Kontron spectrofluorometer (13). Intracellular Adr levels were calculated by subtracting the vales measured at 0°C (extracellular bound Adr) from the corresponding values measured at 37°C (14).

Sulfhydryl Compounds and Enzyme Assays. Cells in the logarithmic phase of growth were harvested 4 days after passage. Cells were washed with ice-cold PBS and resuspended in a relevant concentration in PBS or another solution mentioned in the assay procedure. All measurements were performed under V_{max} conditions. For GSH determination cells were resuspended in ice-cold 5% trichloroacetic acid, vortexed, and centrifuged at 4°C (15 min, 10,000 × *g*). The supernatant was assayed for total GSH by the enzyme recycling method under conditions similar to those described by Tietze (detection limit, 0.1 μg/mg protein) (15). Total sulfhydryl groups were assayed as described by Sedlak and Lindsay (16). Cells were resuspended in Tris-EDTA buffer, pH 8.2, and sonicated. Cell homogenate was incubated with 5,5-dithiobis(2-nitrobenzoic acid), Ellman's reagent, for 15 min followed by centrifugation (15 min, 3000 × *g*). Absorbance of the supernatant was measured at 412 nm. For measurement of the GST activity by the method of Habig *et al.* (17), 1-chloro-2,4-dinitrobenzene was used as substrate and the enzyme reaction was measured by the absorbance change at 340 nm. The catalase activity was measured following the decomposition of H_2O_2 mediated by catalase in a cell suspension of 3×10^7 cells/ml at 240 nm according to the method of Beers and Sizer (18). For protein determination the assay of Lowry *et al.* was used (19). All measurements were performed on a Zeiss PMQ spectrophotometer.

BSO Treatment. Cells were cultured for 65 h in the presence of 50 μM BSO. At that moment GSH was no longer detectable in the three cell lines; no growth delay or loss of viability occurred.

DNA Damage and Repair. Strand breaks in DNA were measured by alkaline unwinding and determination of ethidium bromide fluorescence on a spectrofluorometer with excitation wavelength at 525 nm and emission wavelength at 580 nm as described before (20, 21). For Adr induced DNA damage, cells were incubated with Adr for 1 h. To stop the incubation period the cells were washed 3 times with ice-cold PBS. For determination of X-ray induced DNA damage, cells were irradiated on ice before the fluorometric assay was performed. For repair measurements the DNA damage was induced the same way. After the incubation was stopped, cells were resuspended in fresh medium and allowed to repair at 37°C in 5% CO_2 during a period of 30 to 90 min. Results were expressed as the percentage double-stranded DNA.

Statistics. Statistical significance was determined by use of the unpaired Student's *t* test and for the BSO experiments with the paired Student's *t* test. $P < 0.05$ was considered significant.

RESULTS

Cell survival curves of the three cell lines after exposure to Adr, H_2O_2, cDDP, and radiotherapy are shown in Figs. 1 and 2. GLC_{19} is significantly more sensitive for Adr and H_2O_2 but significantly more resistant to X-ray compared to GLC_{14}. For cDDP minor differences in sensitivity are observed between the three cell lines. GLC_{16} turned out to be the most resistant cell line for Adr and X-ray.

The M_r 170,000 glycoprotein as assessed with the monoclonal antibody JSB-1 was clearly detectable in GLC_{16}, only minimally present in GLC_{14}, and absent in GLC_{19} (Table 1).

Fig. 3 shows the intracellular Adr concentrations for GLC_{14}, GLC_{16}, and GLC_{19} after 1 h incubation with various concentrations of Adr. Intracellular Adr concentrations were significantly decreased in GLC_{16} and GLC_{19} as compared to GLC_{14}.

