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## Establishment of an adriamycin-resistant subline of human small cell lung cancer showing multifactorial mechanisms of resistance.

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## Abstract

A subline highly resistant to Adriamycin (SBC-3/ADM100) was isolated in vitro from the human small cell lung cancer cell line, SBC-3, by culturing in progressively higher concentrations of Adriamycin. The SBC-3/ADM100 cells were 106-fold more resistant to the drug than the parent cells in an inhibitory concentration of 50% determined by the MTT assay. The population-doubling time was much longer in SBC-3/ADM100 than in the parent cells. Northern blot hybridization revealed marked overexpression of the MDR1 mRNA in the resistant cells. P-glycoprotein overexpression and a decrease in intracellular accumulation of Adriamycin were demonstrated in SBC-3/ADM100, indicating that outward drug transport was the major mechanism of resistance in this subline. Additionally, a significant elevation of the intracellular glutathione content coupled with the glutathione S-transferase (GST) pi level and a decrease in DNA topoisomerase II (Topo II) activity were noted in this resistant subline. These results indicate that the mechanism of resistance to Adriamycin is multifactorial; involving altered growth characteristics, an enhanced outward transport, enhanced drug detoxification process, and decreased target enzyme activity. The resistant subline will serve as a useful tool in the search for ways to overcome drug resistance.

**KEYWORDS:** Adriamycin-resistant cell line, MDR1 mRNA, glutathione, glutathione S-transferase, DNA topoisomerase II

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## Establishment of an Adriamycin-Resistant Subline of Human Small Cell Lung Cancer Showing Multifactorial Mechanisms of Resistance

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A subline highly resistant to Adriamycin (SBC-3/ADM100) was isolated *in vitro* from the human small cell lung cancer cell line, SBC-3, by culturing in progressively higher concentrations of Adriamycin. The SBC-3/ADM100 cells were 106-fold more resistant to the drug than the parent cells in an inhibitory concentration of 50 % determined by the MTT assay. The population-doubling time was much longer in SBC-3/ADM100 than in the parent cells. Northern blot hybridization revealed marked overexpression of the *MDR1* mRNA in the resistant cells. P-glycoprotein overexpression and a decrease in intracellular accumulation of Adriamycin were demonstrated in SBC-3/ADM100, indicating that outward drug transport was the major mechanism of resistance in this subline. Additionally, a significant elevation of the intracellular glutathione content coupled with the glutathione S-transferase (GST)  $\pi$  level and a decrease in DNA topoisomerase II (Topo II) activity were noted in this resistant subline. These results indicate that the mechanism of resistance to Adriamycin is multifactorial; involving altered growth characteristics, an enhanced outward transport, enhanced drug detoxification process, and decreased target enzyme activity. The resistant subline will serve as a useful tool in the search for ways to overcome drug resistance.

**Key words :** Adriamycin-resistant cell line, *MDR1* mRNA, glutathione, glutathione S-transferase  $\pi$ , DNA topoisomerase II

Adriamycin (ADM) is an antitumor anthracycline possessing a substantial activity against a wide variety of human neoplasms, and is regarded as one of the most potent drugs in the treatment of small cell lung cancer (SCLC). The development of acquired resistance to ADM, however, is one of the major obstacles to effective chemotherapy. Although a number of possible mechanisms for *in vitro* acquired resistance to the drug have been reported in a variety of tumor cells (1-7) including human SCLC (8-12), one mechanism can not explain complex drug resistance, and much attention has recently been directed to acquired drug resistance with multifactorial mechanisms (13, 14). We obtained a highly ADM-resistant SCLC subline from SBC-3/ADM cells showing a decreased intracellular ADM level (9). Using this highly

resistant subline, we evaluated a) *MDR1* mRNA expression, b) P-glycoprotein (P-gp) expression, c) intracellular drug accumulation, d) glutathione (GSH) and GST- $\pi$  levels (major components concerned in cellular detoxification), and e) DNA Topo II activity, a possible target enzyme of ADM.

