

# Specific Detection of Multidrug Resistance Proteins MRP1, MRP2, MRP3, MRP5, and MDR3 P-Glycoprotein with a Panel of Monoclonal Antibodies<sup>1</sup>

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## ABSTRACT

Tumor cells may display a multidrug resistance phenotype by overexpression of ATP binding cassette transporter genes such as *multidrug resistance (MDR) 1 P-glycoprotein (P-gp)* or the *multidrug resistance protein 1 (MRP1)*. MDR3 P-gp is a close homologue of MDR1 P-gp, but its role in MDR is probably minor and remains to be established. The MRP1 protein belongs to a family of at least six members. Three of these, *i.e.*, MRP1, MRP2, and MRP3, can transport MDR drugs and could be involved in MDR. The substrate specificity of the other family members remains to be defined. Specific monoclonal antibodies are required for wide-scale studies on the putative contribution of these closely related transporter proteins to MDR.

In this report, we describe the extensive characterization of a panel of monoclonal antibodies (Mabs) detecting several MDR-related transporter proteins in both human and animal tissues. The panel consists of P<sub>3</sub>II-1 and P<sub>3</sub>II-26 for MDR3 P-gp; MRPr1, MRpm6, MRpm5, and MIB6 for MRP1; M<sub>2</sub>I-4, M<sub>2</sub>II-12, M<sub>2</sub>III-5 and M<sub>2</sub>III-6 for MRP2; M<sub>3</sub>II-9 and M<sub>3</sub>II-21 for MRP3; and M<sub>5</sub>I-1 and M<sub>5</sub>II-54 for MRP5. All Mabs in the panel appeared to be fully specific for their cognate antigens, both in Western blots and cytospin preparations, as revealed by lack of cross-reactivity with any of the other family members. Indeed, all Mabs were very effective in detecting their respective antigens in cytospins of transfected cell lines, whereas in flow cytometric and immunohistochemical analyses, distinct differences in reactivity and suitability were noted. These Mabs should become valuable tools in studying the physiological functions of these transporter proteins, in screening procedures for the absence of these proteins in hereditary metabolic (liver) diseases, and in studying the possible contributions of these molecules to MDR in cancer patients.

## INTRODUCTION

Tumor cells exposed to cytotoxic drugs can acquire resistance to structurally and functionally unrelated drugs. This phenomenon is known as MDR<sup>3</sup> (reviewed in Ref. 1). In human tumor cells, several transporter proteins can be involved in MDR. These proteins, MDR1 P-gp (ABCB1; reviewed in Ref. 2), MRP1 (ABCC1; reviewed in Ref. 3), MRP2 (ABCC2; Refs. 4–6), MRP3 (ABCC3; Refs. 5 and 7–11), and Breast Cancer Resistance Protein (ABCG2; Refs. 12–14) all belong to the ABC transporter family (15, 16). They act as efflux

pumps, resulting in decreased intracellular concentrations of natural product drugs.

MDR1 P-gp, the prototypic MDR transporter protein, is a glycosylated,  $M_r$  170,000 protein that transports a broad range of substrates, including several anticancer drugs (17). A close homologue of MDR1 P-gp is the MDR3 P-gp protein (ABCB4). This protein is essential for the secretion of phosphatidylcholine into the bile (18). Lack of expression of *MDR3 P-gp* in the liver is responsible for type 3 progressive familial intrahepatic cholestasis (19). Although 77% identical to MDR1 P-gp, most attempts to link MDR3 P-gp to MDR have not been successful (20–23). However, some investigators have reported overexpression of *MDR3 P-gp* in resistant tumor cells (24, 25). Furthermore, recent studies with MDR3 P-gp transfected pig kidney LLC-PK1 cells show that *MDR3 P-gp* is capable of transporting some cytotoxic drugs (26). These studies indicate that the role of MDR3 P-gp in MDR merits further investigation, particularly when taking into account that also the second homologue of MDR1 P-gp, known as sister of P-gp (27) or the bile salt efflux pump (Ref. 28; ABCB11), is capable of transporting the anticancer drug paclitaxel (29).

The  $M_r$  190,000 MRP1 protein was shown to confer a similar resistance phenotype as MDR1 P-gp (30, 31), although these two proteins share only 14% amino acid identity. In humans, at least five homologues of *MRP1* are expressed (5, 32).

MRP2 is responsible for the hepatobiliary excretion of a broad range of organic anions, including glutathione and bilirubin glucuronides (33–35). Mutations in the *MRP2* gene cause the Dubin-Johnson syndrome (36–39). The substrate specificity of MRP2 is very similar to that of MRP1 (35, 40).

Of all MRP family members, *MRP3* is the closest homologue of MRP1 (7–11). Recently, we showed, using MRP3-transfected ovarian carcinoma cells, that MRP3 is capable of transporting several anticancer drugs (7).

For MRP4 (ABCC4) and MRP5 (ABCC5), no major changes were found in mRNA levels in doxorubicin- or cisplatin-resistant cells (5). However, recently it was found that these transporters are able to transport nucleoside analogues (41, 42). In addition, some low level resistance against CdCl<sub>2</sub> and potassium antimonyl tartrate was found in *MRP5*-transfected cell lines (43).

Overproduction of *MRP6* (ABCC6) mRNA in tumor cell lines was found to be invariably associated with the amplification of the adjacent *MRP1* gene, and MRP6 probably does not contribute to the resistance of these cell lines (32). Very recently, it was found that mutations in the *MRP6* gene are responsible for the connective tissue disorder pseudoxanthoma elasticum (44–46).

To study these transporter proteins in clinical material, to reveal their physiological functions and their possible contributions to MDR, specific monoclonal antibodies are essential. Here, we focus on extending the panel of Mabs specifically recognizing members of the P-gp and MRP families of transporter proteins.

Several Mabs have been described that detect internal or external epitopes of MDR1 P-gp (47–50). Mabs that detect external epitopes

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<sup>3</sup> The abbreviations used are: MDR, multidrug resistance; ABC, ATP binding cassette; P-gp, P-glycoprotein; MRP, multidrug resistance protein; BCRP, breast cancer resistance protein; Mab, monoclonal antibody; HRP, horseradish peroxidase; MDCK, Madin-Darby canine kidney; FACS, fluorescence-activated cell sorter.

are widely used to detect the protein on viable cells that have not been altered by fixation. Those binding a functional epitope of the protein, as is the case with, *e.g.*, the UIC2 Mab (48), allow studies of the substrate specificity in blocking experiments.

MDR3 P-gp has thus far mainly been studied by means of polyclonal antisera (51) or cross-reacting MDR1 P-gp Mabs [such as C219 (52)]. To date, two reports have been published describing IgM Mabs detecting the MDR3 P-gp (53, 54).

For MRP1, several well-performing Mabs have been described (55, 56). All described MRP1 Mabs detect internal epitopes that require permeabilization before they can bind to their epitopes. No Mabs detecting external epitopes of the MRP1 protein have been reported.