Table 1 shows the levels of sulfhydryl compounds, the activity of the associated enzyme GST, and the activity of catalase in the three cell lines. GSH levels of GLC_{16} and GLC_{19} were significantly increased compared to GLC_{14}, whereas the amount of total sulfhydryl groups remained the same. The GST activity increased significantly from GLC_{14} to GLC_{16} to GLC_{19}. Also for catalase a sequential increase in activity was observed in the sequence GLC_{14}, GLC_{16}, and GLC_{19}.

BSO increased both Adr and cDDP induced cytotoxicity (Figs. 4 and 5) whereas X-ray induced cytotoxicity remained the same (not shown).

Fig. 6 shows the Adr induced DNA strand breaks for the three lines. After 1 h incubation with Adr, an increased amount of DNA strand breaks was induced in GLC_{19} compared to

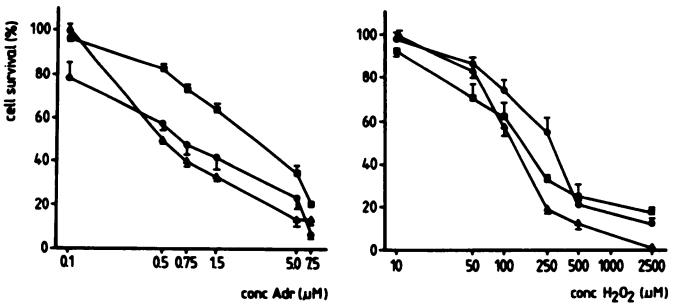

Fig. 1. Microculture tetrazolium assay after 1 h incubation with Adr (*left*) and H_2O_2 (*right*) with GLC_{14} (●), GLC_{16} (■), and GLC_{19} (▲) ($n = 2$–3, in quadruplicate; *bars*, SEM). For Adr, the GLC_{16} values were significantly different from GLC_{14} at 0.1 μM, $P < 0.05$; at 0.5 μM, $P < 0.0005$; at 0.75 μM, $P < 0.0005$; at 1.5 μM, $P < 0.005$; at 7.5 μM, $P < 0.0005$; for GLC_{19} *versus* GLC_{14} at 0.1 μM, $P < 0.005$; at 0.5 μM, $P < 0.05$; at 5.0 μM, $P < 0.05$; at 7.5 μM, $P < 0.005$. For H_2O_2, the GLC_{16} values were significantly different from those for GLC_{14} at 50 μM, $P < 0.025$; at 250 μM, $P < 0.05$; at 1000 μM, $P < 0.05$; at 2500 μM, $P < 0.05$; for GLC_{19} *versus* GLC_{14} at 100 μM, $P < 0.05$; at 250 μM, $P < 0.0005$; at 500 μM, $P < 0.05$; at 1000 μM, $P < 0.05$; at 2500 μM, $P < 0.0025$.