### Materials and Methods

**Chemicals and reagents.** The drugs were provided by the following sources: ADM, epirubicin, by Kyowa Hakko, Tokyo, Japan; vincristine, vindesine, 4-hydroperoxycyclophosphamide (an active metabolite of cyclophosphamide), by Shionogi, Osaka, Japan; cisplatin, carboplatin, by Bristol-Myers Squibb, Tokyo; etoposide, by Nippon Kayaku, Tokyo. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was purchased from Sigma Chemical Co., St. Louis, MO, USA.

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*Cell culture and isolation of a highly ADM-resistant subline.* The parent cell line, SBC-3, was established in our laboratory from bone marrow aspirates of a previously untreated patient with SCLC (15). The growth medium was RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (Gibco), penicillin G (100 units/ml), and streptomycin (100 µg/ml). A subline highly resistant to ADM (SBC-3/ADM100) was isolated from SBC-3/ADM cells (9) by continuous exposure to increasing concentrations of ADM, with subsequent cloning, after a total of 16 months. Briefly, SBC-3/ADM cells were initially passaged continuously in a medium that contained 0.05 µM ADM. Stepwise increments of the drug concentration increased up to 5 µM. The cells were then cloned by limiting dilution in a double-layer soft agar system containing 5 µM ADM; a single colony growing in the soft agar from about 100 plated cells was transferred to one well of a 24-multiwell tissue culture plate (Costar, Cambridge, MA, USA) with ADM-free complete culture medium. The cells growing in the well were then passaged continuously. This clonal subline designated SBC-3/ADM100 was 106-fold more resistant to ADM than the parent cells.

*Assay of cytotoxic effect of drugs.* The cytotoxicity of ADM and other drugs was determined by the MTT assay (16) with a slight modification as described previously (17). Dose-response curves were obtained by calculating the percentage of surviving cells, which was expressed as a percentage of the control absorbance at 560 nm. The concentration of each drug necessary to inhibit tumor cell growth by 50 % (IC<sub>50</sub>) was determined from the dose-response curves. The relative resistance of SBC-3/ADM100 to SBC-3 was expressed as the ratio of IC<sub>50</sub>. Determinations were performed in quadruplicate, and the results were confirmed by two to three separate experiments.

*Measurement of the population-doubling time.* The population-doubling time of the cells was determined by the MTT assay. Cells growing in the exponential phase were seeded in the 96-well microplate at 5,000 cells/well and incubated at 37 °C in a humidified atmosphere with 5 % CO<sub>2</sub>. The population-doubling time was calculated from the time course of cell increments in the wells, which was determined by measuring the mean absorbance of eight wells daily for seven days.

*RNA preparation and Northern blot hybridization.* Total cellular RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction as reported by Chomczynski and Sacchi (18). Cells were washed twice with phosphate buffered saline (PBS) and homogenized in 4 M guanidinium isothiocyanate, 25 mM sodium citrate (pH 7.5), 0.5 % sarcosyl and 0.1 M 6-mercaptoethanol. Total cellular RNA was extracted with 0.2 M sodium acetate (pH 4.0), water-saturated phenol and chloroform, then precipitated with 1 volume of isopropanol. The RNA (15 µg) was electrophoresed on a 1 % agarose-formaldehyde gel and transferred to pretreated nylon membrane (Hybond-N, Amersham, Buckinghamshire, UK) in 20x SCC under vacuum (Vacu-Gene XL, Pharmacia Biotechnology Inc, Piscataway, NJ, USA). The membrane was hybridized at 65 °C for 24 h in 5x Denhardt's solution, 6x SCC, 0.5 % SDS, 200 µg/ml denaturated salmon sperm

DNA. The membrane was washed at high stringency and exposed to Fuji RX-film at -72 °C for seven days using intensifying screens. The probe was a <sup>32</sup>P-labeled *MDR1* probe, a polymerase chain reaction-generated 0.8-kb fragment, which was labeled using the Random Primed Labeling Kit ([ $\alpha$ -<sup>32</sup>P]dCTP, Boehringer, Mannheim, Germany).

