Antigens Associated with Multidrug Resistance in H69AR, a Small Cell Lung Cancer Cell Line

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ABSTRACT

In a previous study (S. E. L. Mirski et al., Cancer Res., 47: 2594-2598, 1987), we described the derivation of a multidrug-resistant small cell lung cancer cell line, H69AR. The H69AR cell line does not overexpress P-glycoprotein and is therefore a useful model for the investigation of alternate mechanisms of drug resistance. In this paper we report the production and preliminary characterization of six murine monoclonal antibodies (MAbs) which react selectively with the H69AR cell line compared to its drug-sensitive parent cell line, NCI-H69. One of these antibodies, MAB 2.54, detects a cell surface epitope and reacts with multiple proteins of molecular weight 24,500-34,500 on immunoblots. Non-cell surface membrane-associated epitopes are detected by the other five antibodies, MABs 3.50, 3.80, 3.177, 3.187, and 3.186. MABs 3.50 and 3.186 immunoprecipitate antigens of molecular weight 55,000 and 36,000, respectively, while MABs 3.80, 3.177, and 3.187 all precipitate a molecular weight 47,000 protein, suggesting that they may detect epitopes on the same antigen. The epitopes detected by all six antibodies are present on greater than 80% of H69AR cells, as determined by flow cytometry. With the exception of MAB 2.54, the MABs cross-react in an enzyme-linked immunosorbent assay with the multidrug-resistant human fibrosarcoma cell line HT1080/DR4. Thus, these MABs react with two drug-resistant cell lines derived from different tumor types in which overexpression of P-glycoprotein is undetectable. These MABs may detect novel markers for drug resistance and thus may have potential diagnostic or therapeutic value.

INTRODUCTION

Patients with SCLC receive regimens of combination chemotherapy or chemotherapy and radiotherapy; however, subsequent relapse is almost inevitable and only 10% of patients survive 2 years (1). This high failure rate of chemotherapy is believed to be due to drug-resistant cells which either exist prior to or arise during treatment.

In a previous study, we have described a MDR human SCLC line, H69AR (2), which, unlike most MDR cell lines, does not overexpress P-glycoprotein (P-gp), a plasma membrane protein which acts as an energy-dependent drug efflux pump to reduce drug accumulation (3). H69AR is also unusual in that overexpression of P-gp is undetectable. These MABs may detect novel markers for drug resistance and thus may have potential diagnostic or therapeutic value.

MATERIALS AND METHODS

Cell Culture. SCLC cell line NCI-H69 (H69) was provided by J. Minna (NIH, Bethesda, MD). A MDR variant of H69, designated H69AR, was obtained by culturing H69 cells in increasing concentrations of ADM up to 0.8 μM and has been described previously (2). These cell lines were routinely maintained in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) that was supplemented with 5-10% heat-inactivated fetal bovine serum (FBS), 4 mM l-glutamine, and 1 mM pyruvate. H69AR cells were challenged with 0.8 μM ADM once a month. The HT1080 human fibrosarcoma cell line and its ADM-selected MDR subline, HT1080/DR4, were provided by M. Slivov and J. Trent (Arizona Cancer Center, Tucson, AZ) (14). These cell lines were grown in Eagle’s minimum essential medium with Earle’s salts supplemented with 10% FBS, 4 mM l-glutamine, and 0.1 mM nonessential amino acids (GIBCO Laboratories). The HT1080/DR4 cell line was maintained continuously in ADM (0.10 μg/mL). All cell lines were cultured in the absence of antibiotics and were negative for Mycoplasma contamination (15).

Generation of Hybrids. Two BALB/C mice (one male, one female) received three i.p. injections of approximately 1.5 × 10^7 viable H69AR cells suspended in PBS at 1-month intervals. Three days before fusion, 5–10 × 10^6 H69AR cells were injected i.v. into a tail vein. Spleen cells were fused with NS-1 myeloma cells (American Type Culture Collection, Rockville, MD) with polyethylene glycol 4000 (Sigma Chemical Co., St. Louis, MO) as per standard procedures (16). The fused cells were plated into 96-well plates with peritoneal exudate cells and irradiated spleen cells from CD1 mice as feeder layers and the cultures were fed with hypoxanthine, aminopterin, and thymidine-containing medium to select for hybrids.

