Detection of the Mr 190,000 Multidrug Resistance Protein, MRP, with Monoclonal Antibodies

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Abstract

MRP is a Mr 190,000 integral membrane phosphoglycoprotein that is overexpressed in some drug-selected resistant cell lines and has been shown to cause multidrug resistance in transfected cells. Five murine hybridoma cell lines (QCRL-1, QCRL-2, QCRL-3, QCRL-4, and QCRL-6) have been generated which secrete monoclonal antibodies (MAbs) that react specifically with membrane proteins of MRP-overexpressing, multidrug-resistant, drug-selected H69AR cells and MRP-transfected HeLa cells (T5) but not the respective parental (H69) and vector-transfected (C1) cells. The ability of three of these MAbs (QCRL-1, QCRL-2, and QCRL-3) to selectively immunoprecipitate a Mr 190,000 protein from Mr-labeled H69AR and T5 membranes indicates that these MAbs are specific for MRP. MAb QCRL-1 is also capable of detecting the low levels of MRP present in revertant H69PR cells by immunoblot analysis. Indirect immunofluorescence analyses show that MAbs QCRL-1, QCRL-2, and QCRL-3 strongly and differentially react with fixed T5 and H69AR cells but not with unfixed cells, suggesting that these MAbs recognize intracellular MRP epitopes. The availability of reagents for the specific and sensitive immunodetection of MRP should greatly facilitate biological and clinical studies of this novel drug resistance protein.

Introduction

The inherent or acquired resistance of tumor cells to a spectrum of structurally and functionally diverse drugs, termed multidrug resistance, is a serious obstacle to the successful chemotherapeutic treatment of many human cancers. Numerous cell lines have been selected in vitro for resistance to a variety of cytotoxic drugs, providing experimental models to study the phenomenon of multidrug resistance (1). Many of these resistant cell lines have been found to overexpress P-gp, a Mr 170,000 integral membrane protein encoded by the MDR1 gene, which is believed to confer resistance by acting as a drug efflux pump (2, 3). However, a number of cell lines have been described, such as the human small cell lung cancer cell line H69AR, which display multidrug resistance but do not overexpress this protein (1, 4, 5). Recently, cDNA clones corresponding to an mRNA markedly overexpressed in H69AR cells were isolated and characterized (6). The previously unidentified Mr 190,000 integral membrane phosphoglycoprotein encoded by this 6.5-kilobase mRNA, designated MRP, belongs to the same superfamily of ATP-binding cassette transporter proteins as P-gp (6, 7). Like P-gp, transfection of drug-sensitive cells with a full-length MRP cDNA is sufficient to confer multidrug resistance (7). However, despite the fact that both P-gp and MRP confer resistance to a similar spectrum of anticancer drugs, these two proteins share less than 15% amino acid identity (6).

MAbs against P-gp have played a critical role in determining the relevance of this protein in clinical drug resistance (8). Some anti-P-gp MAbs and their derivatives are being investigated for their therapeutic potential, particularly those that are able to reverse drug resistance (9–12) or participate in antibody-dependent cell-mediated cytosis (13). P-gp-specific MAbs have also been useful in immunolocalization studies of normal tissues and have provided important clues as to the physiological role of this protein (14, 15). The epitopes of several MAbs have been mapped, providing information about P-gp secondary structure and membrane topology (16, 17).

To date, polyclonal antisera raised against MRP-derived synthetic peptides have been used to quantify MRP protein levels in drug-selected and transfected multidrug resistant cell lines and to examine MRP structure, biosynthesis, and subcellular distribution (7, 18, 19). However, these studies have been hampered by the unsuitability of polyclonal antisera for many experimental applications and by the limited availability of these immunoreagents. To obtain better probes for immunodetection of MRP, we have produced MAbs from mice immunized with membranes from multidrug resistant H69AR cells which express high levels of this Mr 190,000 protein. These MRP-specific MAbs should greatly facilitate ongoing investigations of the biology and clinical relevance of this novel drug resistance protein.