*Detection of P-gp by flow cytometry.* Cells in the exponential growth phase were washed and resuspended in PBS supplemented with 0.2 % bovine serum albumin (BSA) and 0.1 % azide. They were allowed to react on ice for 30 min with 20 µg/ml MRK16 mAb (19), a mouse antihuman P-gp mAb (generous gift from Dr. T. Tsuruo, Applied Microbial Institute, Tokyo University) or mouse IgG2a, as a control, at 1 × 10<sup>6</sup> cells/ml in a volume of 250 µl. After washing twice with cold PBS supplemented with 0.2 % BSA and 0.1 % azide, the cells were allowed to react on ice for 30 min with 40 µg/ml fluorescein-isothiocyanate conjugated goat anti-mouse IgG F(ab')<sub>2</sub> fragment (TAGO, Burlingame, CA, USA). They were washed twice with the same solution, passed through a 60 µm nylon mesh and resuspended. Using a FACStar flow cytometer (Becton Dickinson, Mountain View, CA), fluorescence histograms were analyzed after gating on both forward and rectangular light scatter characteristics to avoid cell debris. Fluorescence intensity was represented in log units over a 4-log scale. All the data are based on a 10,000 gated cell count with the use of 256 channels.

*Uptake study of ADM.* Intracellular accumulation of ADM was determined by flow cytometry as described by Nooter (20). Cells growing exponentially were harvested and incubated at 5 × 10<sup>5</sup> cells/ml in 0.5 µM ADM-containing RPMI-1640 medium at 37 °C for various periods. After incubation, the cells were washed twice with ice cold PBS to remove extracellular ADM, passed through a 60 µm nylon mesh and resuspended. The intracellular ADM level was measured in terms of fluorescence intensity excited with 488 nm laser beam using a FACStar flow cytometer. A Consort 30 program made it possible to determine the provisional mean intracellular ADM level by generating fluorescence histograms. A net intracellular ADM level in treated cells was obtained by subtracting the mean autofluorescence of untreated cells from the provisional mean intracellular ADM level.

*Intracellular GSH and GST- $\pi$  levels.* Cells washed three times in cold PBS were sonicated for 30 sec using a Bioruptor (Model UC-100D, Olympus, Tokyo). The supernatant obtained following 7,000 g centrifugation for 5 min was assayed for intracellular GSH and GST- $\pi$  content. GSH was determined according to the method described by Tietze (21), and the GST- $\pi$  level was measured using a GST- $\pi$  EIA kit (one step sandwich EIA; Dainippon Seiyaku, Osaka). Each experiment was carried out in duplicate and repeated three times.

*DNA Topo II assay.* Crude nuclear extracts for the assay of DNA Topo II activity were prepared according to the method reported by Tsutsui et al. (22), and all the following procedures were performed on ice. Exponentially growing cells were washed twice with PBS and resuspended in 1 ml containing 10 mM MgCl<sub>2</sub> and 0.35 % Triton 100-X. Nuclei were pelleted by centrifugation at

1,000 *g* for 10 min and resuspended to a final concentration of 10 mg DNA/ml in extraction buffer [20 mM Tris-HCl (pH 7.5), 0.35 mM NaCl, 140 mM 2-mercaptoethanol, 50  $\mu$ g/ml BSA (DNase-free, Takara Shuzou, Tokyo)] and then incubated for 30 min with periodic mixing. Nuclear extract was obtained by centrifugation at 12,000 *g* for 5 min. The protein concentration of the extracts was adjusted to an equivalence with the same buffer.

Topo II activity was assayed by decatenation of kinetoplast DNA (kDNA) into free minicircles (23). In brief, decatenation was carried out in a total of 20  $\mu$ l reaction mixture containing 50 mM Tris-HCl (pH 8.0), 120 mM KCl, 10 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, 0.5 mM EDTA (pH 8.0), 0.5 mM ATP, 30  $\mu$ g/ml BSA, 0.5  $\mu$ g catenated kDNA (TopoGEN, Columbus, Ohio,

USA), and 1  $\mu$ l of serially diluted nuclear extract. After a 40 min incubation at 30 °C, the reaction mixture was treated with 0.66 % sodium dodecylsulfate and 33 mg/ml proteinase K (Sigma), and minicircles were resolved by electrophoresis on 0.8 % agarose gels. The gels were stained with 0.5  $\mu$ g/ml ethidium bromide and photographed under UV light.