Screening Procedure. After 2 weeks of growth in hypoxanthine-, aminopterin-, and thymidine-containing medium, binding of MAbs to P-gp was determined.

Received 4/5/89; revised 6/29/89; accepted 7/17/89.

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1 Supported by grants to S. P. C. C. from the Medical Research Council of Canada and the National Cancer Institute of Canada.

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3 The abbreviations used are: SCLC, small cell lung cancer; P-gp, P-glycoprotein; P-gp, overexpression of P-glycoprotein is not detectable; P-gp, detectable overexpression of P-glycoprotein; MAB, monoclonal antibody; PBS, phosphate-buffered saline; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; MDR, multidrug resistance (t); ADM, Adriamycin (doxorubicin); PMSF, phenylmethylsulfonyl fluoride; 2-ME, 2-mercaptoethanol; SDS, sodium dodecyl sulfate; NGS, normal goat serum; Igs, immunoglobulins; NS-1, P3.NS1/As4.1; H69, NCI-H69.

4 J. G. Reeve and P. R. Twentyman, personal communication.
washing with PBS and blocked for 1 h with 1% bovine serum albumin and 5% NGS in PBS (blocking solution). Hybridoma culture supernatants were then added at a 1:5 final dilution in blocking solution. Negative controls included no first antibody and NS-1 supernatant supplemented with irrelevant murine IgG and IgM. MAb HKN-1 served as a positive control (18). Binding of the first antibody was detected using horseradish peroxidase-conjugated goat anti-mouse IgG (6-10+M) affinity-purified F(ab')2 fragments (Cappel, Cooper Biomedical, Inc., Malvern, PA) with o-phenylenediamine and hydrogen peroxide as substrates. Color development was measured at 490 nm, with a reference wavelength of 410 nm, on a Dynatech MR600 microtiter plate reader.

Cloning, Isotyping, and Ascites Production. Hybridomas selected for further study were cloned twice at 1 cell/well, once at 0.5 cell/well, and finally at 0.3 cell/well to ensure monoclonality (19). Immunoglobulin subtype was determined using a mouse subtyping kit (BioRad Laboratories, Mississauga, Ontario). Hybridoma cells (5–10 x 10⁶) were washed, resuspended in PBS, and injected i.p. into pristane-treated BALB/c (nu/nu) mice. Approximately 2 weeks later, mice were killed and the ascites was collected. The ascites preparation was cleared of cells by centrifugation, tested by titration in an ELISA, and frozen at −70°C.

Indirect Immunofluorescence and Flow Cytometry. H69 and H69AR cells were monodispersed by repeatedly passing them, in suspension, through a 16-gauge needle. All subsequent procedures were performed at 4°C. The cells (unfixed or 70% methanol-fixed for 5 min) were dispensed into tubes (1 x 10⁶ cells/tube) and incubated with hybridoma supernatant MAb for 30 min. In some experiments, ethidium bromide was added with the MAb to distinguish between permeable and non-permeable cells. The cells were washed and incubated for 1 h with affinity-isolated fluorescein isothiocyanate-conjugated goat anti-mouse polyvalent IgG (Sigma) diluted 1:25 in RPMI 1640/5% FBS. After washing, the cells were resuspended in medium and either analyzed using a Becton-Dickinson fluorescence-activated cell sorter IV (filtration detectors, 500–550 nm band pass for fluorescein isothiocyanate, 620 nm long pass for ethidium bromide) or used to make cytospin preparations in a Shandon Southern cytospin centrifuge. Cytospin preparations were mounted with 0.2% o-phenylenediamine in 50% glycerol/PBS for coverslipping. Photographs were taken with 40× exposures of 400 ASA Kodak film, using a Leitz Dialux 22 microscope with a Fluoreszenz 50/1.00 W lens and a WILD MPS12 camera.

