Multidrug Resistance in a Human Small Cell Lung Cancer Cell Line Selected in Adriamycin

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INTRODUCTION

Lung cancer is the leading cause of cancer death in American men aged 35 or older and is increasing in incidence in women in whom it is predicted that it will surpass breast cancer as the leading cause of cancer death during the 1980s (1). Twenty-five percent of autopsied lung cancer patients have the histological type designated “small cell.” The histological distinction between SCLC and nonsmall cell lung cancer is of major clinical importance because of the different responses to therapy of these two tumor types. Non-SCLC is treated with surgery and/or radiotherapy, SCLC does not respond well to surgery or radiotherapy alone but regimens of combination chemotherapy or radiotherapy and radiotherapy alone have resulted in increases in survival to 1 year compared to 7 weeks with supportive care only. However, subsequent relapse is common with a 2-year survival of only 10% (2). The high rate of relapse and failure of chemotherapy is believed to be due to a large degree to drug resistant cells either existing prior to or arising during treatment.

A MDR phenotype has been observed in a variety of mammalian cell lines which provides a model for the study of this clinical problem (3). Even though selected by a single agent, these cell lines are resistant to a wide range of chemically and functionally unrelated drugs. In most cases, this cross-resistance has been closely associated with the increased expression of P-glycoprotein, a Mr 170,000 plasma membrane glycoprotein (3, 4). Because of the clinical importance of MDR in SCLC, we have derived a MDR variant (designated H69AR) of the human SCLC cell line NCI-H69 using doxorubicin (ADM) as the selecting drug. In this paper we describe the preliminary characterization of this cell line and show that, although it exhibits the MDR phenotype, enhanced expression of P-glycoprotein is not detectable.

MATERIALS AND METHODS

Drugs. Gramicidin D, 1-dehydrotestosterone, lidocaine, 5ß-pregnane-3α-ol-20-one, 5α-pregnane-3ß-ol-20-one, deoxycoformicine, dexamethasone, dibucaine, tetracaine, and procaine were obtained from Sigma Chemical Co. Acivicin was the generous gift of Dr. R. A. Whitney, Department of Chemistry, Queen’s University. The remaining drugs were obtained from the pharmacy at the Kingston Regional Cancer Centre.

Cell Culture. The human SCLC cell line NCI-H69 was kindly provided by J. Minna (National Cancer Institute, Bethesda, MD). It was routinely cultured in RPMI 1640 medium (GIBCO) supplemented with 5 or 10% heat-inactivated FBS, 4 mM l-glutamine, 50 μM 2-mercaptopethanol, and 1 mM sodium pyruvate. CHO cell lines AuxBl and CHOC5 (5) were cultured in minimal essential medium supplemented with 10% FBS, 4 mM l-glutamine, 50 μM 2-mercaptopethanol, and 1 mM sodium pyruvate. Cultures were checked monthly for Mycoplasma contamination using the 4’,6-diamidino-2-phenylindole DNA-binding assay (6) and found to be negative.

A multidrug resistant subline of H69 was obtained by culturing the cells in gradually increasing doses of ADM. After 8 months, cells which grew in 0.4 μM ADM were obtained. After a further 6 months, cells which grew in 0.8 μM ADM were obtained. This cell line has been designated H69AR and has been maintained by alternate feedings with drug-free medium or medium containing 0.8 μM ADM.

The stability of the resistant phenotype was determined by culturing continuously in medium with either 0.8 μM ADM or no drug and assessing relative resistance after various periods of time up to 5 months.

Attempts to derive multidrug resistant variants of other SCLC lines including MAR (a generous gift of Prof. A. Neville, Ludwig Institute for Cancer Research, London, United Kingdom), NCI-H209, NCI-H128 (generous gifts of J. Minna, National Cancer Institute), and QU-AD (7) in liquid culture or in soft agar have been unsuccessful to date.

