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ORIGINAL ARTICLE

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Generation of a monoclonal antibody to P-glycoprotein peptides using tuberculin-PPD as a carrier

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Abstract A novel immunization protocol together with stringent selection criteria have been employed to generate a new murine monoclonal antibody (''D8'', isotype IgG_1, kappa) which specifically recognizes the human p170 drug resistance glycoprotein. This antibody is directed towards a defined peptide sequence located in the –COOH terminal region of the first external loop of the molecule. It is reactive with its epitope within the intact native glycoprotein in formalin-fixed and conventionally processed histological tissues, in flow-cytometric preparations and by Western blotting. The antibody precipitates its target peptide sequence from solution, and thus may be a useful reagent with which to establish an ELISA, RIMA or other similar assay. The peptide epitope recognized by this monoclonal antibody is restricted to the human *MDR*1 gene product and is not contained within the rodent homologue of the P-170 molecule. Immunohistochemistry has consistently failed to detect this epitope in rodent tissues, thus confirming that it does not exhibit the cross-reactivity of other currently available anti-P-glycoprotein monoclonal antibodies. The experience of this study emphasizes the value of the tuberculin-PPD (purified protein derivative) immunization protocol as a powerful strategy when generating monoclonal antibodies to small synthetic peptides. The resulting monoclonal antibody (D8) will be an invaluable reagent with which to analyse P-170 glycoprotein expression when assessing the role of multidrug resistance in human cancers.

Key words Monoclonal antibody · Multidrug resistance · P-Glycoprotein peptide · Mouse · Human

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Abbreviations HT: hypoxanthine-thymidine, HAT: hypoxanthine-aminopterin-thymidine, PPD: purified protein derivative, PBS: sodium phosphate-buffered saline (120 mM, pH 7.4), BSA: bovine serum albumin

Introduction

P-Glycoprotein is a 140- to 170-kDa ATP-dependent, volume-regulated ion channel expressed by a small family of genes [16] within the ABC (ATP-binding cassette) superfamily of molecular transporters [15]. The channel protein contains six extracellular hydrophilic domains (Fig. 1) and acts as an efflux pump for a range of structurally and functionally unrelated cytotoxic drugs. Expression of P-glycoprotein results in classical multidrug resistance (MDR), one of the more common types of drug resistance that enables tumour cells to withstand increased levels of cytotoxic chemotherapy [13] and is believed to oppose the successful treatment of many cancers [28]. Although three classes of mammalian MDR genes have been described [5], only the proteins encoded by class I and class II genes confer multidrug resistance [33]. The human MDR gene family comprises two genes, belonging to class I (*MDR*1) and to class II (*MDR*2).

Several monoclonal antibodies recognizing P-glycoprotein have already been described (Table 1). Some of these reagents have been employed to investigate multidrug resistance under experimental conditions, and expression of the P-170 glycoprotein has been identified in normal and malignant tissues [40]. Anti-P-170 monoclonal antibodies have been employed to target conjugated cytotoxins [10], for modulation of multidrug resistance [14] and as anti-proliferative agents [8]. However, these different studies have failed to provide consistent data defining the functional relationship between P-glycoprotein and clinical drug resistance [9, 41]. Such problems in correlating the data are due, in part, to incomplete specification of the antibodies and to partial destruction of their target epitopes during tissue processing and fixation [37]. Significant variance in immunohistochemical staining