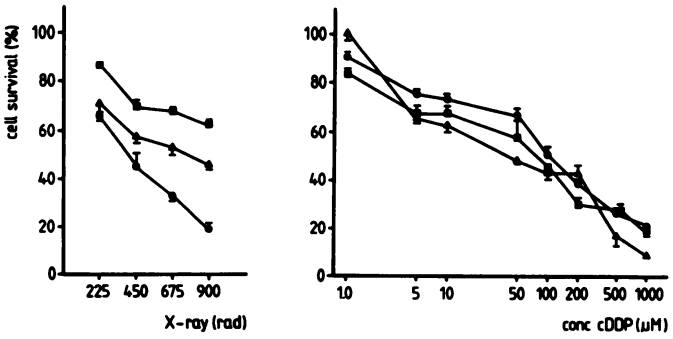

Fig. 2. Microculture tetrazolium assay after X-ray (*left*) and after 1 h incubation with cDDP (*right*) with GLC_{14} (●), GLC_{16} (■), and GLC_{19} (▲) ($n = 2$–3, in quadruplicate; *bars*, SEM). For X-ray, the GLC_{16} values were significantly different from GLC_{14} at 225 rad, $P < 0.0025$; at 450 rad, $P < 0.01$; at 675 rad, $P < 0.0005$; at 900 rad, $P < 0.0025$; for GLC_{19} *versus* GLC_{14} at 225 rad, $P < 0.01$; at 450 rad, $P < 0.025$; at 675 rad, $P < 0.01$; at 900 rad, $P < 0.025$. For cDDP, the GLC_{16} values were significantly different from those for GLC_{14} at 1 μM, $P < 0.05$; at 5 μM, $P < 0.05$; at 50 μM, $P < 0.025$; for GLC_{19} *versus* GLC_{14} at 1 μM, $P < 0.01$; at 10 μM, $P < 0.0025$; at 50 μM, $P < 0.0005$; at 100 μM, $P < 0.05$; at 500 μM, $P < 0.05$; at 750 μM, $P < 0.025$; at 1000 μM, $P < 0.0005$.

Table 1 *Sulfhydryl compounds, the activity of the enzymes GST and catalase, and the presence of the M, 170,000 glycoprotein, as assessed with JSB-1, in the three different cell lines*

	No. of experiments	GLC_{14}	GLC_{16}	GLC_{19}
Total GSH (μg/mg protein)	6	12.9 ± 1.9[a]	15.5 ± 1.4 ($P < 0.05$)[b]	16.6 ± 1.3 ($P < 0.0025$)[c]
Total sulfhydryl groups (μg/mg protein)	8	36.5 ± 9.4	45.7 ± 11.7 (NS)[d]	48.8 ± 19.6 (NS)
GST (nmol CDNB/min/mg protein)	4	13 ± 7	29 ± 4 ($P < 0.0025$)[b]	43 ± 16 ($P < 0.01$)[c]
Catalase (μmol H_2O_2/min/mg protein)	3	0.88 ± 0.19	1.73 ± 0.65 ($P < 0.05$)[b]	3.83 ± 0.81 ($P < 0.0025$)[c]
JSB-1	3	Positive, 0–1%	Positive, 5%	Negative

[a] Mean ± SD.
[b] GLC_{16} *versus* GLC_{14}.
[c] GLC_{19} *versus* GLC_{14}.
[d] NS, not significant; CDNB, 1-chloro-2,4-dinitrobenzene.

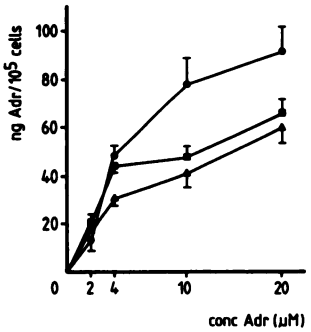

Fig. 3. Intracellular Adr concentrations after 1 h incubation with Adr ($n \geq 6$; *bars*, SEM) for GLC_{14} (●), GLC_{16} (■), and GLC_{19} (▲). For GLC_{16} values were significantly different from GLC_{14} at 10 μM, $P < 0.01$; for GLC_{19} *versus* GLC_{14} at 10 μM, $P < 0.005$; at 20 μM, $P < 0.025$.

