Advances in Brief

Immunohistochemical Detection of the Multidrug Resistance-associated Protein MRP in Human Multidrug-resistant Tumor Cells by Monoclonal Antibodies

Marcel J. Flens, Miguel A. Izquierdo, George L. Scheffer, Jan M. Fritz, Chris J. L. M. Meijer, Rik J. Scheper, and Guido J. R. Zaman

Abstract

We have generated rat and murine monoclonal antibodies against multidrug resistance-associated protein (MRP), a M, 180,000–195,000 membrane glycoprotein involved in a non-P-glycoprotein multidrug resistance of human tumor cells. The antibodies were raised against two different segments of MRP and found to be suitable for protein blot analyses, immunohistochemical and cytochemical studies, as well as flow cytometry of permeabilized cells. The antibodies do not cross-react with the human P-glycoproteins. Immunocytochemistry using MRP-overexpressing tumor cells of different histogenetic origins showed that MRP is predominantly located in the plasma membrane. Immunoelectron microscopy confirmed the plasma membrane location of MRP. The MRP antibodies provide a sensitive and specific tool for studies on MRP-mediated multidrug resistance.

Introduction

Upon exposure to natural product drugs, tumor cells can acquire resistance to structurally and functionally unrelated drugs. This type of drug resistance is called multidrug resistance. The classical form of MDR is caused by the P-glycoprotein encoded by the human MDR1 gene. This protein is inserted in the plasma membrane and acts as an ATP-driven drug efflux pump (1). The presence of MDR1 Pgp in tissues such as colon, liver, and kidney and at the blood-brain barrier suggests that it has a role in the defense against xenobiotics (1). Increased expression of the MDR1 gene has been detected in various human tumors arising from tissues that normally express Pgp, as well as in tumors originating from cells that do not express Pgp, such as myelomas and sarcomas (1). Although Pgp overexpression is likely to suggest that it has a role in the defense against xenobiotics (1). MDR3 is caused by the P-glycoprotein encoded by the human MDR3 gene and normal FVB mice, respectively (18). The MRP sequences in the expression plasmids encoded amino acids 192–360 (FPI) or amino acids 1294–1430 plus 1497–1531 of MRP (FPIII). The fusion proteins were produced in E. coli JM101 and purified by affinity chromatography (19). From 1-liter batches of lysed E. coli cells, 15 mg of soluble FPI (>95% pure) and 100 mg of soluble FPIII (>95% pure) were isolated.

Immunizations. Female Wistar rats and BALB/c mice received footpad injections of 25 mg of purified FPI or 10 mg of FPIII emulsified in Freund’s complete adjuvant (Difco, Detroit, MI). After 2 weeks, a first booster injection was given with the same dose of antigen emulsified in Freund’s incomplete adjuvant (Bacto, Detroit, MI). Four days before fusion, a second booster injection was given without adjuvant.

Materials and Methods

Cell Lines. All multidrug-resistant cell lines used in this study have been described previously (3–6, 13, 16) (Table 1). S1(MRP) and S1(MRP)-2 are sublines of the nonsmall cell lung cancer cell line SW-1573 that stably overexpress MRP. These cell lines were obtained after transfection of SW-1573/S1 cells with an expression vector containing MRP complementary DNA and a neomycin resistance gene, followed by selection with neomycin (16). The MDR3 Pgp expressing cell line V01V01 and the control cell line FVB#C are SV40 immortalized mouse ear fibroblasts obtained from FVB mice transgenic for the human MDR3 gene and normal FVB mice, respectively (18).

MRP Fusion Proteins. Two fusion genes consisting of the gene for the Escherichia coli maltose-binding protein and two different segments of the MRP complementary DNA were constructed in the plasmid vector pMal-c (19). The MRP sequences in the expression plasmids encoded amino acids 192–360 (FPI) or amino acids 1294–1430 plus 1497–1531 of MRP (FPIII). The fusion proteins were produced in E. coli JM101 and purified by affinity resin affinity chromatography (19). From 1-liter batches of lysed E. coli cells, 15 mg of soluble FPI (>95% pure) and 100 mg of soluble FPIII (>95% pure) were isolated.
Table 1 Staining of MDR tumor cell lines with MRPM6