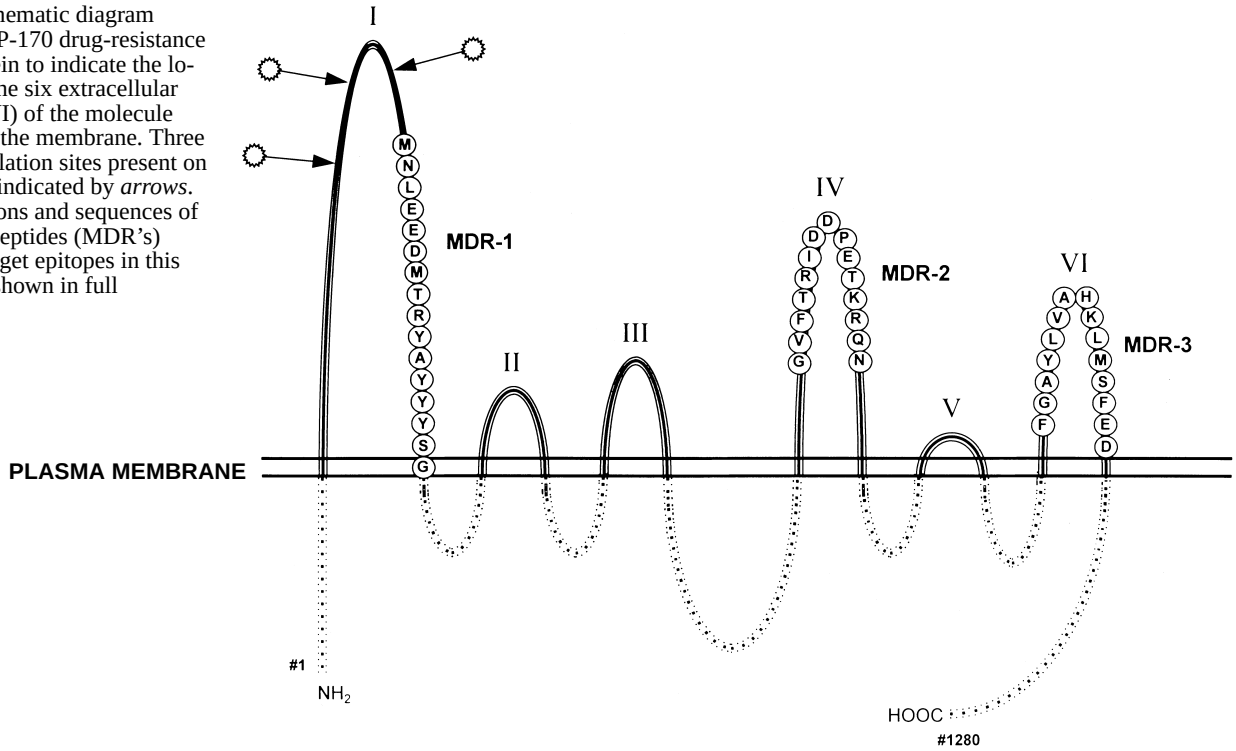

Fig. 1 Schematic diagram of human P-170 drug-resistance glycoprotein to indicate the location of the six extracellular loops (I–VI) of the molecule relative to the membrane. Three N-glycosylation sites present on loop I are indicated by *arrows*. The positions and sequences of the three peptides (MDR's) used as target epitopes in this study are shown in full

patterns has been reported when different monoclonal antibodies have been employed under identical conditions to investigate normal and malignant tissues. Monoclonal antibody C219 recognizes a cytoplasmic epitope of the P-170 molecule and cross-reacts with an epitope expressed in muscle myosin [40]. This antibody also detects a 170-kDa protein that is present in *atypical* multidrug-resistant human leukaemia cells selected in methotrexate but is not recognized by anti-P-glycoprotein monoclonal antibodies MRK16 or JSB-1 [29]. Conversely, monoclonal antibody JSB-1 cross-reacts with a 130-kDa protein present in rat liver, mitochondrial inner membrane/matrix fractions, bovine liver and human skeletal muscle. The protein has now been identified as the enzyme pyruvate carboxylase [32]. The same protein was earlier identified as containing an epitope cross-reacting with anti-P-glycoprotein monoclonal antibody C494 [31].

For our planned studies to analyse a possible association between multidrug resistance and cancer cell phenotype, we required a high-affinity monoclonal antibody that would detect a selected epitope within the *external* domain of the P-170 glycoprotein and would also be unique to the *human* isoform of the molecule. None of the currently available monoclonal antibodies fulfilled our precise criteria. In order to generate lymphocyte hybridomas secreting a monoclonal antibody of the required specificity, we immunized Balb/c mice according to a newly defined protocol using synthetic peptides corresponding to three defined regions within the external loops of human P-glycoprotein. The resulting hybridomas were assayed stringently in order to select only those antibodies fulfilling the predefined criteria. Monoclonal antibody ''D8'', and IgG_{2a} kappa isotype immunoglobulin, is the first reagent of this type reported to be generated to a *predefined* extracellular domain of the human P-170 glycoprotein – rather than by secondary selection following nonspecific immunization with either the whole glycoprotein molecule or with intact P-glycoprotein-expressing tumour cells. This human-specific, selective and high-affinity monoclonal antibody will be an invaluable reagent in forthcoming studies aimed at defining the expression and role of the P-170 drug-resistance glycoprotein in modulating the behavioural phenotype, and also in assessments of chemotherapeutic responsiveness of human cancer cells.

Materials and methods

Cell lines

Cells of the P-glycoprotein-expressing and multidrug-resistant human lymphoid leukaemic cell line CEM/VBL^{1000} and its drug-sensitive parental cell line CCRF-CEM [17] were obtained from Dr. Victor Ling (Ontario Institute of Cancer Research, Toronto, Canada).

Chemicals

BCG vaccine and tuberculin-PPD (purified protein derivative) were obtained from Evans Medical, England. Freund's (incomplete) adjuvant was purchased from Sigma, Poole, England. Hypoxanthine-thymidine (HT), hypoxanthine-aminopterin-thymidine (HAT), myoclone and all tissue culture media were purchased from GIBCO BRL, Paisley, UK. Antibody-isotyping kits were purchased from Sera-Lab, Sussex, UK. P-GlycoCHECK C219 was purchased from CIS (UK), High Wycombe, Bucks, UK. Orthophenylenediamine (OPD) and all other immunological reagents were purchased from Dakopatts, Glostrup, Denmark. Polyethylene glycol (PEG-1500) was obtained from Boehringer Mannheim, East Sussex, UK.

Table 1 P-glycoprotein (170–180 kDa)-specific monoclonal antibodies (*P-gp* P-glycoprotein; *DNR* data not reported)

Antibody	Isotype	Immunogen	Epitope	P-gp isoform recognised	Species	Reference
C219	IgG_{2a}	CH^RB30 & CEM/VBL_{500}	Cytoplasmic	Class I, II, III	All	[17]
C494	IgG_{2a}	CH^RB30 & CEM/VBL_{500}		Class I	Human and hamster	
C32	IgG_{2a}	CH^RB30 & CEM/VBL_{500}		Class I, II	Hamster	
HYB-241	IgG_1	SH-SY5Y/VCR				
HYB-612	IgG_1	SH-SY5Y/VCR	Cell surface	Class I	Human and hamster	[27]
JSB-1	IgG_1	CH^RC5	Cytoplasmic	Class I, II	Human and hamster	[35]
MRK16	IgG_{2a}	K562/ADM	Cell surface	Class I	Human	[14]
MRK17	IgG_1	K562/ADM				
265/F4	IgG_1	CH^RC5	Cell surface	Class II	Hamster, mouse and human	[22]
32G7	IgM	CCRF-CEM & CEM/VBL_{100}	Cell surface	Class I		[7]
9A7	IgG_1	CCRF-CEM & CEM/VBL_{100}				
1F10	IgG_1	CCRF-CEM & CEM/VBL_{100}				
UIC2	IgG_{2a}	3T3-1000	Cell surface/ cytoplasmic	Class I	Human	[25]
4E3	IgG_{2a}	SW-1573/500/ ME180/Dox500	Cell surface/ cytoplasmic	Class I	Human	[1]
Mab57	IgG_{2a}	CEM-VBL100	Cell surface/ cytoplasmic	Class I	Human	[4]
7G4	IgG_{2a}	DNR	DNR	Class I	Human	[36]
MM4.17	IgG_{2a} kappa	CEM-VBL100	Cell surface/ cytoplasmic	Class I	Human	[6]