For earlier MRP2 studies, we used Mabs produced against a fusion protein of the rat Mrp2 protein that also react with the human MRP2 protein (4, 5). For our initial studies on the putative drug transport activity of MRP3 (7), we developed Mabs detecting the MRP3 protein in 2008 ovarian carcinoma cells transfected with the *MRP3* cDNA construct. As yet, no detailed analysis of the performance of these antibodies has been made, whereas no Mabs have thus far been described detecting the other MDR-related ABC transporter molecules.

As the list of related ABC transporters involved in MDR grows, the specificity of the Mabs identifying these transporter molecules becomes exceedingly important. Only incidental information regarding cross-reactivity with family transporter molecules of the above-mentioned Mabs has become available. The epitopes detected by the anti-MRP1 Mabs QCRL-1, MRPr1, and MRpm6 were mapped to amino acids 918–924, 238–247, and 1511–1520, respectively (57, 58). Because these epitope sequences are poorly conserved in the family members examined, it was assumed that these Mabs specifically detect MRP1. Extending these studies, we present here a thorough characterization of a panel of new and previously produced Mabs that detect MDR3 P-gp, MRP1, MRP2, MRP3, or MRP5.

## MATERIALS AND METHODS

**Cell Lines.** The lung cancer cell lines SW1573, GLC4, and GLC4/ADR have been described by Eijndems *et al.* (59) and Zijlstra *et al.* (60) and the leukemia cell lines HL60 and HL60/ADR by Marsh and Center (61). The 2008 ovarian carcinoma cell line (62) was obtained from Dr. Howell from the University of California (San Diego, CA). The MDCK II cell line was obtained from K. Simons (EMBL, Heidelberg, Germany). The pig kidney epithelial LLC-PK1 cells were obtained from the American Type Culture Collection.

All cell lines were grown in DMEM (Life Technologies, Inc., Paisley,

Scotland), supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, penicillin, and streptomycin, except for the LLC-PK1 cells, which were grown in M199 medium (Life Technologies, Inc., Paisley, Scotland). Resistant cell lines were cultured in the presence of drugs until 3–10 days before the experiments. All cells were negative for *Mycoplasma* as tested by the Gene-Probe rapid *Mycoplasma* detection system (Gene-Probe, San Diego, CA).

**Transfections and Transfected Cell Lines.** To make stable cell lines expressing *MRP1*, *MRP2*, or *MRP3*, the full-length cDNAs encoding these proteins were cloned behind the cytomegalovirus promoter in the retroviral vector pCMV-neo. The resulting constructs were subsequently transfected into the amphotropic retroviral packaging cell line Phoenix (kindly provided by G. P. Nolan, Stanford University Medical Center, Stanford, CA; Ref. 63) according to the calcium phosphate coprecipitation method using a transfection kit (Life Technologies, Inc., Gaithersburg, MD). Transduction of human tumor cell lines was as described (64). Positive clones were selected by Western blot analysis with monoclonal antibody MRPr1 for MRP1 (55), M<sub>2</sub>III-5 for MRP2 (4), and M<sub>3</sub>II-9 for MRP3 (7).

The *MRP1* transfected subline of the SW1573, S1(MRP), the pig kidney LLC PK1 cells transfected with *MDR1 P-gp* or *MDR3 P-gp* and the *MRP5* transfected subline of the MDCK II cell line have been described (31, 42, 65).

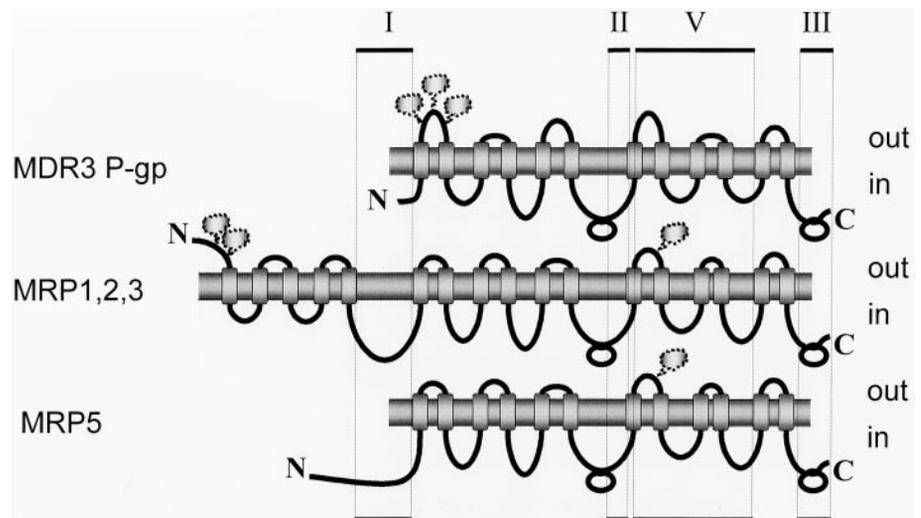
**Fusion Proteins.** A fusion gene consisting of the *glutathione S-transferase* gene and a fragment corresponding to amino acids 629–692 (FP P<sub>3</sub>II) of the human *MDR3* gene was constructed in pGEX-3x vector as described (51). Fusion genes consisting of the gene for the *Escherichia coli* maltose-binding protein and different fragments of the human *MRP1*, *MRP2*, *MRP3*, or *MRP5* were constructed in the pMAL-c vector as described (31). The *MRP1* segment in the expression plasmid encoded amino acids 986–1204 (FP M<sub>1</sub>V). The fragment spans both putative internal and external regions of MRP1. The *MRP2* segments encoded amino acids 215–310 (FP M<sub>2</sub>I) and 852–954 (FP M<sub>2</sub>II). The rat *Mrp2* segment encoded amino acids 1339–1541 (FP M<sub>2</sub>III). The *MRP3* segments encoded amino acids 830–949 (FP M<sub>3</sub>II) and 1338–1527 (FP M<sub>3</sub>III), and the *MRP5* segments encoded amino acids 82–168 (FP M<sub>5</sub>I) and 722–910 (FP M<sub>5</sub>II).

Production and purification of the fusion proteins was as described (55). The positions of the protein fragments of the ABC transporters that are used in the different fusion proteins are indicated in Fig. 1.

**Mab Production.** Immunizations and production of hybridomas were as described before (55). For MRP1 immunizations, both the fusion protein and viable human *MRP1* overexpressing GLC4/ADR cells were used for alternating immunizations. For the other immunizations, just the bacterial fusion proteins were used. Approximately  $2 \times 10^6$  cells or 50  $\mu$ g of protein were used for each injection. All immunizations were in mice, except for the MRP5 immunization, which was carried out in rats.