Growth Curve. The growth curves of H69AR and H69 were determined by seeding 1 x 10⁶ cells/ml in triplicate wells in a 24-well plate (Costar). Cell counts were done on days 3, 4, and 7 using a hemocytometer with trypan blue exclusion as an indicator of viability.

Tumorigenicity in Athymic Mice. The tumorigenicity of H69 and H69AR was determined by s.c. injection of 1 x 10³ viable cells of each type in a volume of 0.2 ml PBS into the left flank of 5- to 6-week-old male BALB/c nu/nu mice. Tumor size was measured in two dimensions, and the area was estimated. Tumors that developed after injection of H69AR were excised, placed into culture, and tested for sensitivity to ADM after 4 weeks. The experiment was performed three times with 3–4 mice in each experimental group.

Chemosensitivity Testing. The resistance of H69AR to other antineo-
plastic agents and its collateral sensitivity to a number of anesthetics and steroids were tested using the MTT assay (8). In brief, H69 and H69AR cells were harvested by centrifugation 48–72 h after feeding and plated at 2.5 x 10^4 cells/well in 96-well plates. Preliminary experiments showed that cultures initiated at this cell density continue to grow exponentially for at least 7 days. After incubation at 37°C for 2–4 h, drugs were added and the plate incubated at 37°C for 7 days. Three h before the end of drug exposure time, 25 µl of MTT (Sigma; 2 mg/ml in PBS) were added and the plate incubated at 37°C for an additional 3 h. Isopropanol:1 HCl (25:1) was added to solubilize the formazan crystals, and then the absorbance at 570 nm was determined using a Dynatech MR600 microtitre plate reader. Within each experiment, determinations were done in quadruplicate, and each drug was tested in at least two separate experiments in most cases. Controls included wells with cells but no drugs (base line) and wells with medium and the highest drug concentration but no cells. The percentage of viability was expressed as a percentage of the base-line absorbance at 570 nm. The relative resistance of H69AR compared to H69 is expressed as the ratio of drug concentrations which decrease the base-line absorbance by 50%. The H69AR cell line was cultured in drug-free medium at least 48 h before testing.

Cell ELISA. The expression of P-glycoprotein as detected by the MAB C219 (9) was assessed in a cell ELISA (10). H69, H69AR, AuxB1, and CH4C5 were washed with PBS and fixed in 70% methanol at −20°C for 5 min. The cell concentration was adjusted such that 5 x 10^4 cells in 50 µl PBS were layered per well in a 96-well plate (Falcon 3912). The plates were dried overnight in a 37°C warm room. The cells were rehydrated in PBS and nonspecific binding was blocked with 1% bovine serum albumin/5% normal goat serum. MAB C219, an irrelevant MAB 1-7-1 (11), or medium were added to the wells and binding of antibody detected using horseradish peroxidase conjugated goat anti-mouse IgG plus IgA plus IgM affinity purified F(ab') fragments (Cappel No. 23470) with o-phenylenediamine and hydrogen peroxide used as substrates. Color development was measured by scanning at 490 nm on a Dynatech MR600 microtitre plate reader.

P-Glycoprotein Detection by Western Blotting. Radioiodinated MAB C219 and C494 were used to detect P-glycoprotein in crude membrane preparations of H69 and H69AR as described previously (9). Membrane preparations of AuxB1 and CH4C5 were included as controls in all steps of the procedure. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using a modification (12) of the procedure of Fairbanks et al. (13) and the gel was replica blotted onto nitrocellulose paper essentially by the method of Towbin et al. (14). Nonspecific binding of antibody was blocked with 10% bovine serum albumin/5% normal goat serum. MAB 1-7-1 (11), or medium were added to the wells and binding of antibody detected using horseradish peroxidase conjugated goat anti-mouse IgG plus IgA plus IgM affinity purified F(ab') fragments (Cappel No. 23470) with o-phenylenediamine and hydrogen peroxide used as substrates. Color development was measured by scanning at 490 nm on a Dynatech MR600 microtitre plate reader.

