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A NEW MONOCLONAL ANTIBODY THAT SPECIFICALLY RECOGNISES THE *MDR-3*-ENCODED GENE PRODUCT

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The *MDR-3*-encoded P-glycoprotein (Pgp) is highly expressed in liver and is thought to function as a hepatic transporter of phospholipids into bile. However its role, if any, in other tissues remains undefined. Although transfection experiments have indicated that it may be unable to confer drug resistance, there is evidence that it may be involved in drug resistance in certain B-cell leukaemias. To date, most work on clinical samples has been performed at the mRNA level; limited work has been performed using polyclonal antibodies raised to *MDR-3* and *mdr-2* (the murine equivalent of *MDR-3*). We have generated a new monoclonal antibody, termed 6/1G, which specifically recognises the human *MDR-3* gene-encoded product. Antibody 6/1G was produced by *in vitro* immunisation of spleen cells from BALB/c mice with a synthetic 12-amino acid peptide. Cells from *MDR-3* transgenic mice showed consistent membranous staining with antibody 6/1G. Immunoblotting with 6/1G identifed a band at 170 kDa on lysates of *MDR-3* transgenic cells. Preliminary results with a range of B-cell leukaemias suggest that *MDR-3* Pgp positivity may be a marker for a more malignant phenotype in B-CLL. Antibody 6/1G may be useful in defining a role for *MDR-3* in malignancy and drug resistance, as well as in certain liver diseases such as progressive familial intracholeostasis. *Int. J. Cancer* 80:265–271, 1999.
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P-glycoproteins (Pgps) belong to the family of ATP-binding cassette (ABC) transporter proteins. Various Pgp isoforms have been identified; all are encoded by a group of closely related genes. Two Pgp genes have been identified in humans, *MDR-1* and *MDR-3,* which show 80% homology. *MDR-3* is thought to be the most highly conserved Pgp gene amongst various mammalian species, and human *MDR-3* is closely related to murine *mdr-2* (91% identity, 95% similarity; for review: Croop, 1993).

The *MDR-1* gene product, P-170, may have a protective role in normal cells, *i.e.,* the transport and excretion of naturally occurring xenobiotics (Schinkel *et al.,* 1994). Classical multiple drug resistance (MDR) is associated with over-expression of P-170; to date, no role for *MDR-3* in MDR has been proven. Specific information regarding the exact role of *MDR-3* in normal or resistant cells and in various tissues is limited.

Like the closely related *MDR-1* gene, a physiological role as a transport pump has been ascribed to *MDR-3.* By generating mice with a disruption of the *mdr-2* gene, this gene has been shown to encode a Pgp located in the canalicular membrane of hepatocytes, where it functions as a transporter of phosphatidylcholine into bile (Smit *et al.,* 1993). On analysis, these mice showed a defect in the transport of phospholipids (mainly phosphatidylcholine) into bile. It is thought that the *MDR-3* and *mdr-2* genes may encode a phosphatidylcholine flippase (Smith *et al.,* 1994); MDR-3 and mdr-2 Pgps have been identifed as specific translocators of phosphatidylcholine (van Helvoort *et al.,* 1996). Given the high conservation of amino acid sequence between these 2 genes and their respective tissue distribution patterns, it is likely that human *MDR-3* fulfils a function similar to murine *mdr-2.* It has been confirmed that human MDR-3 Pgp is functionally homologous to murine mdr-2 Pgp (Smith, 1998). Mutations in the *MDR-3* gene lead to a subtype of progressive familial intracholeostasis (PFIC) with a high serum gamma-glutamyltranspeptidase (GGT) activity. This condition shares histological, biochemical and genetic features with mice lacking *mdr-2* gene expression. Absent or reduced hepatic MDR-3 mRNA levels have been observed in PFIC patients (de Vree *et al.,* 1998).