Synthetic P-glycoprotein peptides

The hydropathic profile of the complete peptide sequence for human *MDR*1-encoded P-glycoprotein was determined [3], and three peptide sequences were identified as the most hydrophilic, and hence likely to be the most immunogenic. These peptides, referred-to as MDR-1, MDR-2, and MDR-3, (Fig. 2) correspond to amino acid sequences within the external domains of three different extracellular loops located at the N- and C-terminal regions of human P-glycoprotein. The synthetic peptides were prepared to >95% purity by Cambridge Research Biochemicals, Cambridge, UK.

Iodination of P-glycoprotein peptides

Radio-iodination of 1-μg aliquots of peptides MDR-1 and MDR-3 was performed using chloramine-T [24]. Free iodine was separated by chromatography on a 20-cm-long Sephadex G10 column equilibated in 100 mM sodium bicarbonate (pH 8.3), and 0.5-ml aliquots containing the peak iodinated peptide fractions were retained.

Peparation of immunogen

The three peptides were separately coupled to tuberculin-PPD as carrier protein [21] using glutaraldehyde. Prior to conjugation, tuberculin-PPD was exhaustively dialysed against 50 mM ammonium bicarbonate and collected by lyophilization. Protein content was determined by colorimetric assay [3]. Then 2.0 mg of each peptide was coupled with tuberculin-PPD at a molar ratio of 2:1 in 100 mM sodium bicarbonate (pH 8.3) using a concentration of 2.0 mg/ml tuberculin-PPD. To confirm coupling efficiency, an aliquot containing 5×10^6 cpm of radiolabelled MDR-3 peptide was added to this mixture. Glutaraldehyde was then added to the three separate peptide solutions to give a final concentration of 0.05% (v/v) in each. Solutions were mixed in glass vials by rolling at 4° C overnight. Conjugated MDR-3 was size-fractionated on a column of G-25 Sephadex, which was equilibrated and eluted in 100 mM sodium bicarbonate (pH 8.3). Radioactivity and protein concentration in 1-ml fractions eluted from the column were determined by gamma counting and by a colorimetric spot test, respectively. Conjugation efficiency was calculated as the radioactivity contained in the peak as a proportion of the total radioactivity eluted from the column. The first peak after the void volume, comprising MDR-3 peptide-coupled tuberculin-PPD, was retained and the sample lyophilized. Tuberculin-PPD conjugated MDR-1 and MDR-2 peptides were extracted by adding glycine ethyl ester (1 M, pH 8.0) to give a final concentration of 100 mM and incubated for 30 min at room temperature. Proteins were then precipitated by incubating with 3–4 volumes of acetone at −70° C for 30 min. Precipitates were separated by centrifugation at 10,000 *g* for 10 min and then air-dried. The three coupled peptides were redissolved in normal saline (120 mM) at a concentration of 1.0 mg protein/ml. Each coupled peptide was emulsified in an equal volume of Freund's incomplete adjuvant, aliquotted at 50 μg protein per injected dose, and stored at −20° C until required. At least two aliquots of each of the coupled peptides were also stored without emulsifying in adjuvant and used to boost immunization.

Immunization of mice

Prior to immunization, 3-week-old female BALB/c mice were primed by s.c. injections of 0.2 ml of BCG vaccine at each of two