Per fusion experiment, draining popliteal lymph nodes of three mice or two rats were used. Supernatants of obtained hybridoma cells containing Mabs were screened either on ELISA plates coated with specific fusion protein and on plates coated with irrelevant fusion protein or on isolated vesicles of

Fig. 1. Indication of regions of ABC transporter proteins used in the fusion proteins. The MRP1 model is based on the work of Bakos *et al.* (79), Hipfner *et al.* (80), and Kast and Gros (81); MRP5 is based on Belinsky *et al.* (10), and MDR3 P-gp is based on Lincke *et al.* (82). Glycosylation sites are indicated by "cloud" symbols. The roman numbers refer to the different fusion proteins (see also "Materials and Methods").



*MRP1*-expressing cells. Antibody binding was detected using peroxidase-conjugated secondary antibodies and an appropriate substrate (see below).

Hybrid cells secreting antibodies of interest were selected and subcloned at least three times by limiting dilution. For large-scale antibody production, clonal hybridomas were cultured in 1.5 L growth medium containing 1% (v/v) Nutridoma serum replacement (Boehringer Mannheim, Mannheim, Germany). Obtained supernatants were concentrated in ST25 capillary flow dialysers (Travenol AG, Baiter, Germany).

The isotype of the selected Mabs was determined using IsoStrips (Boehringer Mannheim) or using isotype-specific, second step reagents. (Nordic, Tilburg, the Netherlands).

**ELISA Screening.** Flat-bottomed 96-well plates (Nunc maxisorp; Life Technologies, Inc., Merelbeke, Belgium) were coated overnight at 37°C with 100  $\mu$ l of ~2  $\mu$ g/ml protein solution in coating buffer (0.05 M sodium carbonate, pH 9.6). Plates were rinsed, blocked with 150  $\mu$ l PBS/1% BSA/0.05% Tween 20, and incubated with hybridoma supernatant and appropriate controls for 1 h at room temperature. HRP-labeled rabbit-antimouse (or -antirat) serum (1:500; Dako, Copenhagen, Denmark) in blocking buffer was used as a second step reagent. Color development was with 5-amino-2-hydroxybenzoic acid (Merck, Darmstadt, Germany) and 0.02% H<sub>2</sub>O<sub>2</sub> as a chromogen.

**Membrane Vesicle Screening.** Plasma membrane vesicles were prepared from parental GLC4 and drug-resistant GLC4/ADR cells as described (66). The final membrane preparations, containing both inside-out and right side-out vesicles, were stored at -80°C at a protein concentration of ~4 mg/ml. The enrichment of Na<sup>+</sup>K<sup>+</sup>-ATPase was ~5-fold.

Fifty  $\mu$ l of hybridoma supernatant was incubated at room temperature for 1 h with 2  $\mu$ g of plasma membrane vesicle preparations. The membranes were dot-blotted on a BSA-preloaded cellulose nitrate membrane filter in a 96-well dot-blot under vacuum suction and washed three times with PBS containing 1% BSA. Mab binding to the membrane preparations was demonstrated using HRP-labeled rabbit-antimouse serum (1:500; Dako) and 5-amino-2-hydroxybenzoic acid, 0.02% H<sub>2</sub>O<sub>2</sub> as a chromogen.

**Uptake of S-(2,4-Dinitrophenyl)-glutathione into Inside-Out Vesicles.** Uptake of tritium-labeled S-(2,4-dinitrophenyl)-glutathione into inside-out membrane vesicles was measured by rapid filtration and liquid scintillation counting, as described previously (66).

**Western Blot Analysis.** Total cell lysates were made as described (31). Protein concentrations were determined with a Bio-Rad protein assay (Bio-Rad, Richmond, CA). Ten to 40  $\mu$ g of cell lysates or crude or purified fusion proteins were fractionated on a 7% polyacrylamide slab gel and transferred onto a nitrocellulose membrane by electroblotting. After blocking, the membrane was incubated for 2 h with primary antibody in appropriate dilution. HRP-labeled-antimouse or -antirat serum (1:1000; Dako) was used as a secondary antibody. Enhanced chemiluminescence (Amersham, Buckinghamshire, United Kingdom) was used to detect Mab binding.

**Immunohistochemistry.** Cytospin preparations and cryosections (4  $\mu$ m) were air dried overnight and fixed for 7 min in acetone at room temperature. Sections of routinely processed, formalin-fixed, paraffin-embedded tissues were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked using 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 min. Antigen was detected either without pretreatment or after microwave antigen retrieval methods using 1 mM EDTA or 0.01 M citric acid (pH 6.0) in distilled water. The slides were incubated with primary antibody for 1 h at room temperature. Biotinylated rabbit-antimouse or -antirat serum (1:150; Zymed, San Francisco, CA) and HRP-labeled streptavidin (1:500; Zymed) were used as secondary reagents. Color development was with 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride and 0.02% H<sub>2</sub>O<sub>2</sub> or 0.4 mg/ml amino-ethyl-carbazole and 0.02% H<sub>2</sub>O<sub>2</sub> as a chromogen.

For tissues with high endogenous biotin activity, incubation with primary antibody was followed by HRP-labeled rabbit-antimouse or -antirat serum (1:200; Dako, Copenhagen, Denmark). Then a 10-min incubation with FITC-labeled tyramine [according to Raap *et al.* (67)] in PBS containing 0.01% H<sub>2</sub>O<sub>2</sub> was performed. The slides were examined under a fluorescence microscope (Leica DMRB, Rijswijk, the Netherlands). To obtain more permanent results and for a better impression of morphology, the slides were further incubated with HRP-labeled rabbit F(ab')<sub>2</sub>-anti-FITC fragments (1:100; Dako) and developed with amino-ethyl-carbazole/H<sub>2</sub>O<sub>2</sub>.

**FACS Analysis.** Cell suspensions at a concentration of 1  $\times$  10<sup>6</sup> viable cells/ml were plated in a V-shaped, 96-well plate in 100  $\mu$ l/well and incubated

with 100  $\mu$ l of hybridoma supernatant or positive or negative control Mab (HLA class I marker W6/32 and mouse IgG1, respectively) for 1 h at room temperature on an ELISA plate shaker (Heidolph Titramax 100; Salm and Kipp, Breukelen, the Netherlands). Antibody binding was detected using FITC-labeled rabbit-antimouse (or -antirat) serum (1:100; 50  $\mu$ l/well; Dako) for another 1 h at room temperature. The samples were analyzed on a FACS Star flow cytometer (Becton Dickinson, San Jose, CA). When Mabs detecting internal epitopes were tested, the cells were permeabilized prior to the experiment by a 10-min incubation in 10% lysing solution G (Beckton and Dickinson) and 0.1% saponin (Merck), or they were permeabilized by a 5-s incubation in 2% formaldehyde in acetone, or they were permeabilized by a 5-min incubation in 70% methanol at -20°C.