In other tissues, a physiological role for human *MDR-3/*murine *mdr-2* has not been established, though gene-expression studies using PCR and the RNase protection assay have revealed details regarding their distribution in various normal tissues. MDR-3 RNA has been detected at high levels in liver and, to a lesser extent, in kidney, spleen, bone marrow, tonsil, striated muscle and adrenal gland, which showed very low levels (Chin *et al.,* 1989; Smit *et al.,* 1994). Murine mdr-2 mRNA and rat mdr-2 mRNA have been shown to have a distribution pattern similar to that of human MDR-3; *mdr-2* has also been detected in mouse lung and rat mammary tissue (Croop *et al.,* 1989; Smit *et al.,* 1994; Zhang *et al.,* 1996). Using mice transgenic for an *MDR-3* mini-gene, Smit *et al.* (1996) found that *MDR-3* over-expression in Schwann cells resulted in dysmyelination of the peripheral nervous system.

There is a possibility that *MDR-3* may encode a functional drug pump in certain B-cell lymphocytic leukaemias. High levels of *MDR-3* gene expression have been observed in cases of B-cell prolymphocytic leukaemia (B-PLL) with no detectable *MDR-1* expression (Nooter *et al.,* 1990; Herweijer *et al.,*1990). *In vitro* drug-uptake studies have shown that in PLL and B-cell chronic lymphocytic leukaemia (B-CLL) cells expressing *MDR-3,* drug accumulation can be significantly increased upon addition of cyclosporine and, to a lesser extent, verapamil (Herweijer *et al.,* 1990). However, Ludescher *et al.* (1993) found no evidence that over-expression of *MDR-3* was related to rhodamine efflux in B-CLL. Increases in intracellular rhodamine levels upon addition of cyclosporin A were observed in MDR-1 Pgp-negative/ MDR-3 Pgp-positive acute leukaemia patients (Arai *et al.,* 1997). Kino *et al.* (1996) reported that a yeast strain transformed with the *MDR-3* gene developed low-level resistance to the fungicide aureobasidin A. Studies on polarised cell systems suggest that *MDR-3* may act as a transporter of certain cytostatic drugs (Smith, 1998).

In the majority of the haematological and tissue studies performed to date, assessment of MDR-3 status was by *MDR-3* gene expression using RT-PCR and the RNase protection assay; *MDR-3*-encoded Pgp levels have not been investigated extensively, with the exception of a report by Arai *et al.* (1997). Some work has been performed using polyclonal antibodies generated to the murine *mdr-2* and human *MDR-3* gene products, where mdr-2 and MDR-3 Pgps have been observed in the canalicular membranes of liver (Buschman *et al.,* 1992; Smit *et al.,* 1994; de Vree *et al.,* 1998). Excluding this work, however, for the most part, detection of the *MDR-3*-encoded gene product has relied on comparison studies with the C219 monoclonal antibody (MAb), which recognises both the *MDR-1* and the *MDR-3*/*mdr-2*-encoded gene products and

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MDR-1-specific MAbs, such as JSB-1 and MRK16. The production and characterisation of a MAb (clone 6/1G) which specifically recognises the MDR-3 Pgp is described. This antibody may prove useful in further defining the role, if any, of the *MDR-3*-encoded Pgp in normal and malignant cells and tissues.

MATERIAL AND METHODS

Immunogen for generation of MAb 6/1G

The peptide used for the production of antibody 6/1G was selected after alignment searches of the EMBL Swiss-Prot protein sequence database using the Mail-FASTA programme. The entire amino acid sequence of the *MDR-3*-encoded Pgp [deduced from the known cDNA sequence (van der Bliek *et al.,* 1988)] was obtained from the data bank above using the NETSERV programme. From this deduced sequence, a 12-amino acid peptide was chosen, corresponding to the intracellularly located amino acids 13–24 (RPTSAEGDFELG) of *MDR-3*-encoded Pgp, which resides in the N-terminal part of the protein. This peptide was chosen because of its insignificant homology with the *MDR-1*-encoded Pgp. The peptide was synthesised by BioSyn (Belfast, Northern Ireland) and purchased in both the free form and the form conjugated to BSA for immunisation and screening purposes.