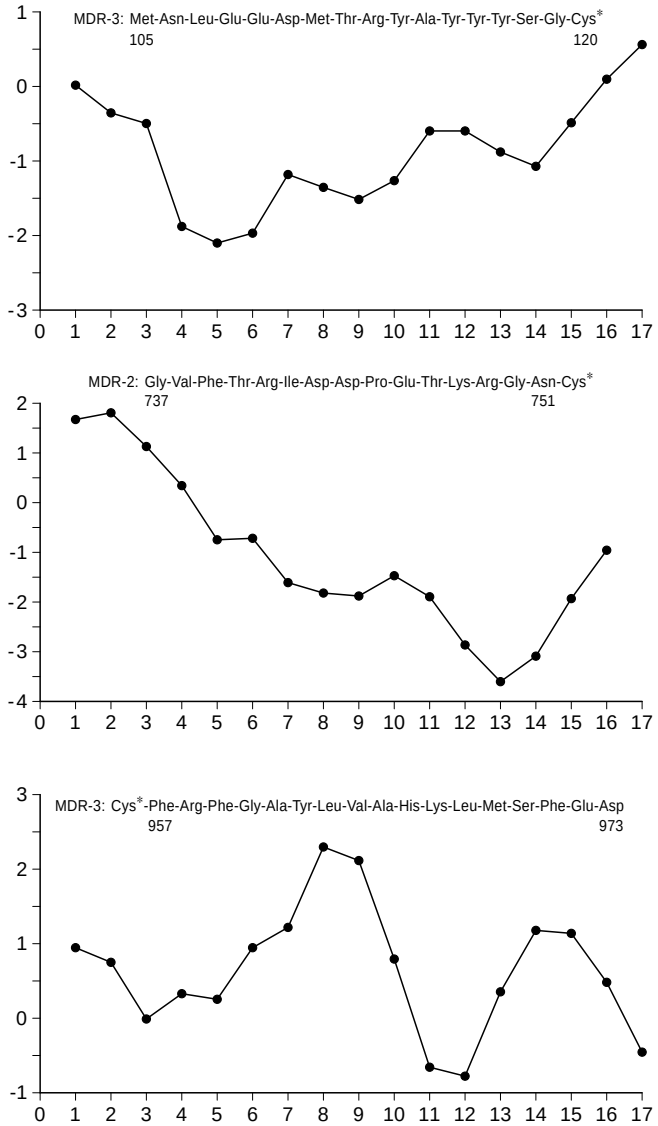

Fig. 2 Hydropathic profiles [19] of the three synthetic peptides used as immunogens, including the effect of the additional cysteines (–Cys*) conjugated to the C-terminal end of MDRs 1 and 2 and to the N-terminal end of MDR-3. Each amino acid sequence is presented in numerical order, as shown above, along the *abscissae*. Increasing relative hydropathy is plotted along the *ordinates*

sites near the tail base. Four weeks after priming, each mouse was immunized with 50 µg of coupled protein in Freund's incomplete adjuvant, using multiple subcutaneous injections. Immunization was continued at 2- to 3-week intervals. Antibody response was monitored by enzyme-linked immunosorbant assay (ELISA) at 10 days after each injection using sera obtained following test tail bleeds. Mice responding with the highest antibody titre were selected for fusion. At 6 days and then at 3 days before fusion, booster doses of coupled peptides without adjuvant were given i.p.

Fusion

Fusion was performed between immune-stimulated splenocytes and Sp/O-Ag14 murine myeloma cells [38] using conventional techniques [11, 18]. Splenocytes and myeloma cells at a ratio of 5:1 were fused using PEG 1500 dissolved in HEPES (75 mM, pH 8.0). Fused cells were resuspended in 10 ml prewarmed Dulbecco's minimal Eagle's medium (DMEM) containing 10% Myoclone, 1×10^{-4} M hypoxanthine, and 1.6×10^{-5} M thymidine. Thymocytes isolated from a 3-week-old BALB/c mouse were used as feeders at a density of 10^4 cells per multiwell during the initial culture of fused cells. Cell suspensions were plated in 24-well tissue culture plates in a volume of 1.0 ml/well. The plates were incubated at 37° C and 5% CO_2 in humid air.

HAT selection of hybridomas

Selection of hybridomas was started 24 h after fusion by feeding cells with culture medium containing 40×10^{-6} M aminopterin in addition to hypoxanthine and thymidine (HAT medium). Aminopterin was included for the first 2 weeks and thereafter omitted. Between the 4th and 6th weeks following fusion, the concentration of HT was progressively reduced and eliminated [11].

Isolation and cloning of hybridomas

On day 10 following fusion, hybridoma colonies were visible in most wells. Each colony was picked under phase-contrast microscopy and transferred to a separate well of a 96-well tissue cultue plate. Cultures were continued at 37° C and 5% CO_2 in humid air. When hybridoma cells were at such a density as to require daily re-feeding, culture supernatants were collected and screened for activity against each individual immunizing peptide. Positive colonies were cloned by limiting dilution and amplified by growing-up on 24-well tissue culture plates. At each stage during amplification, at least one aliquot of hybridoma cells was cryo-preserved in liquid nitrogen.

Screening of hybridomas

Screening was performed against pure synthetic peptides as well as against native P-glycoprotein. Five different screening procedures were used to select antibodies fulfilling the initial specifications.

Enzyme-linked immunosorbant assay

An enzyme-linked immunosorbant assay (ELISA) preoptimized using serum collected during immunization was employed to assess all supernatants obtained from the hybridomas on day 10 after fusion. Immulon-II ELISA plates were coated with peptide antigen (1.25×10^{-8} M concentration) in 50 µl coupling buffer (100 mM sodium carbonate, pH 9.6) at 4° C overnight. Unabsorbed antigen was removed, and the wells washed in TBS (25 mM Tris-buffered saline, pH 8.1) three times for 5 min each and the plates shaken dry. All wells were filled with blocking buffer comprising TBS containing 2% (w/v) bovine serum albumin and left at room temperature for 30 min. Then 50 µl of neat hybridoma supernatant was added to each well, and the plates were wrapped in cling-film and incubated for 1 h at 37° C. In control wells, supernatant was either replaced by blocking buffer or by an irrelevant antibody. After three washes with TBS, 50 µl of peroxidase-conjugated rabbit anti-(mouse immunoglobulin) antiserum diluted 1:500 in blocking buffer was added to each well and incubated for 30 min at room temperature. Unbound antisera were washed from the plates and 100 µl of freshly prepared OPD substrate solution (8 mg of ortho-phenylenediamine in 12 ml of 100 mM citrate-phosphate buffer, pH 5.0) containing 5 µl H_2O_2 was added to each well. Plates were incubated for 10 min in the dark at room temperature. The reaction was stopped by adding 60 µl of 3 M sulfuric acid. The optical density of each well was measured at 492 nm, after blanking against water, using a Titertek Multiskan-Plus plate reader.