RESULTS

A multidrug resistant variant of H69 was obtained by culturing these cells in gradually increasing doses of ADM up to 0.8 µM after a total of 14 months.

Relative Resistance to ADM. The relative resistance to ADM of H69AR compared to H69 was determined using the MTT assay (Fig. 1). In this experiment the ratio of the ID_{50} for each cell line indicates a 32-fold relative resistance to ADM of H69AR compared to H69. The results from additional experiments are presented in Tables 2 and 3.

Growth of H69 and H69AR in Vitro. When grown in RPMI/10% FBS in the absence of ADM and at a starting cell density of 1 x 10^5 cells/ml, H69 and H69AR have the same rate of growth with a doubling time of about 24 h (Fig. 2).

Tumorigenicity. The tumorigenicity of H69 and H69AR was assessed by measuring tumor size in BALB/c nu/nu mice after s.c. inoculation of 1.3 x 10^5 cells (Fig. 3). In three separate experiments, using a total of 11 mice given injections of H69 and 12 mice given injections of H69AR, there was no significant difference in the rate of tumor growth.

Stability of the Resistant Phenotype. The drug resistance phenotype of H69AR was stable when these cells were passaged in BALB/c nu/nu mice as solid tumors and returned to culture for 4 weeks in the absence of ADM before testing for drug resistance (Fig. 1).

The resistance of H69AR which had been grown in the absence of drug was compared to H69AR grown continuously in 0.8 µM ADM using the MTT assay (Table 1). By 35 days of culture without ADM, the ID_{50} of H69AR cells had decreased to about 60% of that of H69AR cultured continuously in the presence of 0.8 µM ADM. Although there was some variation, the resistance of cells cultured without ADM remained at about 60% of those grown continuously in ADM up to 181 days in culture.

Multidrug Resistance of H69AR. To determine whether H69AR exhibited the MDR phenotype, the relative resistance of H69AR compared to H69 was assessed with a panel of drugs (Table 2). These results show that H69AR expressed the MDR phenotype as it is cross-resistant to anthracycline analogues including daunomycin, epirubicin, menogaril, mitoxantrone, as well as to acivicin, etoposide, gramicidin D, colchicine, and the Vinca alkaloids, vincristine and vinblastine.

Although H69AR was resistant to colchicine and vincristine, the dose/response curves to these drugs were unusual. H69 cells were very sensitive to these drugs (ID_{50} < 10^{-5} µM). With H69AR cells, the viability decreased to a certain level (usually less than 50%) and then did not decrease any further with an increase in drug dose. For example, in one experiment, viability remained at 35% of control values from 0.001–100 µM colchi-
**Table 2** Relative resistance of H69AR compared to H69