<table>
<thead>
<tr>
<th>Cell linea</th>
<th>MDR phenotypeb</th>
<th>MRPM6 stainingc</th>
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<tbody>
<tr>
<td>Non-small cell lung cancer</td>
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<tr>
<td>SW-1573</td>
<td>non-Pgp</td>
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<tr>
<td>2R120</td>
<td>Pgp</td>
<td>20 30 50</td>
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<td>1R500-0</td>
<td>Pgp</td>
<td>55 45</td>
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<td>S1 (MRP)</td>
<td>non-Pgp</td>
<td>20 20 50 10</td>
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<tr>
<td>S1 (MRP)-2</td>
<td>non-Pgp</td>
<td>30 50 20</td>
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<tr>
<td>Small cell lung cancer</td>
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<td>GLC4</td>
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<tr>
<td>GLC4/ADR</td>
<td>non-Pgp</td>
<td>10 20 70</td>
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<td>Promyelocytic leukemia</td>
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<td>HL60</td>
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<td>non-Pgp</td>
<td>30 70</td>
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<tr>
<td>HT1080/DR4</td>
<td>non-Pgp</td>
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<td>Breast cancer</td>
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<td>MCF7</td>
<td>non-Pgp</td>
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<td>MCF7/MITOX</td>
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<td>MCF7/D40</td>
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<td>8226</td>
<td>Pgp</td>
<td>98 2</td>
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<tr>
<td>8226/DOX40</td>
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<tr>
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<td>Pgp</td>
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<tr>
<td>2780/AD</td>
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a S1 (MRP) and S1 (MRP)-2 were obtained after transfection of SW-1573 cells with an expression vector containing MRP complementary DNA and a neomycin resistance gene, folowed by selection with doxorubicin. All other MDR cell lines were isolated after selection with doxorubicin except MCF7/MITOX, which was isolated after selection with mitoxantrone.

b MDR phenotype: -, parental cell line (by definition, drug-sensitive); Pgp, overexpression of MDR gene relative to parental cell line; non-Pgp, MDR with reduced drug accumulation but without overexpression of MDR gene.

c Staining intensity for each cell line was determined according to the following scale: no staining, -; weak, ±; positive, +; intermediate, ++; strong, +++. Percentages of staining were determined by counting at least 200 cells/preparation.

(w/v) SDS supplemented with 1 mM phenylmethylsulfonyl fluoride, leupeptide (2 μg/ml), pepstatin (1 μg/ml), and aprotinin (2 μg/ml). DNA was sheared by sonification. Protein concentration was determined with a Bio-Rad protein assay (Bio-Rad, Richmond, CA). Total protein (25 μg) was fractionated on a 7.5% (w/v) polyacrylamide slab gel containing 0.1% (w/v) SDS and transferred onto a nitrocellulose filter by electroblotting. The filter was incubated for at least 2 h in PBS containing 1% (w/v) BSA (PBS/BSA), 1% (w/v) milk powder, and 0.05% (v/v) Tween-20 to prevent nonspecific binding of antibodies. Incubation with MAbs (MRPrl, 1:3000; MRPmo, 1:250) was for 1 h at 4°C in 500 μl PBS/BSA containing either one of the MRP MAbs or rabbit anti-mouse IgG, and 125I-labeled protein A.

Flow Cytometry. Cells were permeabilized in 10% (v/v) lyzing solution G (Becton and Dickinson, San Jose, CA) in dH₂O and incubated for 15 min in PBS/BSA containing 1% (v/v) normal goat serum. Cells (10⁵) were incubated for 1 h at 4°C in 500 μl PBS/BSA containing either one of the MRP MAbs or rabbit anti-mouse IgG, and 125I-labeled protein A.

Immunocytochemistry. Cyto centrifuge preparations of tumor cell lines were air dried overnight and fixed for 10 min in acetone or 4% (v/v) paraformaldehyde in PBS. The slides were incubated for 1 h with MAB diluted in PBS containing 1% (v/v) BSA (PBS/BSA). MAB1 was diluted 1:2000 and MRPM6 1:50. MB binding was detected using biotinylated rabbit anti-mouse IgG (1:100) or anti-mouse IgG (1:150, Dako) and streptavidin conjugated to horseradish peroxidase (1:500, Zymed). Bound peroxidase was developed with 4 mg (w/v) amino-ethyl-carbazole and 0.02% (v/v) H₂O₂ in 0.1 M NaAc (pH 5.0), counterstained with haematoxylin, and mounted with Aquamount. Cells spotted on poly-L-lysine coated slides were fixed and incubated with the MRP MAbs as described above for cyto centrifuge preparations.