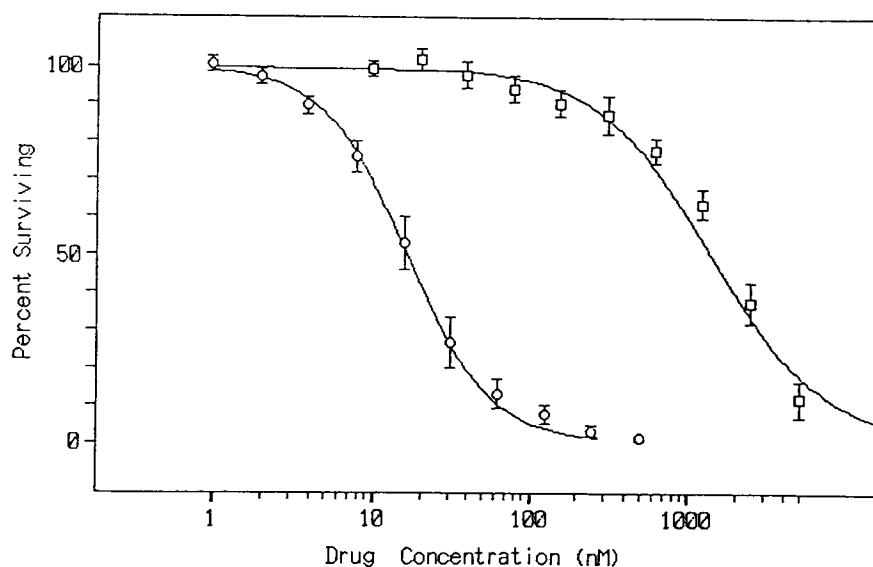
## Results

*Relative resistance to ADM and other drugs in SBC-3/ADM100.* The relative resistance to ADM

**Table 1** Cross-resistance pattern of various cytotoxic agents in SBC-3/ADM100

Drugs	IC <sub>50</sub> ( $\mu$ M $\pm$ SD) <sup>a</sup>		Relative resistance <sup>b</sup>
	SBC-3	SBC-3/ADM100	
Adriamycin	0.0188 $\pm$ 0.0045	1.93 $\pm$ 0.383	106
Epirubicin	0.0205 $\pm$ 0.0098	2.28 $\pm$ 1.15	111
Vincristine	0.00265 $\pm$ 0.00091	1.22 $\pm$ 0.873	538
Vindesine	0.00418 $\pm$ 0.00177	1.90 $\pm$ 1.68	709
Etoposide	0.323 $\pm$ 0.121	13.8 $\pm$ 1.89	47.5
Cisplatin	0.605 $\pm$ 0.154	0.836 $\pm$ 0.485	1.38
Carboplatin	3.62 $\pm$ 1.91	8.6 $\pm$ 5.0	3.81
4-HC <sup>c</sup>	0.710 $\pm$ 0.329	0.918 $\pm$ 0.195	1.39

a: Assessed in quadruplicate by the MTT assay in two or more separate experiments. b: Mean ratio of IC<sub>50</sub> SBC-3/ADM100 to IC<sub>50</sub> SBC-3. c: 4-Hydroperoxycyclophosphamide. SBC-3/ADM100: Subline highly resistant to Adriamycin. SD: standard deviation.



**Fig. 1** Cell survival curves of SBC-3 (circles) and SBC-3/ADM100 (squares) to ADM determined by the MTT assay. In terms of IC<sub>50</sub>, SBC-3/ADM100 were 106-fold more resistant to ADM than SBC-3.

and the other drugs in SBC-3/ADM100 was determined using the MTT assay (Table 1). In terms of  $IC_{50}$ , SBC-3/ADM100 showed a 106-fold relative resistance to ADM compared with SBC-3 (Fig. 1). Marked cross-resistance was found to epirubicin, vincristine, vindesine, and etoposide, while there was much less resistance to cisplatin, carboplatin, and 4-hydroperoxycyclophosphamide.

