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

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ABSTRACT

A multidrug resistant variant (H69AR) of the human small cell lung cancer cell line NCI-H69 was obtained by culturing these cells in gradually increasing doses of Adriamycin up to 0.8 μM after a total of 14 months. H69AR expresses the multidrug resistant phenotype because it is cross-resistant to anthracycline analogues including daunomycin, epirubicin, monarosil, and mitoxantrone as well as to acivicin, etoposide, gramicidin D, colchicine, and the Vinca alkaloids, vincristine and vinblastine. H69AR is also similar to other multidrug resistant cell lines in that it displays little or no cross-resistance to bleomycin, 5-fluorouracil, and carboplatin. It has a slight collateral sensitivity to 1-dehydrotestosterone and lidocaine. H69AR has increased cell-cell adhesiveness compared to H69, but a similar growth rate in vitro and tumorigenicity in nude mice. When cultured in the absence of Adriamycin, there is a 40% decrease in resistance by 35 days of culture, compared to cells in continuous culture in drug, but no further decrease in resistance up to 181 days. Monoclonal antibodies to P-glycoprotein have no detectable reactivity with H69AR cells as determined by enzyme-linked immunosorbent assay and immunoblotting techniques. Thus, unlike most multidrug resistant cell lines, H69AR does not appear to express enhanced levels of P-glycoprotein. H69AR will provide a useful model for the study of multidrug resistance in human small cell lung cancer.

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 % 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 chemotherapy and radiotherapy have resulted in increases in median 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 M, 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, 5β-pregn-3a-ol-20-one, 5β-pregn-3β-ol-20-one, deoxycorticosterone, 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 (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-mercaptoethanol, and 1 mM sodium pyruvate. CHO cell lines AuxB1 and CHFCS (5) were cultured in α-minimal essential medium supplemented with 10% FBS, 4 mM l-glutamine, 50 μM 2-mercaptoethanol, 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 QUAD (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 × 105 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 1.3 × 105 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 sensitivity 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 10^6 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 N 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 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 10^5 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 1-2-1 (11), or medium were added to the wells and binding of antibody detected using horseradish peroxidase conjugated goat antimouse IgG plus IgA plus IgM affinity purified F(ab')2 fragments (Cappel No. 23470) with o-phenylenediamine and hydrogen peroxide used as substrates. Color development was measured by scanning at 490 nm on a Dynatek MR600 microtitre plate reader.

P-Glycoprotein Detection by Western Blotting. Radiiodinated 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). Western blots of cell lysates were exposed to 125I-labeled MAb for 16 h at 4°C with shaking. The blot was washed extensively in PBS, dried, and exposed with intensifying screens on Kodak X-AR5 film at -70°C.

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 ID50 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 10^6 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 passed 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 ID50 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 expresses 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 (ID50 < 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 colchicine.
Multidrug resistant small cell lung cancer

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>
</tr>
<tr>
<td>Daunomycin</td>
<td>3.98</td>
<td>0.825</td>
<td>4.73</td>
</tr>
<tr>
<td>Epirubicin</td>
<td>0.348</td>
<td>&lt;0.001</td>
<td>&gt;348.0</td>
</tr>
<tr>
<td>Menogaril</td>
<td>0.448</td>
<td>0.089</td>
<td>&gt;496.0</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>10.0</td>
<td>0.631</td>
<td>16.8</td>
</tr>
<tr>
<td>Colchicine</td>
<td>0.006</td>
<td>&lt;0.001</td>
<td>&gt;6.0</td>
</tr>
<tr>
<td>Vincristine</td>
<td>5.6 x 10^-7</td>
<td>5 x 10^-8</td>
<td>112.0</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>4.5 x 10^-4</td>
<td>2.82 x 10^-5</td>
<td>15.8</td>
</tr>
<tr>
<td>Etoposide</td>
<td>6.3</td>
<td>0.39</td>
<td>16.2</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>24.0</td>
<td>13.5</td>
<td>1.8</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>1.6</td>
<td>0.63</td>
<td>2.5</td>
</tr>
<tr>
<td>Carboplatin</td>
<td>2.0</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Acivicin</td>
<td>10.0</td>
<td>0.47</td>
<td>21.3</td>
</tr>
<tr>
<td>Gramicidin D</td>
<td>0.010</td>
<td>&lt;0.003</td>
<td>&gt;3.3</td>
</tr>
</tbody>
</table>

* Assessed in quadruplicate by the MTT assay. Each line is the result from one experiment.
* Ratio of ID50 H69AR/ID50 H69.