<table>
<thead>
<tr>
<th>Drug</th>
<th>ID₅₀ (µM) H69AR</th>
<th>ID₅₀ (µM) H69</th>
<th>Relative resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adriamycin</td>
<td>3.16</td>
<td>0.0316</td>
<td>100.0</td>
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<tr>
<td></td>
<td>0.825</td>
<td>0.020</td>
<td>41.3</td>
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<td></td>
<td>1.99</td>
<td>0.025</td>
<td>79.6</td>
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<td>Daunomycin</td>
<td>3.98</td>
<td>0.047</td>
<td>84.7</td>
</tr>
<tr>
<td></td>
<td>0.348</td>
<td>&lt;0.001</td>
<td>&gt;348.0</td>
</tr>
<tr>
<td></td>
<td>0.316</td>
<td>0.0005</td>
<td>632.0</td>
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<tr>
<td>Epirubicin</td>
<td>0.619</td>
<td>&lt;0.001</td>
<td>&gt;619.0</td>
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<td>0.468</td>
<td>0.0003</td>
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<td></td>
<td>0.631</td>
<td>6.3 x 10⁻³</td>
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</tr>
<tr>
<td></td>
<td>0.422</td>
<td>0.008</td>
<td>52.8</td>
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<td></td>
<td>0.398</td>
<td>0.004</td>
<td>99.5</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>0.100</td>
<td>0.631</td>
<td>15.8</td>
</tr>
<tr>
<td></td>
<td>0.015</td>
<td>&lt;0.001</td>
<td>&gt;1.5</td>
</tr>
<tr>
<td>Colchicine</td>
<td>0.0006</td>
<td>&lt;0.001</td>
<td>&gt;6.0</td>
</tr>
<tr>
<td></td>
<td>&gt;1.00</td>
<td>&lt;0.0001</td>
<td>&gt;10⁴</td>
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<tr>
<td></td>
<td>0.0007</td>
<td>10⁻⁷</td>
<td>7,000.0</td>
</tr>
<tr>
<td>Vincristine</td>
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<td>5 x 10⁻⁴</td>
<td>112.0</td>
</tr>
<tr>
<td></td>
<td>7.9</td>
<td>3.2</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>5.5 x 10⁻⁴</td>
<td>&lt;1 x 10⁻³</td>
<td>&gt;55.0</td>
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<td>Vinblastine</td>
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<td>2.82 x 10⁻³</td>
<td>15.8</td>
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<td>Etoposide</td>
<td>6.3</td>
<td>0.39</td>
<td>16.2</td>
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<td>13.2</td>
<td>0.22</td>
<td>59.0</td>
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<td>5-Fluorouracil</td>
<td>24.0</td>
<td>13.5</td>
<td>1.8</td>
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<tr>
<td>Bleomycin</td>
<td>1.6</td>
<td>.63</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
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<td>Carboplatin</td>
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<td>1.0</td>
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<td></td>
<td>8.0</td>
<td>0.7</td>
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</tr>
<tr>
<td></td>
<td>28.2</td>
<td>1.78</td>
<td>15.9</td>
</tr>
<tr>
<td>Gramicidin D</td>
<td>0.010</td>
<td>&lt;0.003</td>
<td>&gt;3.3</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>1.12 x 10⁻³</td>
<td>22,430.0</td>
</tr>
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</table>

* Assessed in quadruplicate by the MTT assay. Each line is the result from one experiment.

* Ratio of ID₅₀H69AR/ID₅₀H69.

**Table 3** H69AR tested for collateral sensitivity to various drugs

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Drug</th>
<th>ID₅₀ (µM) H69AR</th>
<th>ID₅₀ (µM) H69</th>
<th>Relative resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Adriamycin</td>
<td>1.58</td>
<td>0.0158</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>1-Dehydrotestosterone</td>
<td>56.0</td>
<td>79.0</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>5β-Pregn-3α-ol-20-one</td>
<td>32.0</td>
<td>32.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>5β-Pregn-3β-ol-20-one</td>
<td>28.0</td>
<td>28.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Deoxycortisone</td>
<td>38.0</td>
<td>38.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Dexamethasone</td>
<td>100.0</td>
<td>100.0</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>Adriamycin</td>
<td>1.0</td>
<td>0.1</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>Lidocaine</td>
<td>1260.0</td>
<td>2000.0</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>Dibucaine</td>
<td>40.0</td>
<td>40.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Tetracaine</td>
<td>178.0</td>
<td>178.0</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>Adriamycin</td>
<td>0.38</td>
<td>0.006</td>
<td>63.0</td>
</tr>
<tr>
<td></td>
<td>Procaine</td>
<td>1580.0</td>
<td>1580.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* Adriamycin is included in each experiment as a positive control, demonstrating the relative resistance of H69AR to this drug.

* Assessed in quadruplicate by MTT assay.

* Ratio of ID₅₀H69AR/ID₅₀H69.