Immunoblotting. H69 and H69AR cells were homogenized at 4°C in 10 mm Tris-HCl, pH 7.6, 0.15 M NaCl, 1 mm EDTA, 2 mm PMSF, 0.02% NaN₃, 1% Triton X-100, 1% sodium deoxycholate, 0.5% Trasylol, mixed for 20 min at 4°C with NS-1 ascites and mouse immunoglobulins (Cappel, Cooper Biomedical), and rabbit anti-mouse immunoglobulins (DAKO Immunoglobulins, Denmark), and Staphylococcus aureus (Sigma) which had been suspended at 10% (v/v) in buffer A. The suspension was microfuged to obtain a cleared cell lysate. Each subsequent addition was followed by mixing for 20 min at 4°C. MAbs ascites (diluted 1:100 in 1% bovine serum albumin/PBS) was added to the cleared cell lysate and rabbit anti-mouse immunoglobulins were added, followed by the S. aureus suspension. The S. aureus precipitate was washed 5 times in buffer B (10 mm Tris-HCl, pH 7.6, 0.15 M NaCl, 1 mm EDTA, 0.02% NaN₃, 0.2% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS), boiled 5 min in sample buffer with 2-ME, and microfuged, and the supernatant was loaded for SDS-polyacrylamide gel electrophoresis. Dried gels were used to expose Kodak X-AR film at room temperature.

RESULTS

Selection of MAbs. From several hundred hybrids which were initially obtained, six were selected for further characterization and designated MAbs 2.54, 3.80, 3.186, 3.80, 3.187, and 3.186. The selection criteria were as follows: (a) reactivity with H69 cells similar to negative control values in the cell ELISA; (b) a ratio of ELISA absorbance values (H69AR:H69) greater than 5; and (c) consistent specific reactivity with H69AR cells following cloning, expansion, and freezing of hybrids. The intensity of reaction on H69AR cells in the ELISA differed among the MAbs, with MAb 2.54 being the strongest, MAbs 3.80, 3.177, and 3.186 intermediate, and MAb 3.50 the weakest. MAb 3.50 had the isotype μ₅, while the others were either γ₅,κ or γ₂a,κ. These results are presented in Table 1.

Immunofluorescent Microscopy and Flow Cytometry. Indirect immunofluorescent labeling of H69 and H69AR cells by the MAbs was evaluated by microscopy of cytopsin preparations. Two patterns of reactivity were observed and are illustrated in Fig. 1. MAb 2.54 labeled the majority of both unfixed (Fig. 1d) and 70% methanol-fixed H69AR cells (not shown). In contrast, the other five MAbs labeled few of the unfixed H69AR cells (Fig. 1b); however, the majority of H69AR cells were labeled after fixation with 70% methanol (Fig. 1c). None of the MAbs had detectable reactivity on cytopsins of unfixed or fixed H69 cells.

The percentage of H69 and H69AR cells which were labeled by the MAbs and the relative antigen density as reflected by mean fluorescence intensity was quantitated by flow cytometry. The results of these experiments are shown in Fig. 2 and Table 2. Viable cells were selected for analysis on the basis of their ability to exclude ethidium bromide (i.e. fluorescence-negative population at 620 nm). The percentage of cells labeled with each MAb was determined by setting a window with lower limits in a channel such that 5% of each cell line was positive with the negative control (NS-1 + irrelevant mouse immuno-

Table 1 Monoclonal antibodies to H69AR cells

<table>
<thead>
<tr>
<th>MAb</th>
<th>H69</th>
<th>H69AR</th>
<th>Isotype</th>
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<tr>
<td>2.54</td>
<td>0.002</td>
<td>0.475</td>
<td>γ₅κ₂a</td>
</tr>
<tr>
<td>3.50</td>
<td>0.009</td>
<td>0.164</td>
<td>μ₅</td>
</tr>
<tr>
<td>3.80</td>
<td>0.016</td>
<td>0.302</td>
<td>γ₅κ</td>
</tr>
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<td>3.177</td>
<td>0.066</td>
<td>0.358</td>
<td>γ₅κ</td>
</tr>
<tr>
<td>3.187</td>
<td>0.012</td>
<td>0.279</td>
<td>γ₅κ</td>
</tr>
<tr>
<td>3.186</td>
<td>0.011</td>
<td>0.220</td>
<td>γ₅κ</td>
</tr>
</tbody>
</table>

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Drug resistance-associated antigens in H69AR cells

Fig. 1. Cytocentrifuge preparations of H69AR cells labeled with monoclonal antibody. a, Phase contrast of unfixed H69AR cells; b, same field as a labeled with MAb 3.50; c, methanol-fixed H69AR cells labeled with MAb 3.50; d, unfixed H69AR cells labeled with MAb 2.54.