Materials and Methods

Cell Culture. The parental H69; doxorubicin-selected, multidrug-resistant H69AR; and revertant H69PR small cell lung cancer cell lines were maintained as described previously (4, 20). T5 cells are HeLa cells that have been made multidrug resistant by transfection with a full-length MRP cDNA expression vector, pRe/CMV-MRP1, and C1 cells are HeLa cells that have been transfected with pRe/CMV vector alone (7). These cells were maintained in the same medium as the lung cancer cells, supplemented with 400 µg/ml G-418 (Sigma Chemical Co., St. Louis, MO). SP2/0-Ag14 myeloma cells were maintained in Dulbecco’s modified Eagle’s medium (Hybri-Max, Sigma) supplemented with 4 mm l-glutamine and 5% heat-inactivated bovine calf serum. Approximately 1 week prior to fusion, Sp2/0 cells were challenged with 0.132 mm 8-azaguanine (Sigma) for one passage.

Generation of Hybridomas. Membrane-enriched fractions were prepared as described previously and resuspended in Tris-sucrose buffer (10 mm Tris-HCl, pH 7.5–0.25 m sucrose) containing protease inhibitors (7). Female BALB/c mice (6–8 weeks old) received three i.p. injections of 150 µg H69AR


membrane protein (without detergent) in PBS and mixed 1:1 with RIBI MLS-TDM+CWS emulsion (Cedarlane Laboratories, Hornby, Ontario, Canada) at approximately 3-week intervals. Three days before fusion, 100 μg H69AR membrane protein was injected i.v. into a tail vein. Spleen cells were fused with SP2/0 myeloma cells with polyethylene glycol 4000 (Sigma) according to standard methods (21, 22). Cultures were fed with Dulbecco's modified Eagle's medium containing 100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine, 20% heat-inactivated fetal bovine serum, and 25 μg/ml gentamicin (ICN Biomedicals, St. Laurent, Quebec, Canada). After initial screening, aminopterin was omitted from the growth medium.

Screening, Cloning, Isotyping, and Ascites Production. After 11 days of growth in selective medium, 459 hybridoma supernatants were tested for the presence of MRP-specific antibody by immunodot blot analysis. H69, H69AR, and H69PR membrane proteins in TBS were blotted (4 μg protein/dot) onto Immobilon-P polyvinylidene fluoride membrane (Millipore, Mississauga, Ontario, Canada) using a 96-well vacuum manifold, and blots were kept wet at all times. The blots were cut into strips such that each strip had spots of membrane proteins from each of the three cell lines. After transfer to 24-slot incubation trays, strips were blocked for 1 h in blocking solution (5% bovine serum/5% normal goat serum/1% bovine serum albumin in TBS-T). Hybridoma supernatants were added directly to the blocking solution at a final dilution of 1:9. After 90 min, the strips were washed three times for 5 min in TBS-T, and secondary antibody [horseradish peroxidase-conjugated goat anti-mouse IgG + IgM (H+L) F(ab')2 fragment; Pierce, Edmonton, Alberta] was added (diluted 1:10,000 in blocking buffer). After 1 h, the strips were washed five times for 5 min in TBS-T, and antibody binding was determined by enhanced chemiluminescence detection (Amersham, Oakville, Ontario, Canada) with exposure on Kodak X-OMAT film.

Hybridomas which showed preferential reactivity with H69AR membrane dots were subjected to a second immunodot blot, using strips with C1 and T5 membrane protein dots in addition to the H69, H69AR, and H69PR dots. Hybridomas which reacted preferentially with H69AR and T5 membrane dots compared to H69, H69PR, and C1 dots were cloned twice by limiting dilution and then expanded. Immunoglobulin subtypes of the MAb6 produced by the five stable hybridoma clones obtained were determined using an isotyping reagent kit (Sigma). To produce ascites, 5 × 106 hybridoma cells resuspended in PBS were injected i.p. into pristane-pretreated BALB/c nu/nu mice. Ascites fluid was collected over the next 1–2 weeks, and Mabs were purified by passage over Econo-Pac DEAE Blue cartridges (Bio-Rad, Mississauga, Ontario, Canada) according to the manufacturer's instructions.