Collateral Sensitivity. To determine whether H69AR exhibited collateral sensitivity, the effect of a panel of steroids and anesthetics on cell viability was assessed (Table 3). H69AR showed a slightly enhanced sensitivity to 1-dehydrotestosterone and to lidocaine compared to H69 (relative resistance 0.71 and 0.63, respectively). H69 and H69AR were equally sensitive to 5β-pregn-3β-ol-20-one, 5β-pregn-3α-ol-20-one, deoxycorticosterone, dexamethasone, dibucaine, tetracaine, and procaine.

P-Glycoprotein. P-Glycoprotein is recognized in Chinese and Syrian hamster, mouse, and human MDR cell lines by MAb C219, and in Chinese and Syrian hamster and human cell lines by MAB C494 (9). To determine whether H69AR expressed P-glycoprotein, H69AR and H69 were tested in a cell ELISA using MAB C219 (Fig. 4). AuxBl was negative and CHRC5 was positive for P-glycoprotein as expected. Expression of P-gly-

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**Fig. 2.** Growth of H69 and H69AR in vitro in the absence of ADM. Cultures were set up in triplicate wells at 1 x 10⁶ cells/ml on day 0. Results are from one experiment. O, H69AR; •, H69, mean ± SE (bars). **Fig. 3.** Tumorigenicity of H69 and H69AR cells in nude mice. Each mouse received a s.c. inoculation of 1.3 x 10⁷ cells in 0.2 ml PBS on day 0. O, H69; •, H69AR. Curves, tumor growth in an individual mouse. Results are from one experiment. Similar results were obtained in two additional experiments.
The blot was incubated with "I-labeled C219 or C494. Auto-

coprotein was not detectable above background levels in either

iiM ADM. When compared to its parent line, H69AR is 10- to

gradually increasing doses of ADM we have obtained over a

extracts of CHRC5 as expected, but not in AuxBl, H69, or

radiographs showed a band corresponding to P-glycoprotein in

phoretic gels, and replica blotted on to nitrocellulose paper.

CHRC5, H69, and H69AR were made, separated on electro-

immunoblotting. Crude membrane fractions of AuxBl,

included wells with an irrelevant MAb (1-7-1) or no first antibody.

ELISA. Bars, mean absorbance of tests performed in duplicate. Negative controls

cross-resistance to vincristine when cell survival was assessed

cell carcinoma line and its VP 16-213 resistant variant VPR,

DISCUSSION

H69AR (results not shown).

phenotype which has been described in numerous rodent and

acivicin, and etoposide (Table 2) and thus possesses the MDR

panel of anthracycline analogues, Vinca alkaloids, colchicine,

patterns of growth including increased cell-substrate and cell-
cell adhesiveness and weak tumorigenicity in vivo compared to

sensitive cells. H69AR also displayed increased cell-cell adher-
siveness, growing in spheroids rather than loose aggregates as
did the parent H69. However, in contrast to the results of

Biedler et al. (29), there was no significant difference between

H69 and H69AR growth rates in vitro or tumorigenicity in vivo.

Similar in vitro growth characteristics for paired sensitive and

resistant human leukemia cell lines have been observed previ-

ously (17, 19). In contrast, the human sarcoma MDR cell line,

MES-SA, described by Harker and Sikic (21), and the SCLC

MDR cell line, H69/LX4, described by Twentyman et al. (22)
grew at about 70% of the rate of the parent line in vitro. Whether

the altered growth characteristics observed in these cells reflect

cell membrane changes which may be involved in the mecha-
nism of MDR is unknown and remains to be determined.

The experiments aimed at determining the stability of mul-
tidrug resistance suggest that the drug resistant phenotype of

H69AR may be complex and possibly has two components.

One component is lost within 35 days of culture in the absence

of ADM, resulting in a 40% decrease in resistance compared
to cells in continuous culture in drug. The second component

is relatively stable, so that cells cultured in drug free medium

for up to 181 days are still 60% as resistant as those cultured

with ADM. Similarly, Twentyman et al. (22) found a partial

loss of resistance in H69/LX4 within 3 weeks, but no further

loss up to 9 weeks in drug-free medium.