MAb 2.54 labeled equal numbers of both viable and 70% methanol-fixed H69AR cells (83% positive). In contrast, labeling by the other MAbs was increased by 40–70% after fixation of the H69AR cells in methanol. Neither viable nor fixed H69 cells were labeled with MAbs 2.54 or 3.50. The percentage of positive H69 cells with MAbs 3.186, 3.80, and 3.177 was increased slightly by fixation (3, 16, and 17%, respectively). MAB 3.186 labeled approximately 33% of viable H69 cells and the percentage was not increased by fixation of the cells; in contrast, fixation of H69AR cells increased the number of cells labeled by MAb 3.186 from 53 to 95%. The relative antigen density (fluorescence intensity) for each MAB was much greater on fixed H69AR cells than on fixed H69 cells, with the difference being greatest for MAbs 2.54 and 3.50 (Fig. 2 and Table 2).

Immunoblots. The antigens recognized by MAbs 2.54 and 3.186 were identified by immunoblotting (Fig. 3). Equal amounts of protein from extracts of the H69 or the H69AR cell line were loaded for each set of conditions. In the absence of 2-ME, MAB 2.54 reacted with a series of protein bands of molecular weight 24,500–34,500 in extracts of H69AR cells. Inclusion of 2-ME in the sample buffer abolished immunoreactivity, in spite of loading more reduced protein (69 µg/lane) than nonreduced protein (47 µg/lane). MAB 3.186 reacted with an antigen of apparent molecular weight 36,000 in both reduced (69 µg/lane) and nonreduced (54 µg/lane) H69AR samples. When the blots with MAB 3.186 were overdeveloped, a band of the same apparent molecular weight became visible in H69 cell lysates, suggesting a quantitative difference in the expression of this protein. Results of immunoblots with MAbs 3.177, 3.80, and 3.187 were equivocal and immunoblots with MAB 3.50 were negative.

Immunoprecipitations. The molecular weights of the antigens precipitated by MAbs 2.54 and 3.186 were 24,000 and 36,000, respectively (Fig. 4), confirming the results obtained by immunoblotting. In control experiments (not shown), it was clear that the band that was nonspecifically precipitated by all MAbs and which is an abundant protein in H69AR cell extracts was of slightly higher molecular weight than the protein specifically precipitated by MAB 3.186. MAbs 3.80, 3.177, and 3.187 precipitated proteins of the same apparent molecular weight, 47,000, while a 55,000 protein was precipitated by MAB 3.50 (Fig. 4). When NS-1 ascites with irrelevant mouse Ig was used as a negative control first antibody, the same bands which were precipitated nonspecifically by all the MAbs were invariably observed. Immunoprecipitations from membrane and cytosol fractions indicated that all of the antigens were membrane associated (results not shown).

Cross-Reactivity. The cross-reactivity of the MAbs with the P-gp- MDR fibrosarcoma line HT1080/DR4 (14) and its drug sensitive parent HT1080 was measured using a cell ELISA and the results are shown in Table 3. Resistance-associated reactions were observed with five of the six MAbs on HT1080/DR4 cells; the exception, MAB 2.54, did not react with either HT1080 cell line.

Discussion

Acquired resistance to multiple chemotherapeutic agents is the major impediment to effective treatment of patients with
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SCLC. In order to study this phenomenon we have derived a MDR SCLC cell line, H69AR (2). This cell line does not overexpress P-gp and thus provides a useful model in which to investigate alternate mechanisms of MDR. We have produced a panel of six mouse MAbs which, in a number of in vitro assays, react specifically with the H69AR cell line but not with its drug-sensitive parental cell line H69.