In vitro *immunisation regime*

The spleen was removed from a BALB/c mouse, and the spleen cells were immunised *in vitro* with 60 µg of conjugated peptide following instructions provided with the *in vitro* immunisation kit Cell-Prime (supplied by Immune Systems, Bristol, UK). Three days after *in vitro* immunisation, spleen cells were fused with SP2 myeloma cells according to a modification of the method of Kohler and Milstein (1975). Initially, all clones produced were screened by ELISA against the peptide/BSA complex and against BSA alone. Only clones positive for the peptide/BSA complex and negative for BSA alone were chosen for further expansion. Antibody 6/1G was cloned by limiting dilution to ensure its monoclonal status. The resultant supernatant was used for characterisation of the antibody by Western blotting and immuno-cytochemical methods.

Antibodies

MDR-1-encoded Pgp was detected using the MDR-1-specific BRI MAb 6/1C (Moran *et al.,* 1997) obtained from Bioresearch Ireland. The C219 MAb was obtained from Centocor (Malvern, PA).

Control cell lines

Western blotting studies and initial immuno-cytochemical studies to test the specificity of antibody 6/1G were performed on the *MDR-3* transgenic mouse fibroblast cell lines Vim 1 and V01-V01 (along with the parental cell line FVB c), which were kindly provided by Dr P. Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). The MDR non-small cell carcinoma cell line DLKPA (Clynes *et al.,* 1992), which over-expresses *MDR-1*-encoded Pgp and its sensitive counterpart DLKP (Law *et al.,* 1992), were also investigated. The ovarian carcinoma cell line A2780 was kindly provided by Dr R.J. Scheper (Free University Hospital, Amsterdam, The Netherlands).

Additional cell lines from different species whose MDR-3 status was unknown were also screened by Western blotting with antibody 6/1G. The African green monkey kidney cell line BS-C-1 and the Buffalo rat liver cell line BRL 3A were obtained from the ATCC (Rockville, MD).

Cell lines were cultured at 37°C in DMEM (DMEM/Ham's F12 1:1 was used for the A2780, DLKP and DLKP cell lines), supplemented with 5% FCS, L-glutamine (2 M) and Hepes (1 M).

Haematological samples

Cytospin preparations of peripheral blood, bone marrow and pleural fluid were kindly provided by the Department of Haematology, Belfast City Hospital.

Tissue sections

Formalin-fixed, paraffin-embedded tissue was kindly provided by the Histopathology Departments of St. Vincent's Hospital (Dublin). Sections (5 µm) of tissue blocks were cut, mounted onto poly-L-lysine-coated slides and dried overnight at 37°C. Slides were stored at room temperature until required.

Immuno-cytochemical studies

An ABC detection system employing an avidin-biotin horseradish peroxidase (HRP)-conjugated kit plus an appropriate secondary antibody from Dako (High Wycombe, UK) was used for all immuno-cytochemical studies on cell lines, haematological samples and paraffin-embedded archival material.

Briefly, cytospin preparations were fixed for 2 min in ice-cold acetone and allowed to air dry for at least 15 min prior to immunostaining. Tissue sections were dewaxed in xylene (2×5 min), rehydrated in graded alcohols and placed in Tris-buffered saline (TBS)/0.05% (vol/vol) Tween 20. Endogenous peroxidase activity was quenched by placing cytospins in 0.6% (vol/vol) H_2O_2/ methanol and tissue sections in 3% (vol/vol) H_2O_2/ distilled H_2O for 5 min. All slides were blocked for non-specific staining with 20% (vol/vol) normal rabbit serum for 20 min. Primary antibodies were applied to each sample [antibody 6/1G diluted 1:2 to 1:10 in TBS/0.05% (vol/vol) Tween 20, the MDR-1-specific antibody 6/1C used as neat supernatant or as ascites diluted 1:40 in TBS/0.05% (vol/vol) Tween 20] for 2 hr. This was followed by a 30-min incubation with biotinylated rabbit anti-mouse Igs [1/300 dilution in TBS/ 0.05% (vol/vol) Tween 20]. Finally, ABC (HRP-conjugated) was applied for 25 min, and the peroxidase substrate 3′-3 diaminobenzidine tetrahydrochloride (DAB, Dako) was then applied for 5 to 7 min. All incubations were carried out at room temperature, and slides were washed after each incubation in 3 changes of TBS/0.05% (vol/vol) Tween 20 over 15 min. Tissue sections and cells were lightly stained with haematoxylin, differentiated in 1% (vol/vol) acid alcohol and "blued" in Scott's tap water. Following dehydration in graded alcohols, slides were cleared in xylene and mounted in DPX (BDH, Poole, UK). Negative control slides in which primary antibody was replaced by control mouse immunoglobulins (Vector, Peterborough, UK; used within the manufacturer's recommended concentration range) were included in all experiments.