Radio-immunometric assay

ELISA-reactive hybridomas were further assayed using a radio-immunometric assay (RIMA) to identify those monoclonal antibodies that would be capable of binding and immobilizing labelled antigens from a solution. The assay was performed to detect monoclonal antibodies recognizing MDR-1 and MDR-3 peptides, since only these were radio-iodinated. Immulon-II plates were coated with protein-A at a concentration of 2 µg/well in 200 µl of coupling buffer for 18 h at 4° C. Wells were washed three times with TBS for 5 min each. Nonspecific binding sites were blocked using TBS blocking buffer for 30 min at room temperatue. After shaking dry, 150 µl of tissue culture supernatant was added to each well and incubated for 1 h at room temperature. In negative control wells, an unrelated monoclonal antibody was added. After washing and shaking dry, 150 µl of each of the ^{125}I-labelled synthetic peptides MDR-1 and MDR-3, each containing 1×10^5 cpm, was added separately to triplicate wells and incubated for 1 h at room temperature. Plates were washed with three changes of TBS and shaken dry. Each well was then eluted with 200 µl of 0.1 N sodium hydroxide containing 1% (v/v) Sarkosyl, and the radioactivity contained in each eluate was measured. Readings were corrected for background radioactivity and those at least 5-fold higher than the controls were regarded as positive.

Flow cytometry

The drug-sensitive parental cell line CCRF-CEM was grown as supension cultures in RPMI-1640 medium supplemented with 10% FCS. P-Glycoprotein-expressing and multidrug-resistant human lymphoid leukaemic CEM/VBL^{1000} cells were grown in identical medium but also containing 1000 ng/ml of vinblastine. Cultures were maintained at 37° C and 5% CO_2 in humid air, split each week, and propagated by subculturing at a plating density of 1:10. Multidrug-resistant cells were grown in the absence of vinblastine for 1 week prior to performance of each experiment. Flow-cytometric assays were optimized with human lymphoid leukaemic CCRF-CEM cells and their vinblastine-resistant CEM/VBL^{1000} derivative. P-Glycoprotein expression was confirmed by staining the cells with monoclonal antibody C219 after permeabilization achieved by incubating 10×10^6 cells in 1.0 ml of 70% (v/v) methanol in water at –20° C for 5 min. The permeabilized cells were then washed three times with TBS washing buffer. An anti-CD4 monoclonal antibody was chosen as a positive control for cell-surface markers of leucocyte differentiation with which to screen the hybridomas. Cells were harvested and washed in sodium phosphate-buffered saline (PBS; 120 mM, pH 7.4) containing 0.1% (w/v) bovine serum albumin, (BSA). Aliquots containing 1×10^6 cells each were incubated with monoclonal antibody C219 at a concentration of 250 ng in 100 µl of PBS containing 0.1% (w/v) BSA for 1 h on ice. After three washes, cells were incubated with 100 µl of FITC-labelled rabbit anti-(mouse immunoglobulin) diluted 1:50 in washing buffer, for 30 min on ice. After washing, cells were resuspended in 500 µl of washing buffer and were analysed at 517 nm in a precalibrated Coulter EPICS-Profile flow cytometer fitted with a 15-mW laser at 488 nm excitation. At least 10,000 events were recorded. For cell-surface antigens, the permeabilizing step was omitted. Anti-CD4 monoclonal antibody was used at a dilution of 1:100 in identical procedures. Test supernatants were used at a dilution of 1:5 in washing buffer.

Western blotting

The molecular size of the antigens reactive with the generated monoclonal antibodies was determined by Western blotting of denatured crude membrane preparations from multidrug-resistant CEM/VBL^{1000} cells and from parental drug-sensitive CEM-CCRF cells. Crude membrane fractions were isolated from CEM/VBL^{1000} and CCRF-CEM cells [13]. Protein concentrations of membrane samples were determined colorimetrically [3]. Membrane proteins, loaded at a protein concentration of 50 µg/lane, were separated by polyacrylamide gel electrophoresis in 5.8% (w/v) gels and thereafter transferred electrophoretically to nitrocellulose membranes. Following transfer, the blots were incubated in blocking buffer comprising 150 mM PBS (pH 7.4) containing 5% (w/v) BSA and 5% (w/v) fat-free milk (Marvel) at room temperature to block nonspecific binding sites. Blots were cut into strips and each incubated with 2.5 ml supernatant (diluted 1:5 in freshly prepared blocking buffer) overnight at 4° C. Monoclonal antibody C219 was used as the positive control antibody at a dilution of 100 ng/ml. Monoclonal antibody M8, an IgG kappa immunoglobulin raised to the human milk fat globule membrane and directed towards a peptide epitope contained within epithelial membrane antigen [12] was used as the negative control. Thereafter, blots were washed three times in PBS for 5 min each before incubation with biotinylated rabbit anti-(mouse immunoglobulin), diluted 1:1000 in PBS containing 2.5% BSA, for 1 h at room temperature. Unbound immunoglobulins were removed by washing the membranes three times in PBS for 5 min each. Membranes were incubated with horseradish peroxidase-conjugated strepavidin–biotin complex (SABC) diluted in PBS for 1 h at room temperature. After washing in PBS, bound peroxidase activity was developed by incubation in 50 mM, Tris-HCl (pH 7.6) containing 0.025% (w/v) DAB and 0.03% (v/v) H_2O_2 for 5 min at room temperature. $NiCl_2$ (0.3% w/v) was added to enhance sensitivity. The reaction was stopped by transferring blots to distilled water.