## RESULTS

### Mabs

To generate a broad panel of Mabs detecting MDR-related proteins of the P-gp and MRP family, a series of Mabs was produced. ELISA-based screening of hybridoma supernatants of mice immunized with MDR3 P-gp fusion proteins resulted in the isolation of two anti-MDR3 P-gp Mabs: P<sub>3</sub>II-1 and P<sub>3</sub>II-26. Similar fusions and screenings resulted in two anti-MRP2 Mabs: M<sub>2</sub>I-4 and M<sub>2</sub>II-12; two anti-rat-Mrp2 Mabs: M<sub>2</sub>III-5 and M<sub>2</sub>III-6; two anti-MRP3 Mabs: M<sub>3</sub>II-9 and M<sub>3</sub>II-21; and two anti-MRP5 Mabs: M<sub>5</sub>I-1 and M<sub>5</sub>II-54 (Table 1). All Mabs are mouse Mabs, except for the two anti-MRP5 Mabs, which are derived from a rat.

The three pairs of anti-MDR3 P-gp, -Mrp2 and -MRP3 Mabs were each raised against the same fusion protein, *i.e.*, FP P<sub>3</sub>II, FP M<sub>2</sub>III, and FP M<sub>3</sub>II, respectively. To exclude the possibility that the two MDR3 P-gp Mabs and the two Mrp2 Mabs detected the same epitopes on their respective antigens, blocking experiments in an ELISA system were performed. No significant decrease in signal was observed when wells were preincubated with one Mab of the couple and then stained for the other with isotype-specific, second step reagents (data not shown). Because the two MRP3 Mabs are both of IgG1 isotype, this type of blocking experiment could not be performed; and therefore, reactivity of both Mabs toward the same epitope of MRP3 cannot fully be excluded at this stage.

For MRP3, hybridoma supernatants of mice immunized with the COOH-terminal fusion protein M<sub>3</sub>III reacted with the MRP3 fusion protein, but they also reacted with the COOH-terminal fusion proteins of MRP1, MRP2, and MRP5 in ELISA (data not shown).

Regarding MRP1, for which already several good quality Mabs are available, we aimed at Mabs that react with functional epitopes. For immunizations, we used fusion protein M<sub>1</sub>V, containing both internal and external regions of MRP1, as well as viable *MRP1*-overexpressing GLC4/ADR cells. Mabs were selected on membrane fractions, consisting of a mixture of inside-out and right side-out membrane vesicles. A mouse Mab, named MIB6, was selected, reactive to an internal epitope of MRP1 as determined by FACS analysis with viable and permeabilized cells and immunoprecipitation experiments (not shown). Functional assays were performed with membrane vesicles in the presence or absence of the Mab. Addition of MIB6 supernatant caused an ~70% reduction of uptake of radiolabeled S-(2,4-dinitrophenyl)-glutathione into inside-out membrane vesicles of MRP1-positive GLC4/ADR cells. The blocking effect was not observed when ATP was left out or was replaced by AMP, nor with MRPr1, MRpm6, or MRpm5, nor in inside-out vesicles of parental GLC4 cells (data not shown). In other experiments, MIB6 was also found to inhibit MRP1-mediated transport of antifolates and substrate induced ATPase activity of MRP1 (68, 69), both in *MRP1*-overexpressing tumor cells and in *MRP1* transfectants.

All selected Mabs were tested for their reactivity and specificity in

Table 1 Specificities and performance of monoclonal antibodies detecting MDR transporter proteins

Transporter protein, Mab name, species, isotype, fusion protein used for immunization, and smallest reactive amino acid sequence known are indicated in the columns on the left. Reactivity in different techniques is indicated in the middle columns. The columns on the right show reactivities towards the respective antigens in frozen sections of different species. For reasons of comparison, the anti-MDR1 P-gp Mabs JSB-1 and C219 are included in the table.

Transporter protein	Mab			Antigen		Reactivity <sup>a</sup>							
	Name	Species	Isotype	Fusion protein	Amino acid sequence	Cytospins/ Frozen sections	Paraffin sections	Western blot	FACS	Human	Mouse	Rat	Guinea Pig
MDR1 P-gp	JSB1	Mouse	IgG1	NA <sup>b</sup>	?	+++	+	+	++	+++	-	-	-
	C219	Mouse	IgG2a	NA	569-74 + 1214-19	+++	++	+++	++	+++	++	++	++
MDR3 P-gp	P <sub>3</sub> II-1	Mouse	IgG1	P <sub>3</sub> II	629-692	++	+/-	+	ND	++	-	+/-	+/-
	P <sub>3</sub> II-26	Mouse	IgG2b	P <sub>3</sub> II	629-692	+++	+/-	+++	ND	+++	+/-	+	++
MRP1	MRPr1	Rat	IgG2a	M <sub>1</sub> I	238-247	+++	++	+++	++	+++	++	-	++
	MRPm5	Mouse	IgG2a	M <sub>1</sub> V	986-1204	++	+	+++	++	++	-	-	-
	MRPm6	Mouse	IgG1	M <sub>1</sub> III	1511-1520	+++	+	+++	++	+++	-	-	-
	MIB6	Mouse	IgM	M <sub>1</sub> V <sup>c</sup>	?	+	+	-	++	++	-	-	ND
MRP2	M <sub>2</sub> I-4	Mouse	IgG1	M <sub>2</sub> I	215-310	+++	+	++	ND	+++	-	-	-
	M <sub>2</sub> II-12	Mouse	IgG2a	M <sub>2</sub> II	852-954	++	-	+	ND	++	-	-	-
	M <sub>2</sub> III-5	Mouse	IgG2b	M <sub>2</sub> III	1339-1541	++	-	++	ND	++	-	++	+
	M <sub>2</sub> III-6	Mouse	IgG2a	M <sub>2</sub> III	1339-1541	+++	+	+++	+	++	-	+++	++
MRP3	M <sub>3</sub> II-9	Mouse	IgG1	M <sub>3</sub> II	830-949	+++	-	+++	+	+++	-	-	-
	M <sub>3</sub> II-21	Mouse	IgG1	M <sub>3</sub> II	830-949	+++	-	+++	ND	+++	-	-	-
MRP5	M <sub>5</sub> I-1	Rat	IgG2a	M <sub>5</sub> I	82-168	+++	-	++	+	+++	ND	ND	ND
	M <sub>5</sub> II-54	Rat	IgG2a	M <sub>5</sub> II	722-910	+	-	+	ND	+	ND	ND	ND

<sup>a</sup> -, no reactivity; +/-, very weak reactivity; +, weak reactivity; ++, good reactivity; +++, very good reactivity.

<sup>b</sup> NA, not applicable; ?, unknown; ND, not determined.

<sup>c</sup> For this immunization, booster injections with GLC4/ADR cells were also given.

Western blotting and on cytopins with transfected cell lines. In addition to the selected Mabs, we included the previously produced anti-MRP1 Mabs MRPr1, MRPm5, and MRPm6 in the panel. Furthermore, we studied the performance of this panel of Mabs on sections of frozen and formalin-fixed, paraffin-embedded tissues and in FACS analysis. In addition, we determined whether the antigen was detected in tissues from mice, rats, and guinea pigs. A summary of the results is presented in Table 1.