Immunofluorescence studies on live cells

Viable *MDR-3* transgenic cells, VIM 1, and their parental cells, FVB c, were tested for reactivity with antibody 6/1G by indirect immunofluorescence. When immunofluorescence studies are performed on viable cells, only cell-surface antigens are recognised. Briefly, test cells were adjusted to a concentration of 1×10^6 cells/ml in PBS; 100 µl of the cell suspension was pipetted into each of 2 Eppendorf tubes and 100 µl of antibody 6/1G added (neat supernatant) to one and 100 µl of PBS added to the other. Tubes were mixed and incubated for 30 min at room temperature. The primary antibody was removed by centrifugation of cells at 800 *g* for 4 min. Cells were washed 3× with PBS by the same procedure; 100 µl of secondary antibody, fluorescein isothiocyanate-linked (FITC) sheep anti-mouse Ig (Boehringer-Mannheim, Lewes, UK) diluted 1/50 in PBS were added to the tubes, mixed and incubated for 30 min at room temperature. Secondary antibody was removed and cells washed as above. Each cell pellet was resuspended in a mimimum amount of Vectashield (Vector) mounting medium and cells viewed using a Nikon phase contrast microscope fitted with an FITC filter.

Preparation of murine tissues and cell lysates for Western blotting

Kidney and liver were removed from a normal healthy BALB/c mouse and snap-frozen in liquid N_2 until required. Briefly, tissue samples were allowed to thaw whilst being maintained on crushed ice before being homogenised in ice-cold PBS containing a cocktail of protease inhibitors (Boehringer-Mannheim). The homogenate was centrifuged at 12,000 *g* for 10 min and the resultant

supernatant centrifuged at 61,000 *g* for 1hr in an ultracentrifuge (Optima, model XL-80; Beckman, Carlsbad, CA). The resultant pellet was resuspended in 0.5 ml PBS plus 0.5 ml 2× reducing loading buffer [1.25 M Tris-HCL (pH 6.8), 4% SDS, 10% 2-mercaptoethanol, 20% glycerol and 0.02% bromophenol blue].

Whole-cell lysates of cell lines were prepared by trypsinisation of cells from culture flasks (1 × 75-cm^2 flask), washing 3× in PBS, sonicating cells with a Labsonic U sonicator (Braun, Melsungen, Germany) in 250 to 500 µl PBS containing a cocktail of protease inhibitors until all cells were disrupted (when viewed microscopically) and finally resuspending 1:1 in 2× reducing loading buffer (as above) prior to performing SDS-PAGE (10% gels). Before resuspension in 2× loading buffer, samples of the cell lysates and murine tissues in PBS (25 to 50 µl) were taken for determination of protein concentration by the BCA protein assay (Pierce, Rockford, IL). Equal concentrations (20 µg) of all cell membranes and tissues were applied to 10% SDS-PAGE gels. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting for the detection of the antigen recognised by antibody 6/1G were performed by the methods of Laemmli (1970) and Towbin *et al.* (1979), respectively.