Immunohistochemistry

Immunohistochemistry was performed on routinely processed and paraffin-wax-embedded human normal kidney and adrenal glands. An indirect SABC method was employed. The DAB reaction product was intensified by incubating slides in a solution of nickel ammonium sulfate containing H_2O_2. Tissue sections 5 µm thick were dewaxed and taken to water. Endogenous peroxidase was blocked using 0.3% H_2O_2 in distilled water for 30 min. After three washes in PBS, non-specific binding sites were blocked by incubating sections with normal rabbit serum (5% (v/v) in PBS) for 10 min. Excess PBS was wiped from around the sections which were then incubated with 100 µl of hybridoma supernatants for 1 h at room temperature. The slides were then washed three times with TBS before biotinylated rabbit anti-(mouse immunoglobulin), diluted 1:200, was added to each section. The slides were incubated at room temperature for 30 min before being washed and incubated with streptavidin–biotin complex for 30 min at room temperature. Slides were rinsed in 0.1 M sodium acetate buffer (pH 6.0) before bound peroxidase activity was identified by incubation with a solution comprising 2.5% (w/v) nickel ammonium sulfate, 0.05% DAB, 0.2%-D-glucose, 0.04% (w/v), NH_4Cl, and 0.01% (w/v) glucose oxidase for 2 min. Slides were rinsed again in acetate buffer and then in water. Slides were counterstained in Gill's haematoxylin, dehydrated and mounted in Pertex.

Isotyping monoclonal antibodies

Immulon-II ELISA plates were coated with peptide antigen (1.25×10^{-8} M concentration) in 50 µl coupling buffer (100 mM sodium carbonate, pH 9.6) at 4° C overnight. Unabsorbed antigen was removed and the wells washed in TBS (25 mM Tris-buffered saline, pH 8.1) three times for 5 min each and the plates shaken dry. All wells were filled with blocking buffer comprising TBS containing 2% (w/v) BSA and left at room temperature for 30 min, after which 50 µl of neat hybridoma supernatant was added to each well and the plates were wrapped in cling-film and incubated for 1 h at 37° C. In control wells, supernatant was replaced either by blocking buffer or by an unrelated antibody. Bound immunoglobulins were isotyped by reacting each with a panel of peroxidase-conjugated isotype-specific antibodies (Sera Lab, Sussex, UK). The subsequent ELISA technique was identical to that used for screening the hybridoma superantants, as described above.

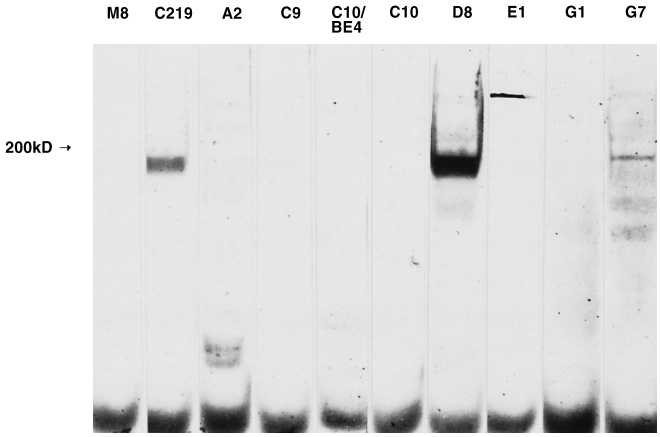

Fig. 3 Electrophoretic separation of crude membrane fractions derived from human multidrug-resistant lymphoid leukaemic (CEM/VBL^{1000}) cell line using 5.8% (w/v) polyacrylamide gel (PAGE). Western blotting was then performed with eight hybridoma supernatants which reacted strongly in ELISA and flow cytometry. Monoclonal antibodies M8 and C219 are included as negative and positive controls, respectively. Supernatant D8 identifies the same band at 170–180 kDa as is recognized by monoclonal antibody C219

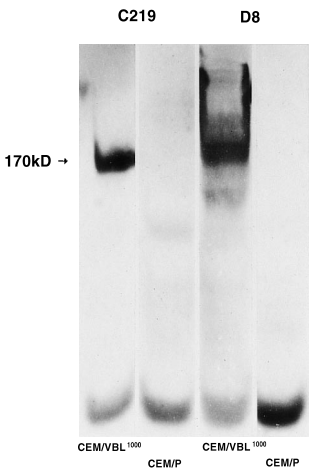

Fig. 4 Electrophoretic separation using 5.8% (w/v) PAGE, followed by Western blotting of crude membrane fractions derived from multidrug-resistant lymphoid leukaemic (CEM/VBL^{1000}) cell line and its drug-sensitive parental counterpart (CEM/P) using monoclonal antibodies C219 and D8. Monoclonal antibody D8 identifies the 170- to 180-kDa P-glycoprotein molecule recognized by C219 in the drug-reistant cell line but is nonreactive with proteins extracted from the drug-sensitive (CEM/P) parental cells

Results

Yield of hybridomas

Two separate fusions were performed, which together yielded more than 1000 hybridoma colonies. Of these, 371 were stable hybridomas producing monoclonal antibodies reactive in ELISA with at least one of the immunizing peptides. The majority of reactive supernatants were against MDR-1, with a smaller number against MDR-3 and a few weakly positive for MDR-2.

Screening of hybridoma superantants

Reactivity with peptides

Supernatants from hybridomas reactive on ELISA with MDR-1 and MDR-3 were cross-checked for reactivity with immunizing peptides by radio-immunometric assay (RIMA). Of the ELISA-positive hybridomas, 22 of 38 MDR-1-reactive supernatants and 4 of 6 MDR-3-reactive supernatants were also positive on RIMA. Of the resulting 26 positive supernatants selected, D8 was the most strongly reactive with MDR-1 peptide on both ELISA and RIMA.