*Growth of SBC-3 and SBC-3/ADM100.* When grown in RPMI-1640 complete medium without ADM, the population-doubling time of SBC-3/ADM100 was 46

h, much longer than that of SBC-3 (22h).

*MDR1 mRNA expression.* Expression of *MDR1* mRNA in both SBC-3 and SBC-3/ADM100 was determined by Northern blotting. As shown in Fig. 2, SBC-3/ADM100 displayed an abundant mRNA for *MDR1*, whereas SBC-3 showed no detectable expression of the *MDR1* mRNA.

*P-gp expression.* Expression of P-gp in SBC-3 and SBC-3/ADM100 was determined by flow cytometry using MRK16 mAb (Fig. 3). Whereas the fluorescence

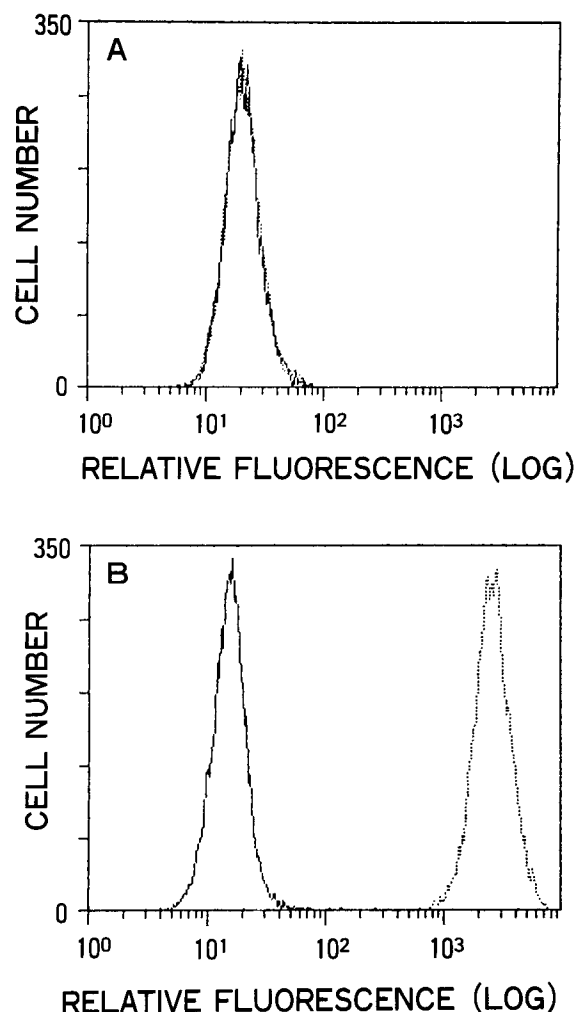
**Table 2** Intracellular GSH and GST- $\pi$  levels in SBC-3 and SBC-3/ADM100

	SBC-3	SBC-3/ADM100
Intracellular GSH ( $\mu\text{g}/\text{mg}$ protein)	< 0.14	3.42
Intracellular GST- $\pi$ ( $\mu\text{g}/\text{mg}$ protein $\pm$ SD)	$3.31 \pm 1.33^a$	$5.11 \pm 1.82^b$

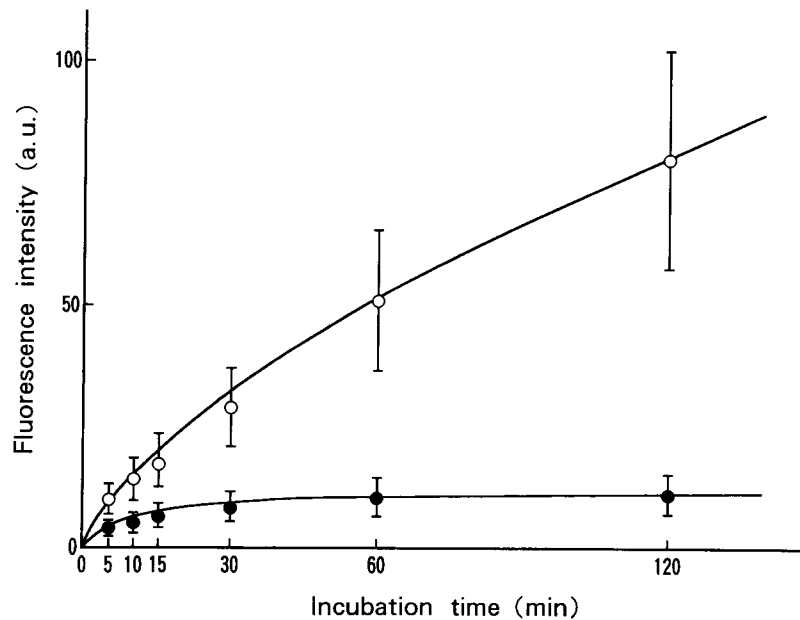
*a vs b:* significant ( $p < 0.01$ ). GSH: glutathione. GST: glutathione S-transferase. SBC-3/ADM100: See Table 1.