Electron Microscopy. Tumor cells were incubated for 15 min on poly-L-lysine coated glass slides, washed with PBS, and fixed for 15 min at room temperature in 0.1% (v/v) glutaraldehyde-1.5% (v/v) paraformaldehyde in PBS. Incubation with MAbs was for 1 h in PBS/BSA. MB binding was detected with biotinylated rabbit anti-rat IgG (1:100; 1 h) or biotinylated rabbit anti-mouse IgG (1:150; 1 h Dako). The gold particles were visualized with silver as described by the manufacturer (Aurion). The slides were incubated in 2% (v/v) glutaraldehyde for 30 min and 1.5% (w/v) osmium tetroxide for 10 min, dehydrated with acetone, and embedded in Epon 812. Ultra-thin sections were collected on 300-mesh Formvar-coated nickel grids, and if necessary, the reaction was further enhanced with silver by incubation with silver acate in sodium citrate-buffered 50% (w/v) gum Arabic, catalyzed by hydroquinone. The sections were contrasted with uranyl acetate and lead citrate and examined in a Jeol 1200EX electron microscope.

Immunohistochemistry. Human tumor tissues were taken from the tissue bank of the Department of Pathology, Free University Hospital. All tissues were sampled from surgical specimens or from autopsies with short (<8 h) postmortem delays. Cryostat sections of tissue samples were cut 4-μm thick, air-dried overnight, and fixed in acetone for 10 min at room temperature. The sections were incubated for 20 min with normal rabbit serum (1:50 Dako) followed by MAb (MRPrl, 1:100; MRPM6, 1:10) and conjugates diluted in PBS/BSA. The method of staining was an indirect immunoperoxidase method using peroxidase conjugated rabbit anti-rat or rabbit anti-mouse immunoglobulins (1:25, Dako). In formalin-embedded tissue samples, endogenous peroxidase activity was blocked by 0.3% (v/v) H₂O₂ in methanol. After preincubation with normal goat serum during 15 min (1:50), the slides were incubated with the MAbs (MRPrl, 1:100; MRPM6, 1:10) for 60 min. MB binding was detected using biotinylated rabbit anti-mouse IgG (1:150) or biotinylated rabbit anti-rat IgG (1:100, Dako) and streptavidin conjugated to horseradish peroxidase (1:500) (Zymed). Bound peroxidase was developed with 4 mg (v/v) 3,3'-diaminobenzidine tetrahydrochloride and 0.02% (v/v) H₂O₂ in PBS. The slides were counterstained with haematoxylin and mounted.

Results

To examine the expression of MRP in human tumor cells, we generated MAbs against two bacterial fusion proteins containing different segments of MRP. The first fusion protein (FPI) contained a relatively hydrophilic segment of 168 amino acids in the amino-proximal half of the protein (Ref. 7, Fig. 1). The second fusion protein (FPPII) contained...
Fig 2. Western blot analysis and flow cytometric analysis of MRP in tumor cell lines with anti-MRP MAbs. a, protein blot of SDS-polyacrylamide gel electrophoresis gels of total protein isolates of cell lines with increased levels of MRP [GLC4/ADR, HT1080/DR4, HL60/ADR, S1(MRP), and S1(MRP)-2] and the corresponding parental drug-sensitive cell lines (GLC4, HT1080, HL60, and SW-1573), incubated with MRP1. Some nonspecific low molecular bands are detected with MRP1. b, protein blot of SDS-polyacrylamide gel electrophoresis gels of total protein isolates of cell lines with increased levels of MRP [GLC4/ADR and S1(MRP)] and the corresponding parental drug-sensitive cell lines (GLC4 and SW-1573) incubated with MRPm. c, d and e, immunoblot analysis of SDS-polyacrylamide gel electrophoresis gels of total protein isolates of cell lines with increased levels of MRP (GLC4/ADR), MDR1 Pgp (1R500-9), or MDR3 Pgp (V01V01) and the corresponding parental cell lines (GLC4, SW-1573, and FVB/C). Protein blots incubated with c, MRP1, d, MRPm or e, C219. f, flow cytometric analysis of permeabilized small cell lung cancer GLC4 and GLC4/ADR cells detected with MRPm, MRP1, and an isotype-matched control rat MAbs.
Fig 3. a–h Detection of MRP in cytocentrifuge preparations of human tumor cell lines (a–f) and frozen sections of human tumors (g and h). a, drug-sensitive small cell lung cancer GLC4 cells, b, multidrug-resistant GLC4/ADR cells. c, drug-sensitive leukemic HL60 cells. d, multidrug-resistant HL60/ADR cells. e, drug-sensitive nonsmall cell lung cancer SW-1573 cells. f, S1(MRP), a multidrug-resistant subline of SW-1573, obtained after transfection with an expression vector containing the MRP complementary DNA. a–f (×40), stained with MRP11. g, normal esophagus with an adenocarcinoma of the stomach stained with a control rat MAb (×40). h, the same tumor stained with MRP11 (×40).