### Specificity of the Panel of Mabs Detecting MDR-related Proteins

The specificity of the panel of Mabs was determined in two types of experiments: (a) immunoblots with extracts of the parental and transfected 2008, LLC PK1, and MDCK II cells; and (b) cytopins of these cell lines. The results as depicted in Figs. 2 and 3, and Table 1 show that all Mabs perform well in either one or both of these techniques.

In the Western blots, specific bands of expected molecular weight are seen in the lanes containing the cell line transfected with the transporter against which the Mab was raised. Also on the cytopins, staining is only observed in the corresponding cell lines. In either test, none of the Mabs cross-reacts with one of the other transfected cell lines.

Some differences were observed, however, when the Western blot results were compared with the staining results on cytopins of the same cell lines. MIB6 shows no reactivity in Western blots but performs well on cytopins of MRP1-expressing cells. Apparently, the MIB6 Mab detects a conformational epitope that is lost during sample treatment for the Western blot technique.

Also some other Mabs (e.g., M<sub>2</sub>II-12 and M<sub>5</sub>II-54) performed better in staining the protein on cytopins than on the Western blots, whereas others performed equally well in both assays. In Table 1, a rating system with pluses is used for performance in the different techniques.

Another observation in the Western blots was that some of the Mabs also detected endogenously expressed transporter proteins. At prolonged exposure times (1-5 minutes), all anti-MRP1 Mabs detected a low level of MRP1 in the 2008 ovarian carcinoma sublines, and Mab M<sub>5</sub>I-1 detected a low level of MRP5 in these sublines as

well. From previous mRNA data, we know that these genes are indeed expressed in the 2008 sublines (5). Furthermore, both M<sub>2</sub>III-5 and M<sub>2</sub>III-6 detected a protein with a molecular weight comparable with the molecular weight of human MRP2 in some of the MDCK II cell lines. Most likely, the protein represents the canine MRP2 protein. Levels of this protein appeared to markedly vary between different sublines; parental and MDR1 P-gp transfected cell lines have relatively high levels, whereas the MRP5 transfected subline has only low

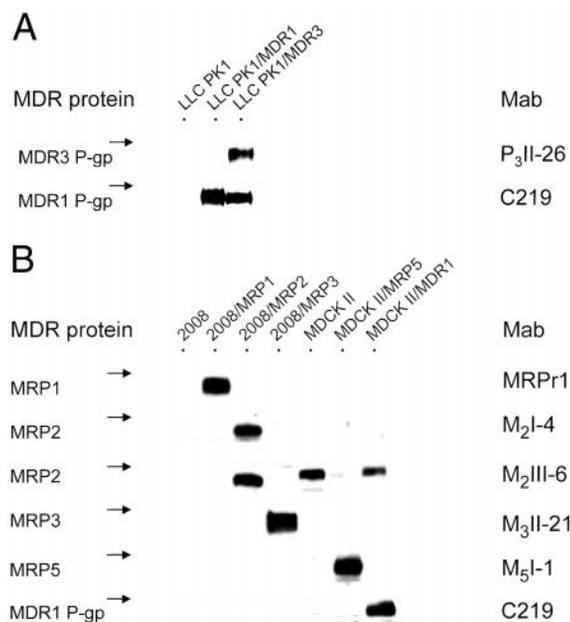


Fig. 2. Western blot with Mabs of the panel, detecting MDR-related proteins in transfected cell lines. Blots were developed with chemiluminescence and exposed to Kodak film. A, parental pig kidney LLC PK1 cells and LLC PK1 cells transfected with MDR1 P-gp or MDR3 P-gp cDNA, probed for MDR3 P-gp with P<sub>3</sub>II-26 Mab and probed for MDR1 P-gp with C219 Mab. Arrow, M<sub>r</sub> 200,000 marker. P<sub>3</sub>II-26 specifically detects MDR3 P-gp, whereas C219 detects both MDR1 P-gp and MDR3 P-gp. B, parental and transfected ovarian 2008 and canine kidney MDCK II cells, probed for MRP1, MRP2, MRP3, and MRP5 and MDR1 P-gp with MRPr1, M<sub>2</sub>I-4, M<sub>2</sub>III-6, M<sub>3</sub>II-21, M<sub>5</sub>I-1, and C219 Mabs. Arrow, M<sub>r</sub> 200,000 marker. Each of the transporters is specifically recognized by its corresponding Mab. The M<sub>2</sub>III-6 Mab also recognizes a band in the parental and MDR1 P-gp-transfected MDCK II cell line, presumably endogenous canine MRP2.