Immunoblotting and Immunoprecipitation of MRP. Membrane protein was solubilized in Laemmli buffer (23) and subjected to SDS-PAGE and electroblotting as described previously (7). Immunoblotting was performed as described above for dot blot strips. In some experiments, a polyclonal anti-Goat serum (MRP-2) that was raised against a peptide corresponding to amino acids 1418 to 1432 of MRP, and which is known to cross-react with P-gp, was used as a positive control for MRP detection.

Immunoprecipitations were carried out as follows. Cells were incubated for 24 h in L-methionine-deficient RPMI 1640 (Sigma) supplemented with 10% dialyzed bovine calf serum and 50 μCi/ml [35S]methionine (1110 Ci/mmol, cell labeling grade; DuPont NEN, Markham, Ontario, Canada). Cells were washed twice with PBS and resuspended at approximately 6 × 106 cells/ml in solubilizing buffer [1% 3-(3-cholamidopropyl)dimethylamino-1-propanesulfonate, 100 mM KCl, and 50 mM Tris-HCl (pH 7.5)] containing protease inhibitors (7). After 1 h on ice, insoluble matter was removed by ultracentrifugation. Aliquots of the supernatant were brought up to 250 μl with solubilizing buffer and incubated for at least 2 h at 4°C with hybridoma supernatant diluted 1:6. Antibody-MRP complexes were recovered by incubation with 25% w/v GammaBind Plus Protein G Sepharose (30 μl) or 10% w/v protein A-Sepharose CL-4B (25 μl) (Pharmacia, Baie D'Urfe, Quebec, Canada) in solubilization buffer for at least 2 h at 4°C. The samples were sequentially washed (24), and precipitated proteins were eluted from the beads with Laemmli buffer and analyzed by SDS-PAGE and fluorography.

Indirect Immunofluorescence and Flow Cytometry. Cells were washed twice with cold PBS and fixed with either 0.5% paraformaldehyde (Sigma) in PBS for 30 min at 4°C or with 70% methanol at −20°C for 10 min. All subsequent procedures were done at 4°C. Cells were washed once with blocking solution (1% bovine serum albumin/5% normal goat serum/PBS). For MAbs QCRL-2 and QCRL-3, the cells were incubated in blocking solution with 0.1% Triton X-100 for 30 min, followed by direct addition of hybridoma supernatant or ascites diluted as required. After incubation for 1 h, the cells were washed once in blocking solution with 0.1% Triton X-100, followed by a wash in blocking solution alone. The washed cells were incubated with fluorescein-conjugated goat anti-mouse IgG (H+L) F(ab')2 fragment (Pierce) diluted 1:50 in blocking solution for 30 min and then washed twice in blocking solution with 0.1% Triton X-100. For MAb QCRL-1, cells were treated similarly except Tween-20 was used at 0.1% instead of Triton X-100 and was included in all washes and incubations. Finally, cells were resuspended in 1% paraformaldehyde in PBS and either analyzed on a Coulter Epic flow cytometer or cytopsin were prepared for examination by fluorescence microscopy.

Results and Discussion

Using spleens from mice immunized with MRP-enriched membranes, murine hybridomas were generated and screened for their ability to detect MRP in non-denatured membranes. In the end, five stable hybridoma cell lines, designated QCRL-1, QCRL-2, QCRL-3, QCRL-4, and QCRL-6, were obtained. MAb QCRL-1, QCRL-4, and QCRL-6 were determined to be of the IgG1 subclass; MAb QCRL-2 was an IgG2α; and MAb QCRL-3 was an IgG2a. The
MABs reacted strongly with MRP-rich membrane fractions from both drug-selected H69AR cells and MRP-transfected T5 cells and weakly or not at all with parental H69, revertant H69PR, or control C1 cell membranes (Fig. 1). These data, particularly the specificity for the MRP-transfected T5 cells, strongly suggested that the MAbs were reacting with MRP. None of the MABs cross-react with P-gp, since they showed no reactivity with membrane fractions from 8226/Dox40 myeloma cells, which are known to overexpress this MRP (6). When corrected for protein loading of gels, the H69AR cells were estimated to express 5-fold higher levels of MRP than T5 cells by densitometry. Middle panel, the gel was loaded with proteins from MRP-transfected T5 and vector-transfected C1 cells (10 μg/lane), multidrug-resistant H69AR cells, parental H69 and revertant H69PR cells (5 μg/lane), and from P-gp overexpressing 8226/Dox40 myeloma cells (25 μg/lane). The blot was probed with MAB QCRL-1 hybridoma supernant (diluted 1:5000). When corrected for protein loading of gels, the H69AR cells were estimated to express 5-fold higher levels of MRP than T5 cells by densitometry. Middle panel, the gel was loaded with protein from T5 cells (15 μg/lane) and H69PR cells (75 μg/lane), and the blot was probed with Mab QCRL-1 ascites (diluted 1:5000). Right panel, the gel was loaded as in the left panel, and the blot was probed with anti-P-gp Mab C219 (1 μg/ml). Antibody binding was visualized by an enhanced chemiluminescence detection system. The immunoblots shown are from gels run under reducing conditions. Identical results were obtained with nonreducing conditions.