Fig 3. i–l Detection of MRP in drug-sensitive (GLC4) and MRP-overexpressing (GLC4/ADR, HL60/ADR, and S1(MRP)) cells by immunoelectron microscopy with MAb MRPm6. i, small cell lung cancer GLC4 cells. j, GLC4/ADR. k, S1(MRP). l, HL60/ADR. (×7000). In the parental cell lines HL60 and SW1573, no MAb binding could be detected (data not shown).
tained a 170-amino acid-long segment of MRP that corresponds to the carboxy-terminal end and part of the carboxy-proximal nucleotide-binding domain of MRP (Ref. 7, Fig. 1). Hybridomas secreting antibodies that reacted with MRP were selected on the basis of strong immunocytochemical staining with GLC4/ADR cells, a non-Pgp MDR subline of the small cell lung cancer cell line GLC4 (3). This subline overexpresses MRP 25-fold (8). About 15% of the hybridomas analyzed (24 of 160 against FPI and 29 of 200 against FPIII) secreted antibodies that reacted strongly with GLC4/ADR and not with GLC4 cells. On protein blots, these antibodies recognized a protein of Mr 195,000 in isolates of GLC4/ADR cells, indicating that they reacted with MRP (Fig. 2, a and b). One rat MAb of the IgG2a subclass against FPI (MRPrl) and one mouse MAb of the IgG1 subclass against FPIII (MRPm6) were subcloned and further characterized.

On protein blots of SDS-polyacrylamide gels, both MAbs reacted with the Mr 180,000 to 195,000 MRPs from GLC4/ADR, the leukemic cell line HL60/ADR, and S1(MRP), a stable MRP-overexpressing transfectant of the nonsmall cell lung carcinoma cell line SW1573 (Fig. 2, a and b). MRPrl even detected a low basal level of MRP present in the drug-sensitive parental cell lines (Fig. 2a). The MAbs did not cross-react with the MDR1 (1R500-0) or MDR3 (V01V01) Pgps (Fig. 2, c and d). However, MRPrl also reacted with a band of Mr 185,000 in the two control mouse fibroblast cell lines FVB#C and V01V01 (Fig. 2c). This band does not comigrate with Pgp (Fig. 2, c and e) and might be mouse MRP. In protein isolates of cell lines with increased amounts of MRP, MRPrl also detected a band of Mr 90,000 and MRPm6 a band of about Mr 70,000 (Fig. 2, a-d). Most likely, these extra bands are degradation products of MRP.

In flow cytometry, both MAbs reacted with permeabilized cells (Fig. 2f). The MAbs did not bind to living, untreated cells. In agreement with this, cells spotted on poly-L-lysine-coated slides had to be fixed before immunocytochemical staining with MRPrl or MRPm6 could be observed. Fixation conditions that allowed MRPrl and MRPm6 access to their epitope were incubation in acetone, methanol, or ethanol for 10 min at room temperature; or in 4% (v/v) formaldehyde or paraformaldehyde; or 0.1% (v/v) glutaraldehyde in PBS for 15 min at room temperature.

In Fig. 3, a–f the immunocytochemical staining of parental and MDR tumor cells with MRPm6 is shown. The staining results on a broader panel of cell lines are summarized in Table 1. MRPrl showed the same specificity and staining pattern for cells with increased MRP expression as MRPm6. Strong staining with MRPrl was still observed at 1 μg/ml and with MRPm6 at 40 μg/ml, indicating highest affinity for the rat MAb. The non-Pgp MDR lines GLC4/ADR, HL60/ADR, and 2R120 were strongly stained with MRPrl and MRPm6, whereas their parental drug-sensitive lines were not stained or only weakly stained (Fig. 3; Table 1). The strong reaction with the stable MRP transfectants S1 (MRP) and S1 (MRP)-2 proves that the MAbs react with MRP (Fig. 3f; Table 1). The breast cancer cell line MCF7/MITOX was the only non-Pgp MDR line that did not show increased
staining compared to its parental line. This is in agreement with the lack of MRP mRNA overexpression reported for this cell line (13).