Reactivity with native P-glycoprotein

Twenty hybridomas reactive with MDR-1 peptide were analysed by flow cytometry. Monoclonal antibody C219 was used as the positive control reagent. More than 80% of multidrug-resistant CEM/VBL^{1000} cells were reactive to C219 monoclonal antibody, as opposed to <1% of parental CEM-CCRF cells (CEM/P). Cell-surface marker CD4 was expressed almost equally by multidrug-resistant and parental cell lines (97.8% vs 95.4%, respectively). Of the hybridomas analysed, D8 was significantly more reactive with multidrug-resistant CEM/VBL^{1000} cells than with parental CEM/P cells (17% vs 2.9%). All other hybridomas exhibited little reactivity (<3%) against CEM/VBL^{1000}.

Western blotting

Eight hybridomas, including D8, screened by ELISA and flow cytometry were also assayed by Western blotting of crude membrane fractions derived from parental CEM/P cells and from MDR CEM/VBL^{1000} cells. Control monoclonal antibody C219 and supernatant D8 reacted strongly with a common protein of molecular weight 170–180 kDa (Fig. 3) in membranes extracted from MDR CEM/VBL^{1000} cells but not in their drug-sensitive parental CEM/P cells (Fig. 4). Three additional hybridomas showed borderline reactivity (data not shown). Negative control monoclonal antibody M8 consistently failed to identify protein bands in these preparations.

Immunohistochemistry

Reactivity of monoclonal antibody D8 revealed strong staining of proximal convoluted and distal tubules of human kidney (Fig. 5a). Staining was diffuse and granular throughout the cytoplasm of tubular epithelial cells, although binding to luminal borders of tubular epithelial

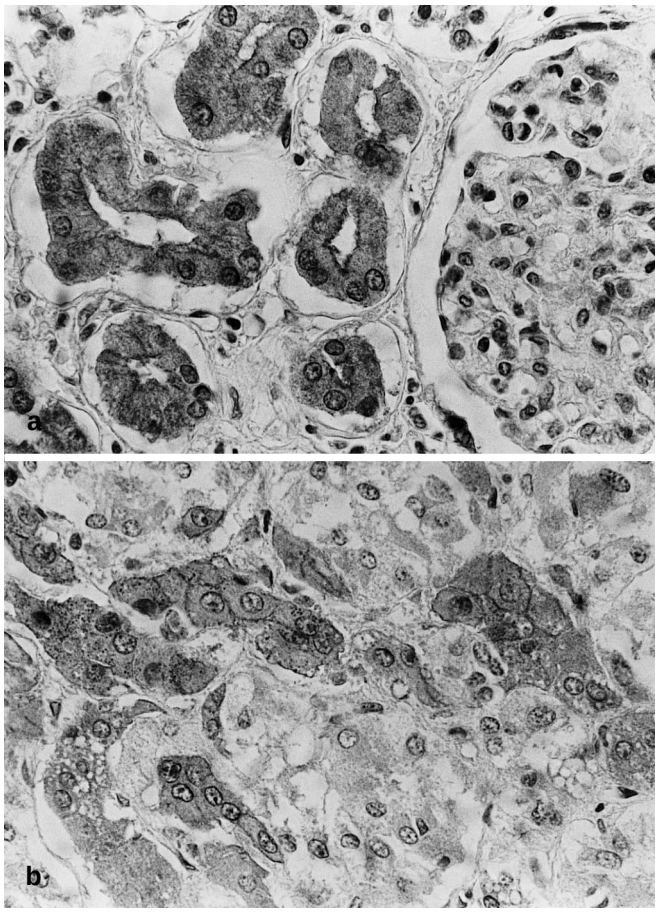

Fig. 5a, b Immunohistochemistry using monoclonal antibody ''D8''. **a** Normal human kidney. Typically, expression of P-glycoprotein is predominantly confined to the epithelia of proximal convoluted tubules. Although expression along luminal plasma membranes is characteristic, co-localization of the molecule within the cytoplasm of these cells is also commonly found. DAB, ×300. **b** Normal human adrenal gland. Characteristically, there is strong cytoplasmic and linear plasma membrane localization of staining to cells in all zones of the cortex, particularly the zona glomerulosa and zona reticularis. DAB, ×300

cells was also present. No staining of glomeruli or other structures were seen. In the adrenal gland, strong staining of cortical epithelial cells was observed (Fig. 5b). Heterogeneous patterns of staining were seen in the medulla and in all zones of the cortex, where staining was dominant in the zona glomerulosa and in the zona reticularis. Immunohistochemical staining of identically fixed and processed rat tissues was negative under the conditions employed.

Isotyping of monoclonal antibodies

Supernatant from hybridoma D8 reacted only with anti-IgG, anti-IgG_{2a}, and anti-kappa-chain immunoglobulins, thus demonstrating monoclonal antibody D8 to be of immunoglobulin-G, subclass 2a and kappa light chain isotype.

Discussion

At the outset of this study, several monoclonal antibodies to P-glycoprotein had already been reported, although when critically none was a satisfactory reagent for our proposed assessment of this molecule in human cancers. The available antibodies either cross-reacted with other cellular proteins [40], were not directed to the extracellular domain of the intact P-glycoprotein molecule [17, 35], or did not identify their epitopes are conventional tissue fixation [30]. An additional group of similar antibodies had been reported in the literature but either they were not available commercially or their use as analytical or diagnostic reagents had not been developed (Table 1). In contrast, the monoclonal antibody we now describe (D8) has been specifically generated using a novel immunization protocol which, together with strigent selection criteria, has resulted in selection of a reagent that specifically recognizes only the human isoform of the P170 drug-resistance gylcoprotein. This antibody is directed towards a defined peptide sequence located in the first external loop of the intact glycoprotein molecule and is reactive in conventionally processed histological tissues, and also in flow-cytometric preparations and by Western blotting. The peptide epitope recognized by this monoclonal antibody is not contained within the rodent homologue of the P-170 molecule, and immunohistochemistry has failed to detect this epitope in any rodent tissues.