Following incubation of blots with clone 6/1G [supernatant diluted 1:2 or ascites diluted 1:50 in TBS/0.1% (vol/vol) Tween 20] overnight at 4°C, blots were incubated with HRP-labelled rabbit anti-mouse Igs (Dako) diluted 1:2,000 in TBS for 90 min at room temperature. Blots were developed using the enhanced chemiluminesence (ECL) system obtained from Amersham (Aylesbury, UK). In all cases, negative control blots were included in which antibody 6/1G was substituted with diluent or irrelevant supernatant.

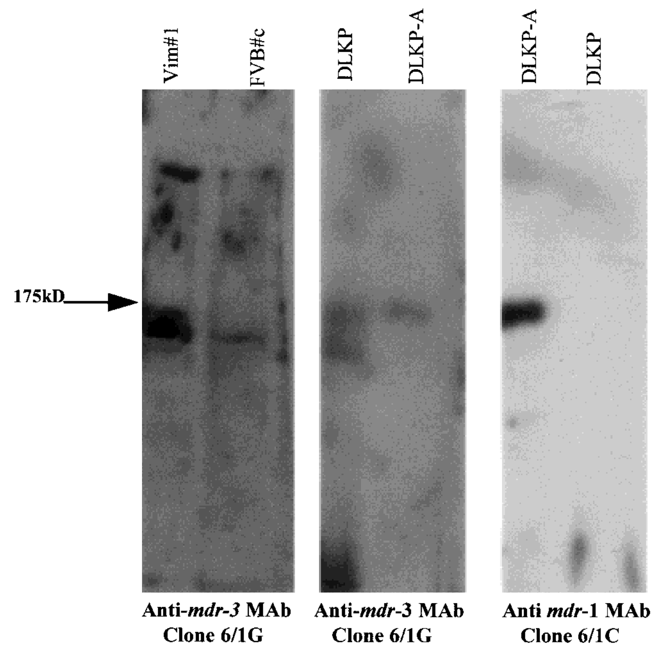

FIGURE 1 – Western blot analysis of crude cell lysates (20 µg) of the *MDR-3* transgenic cell line Vim 1, its parental cell line FVB c, the multidrug resistant *MDR-1* over-expressing DLKP-A cell line and its sensitive counterpart DLKP with MAbs clone 6/1G (MDR-3-specific) and clone 6/1C (MDR-1-specific).

RESULTS

Fusion results

Following fusion, a total of 384 hybridoma supernatants (100% fusion) were screened by ELISA. Supernatants from 48 clones which were positive for the peptide/BSA complex alone were chosen for further expansion. Eleven of these hybridomas were studied in detail, based on reactivity patterns with control cell lines. MAb 6/1G was cloned by limiting dilution prior to further characteristion. Isotype was determined to be subclass IgM (Isostrip; Boheringer-Mannheim).

Western blotting studies of control cell lines

On Western blotting of cell lysates of Vim 1, antibody 6/1G reacted with a band at 170 kDa. This band was only very faintly detected on preparations of parental FVB c cells (Fig. 1). A 170-kDa band was also detected on V01-V01 cells (data not shown). A faint band was detected on cell lysates of the sensitive DLKP cell line and its adriamycin-resistant variant, DLKPA. In contrast, a distinct band on cell lysate preparations from DLKP-A cells, which over-express *MDR-1*-encoded Pgp, can be seen with MAb 6/1C, which is specific for the *MDR-1* gene product (Fig. 1).

Previous results showed lack of positivity of the *MDR-3* transgenic cell lines V01-V01 and Vim 1 with MAb 6/1C (specific for the *MDR-1*-encoded gene product) (Moran *et al.,* 1997). Taken together, these results indicate that 6/1G detects the *MDR-3,* but not the *MDR-1* -encoded protein.

Immuno-cytochemical studies of control cell lines and tissues

Immuno-cytochemical studies on cytospins of both Vim 1 and V01-V01 cells showed consistent cytoplasmic and plasma membranous staining with MAb 6/1G (Fig. 2*a,b*); negligible staining was observed on FVB c cells (Fig. 2*c*). The C219 MAb, which detects both the *MDR-1* and the *MDR-3* gene products, gave a similar pattern of reactivity on Vim 1 (Fig. 2*d*) and V01-V01 cells.