MAb 2.54 reacts strongly with H69AR cells in the ELISA and, in marked contrast to the other five MAbs, detects a cell surface epitope, since it labels both unfixed and fixed H69AR cells in indirect immunofluorescence assays. MAb 2.54 reacts with a series of bands from molecular weight 24,500 to 34,500 on immunoblots of H69AR cell lysates. This reaction is abolished by reduction of disulfide bonds with 2-ME, indicating that the antibody detects an epitope which is dependent on the tertiary structure of the protein. There are various possible explanations for the multiple bands observed with MAb 2.54, including presence of the epitope on otherwise unrelated proteins, differential glycosylation of a single protein, or degradation of the 34,500 molecular weight protein to smaller molecules. The latter explanation seems unlikely, since three protease inhibitors were included in the lysis buffer. Only one 24,000 molecular weight protein band was detectable by immunoprecipitation; however, the faintness of this band suggests that immunoprecipitation might have been less sensitive than immunoblotting with MAb 2.54. Alternatively, antigens might have been differently solubilized or degraded in the different lysis buffers used in each of these techniques.

MAb 3.50 reacts specifically with H69AR cells in the ELISA but weakly compared to the other five MAbs. In contrast, the labeling observed in indirect immunofluorescence assays of fixed H69AR cells with MAb 3.50 was very intense. The antigen precipitated by this MAb is a membrane-associated 55,000 molecular weight protein. It was found that this antigen was relatively labile, since cell lysates stored at −20°C for 1 month lost their immunoreactivity. Thus the low reaction of MAb 3.50 in the ELISA might be due to the relative instability of this antigen.

MAb 3.186 precipitates and detects on immunoblots a 36,000 molecular weight membrane-associated protein. This MAb reacts with H69AR cells with intermediate intensity on the ELISA and with high intensity on 95% of fixed H69AR cells, as assessed by flow cytometry. It also labels a higher proportion of the drug-sensitive H69 cells than do the other MAbs; however, the fluorescence intensity is low. This observation does not exclude the possibility that the increased expression of this protein might be involved in the mechanism of resistance in the H69AR cell line. In this regard, it should be noted that P-gp is expressed at low levels in the drug-sensitive Chinese hamster ovary Aux B1 cell line (8).

MAbs 3.80, 3.177, and 3.187 all show an intermediate intensity of specific reaction with H69AR cells in the ELISA and display very similar profiles in fluorescence-activated cell sorting analyses. In addition, all three antibodies precipitate a 47,000 molecular weight protein from membrane fractions but not cytosol. Taken together, these data suggest that MAbs 3.80, 3.177, and 3.187 may detect epitopes on the same antigen. Experiments are underway to determine if this is in fact the case.

To our knowledge, membrane-associated proteins of molecular weights 24,000, 36,000, 47,000, or 55,000 have not previously been reported to be associated with the acquisition of drug resistance. Sorcin/V19 is a calcium-binding protein that has been associated with resistance in P-gp+ MDR cell lines.

Table 2 Flow cytometry of H69 and H69AR cells

<table>
<thead>
<tr>
<th>MAb</th>
<th>Viable cells</th>
<th>Fixed cells</th>
<th>Fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H69</td>
<td>H69AR</td>
<td>H69</td>
</tr>
<tr>
<td>2.54</td>
<td>6</td>
<td>83</td>
<td>5</td>
</tr>
<tr>
<td>3.50</td>
<td>4</td>
<td>18</td>
<td>8</td>
</tr>
<tr>
<td>3.187</td>
<td>13</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>3.80</td>
<td>9</td>
<td>23</td>
<td>25</td>
</tr>
<tr>
<td>3.177</td>
<td>7</td>
<td>24</td>
<td>20</td>
</tr>
<tr>
<td>3.186</td>
<td>33</td>
<td>53</td>
<td>40</td>
</tr>
</tbody>
</table>

Table 2 Flow cytometry of H69 and H69AR cells

<table>
<thead>
<tr>
<th>% of cells labeled*</th>
<th>Fluorescence intensity*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Fixed cells</td>
</tr>
<tr>
<td>MAb 2.54</td>
<td>H69</td>
</tr>
<tr>
<td>MAb 3.50</td>
<td>4</td>
</tr>
<tr>
<td>MAb 3.187</td>
<td>13</td>
</tr>
<tr>
<td>MAb 3.80</td>
<td>9</td>
</tr>
<tr>
<td>MAb 3.177</td>
<td>7</td>
</tr>
<tr>
<td>MAb 3.186</td>
<td>33</td>
</tr>
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</table>

* Percentage of cells with greater fluorescence intensity than a window set so that approximately 5% of the negative control (NS-1 + Ig) were positive. Twenty thousand events were analyzed per histogram. Results are from one experiment. Similar results on fixed cells were obtained in an additional experiment.