Also, the Pgp-overexpressing myeloma cell line 8226/Dox40 and ovarian carcinoma cell line 2780/AD did not show increased MRP staining, indicating that the MAbs also lack cross-reactivity with MDR1 Pgp on cytological preparations. The SW-1573 sublines 1R500-0 and 2R160 that highly overexpress MDR1 Pgp (5, 6) contained slightly higher amounts of MRP as compared to the drug-sensitive parental cell line (Table 1). This is not unexpected since both cell lines were derived by additional selection from non-Pgp MDR variants with low-level drug resistance.

Both MAbs predominantly stained the plasma membrane of the MRP-overexpressing cells. This confirms our previous results obtained with anti-MRP polyclonal antisera (16). Where detectable, MRP staining of drug-sensitive and Pgp-overexpressing cells was also mainly on the plasma membrane. In most MRP-overexpressing cells, we also found a densely stained spot next to the nucleus. This spot may correspond to the Golgi network. In the HL60/ADR cell line, a larger intracytoplasmatic spot was present in some of the cells (Fig. 3d).

The subcellular location of MRP was studied by immunoelectron microscopy using a preembedding labeling procedure. MRP was detected in GLC4/ADR, HL60/ADR, and S1 (MRP) cells by silver-enhanced, ultra-small gold conjugates (Ref. 20, Fig. 3, i-o). Both MAbs reacted on the plasma membranes of cells with increased MRP, in agreement with the immunocytochemical data (Fig. 3, j-k). We have not been able to detect intracytoplasmatic MRP.

MRP expression was also studied in a small panel of seven human tumors consisting of two breast carcinomas, one adenocarcinoma of the stomach, and four squamous cell carcinomas of the esophagus. None of the patients from which these tumors were derived had received chemotherapy. Staining of MRP was detected both in frozen and formalin-fixed, paraffin-embedded sections from one breast carcinoma, one adenocarcinoma of the stomach, and two squamous cell carcinomas of the esophagus. Staining with control, isotype-matched irrelevant antibodies was negative (Figs. 3g and 2h). These results indicate that both MRP MAbs can be used for immunohistochemical studies.

Discussion

We have generated anti-MRP-specific MAbs that are suitable for protein blot analyses, immunocytochemical and histochemical studies, and flow cytometry of permeabilized cells. The MAbs did not cross-react with the human MDR1 and MDR3 Pgps. The MAbs did not react with living cells as studied by flow cytometry or with unfixed cells in immunocytochemistry, indicating that they recognize internal epitopes. This was expected for MRP, which was raised against the carboxy-end of MRP (FPPI; Fig. 1). However, on the basis of secondary structure analyses and comparisons with other ATP-binding cassette transporters (7), we had expected the segment to which MRP is raised to encompass an extracellular loop (FPPI; Fig. 1). The lack of staining of MRP with unpermeabilized cells could mean that the present secondary structure model for MRP is not correct or that MRP reacts with an internal epitope at one of the ends of the segment. Alternatively, the reaction of MRP with its epitope on living cells may be inhibited by glyclosylation or tertiary structure (7, 10, 16). Peptide libraries will be screened to identify the epitopes recognized by the MRP MAbs. In addition, other MAbs raised against FPI will be tested further for possible reactivity with viable cells.

Immunocytochemistry showed that MRP is predominantly located in the plasma membrane of MRP-overexpressing cells. Immunoelectron microscopy confirmed the plasma membrane location of MRP. In immunocytochemistry, we also detected some cytoplasmic staining, mainly concentrated in a spot next to the nucleus. This spot may correspond to the Golgi network. MRP in the Golgi apparatus may represent MRP en route to the plasma membrane. MRP present in membranes of the endoplasmic reticulum, the Golgi network, and vesicles might contribute to drug resistance by sequestering cytotoxics from their site of action. Such a redistribution of cytotoxic drugs has been reported for several MDR1 Pgp and MRP-overexpressing cell lines, including GLC4/ADR and HL60/ADR (10, 21). However, on the basis of the predominant plasma membrane location of MRP, as observed both by immunocytochemistry and immunoelectron microscopy and recent pharmacological studies on the MRP transfectant S1 (MRP), we conclude that the major function of MRP in drug resistance is that of a plasma membrane drug efflux pump (16). Interestingly, we could detect MRP in four of seven tumors from untreated patients. The expression of MRP at the protein level will be studied further in a large panel of primary and relapsed human tumors. We expect that the MRP MAbs described in this study will provide specific and sensitive reagents to study the putative role of MRP in clinical multidrug resistance at the protein level by immunocytochemical and histochemical techniques.

Acknowledgments

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References


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