Immuno-cytochemical analysis of resistant DLKP-A and sensitive DLKP cells showed equivalent low-level staining with MAb 6/1G (Fig. 2*e,f*). In contrast, previous results showed strong staining on DLKP-A cells and negligible staining on DLKP cells with both the MDR-1-specific MAb 6/1C and the widely used anti-*MDR-1*/*MDR-3* gene product MAb C219 (Heenan *et al.,* 1997; Moran *et al.,* 1997). Analysis of the ovarian carcinoma cell line A2780 showed negligible reactivity with antibody 6/1G.

Immunofluoresence studies on live cells revealed no detectable staining on Vim 1 or parental FVB c cells, indicating that the epitope recognised by antibody 6/1G was not expressed on the surface of these cells.

Formalin-fixed, paraffin-embedded normal liver tissue showed 6/1G-positive staining in hepatocytes throughout the entire liver lobule, with specific staining observed on canalicular membranes.

Immuno-cytochemical analysis of MDR-1 *and* MDR-3*-encoded Pgp expression in B-cell leukaemias*

B-CLL. Two cases of B-CLL showed MDR-1 positivity using BRI MAb MDR-1(6/1C) in normal and malignant cell types. Anti-MDR-3 MAb 6/1G showed a differential pattern of staining in both cases of B-CLL, with the larger malignant cells (more pleiomorphic, possibly associated with higher-grade/more malignant phenotype) showing more intense MDR-3 positivity than the smaller malignant cells (Fig. 3*a*). Both samples were from patients who had received prior treatment.

Acute myeloid leukaemia (AML). Of 3 AML cases included in our study, a specimen from a patient presenting with AML [French–American–British (FAB) classification M0] was negative for MDR-1 and MDR-3 protein expression. One AML sample, FAB M1, showed MDR-1 positivity but did not show any MDR-3 positivity. The third sample, FAB M5a, exhibited weak MDR-1 positivity in monoblasts, with very intense MDR-3 positivity (which was very granular in nature) also observed in these monoblasts (Fig. 3*b*). None of these patients had received prior treatment.

B-cell non-Hodgkins lymphoma (B-NHL). Peripheral blood from one B-NHL patient (diagnosis not further classified) was negative for both MDR-1 and MDR-3 Pgp expression. One case of mantle cell lymphoma showed MDR-3 positivity and was negative for MDR-1; a second case, however, showed a reversal of these results. A small number of follicle centre lymphomas gave conflicting