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>0.77</td>
</tr>
<tr>
<td></td>
<td>5β-Pregnan-3α-ol-20-one</td>
<td>32.0</td>
<td>32.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>5β-Pregnan-3β-ol-20-one</td>
<td>28.0</td>
<td>28.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Deoxycorticosterone</td>
<td>38.0</td>
<td>38.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Dexmethasone</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 ID50 H69AR/ID50 H69.

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-pregnan-3β-ol-20-one, 5β-pregnan-3α-ol-20-one, deoxycorticosterone, dexmethasone, 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 CHC5 was positive for P-glycoprotein as expected. Expression of P-gly-
The blot was incubated with "I-labeled C219 or C494. Auto-
mpcoprotein was not detectable above background levels in either
in ADM. When compared to its parent line, H69AR is 10- to
period of 14 months a cell line, H69AR, which will grow in 0.8
ch 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,
H69 or H69AR.
cluded wells with an irrelevant MAb (1-7-1) or no first antibody.
ELISA. Bars, mean absorbance of tests performed in duplicate. Negative controls
xress to vincristine when cell survival was assessed
urine carcinoma line and its VP 16-213 resistant variant VPR,
DISCUSSION
H69AR (results not shown).
H69AR is similar to other human MDR lines in that it
displays little or no cross-resistance to bleomycin, 5-fluorouracil,
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
a cellular sensitivity to 1-dehydrotestosterone and to lidocaine
and is equally as sensitive as H69 to the remaining steroids
and anesthetics tested (Table 3).
Multidrug resistant Chinese hamster lung cells described by
Biedler et al. (29) displayed an altered cell morphology and
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 adhe-
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
within the boundaries recognized by this probe (37). Taken together, these results appear

Fig. 4. Immunological detection of P-glycoprotein with MAb C219 in a cell
ELISA. Bars, mean absorbance of tests performed in duplicate. Negative controls
included wells with an irrelevant MAb (1-7-1) or no first antibody.

DISCUSSION
By continuous culture of the human SCLC cell line H69 in
growing doses of ADM we have obtained over a
period of 14 months a cell line, H69AR, which will grow in 0.8
µM ADM. When compared to its parent line, H69AR is 10- to
100-fold more resistant to ADM. H69AR is also resistant to a
panel of anthracycline analogues, Vinca alkaloids, colchicine,
acivicin, and etoposide (Table 2) and thus possesses the MDR
phenotype which has been described in numerous rodent and
human cell lines (3, 4, 15–26).
Variation in the level of resistance of H69AR (but not the
pattern of resistance) was observed with all drugs tested. Such
variation has been noted by others (22). We have no explanation
for this phenomenon but note that it is composed of variations
in the ID90 for both the parent (H69) and resistant line
(H69AR). The unusual dose response curves with the Vinca
alkaloids in which total cell kill is not achieved even at high
drug concentrations have been observed by others. For example,
Hill and Bellamy (27) have described a HN-1 human squamous
cell carcinoma line and its VP16-213 resistant variant VP8,
which exhibited a plateau survival fraction after exposure to
vincristine. The VP8 cells had a decreased slope in the expo-
nential region and a higher plateau level of survival, indicating
cross-resistance to vincristine when cell survival was assessed
by colony formation in soft agar.

H69AR may be complex and possibly has two components.
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within the boundaries recognized by this probe (37). Taken together, these results appear
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 MAb's may prove to be valuable aids in the detection of drug-resistant cells in SCLC patients.

ACKNOWLEDGMENTS

We wish to thank Norbert Kartner for his generous provision of MAb's, C219 and C494 and Dr. Victor Ling and Dr. Jeff Trent for helpful discussions. The excellent technical assistance of Ingrid Louwman, Elisabeth Vreeken, and Deanna Evernden-Porelle is gratefully acknowledged. This paper is dedicated to the memory of Ingrid Louwman.

REFERENCES

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