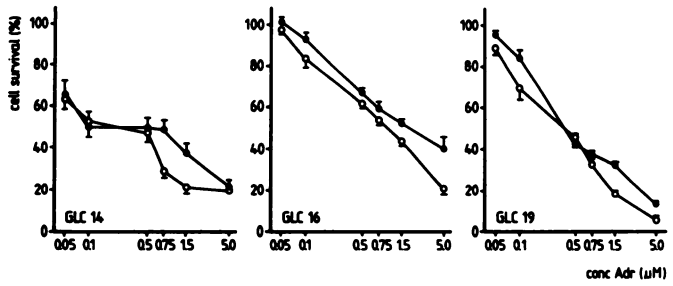

Fig. 4. Effect of pretreatment of the three cell lines with 50 μM BSO for 65 h on Adr induced cytotoxicity measured by microculture tetrazolium assay, without BSO (●), following BSO pretreatment (○) ($n \geq 6$; *bars*, SEM). For GLC_{14} significantly different values were measured at 0.75 μM, $P < 0.005$; and at 1.5 μM, $P < 0.01$. For GLC_{16} at 0.5 μM, $P < 0.05$; at 1.5 μM, $P < 0.0005$; and at 5.0 μM, $P < 0.01$. For GLC_{19} at 0.05 μM, $P < 0.05$; at 0.1 μM, $P < 0.025$; at 0.75 μM, $P < 0.05$; at 1.5 μM, $P < 0.005$; and at 5.0 μM $P < 0.0025$.

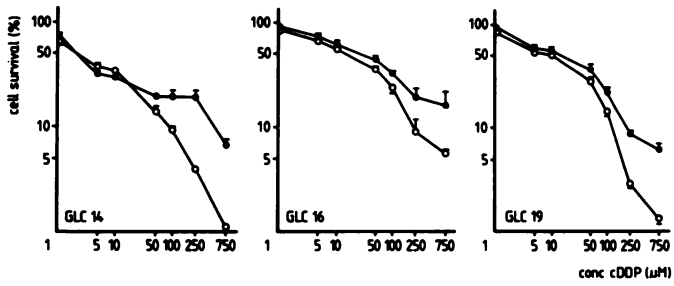

Fig. 5. Effect of pretreatment of the three cell lines with 50 μM BSO for 65 h on cDDP induced cytotoxicity measured by microculture tetrazolium assay, without BSO (●), following pretreatment with BSO (○) ($n \geq 6$; *bars*, SEM). For GLC_{14} significantly different values were observed at 50 μM, $P < 0.005$; at 100 μM, $P < 0.0005$; at 250 μM, $P < 0.0005$; and at 750 μM, $P < 0.0025$. For GLC_{16} at 50 μM, $P < 0.01$; 100 μM, $P < 0.01$; at 250 μM, $P < 0.0125$; and at 750 μM, $P < 0.05$. For GLC_{19} at 50 μM, $P < 0.05$; at 100 μM, $P < 0.0025$; at 250 μM, $P < 0.0005$; and at 750 μM, $P < 0.0025$.

GLC_{14} and GLC_{16}. In none of the three cell lines, Adr induced damage (10 μM, 1 h) could be repaired within 90 min (Fig. 6). For X-ray an identical amount of DNA strand breaks was induced in the three cell lines (Fig. 7). All three cell lines were capable to repair the 900-rad X-ray induced DNA damage completely within 90 min.

DISCUSSION

The three cell lines GLC_{14}, GLC_{16}, and GLC_{19} which were established from one patient during clinical follow-up (7) may permit the study of *in vivo* acquired resistance to cytotoxic drugs and irradiation, provided that the mechanisms concerned remain present during *in vitro* culturing and that *in vivo* conditions do not select out for other clones of cells. The sensitivity of the patient's tumor at the time GLC_{14} was developed is not reflected in a pronounced sensitivity of the cell line for the drugs tested, neither in comparison with the cell lines estab-