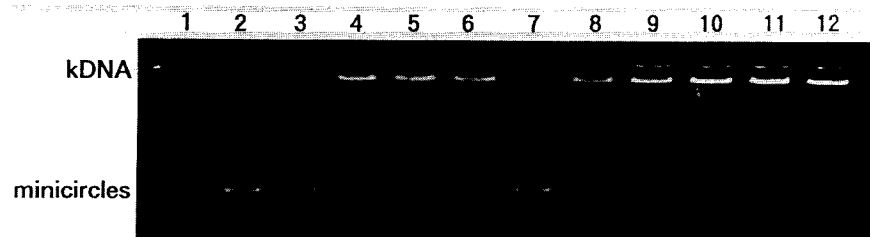
**Fig. 2** Expression of *MDR1* mRNA in SBC-3 and SBC-3/ADM100 was determined by Northern blotting. SBC-3/ADM100 overexpressed *MDR1* mRNA, while in SBC-3 it was undetectable.



**Fig. 3** Expression of P-gp in SBC-3 (A) and SBC-3/ADM100 (B) was analyzed by flow cytometry. A solid line represents a fluorescence histogram by control Ab (mouse IgG2a), and a dotted line represents a fluorescence histogram by MRK16 mAb. SBC-3 had almost identical fluorescence histograms for control Ab and MRK16 mAb, while SBC-3/ADM100 displayed a definite increment in fluorescence for MRK16 mAb alone.



**Fig. 4** Time course of intracellular accumulation of ADM in SBC-3 (open circles) and SBC-3/ADM100 (closed circles). Intracellular ADM levels were determined by flow cytometry. In the presence of  $0.5 \mu\text{M}$  ADM in the incubation medium, the intracellular ADM level at 60 min was 5-fold lower in SBC-3/ADM100 than in SBC-3, and it was 8-fold lower in SBC-3/ADM100 than in SBC-3 at 120 min.



**Fig. 5** Topo II activity in nuclear extracts from SBC-3 and SBC-3/ADM100 was monitored by the decatenation assay. Reaction mixtures containing kDNA and various dilutions of nuclear extracts from SBC-3 (lanes 1-6) or SBC-3/ADM100 (lanes 7-12) were incubated for 40 min at  $30^\circ\text{C}$  and processed for gel electrophoresis. The amounts of nuclear extract protein added to the reaction mixture were: 1,500 (lanes 1 and 7), 750 (lanes 2 and 8), 375 (lanes 3 and 9), 188 (lanes 4 and 10), 94 (lanes 5 and 11), and 47 ng (lanes 6 and 12).

histograms of SBC-3 showed almost identical patterns for MRK16 mAb and control antibody (mouse IgG2a), that of SBC-3/ADM100 for MRK16 mAb displayed a definite increment in fluorescence, indicating overexpression of P-gp on the resistant cells.

**Intracellular uptake of ADM.** The time course of intracellular ADM accumulation of SBC-3 and SBC-3/ADM100 was compared in terms of the fluorescence intensity determined by flow cytometry (Fig. 4). The

ADM level in SBC-3/ADM100 reached a steady state of 10 arbitrary units (a.u.) at 60 min, whereas that in SBC-3 increased almost linearly during the period tested and reached 90 a.u. at 120 min. The intracellular ADM level in SBC-3/ADM100 was 5-fold lower at 60 min, and 8-fold lower at 120 min than that in SBC-3.