* Ungated mean log channel number of ethidium bromide-positive, 70% methanol-fixed cells.

* Gates set to exclude cells which stained with ethidium bromide.

* Cells were fixed with 70% methanol (4°C) in PBS and gates were set to exclude ethidium bromide-negative events.
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Table 3 Reactivity of monoclonal antibodies with drug-sensitive and -resistant human fibrosarcoma cell lines

Reactivity was measured using a cell ELISA as described in “Materials and Methods.” Values given are mean absorbances at 490 nm of duplicate determinations. Similar values were obtained in at least one additional experiment.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MAb 2.54</th>
<th>MAb 3.50</th>
<th>MAb 3.80</th>
<th>MAb 3.177</th>
<th>MAb 3.186</th>
<th>MAb 3.187</th>
</tr>
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<tbody>
<tr>
<td>HT1080</td>
<td>0.007</td>
<td>0.082</td>
<td>0.010</td>
<td>0.015</td>
<td>0.078</td>
<td>0.014</td>
</tr>
<tr>
<td>HT1080/DR4</td>
<td>0.021</td>
<td>0.439</td>
<td>0.212</td>
<td>0.236</td>
<td>0.853</td>
<td>0.240</td>
</tr>
</tbody>
</table>

Fig. 3. Immunoblot of H69 and H69AR (AR) cell extracts with MAbs 2.54 and 3.186. Extracts were reduced with 2-ME (R) or not (NR) prior to SDS-polyacrylamide gel electrophoresis, replica-blotted onto Immobilen, and reacted with MAb 2.54 or MAb 3.186. Arrows at right, positions of molecular weight markers, in descending order, 66,000, 45,000, 36,000, 29,000, 24,000, 20,100.

Fig. 4. SDS-polyacrylamide gel electrophoresis of immunoprecipitates from extracts of [35S]methionine-labeled H69AR cells. Numbers under each lane indicate the MAb used to immunoprecipitate. Arrows and numbers between the two gels indicate position and weight (× 1000) of molecular weight markers. Arrows and numbers to left and right of the gels indicate positions and calculated molecular weight (× 1000) of specifically precipitated proteins.

The epitope detected by MAb 2.54 is not expressed on the drug-sensitive HT1080 or P-gp− MDR HT1080/DR4 human fibrosarcoma cell lines. In contrast, the other five antibodies have considerably greater reactivity with the resistant fibrosarcoma cells than with the drug-sensitive cells. This indicates that these epitopes are not restricted to the immunizing SCLC cell line, nor are they restricted to one tumor type. Of particular interest is the finding that reaction with MAb 3.186 and 3.50 and the trio of MAb 3.80, 3.177, and 3.187 (which appear to detect the same antigen) is associated with the acquisition of drug resistance in two cell lines in which overexpression of P-gp is not detectable. Thus, these MAbs may detect novel markers of MDR. Whether these antibodies detect the same proteins on both HT1080/DR4 and H69AR cells is currently under investigation.

The antigens detected by this panel of MAbs may be involved in the mechanism(s) underlying drug resistance in some P-gp− cell lines or may only be markers associated with this type of resistance. However, expression of the antigens is not restricted to P-gp− cell lines, since cross-reactivity has been noted with some but not all P-gp+ cell lines. Because P-gp has been detected in tumors from only a subset of drug-resistant cancer patients (36-40), other mechanisms of MDR are likely to be clinically relevant. The availability of markers of MDR in addition to P-gp would have obvious diagnostic value. Ultimately, antibodies to such markers may be useful in immunotherapy. Further characterization of the antigens described in this study and a survey of their expression in MDR cell lines and clinical samples are the subjects of ongoing studies.

ACKNOWLEDGMENTS

The authors wish to thank E. Vreken and C. MacDougall for expert technical assistance, Drs. J. Johnson and S. Stewart for their advice and assistance in the fluorescence-activated cell sorting analyses, Drs. M. Slovak and J. Trent for their generous provision of cell lines, and Drs. J. Gerlach and R. Deeley for helpful discussions. The assistance of Bryn Harris in the preparation of this manuscript is gratefully acknowledged.

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