The MDR phenotype has been associated with changes in

cellular protein composition and gene amplification (30, 31).

In particular, the expression of P-glycoprotein, a M, 170,000

plasma membrane-associated glycoprotein, is elevated in MDR

cell lines (4, 16, 21, 25, 26, 32–34). In addition, P-glycoprotein

overexpression has been detected in tumor samples from pa-

tients with ovarian carcinoma who were resistant to multidrug

therapy (35). Although the exact function of P-glycoprotein is

unknown, it is the molecular alteration found to be most

consistently associated with the MDR phenotype (4). The level

of P-glycoprotein expression has been correlated with the de-

gree of resistance (32), and the transfer of genomic DNA from

an MDR line to a sensitive line resulted in the acquisition of

both the MDR phenotype and P-glycoprotein (12). Recently, a

complementary DNA has been isolated which confers multi-
drug resistance in a drug-sensitive cell, clearly demonstrating a

functional role for P-glycoprotein in the expression of the MDR

phenotype (36). To our knowledge, H69AR is the first MDR

cell line in which P-glycoprotein is not detectable using immu-
nological detection methods. The possibility exists that H69AR

expresses a form of P-glycoprotein that is not detected by MAbs

C219 and C494. However, Southern and RNA blot analysis

with the pCHPl probe (31) for P-glycoprotein indicates that

the gene for P-glycoprotein is not amplified, rearranged, or

overexpressed in H69AR cells (within the boundaries recog-
nized by this probe) (37). Taken together, these results appear

H69AR is similar to other human MDR lines in that it

displays little or no cross-resistance to bleomycin, 5-fluoroura-
cil, and platinum-containing drugs (3, 4, 16, 21, 22) (Table 2).

In the CHO derived MDR cell lines, a collateral sensitivity has

been observed to some local anesthetics and hormones (28). In

apparent contrast to the CHO cells, H69AR has only a slight

collateral sensitivity to 1-dehydrotestosterone and to lidocaine

and is equally as sensitive as H69 to the remaining steroids and

anesthetics tested (Table 3).

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consistently associated with the MDR phenotype (4). The level

of P-glycoprotein expression has been correlated with the de-

gree of resistance (32), and the transfer of genomic DNA from

an MDR line to a sensitive line resulted in the acquisition of

both the MDR phenotype and P-glycoprotein (12). Recently, a

complementary DNA has been isolated which confers multi-
drug resistance in a drug-sensitive cell, clearly demonstrating a

functional role for P-glycoprotein in the expression of the MDR

phenotype (36). To our knowledge, H69AR is the first MDR

cell line in which P-glycoprotein is not detectable using immu-
nological detection methods. The possibility exists that H69AR

expresses a form of P-glycoprotein that is not detected by MAbs

C219 and C494. However, Southern and RNA blot analysis

with the pCHPl probe (31) for P-glycoprotein indicates that

the gene for P-glycoprotein is not amplified, rearranged, or

overexpressed in H69AR cells (within the boundaries recog-
to suggest that cellular changes other than enhanced expression of P-glycoprotein are responsible for the MDR phenotype observed in H69AR cells.

The development of drug resistance has been associated with specific chromosomal alterations: homogeneously staining regions or double minute chromosomes (38, 39). Karyotypic analysis of H69 and H69AR showed a marked increase in the number of double minute chromosomes per cell in the drug resistant cells compared to the parental cells, with the number of double minute chromosomes per cell returning to near parental levels in cells cultured in the absence of drug (37). The relationship of this observation to the multidrug resistance of H69AR is unknown at the present time.

We have recently produced a panel of murine Mabs specific for H69AR cells. These MAbs will be useful in the isolation and characterization of membrane components involved in the MDR phenotype in H69AR SCLC cells. Furthermore, these MAbs may prove to be valuable aids in the detection of drug-resistant cells in SCLC patients.

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Multidrug Resistance in a Human Small Cell Lung Cancer Cell Line Selected in Adriamycin

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