**Intracellular GSH and GST- $\pi$  levels.** The intracellular GSH and GST- $\pi$  levels in SBC-3/ADM100 were significantly higher than those of the parent cells,

SBC-3 (Table 2).

**DNA Topo II activity.** Fig. 5 shows the electrophoretic patterns of decatenated kDNA. Topo II activity was one-half or less in SBC-3/ADM100 than that in SBC-3 by comparing band intensities of minicircles in the serial dilutions. In SBC-3, minicircles were formed in the presence of 375 ng of the nuclear extract (Fig. 5: lane 3), whereas in SBC-3/ADM100 minicircle formation was scarcely observed in the presence of 750 ng of the nuclear extract (Fig. 5: lane 8).

## Discussion

Continuous culture of SBC-3/ADM (9) with further increasing concentrations of ADM produced a subline highly resistant to ADM, SBC-3/ADM100. The resistant subline was 106-fold more resistant to ADM than its parent cell line, SBC-3. SBC-3/ADM100 displayed the typical multiple drug resistance (MDR) genotype and phenotype, showing overexpression of *MDR1* mRNA and P-gp. This resistant subline had a decreased intracellular drug level and marked cross-resistance to a panel of anthracyclines (data not shown, except for epirubicin), vinca alkaloids and etoposide. Active outward drug transport plays a major role in this resistant subline as described previously in other reports on SCLC (1-3, 9-12). However, there appeared to be a discrepancy between the relative resistance to ADM (106-fold) determined by the MTT assay, and the decreased intracellular drug level (one eighth or less in SBC-3/ADM100 than in SBC-3) determined by flow cytometry, which prompted us to search for other factors to which the resistance could be attributed.

Mechanisms other than alternation responsible for drug accumulation in ADM resistant cells are reported to be a changed intracellular enzyme capacity to detoxify free radicals and a change in Topo II activity (24). Detoxifying systems often increase in capacity during exposure to antitumor therapy (4-6). The increase in GSH and GST- $\pi$  can explain resistance to alkylating agents, platinum compounds and drugs that act by releasing free radicals (4, 5, 25). The extent to which the detoxifying system is responsible for the development of ADM-resistance in SBC-3/ADM100 is unclear. However, this system appears to be implicated in the relative resistance (1.4 to 3.8) of SBC-3/ADM100 to platinum compounds (cisplatin and carboplatin) and to alkylating agents such as

4-hydroperoxycyclophosphamide.

Topo II is a common target for a number of intercalative and non-intercalative antitumor agents (26-28). ADM is capable of stabilizing the Topo II-DNA cleavage complex, which can lead to the formation of DNA breaks and DNA-protein crosslinks (26, 27). In the present study, we found that Topo II activity was one-half or less in SBC-3/ADM100 than in SBC-3. However, changes in the growth kinetics of SBC-3/ADM100, the longer population-doubling time, may account for the reduction in Topo II activity, because the activity and amount of the enzyme fluctuate with cell cycle progression, and the peak activity and the maximal content occur during the G<sub>2</sub>/M phase (29, 30). SBC-3/ADM100 was notably resistant to etoposide, a Topo II inhibitor. However, the resistance might be interpreted as a consequence of decreased intracellular accumulation rather than decreased Topo II activity. Nevertheless, it is evident that the decreased activity of the enzyme is a definite determinant of ADM-resistance. Deffie *et al.* reported a direct correlation between DNA Topo II activity and cytotoxicity using ADM-sensitive and ADM-resistant P388 leukemia cells (7). De Jong *et al.* described ADM-resistant human SCLC cells which had a reduced DNA Topo II activity and drug induced cleavage (8).

The mechanisms of resistance in SBC-3/ADM100 are multifactorial, involving decreased intracellular drug accumulation, enhanced detoxification and decreased Topo II activity. Drug resistance is a timely topic, and it is our hope that the resistant cells reported here will serve as a useful tool in the search for ways to overcome drug resistance.

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