To confirm the MRP specificity of these MABs, immunoprecipitation and immunoblot analyses were carried out. MAB QCRL-3 immunoprecipitated a single M, 190,000 protein from [35S]methionine-labeled H69AR cells when protein A was used to bind immune complexes (Fig. 2A, left panel). MAB QCRL-2 also precipitated this M, 190,000 protein but only very poorly (data not shown). This precipitated protein had the same electrophoretic mobility as the precipitated protein by the polyclonal antiserum MRP-2, which was raised against an MRP-derived peptide.4 To immunoprecipitate MRP with MAB QCRL-1, protein G was required to bring down immune complexes (Fig. 2A, right panel). Protein G was also effective in precipitating MRP with MAB QCRL-2 and QCRL-3. A M, 190,000 protein was precipitated with all three MABs from the MRP-transfected T5 cells but not from C1 control cells (data not shown). Taken together, these data provide confirmation of the MRP specificity of MABs QCRL-1, QCRL-2, and QCRL-3. None of the membrane solubilization conditions tested to date have enabled the precipitation of MRP (or other membrane proteins) with MABs QCRL-4 and QCRL-6, although the MABs themselves can be precipitated by protein G. Thus, at the present time, the MRP specificity of these MABs cannot be claimed with absolute certainty.

Immunoblot analyses with the MABs were carried out under both reducing and nonreducing conditions. In both cases, only MAB QCRL-1 detected a protein of M, 190,000 (Fig. 2B, left panel). This protein is easily detectable at high levels in membranes from H69AR and T5 cells using QCRL-1 hybridoma supernant, and the relative levels in the two cell types are approximately the same as those we have reported previously using MRP-specific polyclonal antisera (7).4 The very low levels of the M, 190,000 protein found in the drug-sensitive revertant H69PR cells could also be detected with a very high degree of specificity using MAB QCRL-1 ascites (Fig. 2B, middle panel). The M, 170,000 P-gp, detectable in 8226/Dox40 cells with MAB C219 (Fig. 2B, right panel), was not detected in immunoblots with MAB QCRL-1, consistent with the immunodot analyses.

To be widely useful for the detection of MRP in many experimental applications and in the analysis of clinical samples, it is important that MRP-specific MABs are able to recognize MRP epitopes in fixed cells and tissues. For this reason, labeling of H69, H69AR, H69PR, C1, and T5 cells with MAB QCRL-1, QCRL-2, and QCRL-3 was examined by flow cytometry and indirect immunofluorescence microscopy. None of the MABs reacted with unfixed cells (data not shown), suggesting that the MRP epitopes detected by these MABs are not exposed on the cell surface. However, the epitopes recognized by these three MABs remained intact after fixation of cells with either 0.5% paraformaldehyde or 70% methanol. MRP reactivity with MABs QCRL-1 and QCRL-3 also remains intact after fixation with 10% formalin.6 Representative flow cytometry histograms obtained with MAB QCRL-3 and cells fixed with 0.5% paraformaldehyde are shown in Fig. 3. MAB QCRL-3 clearly discriminated between H69AR cells, in which high levels of MRP are detected in essentially all cells, and parental H69 cells, in which the M, 190,000 protein is not detected (Fig. 3A). A small difference in immunofluorescent labeling was also observed between the parental H69 cells and revertant H69PR cells, which express slightly higher levels of MRP than H69 cells (6). When MRP-transfected T5 cells were labeled with MAB QCRL-3, an asymmetric distribution of relative fluorescence intensity was observed (Fig. 3B). These findings were not unexpected since T5 cells are an unclooned population and, therefore, individual cells within this population are likely to express different levels of MRP (7). Similar histograms were obtained with MABs QCRL-1 and QCRL-2 (data not shown).