With respect to the strategy adopted during this study, monoclonal antibody D8 was generated against synthetic peptides following a novel immunization protocol. Tuberculin-PPD a *Mycobacterium tuberculosis* extract used to sensitize humans against tuberculosis, was used as a carrier protein and hapten for the synthetic peptides. Tuberculin-PPD is a partially characterized molecule known to elicit a potent immune response in previously tuberculin-sensitized animals, but without inducing an immune response against itself [20, 21]. Tuberculin-PPD is a potent T-cell hapten and stimulates antigen-primed B-lymphocytes to produce antibodies [39]. This property was exploited, and a potent response was observed after the first immunization.

When the three peptides were used to immunize mice, the predominant response, defined as the number of hybridomas producing reactive monoclonal antibodies, was obtained to peptide MDR-1, which corresponds to the –COOH terminal region of the first loop of P-glycoprotein in the extracellular N-terminal domain (Fig. 1). Since this region of the intact molecule is variable among animal species, but is highly conserved in humans, we suggest that monoclonal antibody D8 is likely to be more specific than other anti-human P-glycoprotein monoclonal antibodies and hence particularly valuable in studying expression of P-glycoprotein in human diseases. Lerner et al. described guidelines for selecting appropriate peptide sequences of a protein against which to raise antibodies and included the use of a hydrophilic sequence of at least 10 amino acids, which should preferably reside on the cell surface [23]. Com-

parison of the hydrophilicity of each individual immunizing peptide with the numbers of reactive hybridomas revealed that while the strongest response was obtained to the moderately hydrophilic sequence MDR-1, few antibodies were obtained to MDR-2, the most hydrophilic of the three chosen peptides. In contrast, significantly more immunoreactive hybridomas were generated to MDR-3, a relatively hydrophobic molecule compared with MDR-1 or MDR-2. The reasons for this apparent discrepancy are not immediately apparent, but are likely to include the probable conformational presentation of each peptide in solution and the relative lack of similarity between the individual immunizing peptide and amino acid sequences within host proteins – not just homologous P-glycoprotein molecules. The findings of our current study suggest that, in addition to Lerner's recommendations, an amino acid sequence that is different to that occurring in the species of animal being immunized should also be chosen, to increase the likelihood of obtaining a strong immune response. Investigation of the precise epitopes within the immunizing peptides recognized by individual monoclonal antibodies would be interesting and informative and could be obtained by competitive peptide-deletion assays. However, such a detailed analysis falls outside the remit of the present study. Conversely, whenever a peptide is chosen as an immunogen, its sequence homology within other host proteins should be determined.

With respect to its identified specificity, monoclonal antibody D8 recognizes a 170- to 189-kDa protein in the multidrug-resistant human lymphoid leukaemic cell line (CEM/VBL^{1000}), but not in the parental cell line (CCRF-CEM). It also has demonstrable reactivity against viable multidrug-resistant cells using flow cytometry. When employed to analyse a range of normal human tissues, monoclonal antibody D8 reacted against P-glycoprotein in a pattern similar to that described by other groups using different anti-P-glycoprotein monoclonal antibodies [30].

Although D8 was raised using a peptide from the extracellular domain of the P-glycoprotein molecule, its reactivity has been seen predominantly in the cytoplasm of adrenal cells with variable luminal staining of renal tubular cells, which might indicate D8 reactivity with its epitope during intracellular trafficking of the nascent molecule. Preliminary immunohistochemical screening of D8 against a range of human and rodent normal tissues failed to show binding to human liver or skeletal muscle and did not show staining of rat liver, skeletal muscle, adrenal or kidney, thus revealing no immunohistological cross-reactivity with proteins such as pyruvate carboxylase, which occurs with antibody C219.

As a consequence of its human-only epitope specificity, monoclonal antibody D8 is likely to provide valuable information about the status of P-glycoprotein expression in human cancer. Expression of P-glycoprotein is one of the few well-known markers of difficult malignancies. An important application might be identification of tumours expressing P-glycoprotein before and during irradiation or drug treatment so that suitable tailored chemotherapy might be administered. Recently, monoclonal antibody MRK-16 was shown to reverse drug resistance in an *MDR*1-expressing human colon cancer xenograft in nude mice following in vivo administration. Similar results were also reported with monoclonal antibody HYB-241 [34]. Since monoclonal antibody D8 specifically recognizes a cell-surface epitope, it could be useful in vivo in immunotargetting P-glycoprotein-expressing cells for tumour imaging [26], or for modulating or killing drug-resistant cells [2]. *Pseudomonas* exotoxin-conjugated MRK-16 antibody has already been shown to be selectively toxic for cells expressing P-glycoprotein in vitro [10]. Some antibodies to P-glycoprotein have been reported to have direct antiproliferative acitivity [8, 14]. The problem of toxicity to normal tissues expressing high levels of P-glycoprotein requires resolution before such clinical applications can be developed. While it is not known whether monoclonal antibody D8 exhibits such in vivo activities, the ability to modulate the behavioural phenotype of human carcinomas would be an important therapeutic application of this new antibody.

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