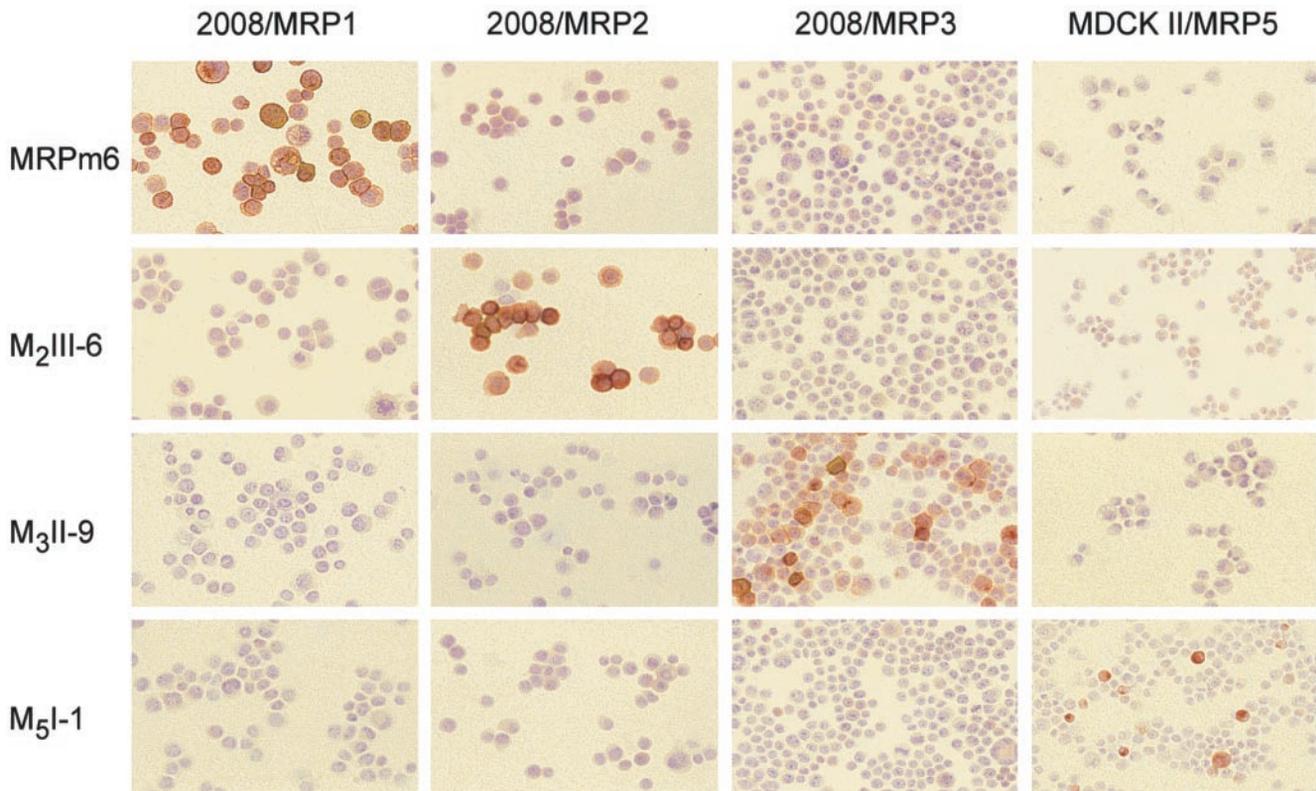


Fig. 3. Staining of cytopins of the ovarian 2008 and canine kidney MDCK II cells transfected with *MRP1*, *MRP2*, *MRP3*, or *MRP5* cDNA with MRPm6, M<sub>2</sub>III-6, M<sub>3</sub>II-9, and M<sub>5</sub>I-1 Mab. Color development was with amino-ethyl-carbazole. Strong staining of transporter molecules by their corresponding Mabs is observed.

levels. Apparently, the canine MRP2 is not detected by the other two anti-MRP2 Mabs, M<sub>2</sub>I-4 and M<sub>2</sub>II-12; they selectively detect the human MRP2.

### Immunohistochemistry

**Frozen Sections of Normal Human Tissues.** To further characterize this panel of Mabs, we tested the Mabs for applicability in standard immunohistochemical stainings. Using this technique, the (intra)cellular localization of the antigen recognized by the Mab can be identified.

In frozen sections of normal human tissues, the MDR3 P-gp Mabs P<sub>3</sub>II-1 and P<sub>3</sub>II-26 prominently stained the canalicular membranes of the hepatocytes and P<sub>3</sub>II-26 was strongest in staining the glomeruli of the kidney (Fig. 4). In liver sections from type 3 cholestatic patients, which lack MDR3 P-gp, no staining with the MDR3 P-gp Mabs was seen (data not shown). This confirms the lack of cross-reactivity with MDR1 P-gp and another P-gp homologue, sister of P-gp, because patients with progressive familial intrahepatic cholestasis type 3 have normal *MDR1 P-gp* and *sPgp* expression [as shown by C219 staining (19)]. None of the other tissues examined, including adrenal gland, pancreas, and lung showed MDR3 P-gp staining.

Also, the MRP2 Mabs, M<sub>2</sub>I-4, M<sub>2</sub>II-12, M<sub>2</sub>III-5, and M<sub>2</sub>III-6 brightly stained the canalicular membranes of the hepatocytes and showed clear staining of the apical membranes of proximal tubules of the kidney (Fig. 4). As expected (5), most other tissues examined, including stomach, bladder, spleen, tonsil, pancreas, heart, skeletal muscle, salivary gland, brain, placenta, testis, and ovary, were negative for MRP2. Interestingly, many tissues showed positive staining of small nerves with the M<sub>2</sub>I-4 Mab (not shown). Nerve tissue has been reported to contain significant levels of *MRP2* mRNA (5). Staining of the apical membrane of bronchi in the lung was only observed with the M<sub>2</sub>III-6 Mab. In frozen sections of normal human liver, the MRP3

Mabs M<sub>3</sub>II-9 and M<sub>3</sub>II-21 demonstrated clear staining of the bile ducts. Also, the basolateral membranes of the hepatocytes displayed appreciable amounts of MRP3. In the kidney, there was a substantial amount of MRP3 in the thinner tubules, most likely the loops of Henle and/or the collecting tubules (Fig. 4). This staining pattern in the kidney was markedly different from that observed for MRP2.

The MRP5-specific Mabs, M<sub>5</sub>I-1 and M<sub>5</sub>II-54, readily detected MRP5 in cytopsin preparations of *MRP5*-transfected MDCK II cells (Fig. 3). However, staining of MRP5 in sections of a broad panel of normal human tissues was not found, even when a very sensitive detection method (FITC-labeled tyramine followed by HRP-labeled rabbit-anti-FITC) was used. The tissues examined included brain and skeletal muscle, tissues that have relatively high *MRP5* mRNA levels (5, 10, 43).

**Formalin-fixed, Paraffin-embedded Normal Human Tissues.** To study whether, and to which extent, the panel of Mabs could be used on formalin-fixed, paraffin-embedded material, we performed experiments without pretreatment or using two different pretreatment methods, citrate or EDTA (see "Materials and Methods"). The MRP1 Mabs and two of the MRP2 Mabs, M<sub>2</sub>I-4 and M<sub>2</sub>III-6, performed well on this material. Staining results were best when citrate was used as a pretreatment. The MDR3 P-gp Mabs P<sub>3</sub>II-1 and P<sub>3</sub>II-26 very weakly stained slides of formalin-fixed, paraffin-embedded liver tissue. The MRP3 and MRP5 Mabs, M<sub>3</sub>II-9, M<sub>3</sub>II-21, M<sub>5</sub>I-1, and M<sub>5</sub>II-54, showed no staining when applied on, respectively, liver and brain and skeletal muscle, processed in this way.

**Reactivity with Antigens in Different Species.** To study the applicability of the panel of Mabs in animals frequently used for experimental MDR studies, notably mouse, rat, and guinea pig, we tested the panel (except for the MRP5 Mabs) on frozen sections of liver and kidney of these animals. On the basis of the presence or absence of the expected expression profile(s) in the examined tissues, we conclude