Using indirect immunofluorescence microscopy, all three MABs were observed to react intensely with resistant T5 and H69AR cells but not with C1 and H69 cells. Representative results obtained with MAB QCRL-3 are shown in Fig. 4. Labeling of H69AR cells was uniform while staining of T5 cells was somewhat heterogeneous, consistent with the flow cytometric analyses. Both MRP-positive T5 cells and H69AR cells showed predominantly plasma membrane labeling. These data are consistent with subcellular fractionation studies, which also indicate a predominantly plasma membrane localization of MRP in these cells.4 However, some granular cytoplasmic

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MRP-SPECIFIC MONOCLONAL ANTIBODIES

Figs. 3. Flow cytometric analyses of fixed cells with MAb QCRL-3. Cells were fixed with 0.5% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with MAb QCRL-3 (ascites, diluted 1:1000), followed by incubation with fluorescein-conjugated goat anti-mouse IgG. Shown are the histograms of relative fluorescence intensity for H69, H69PR and H69AR cells (A) and MRP-transfected T5 and vector-transfected Cl HeLa cells (B).

staining was also evident in the T5 and H69AR cells, suggesting that some MRP may be associated with intracellular membranes.

In our attempts to produce and isolate hybridomas secreting MAbs specific for conformation-dependent epitopes, we deliberately omitted denaturing detergents from the membranes used for immunization and from the immunoblotting screening procedure. This strategy has been relatively successful since it appears likely that only one of the five MAbs obtained, MAb QCRL-1, recognizes a linear epitope, as demonstrated by its reactivity with denatured protein in immunoblots. In contrast, the other four MAbs only detect MRP in nondenaturing immunoblotting procedures or under relatively nondenaturing conditions in immunoprecipitations and in fixed cells. These observations strongly suggest that these latter MAbs recognize conformation-dependent epitopes. Because of its unique ability to detect MRP in immunoblots, it may be inferred that MAb QCRL-1 reacts with an MRP epitope distinct from those recognized by the other four MAbs. The ability of

Fig. 4. Indirect immunofluorescence labeling of cells with MRP-specific MAb QCRL-3. Cells fixed in 0.5% paraformaldehyde were labeled using MAb QCRL-3 ascites (diluted 1:1000) and fluorescein-conjugated goat anti-mouse IgG. Cells were centrifuged onto microscope slides and examined using a Leitz Aristoplan microscope. A, parental H69 cells; B, multidrug resistant H69AR cells; C, vector-transfected control Cl cells; D, MRP-transfected multidrug-resistant T5 cells.
MAbs QCRL-2 and QCRL-3 to immunoprecipitate MRP, while MAbs QCRL-4 and QCRL-6 are unable to do so under the same conditions, suggests that these two pairs of MAbs also recognize at least two different epitopes. Mapping studies to identify the epitope sequences of these MAbs are in progress.

Most studies to date on MRP detection in normal and malignant cells have relied solely on the measurement of MRP mRNA (26–30). Interpretation of results from such studies may be limited because levels of expression of MRP mRNA may not always correlate with levels of M, 190,000 protein (7). Therefore, the MAbs described in this report will be valuable reagents for clinical and basic research investigations requiring the specific detection of the protein encoded by the MRP gene. The high degree of sensitivity of MAb QCRL-1 in immunoblot analysis and the ability of MAbs QCRL-1, QCRL-2, and QCRL-3 to recognize MRP in cells treated with several common fixatives enables the detection and quantitation of low levels of MRP. These MAbs should, therefore, be useful for detecting the presence of MRP in tumor samples and normal tissues. The availability of MAbs against different epitopes on the MRP molecule should also facilitate mapping studies to identify the epitope sequences of these MAbs.

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References

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