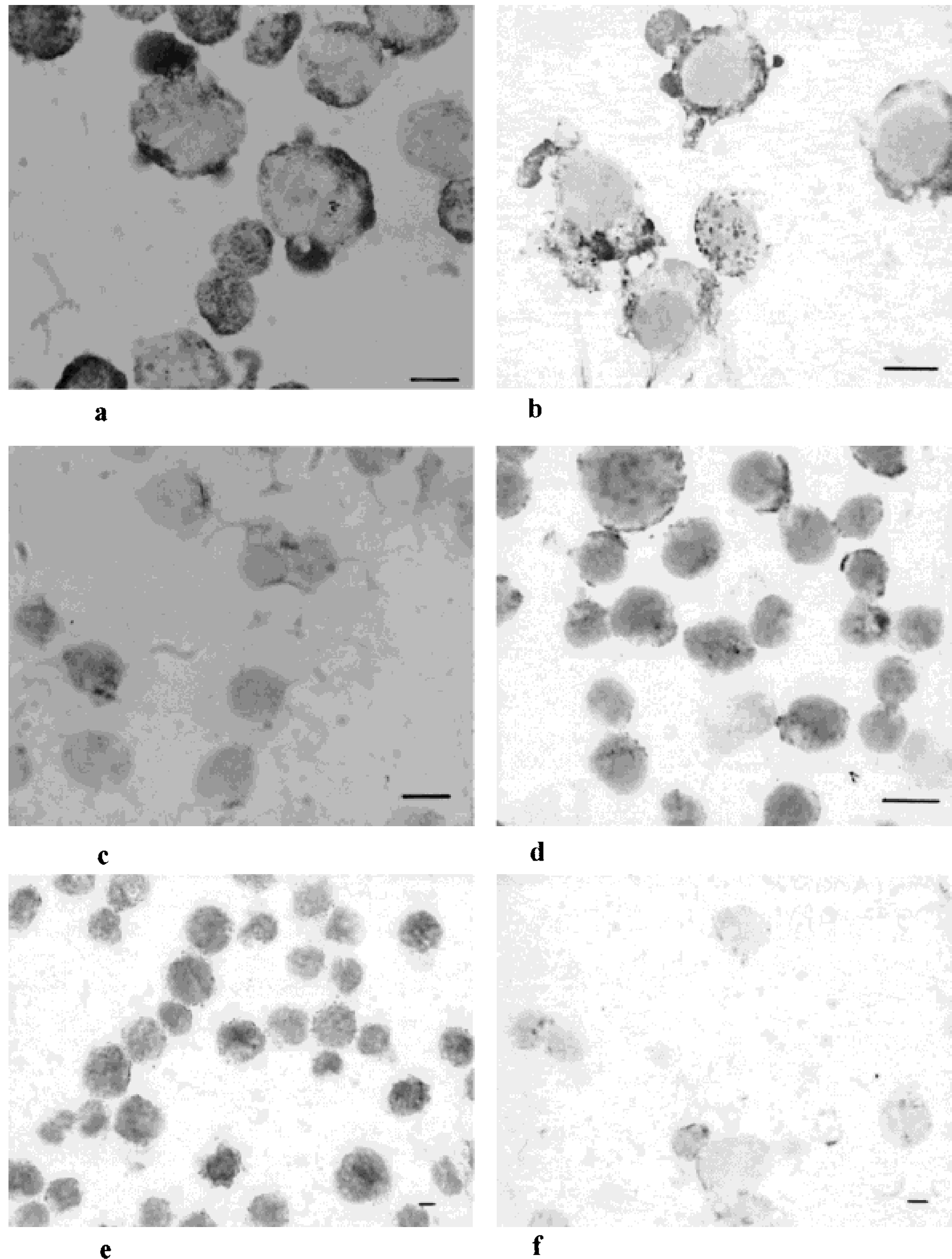

FIGURE 2 – Immuno-cytochemical analysis of antibody 6/1G on the *MDR-3* transgenic cell lines Vim 1 and V01-V01, their parental cell line, FVB c, the multidrug resistant non-small cell carcinoma cell line DLKPA and its sensitive counterpart DLKP and of MAb C219 on the Vim 1 cell line. *(a)* Vim 1 cells showing intense cytoplasmic and plasma membranous positivity with antibody 6/1G (scale bar: 10 μm). *(b)* V01-V01 cells showing intense cytoplasmic and plasma membranous positivity with antibody 6/1G (scale bar: 10 μm). *(c)* FVB c cells showing negligible reactivity with antibody 6/1G (scale bar: 10 μm). *(d)* Vim 1 cells showing plasma membranous positivity with MAb C219 (scale bar: 10 μm). *(e)* DLKP-A cells showing low-level positivity with antibody 6/1G (scale bar: 10 μm). *(f)* DLKP cells showing low-level positivity with antibody 6/1G (scale bar: 10 μm).

results in our study, though in 2 cases there appeared to be co-expression of MDR-1 and MDR-3. One case of follicle centre lymphoma (atypical $CD5^+$ type) showed weak positivity for MDR-1 and did not show any MDR-3 positivity prior to treatment (Fig. 3*c*). At 16 days post-treatment, MDR-1 expression was increased significantly; intense MDR-3-specific staining was also observed (Fig. 3*d*). Further post-treatment samples from this patient are being investigated.

Screening of simian and rat cell lines of unknown MDR-3 status by Western blotting

A distinct band at 170 kDa was observed with cell lysate preparations of the Buffalo rat liver cell line BRL-3A. A very faint band was visible at this m.w. on the African green monkey kidney cell line BSC-1 (data not shown).