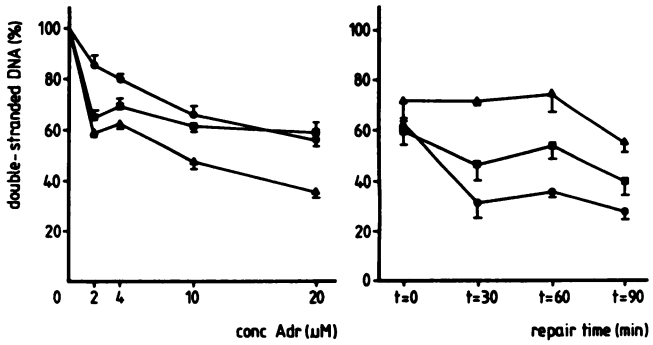

Fig. 6. DNA strand breaks as determined by alkaline unwinding technique, GLC_{14} (●), GLC_{16} (■), and GLC_{19} (▲) after 1 h incubation with Adr ($n \geq 3$; *bars*, SEM). Repair measurements were done after 10 μM Adr for 1 h ($n \geq 3$). For GLC_{16} values were significantly different *versus* GLC_{14}, for DNA breaks, at 2 μM, $P < 0.0005$; and at 4 μM, $P < 0.0025$; for GLC_{19} *versus* GLC_{14} at all incubation points $P < 0.0025$.

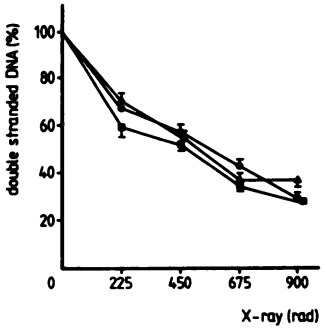

Fig. 7. DNA strand breaks as determined by alkaline unwinding technique, GLC_{14} (●), GLC_{16} (■), and GLC_{19} (▲) were irradiated with different doses ($n \geq 3$; *bars*, SEM).

lished later nor with other cell lines with a known clinical pedigree (1). However, clinically relevant concentrations of Adr may be limited to the lower range of the concentrations tested in Fig. 1, where the sequence of sensitivity correlates with the clinical findings. Mechanisms that could be relevant to the development of resistance that were tested in this study included decreased intracellular drug levels, which are usually a result of an altered membrane transport (2, 22, 23); a changed intracellular enzyme capacity to detoxify electrophilic agents and drug induced free radicals (24, 25); and/or changes in DNA damage or repair capacity (1, 6, 26).

Intracellular Adr levels decrease in the cell lines concomitantly with the emergence of clinical resistance. This is a characteristic of MDR, and the patient was treated with so-called natural products. The cell line GLC_{16} turned out to be more resistant for Adr and X-ray in comparison with GLC_{14} and GLC_{19}. Analysis of the proteins present in multidrug resistant cell lines has revealed one consistent change, overproduction of a M_r 170,000 protein, or P-glycoprotein (27). The stain with JSB-1 antibody detecting the M_r 170,000 glycoprotein (12) was most pronounced in GLC_{16}. Surprisingly no reactivity was seen in GLC_{19}. This last finding does not exclude that MDR plays a role in all three cell lines, but it does show that using only a stain with monoclonal antibody against M_r 170,000 antigen does not predict clinical tumor drug resistance, although we are aware of the fact that, *e.g.*, GLC_{19} could be just another clonal line which has been selected for which handles Adr differently.

Detoxifying systems often increase in capacity during exposure to antitumor therapy (4, 5). The elevated amount of GSH and the increased activity of GST can explain resistance to alkylating agents and agents that act by releasing free radicals. Accordingly, sensitivity increased after exposure of the cell lines to BSO (28, 29). The observed elevation of the catalase activity in the sequence GLC_{14}, GLC_{16}, and GLC_{19} shows that there is in this sequence also an increased capacity to detoxify free radicals, especially hydrogen peroxide.

The amount of X-ray induced DNA strand breaks is not different in the three cell lines, and all lines have an efficient repair of this damage. In contrast, Adr induced DNA strand breaks are not repaired in any of the lines within the observation period of 90 min. The high amount of DNA damage in GLC_{19} did not correlate with the low intracellular drug levels in that line. Probably also the compartmentalization and differences in efflux or metabolism of drugs in the cell can influence their effect.

It is concluded that the exposure of this patient's tumor to combination chemotherapy and radiation coincided with the emergence of tumor cell clones with a number of adaptation mechanisms. From these mechanisms, the intracellular drug restriction and the capacity to detoxify electrophilic agents and drug induced free radicals correlate with the clinical development of resistance of the tumor.

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