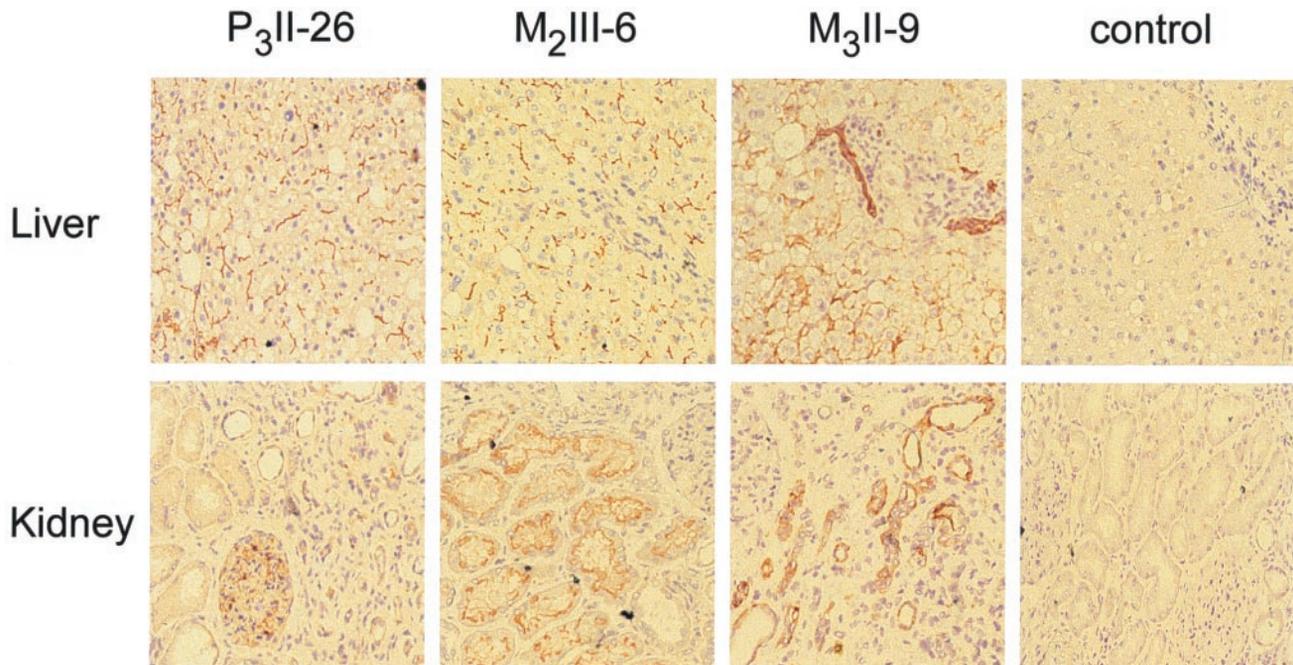


Fig. 4. Staining of frozen sections of normal human liver (*upper panel*) and kidney (*lower panel*) with P<sub>3</sub>II-26, M<sub>2</sub>III-6, M<sub>3</sub>II-9, and control Mabs. P<sub>3</sub>II-26 and M<sub>2</sub>III-6 show that both MDR3 P-gp and MRP2 are present in the canalicular membranes of the hepatocytes, whereas M<sub>3</sub>II-9 shows that MRP3 is present in the bile ducts and the basolateral membranes of the hepatocytes. In kidney, P<sub>3</sub>II-26 shows the presence of MDR3 P-gp in the glomeruli, M<sub>2</sub>III-6 shows the presence of MRP2 in the apical membrane of the proximal tubules, and M<sub>3</sub>II-9 shows the presence of MRP3 in the thinner tubules, most likely the loops of Henle and/or the collecting tubules. Slides were stained with HRP-labeled rabbit-antimouse, FITC-labeled tyramine, HRP-labeled rabbit-anti-FITC, and amino-ethyl-carbazole.

that some of the Mabs are specific for the human antigen (*e.g.*, MRPm6), whereas others (*e.g.*, P<sub>3</sub>II-26) are likely to react with the orthologues of all species tested. Also, affinity differences were sometimes observed. For instance, the M<sub>2</sub>III-5 and M<sub>2</sub>III-6 Mabs that were raised against a fusion protein containing the rat Mrp2 clearly showed a higher affinity for the rat Mrp2 than for the human MRP2 (data not shown). The M<sub>2</sub>I-4 and M<sub>2</sub>II-12 Mabs, raised against a fusion protein containing the human MRP2, were clearly human specific. Of course, with mouse Mabs on mouse tissues and rat Mabs on rat tissues, staining results were more difficult to interpret because of the higher background staining. In Table 1, a rating in plusses is used to rate the reactivity of the Mabs to the antigen orthologues.

### FACS Analysis

Most Mabs of the panel were tested for applicability in the FACS technique. Because all tested Mabs in the panel failed to stain viable cells, the cells were permeabilized prior to testing in the subsequent experiments. As shown in Table 1, all MDR molecules, except for MDR3 P-gp which was not tested, can be detected with this technique. Best results were obtained when Lysing Solution G was used for MIB6, MRPm6, MRPm5, M<sub>2</sub>III-6, M<sub>3</sub>II-9, and M<sub>5</sub>I-1 staining and formaldehyde in acetone for MRPr1 staining.

### DISCUSSION

To date, the contribution of two ABC transmembrane transporter proteins, MDR1 P-gp and MRP1, to the MDR phenotype of human tumor cells is well established. Although the contribution of family members of these transporter proteins to MDR is still under investigation, it has become clear that at least some of these members are able to transport anticancer drugs (7, 64, 70). Whether they also play a role in drug resistance in patients remains to be established. If so, detection of these transporter proteins will be very important for monitoring chemotherapy. For that purpose and to study the physio-

logical function of the transporter proteins, specific monoclonal antibodies are essential. Because the different transporter proteins have a significant homology with each other, it is imperative to know whether the Mabs are specific in detecting only the transporter of interest. In this study, we have tested a panel of new and formerly produced Mabs to MDR3 P-gp, MRP1, MRP2, MRP3, and MRP5 for specificity in detecting their cognate antigens and for reactivity in commonly used techniques, such as immunohistochemistry and Western blotting. As shown in Figs. 3 and 4 and Table 1, all Mabs in this panel specifically detect only the transporter molecule to which they were raised. Also, the previously produced anti-MRP1 Mabs MRPr1, MRPm5, and MRPm6 appeared not to cross-react with any of the related transporter proteins tested. At this stage, cross-reactivity of the panel of Mabs with MRP4, MRP6, and BCRP cannot be excluded, but this is, given the respective sequence differences in the regions used in the fusion proteins, very unlikely. Moreover, staining of cytopins of the mitoxantrone-resistant MCF-7 MR cell line, a cell line with reported high levels of *BCRP* mRNA (71), did not indicate cross-reactivity of any of the Mabs with this transporter (data not shown).

Nevertheless, when the staining results of the panel of Mabs on frozen sections of normal human tissues were analyzed, some discrepancies were noted when Mabs detecting a given transporter were compared. For example, although we have described the presence of *MRP2* mRNA in nerve tissue (5), of the four anti-MRP2 Mabs only M<sub>2</sub>I-4 strongly reacts with nerves in several tissues. Additional studies need to investigate whether this reactivity is based on, *e.g.*, alternative protein processing and masking the epitopes of the other MRP2 Mabs, or is attributable to cross-reactivity with a protein present in nerve tissue.

This will have to be determined for the observed M<sub>2</sub>III-6 staining of the bronchial epithelium in the lung as well. Also, the MDR3 P-gp staining in the glomeruli of the kidney was only observed with the P<sub>3</sub>II-26 Mab. Although a similar explanation as presented above could apply here as well, a difference in affinity of the Mabs might also play

a role. Apparently, the P<sub>3</sub>II-26 Mab detects MDR3 P-gp with a higher affinity than the P<sub>3</sub>II-1 Mab, as judged from the staining results observed in liver tissue. Small differences in expression of the transporter molecules between samples was observed. These differences can probably be explained either by differences in expression of these highly adaptable proteins or by differences in the tissue (*e.g.*, length of storage time or autopsy material *versus* surgical material).

Basically, all Mabs performed very well in immunohistochemical staining techniques with cytopspins or frozen tissue sections. As expected, in the other techniques differences in reactivity and suitability were noted. Epitope conformation is known to depend on the way the antigen is treated. Generally, Mabs selected with linear (poly)peptides in ELISA systems perform relatively well in Western blot techniques, in which the antigen is fully linearized. Mabs selected with more native proteins (*e.g.*, as present on viable cells or cell membranes) are more likely to detect nonlinear epitopes and usually are less suited for Western blot techniques. A typical example is the MIB6 Mab. The Mab was selected on MRP1-positive membrane vesicles and detects MRP1 in cytopspins and in the FACS but is unreactive in Western blots. Nevertheless, together with the earlier produced anti-MRP1 Mabs, for all of the MDR-related transporter proteins studied thus far, Mabs have become available performing well in Western blotting.

Given the fact that formalin fixation causes cross-linking of proteins, frequently leading to altered epitopes, it is not surprising that most of the currently produced Mabs need some method for antigen-retrieval to restore accessibility of the epitopes, when used on paraffin-embedded materials. Using routine EDTA or citrate pretreatments, all MRP1 and two of the MRP2 Mabs (M<sub>2</sub>I-4 and M<sub>2</sub>III-6) could be made effective on this material. It may well be that the epitopes of the other Mabs can be retrieved to satisfactory levels with other retrieval methods.

As summarized in Table 1, most MDR-related molecules studied can now be detected using flow cytometric analyses. Because the available Mabs recognize internal epitopes, a permeabilization step is required. Surprisingly, despite numerous attempts,<sup>4</sup> we nor, to our knowledge, others have succeeded in raising Mabs detecting external epitopes of MRP1 or any MDR-related transmembrane transporter molecule other than MDR1 P-gp. Of course, Mabs detecting external epitopes would be particularly useful for FACS analyses and, if detecting functionally relevant epitopes, for functional studies. Thus far, only MDR1 P-gp Mabs detecting external epitopes of the transporter molecule have been described [*e.g.*, MRK-16 (50) and UIC2 (48)]. Although most MDR molecules may not present antigenic epitopes protruding from the outer cell membrane, *e.g.*, through masking by sugar moieties, at least for MRP2 a polyclonal antiserum detecting the external NH<sub>2</sub> terminus of rat Mrp2 has been reported (70). Notably, the presently described anti-MRP1 Mab MIB6, recognizing an internal epitope, was obtained after immunizations of mice with a fusion protein containing both internal and external regions of MRP1 and with MRP1 positive cells and functional selection on a mixture of inside-out and right-side-out membrane vesicles. This approach led to the development of a potentially important Mab, because MIB6 strongly interfered with MRP1 function. Thus, this Mab can be used to perform functional studies on the transporter protein, to study the drug transport of the transporter in more detail, and possibly to identify the drug binding regions in the transporter. Similar blocking characteristics have been described for the anti-MRP1 Mabs QCRL-2, QCRL-3, and QCRL-4 (72–74), which bind to the regions 617–932 and 1294–1531. Experiments to determine the MIB6 epitope are currently under way.

<sup>4</sup> G. L. Scheffer and R. J. Scheper, unpublished data.

Some points should be emphasized in regard to the individual transmembrane transporter molecules studied here. The possible role of the MDR1 P-gp homologue, MDR3 P-gp, in MDR is still unsolved but deserves further investigation. Thus far, MDR3 P-gp has been detected by means of polyclonal antisera (51) or Mabs against MDR1 P-gp that cross-react with the MDR3 P-gp [such as C219 (52)]. We have described here MDR3 P-gp-specific IgG Mabs that should facilitate further clarification of the putative contribution of MDR3 P-gp to drug resistance in different tumor types. Furthermore, the Mabs can be used to diagnose patients suffering from type 3 progressive familial intrahepatic cholestasis, a disease characterized by the absence of *MDR3 P-gp* expression (19). When diagnosed at an early stage, these patients could be treated more adequately. As reported earlier, the MRP2 Mabs allow the diagnosis of still another liver disease, the Dubin-Johnson syndrome (4, 36).

As to the other MRP family members, erroneous results have been obtained in the recent past with polyclonal antibodies, which appeared to lack adequate specificity. For example, MRP1 was detected in the canalicular membrane of the hepatocyte with a polyclonal antiserum (75), but staining with MRP1-specific Mabs yields contrary results (76). Most probably, the polyclonal used cross-reacted with MRP2, which is easily demonstrated in the canalicular membrane of the hepatocyte (Ref. 4 and this report). The lack of specificity frequently observed with polyclonal antisera may also explain a recent report on the presence of MRP3 in liver canalicular membranes (77), which was not confirmed in other studies (7, 11), and by the results presented here. Both MRP3-specific Mabs revealed high levels of MRP3 in the liver bile ducts and occasional MRP3 protein in the basolateral membranes of the hepatocyte but not in the canalicular membranes.

Regarding MRP5, many questions have yet to be answered. On the basis of its cDNA sequence, MRP5 appears, together with MRP4, to differ from the other MRP family members in lacking the extra NH<sub>2</sub>-terminal domain (10). The anti-MRP5 antibodies described in this report perform well in Western blots and on cytospin preparations of the transfected MDCK II cell line. Surprisingly, all attempts to detect MRP5 protein in frozen sections of normal human tissues have failed, despite readily detectable *MRP5* mRNA levels in such tissues as muscle and brain (5, 10, 43). The possibility that endogenous *MRP5* mRNA is inefficiently translated derives some support from our Western blot results, which show high levels of MRP5 in the *MRP5*-transfected MDCK II cell line but only low endogenous levels of MRP5 in some of the 2008 sublines, which we found earlier to have relatively high *MRP5* mRNA levels (5).

The panel of Mabs described here, together with our recently developed Mabs against BCRP (78),<sup>5</sup> provides for high specificity in detecting most currently known MDR-related ABC transporter proteins in a variety of techniques. Moreover, those detecting MDR3 P-gp, MRP1, and MRP2 also detect orthologues in species other than humans, facilitating experimental approaches. Nevertheless, more work has to be done before a truly complete and multi-purpose set of MDR Mabs will be available. In particular, applicability of the panel in analyzing formalin-fixed, paraffin-embedded patient tissues should be further improved by optimizing pretreatment methods. If necessary, second generation Mabs can be selected for performance on this type of material. In addition, development of Mabs for other members of the transmembrane transporter family, such as MRP4 and MRP6, is important. A complete set of Mabs for MDR proteins will be a valuable tool for investigating clinical multidrug resistance in cancer

<sup>5</sup> M. Mahepaard, G. L. Scheffer, J. F. Faneyte, M. A. van Gastelen, A. C. L. M. Pijnenborg, A. H. Schinkel, M. J. van de Vijver, R. J. Scheper, J. H. M. Schellens. Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues, submitted for publication.

patients and may potentially be an important factor in the diagnosis and treatment of drug-resistant tumors.

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## Specific Detection of Multidrug Resistance Proteins MRP1, MRP2, MRP3, MRP5, and MDR3 P-Glycoprotein with a Panel of Monoclonal Antibodies

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