Screening of normal murine tissues by Western blotting

Kidney and liver tissue preparations showed a single band at 170 kDa on Western blot (data not shown).

DISCUSSION

We describe here the generation and preliminary characterisation of a novel MAb produced by *in vitro* immunisation of murine spleen cells. This antibody, designated 6/1G (subclass IgM),

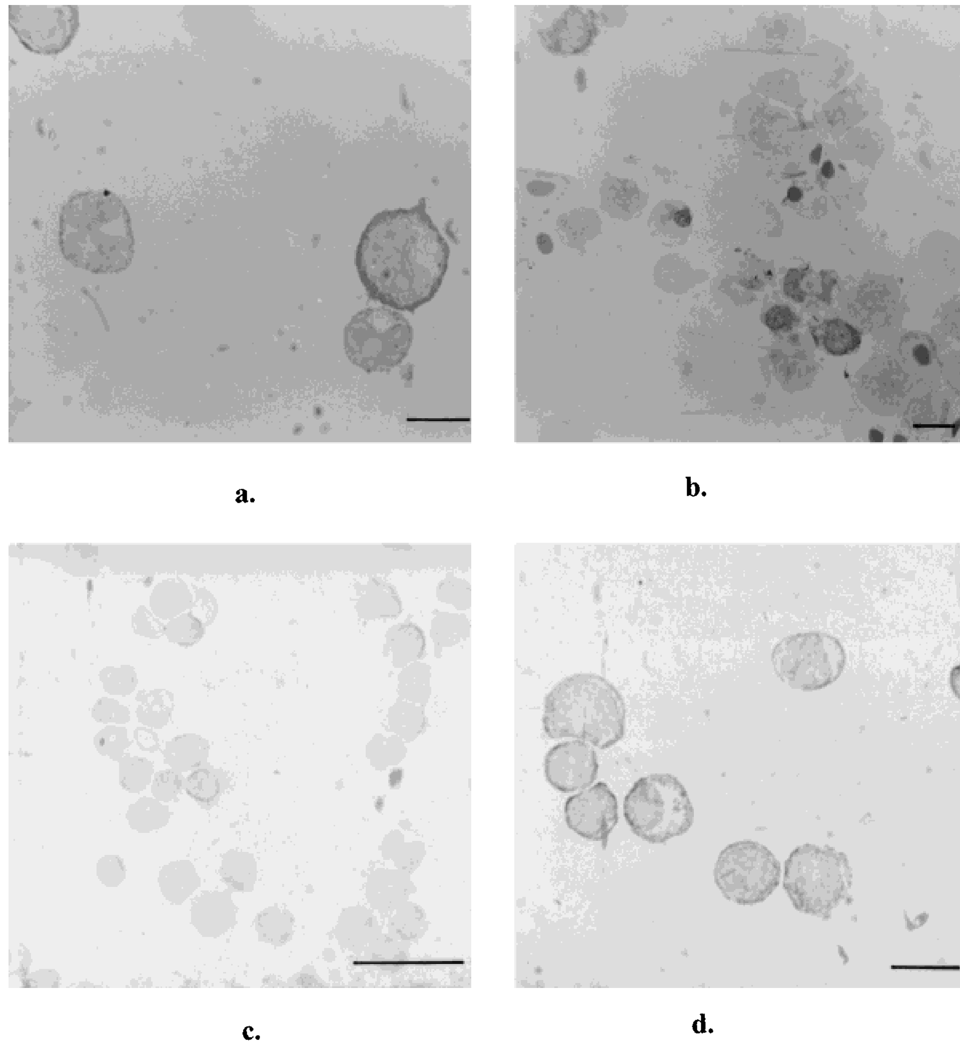

FIGURE 3 – Immuno-cytochemical analysis of MDR-3 Pgp levels (as assessed by antibody 6/1G) in B-cell leukaemias. *(a)* Peripheral blood from a B-CLL patient stained with antibody 6/1G; intense MDR-3-positive staining can be seen in the larger, possibly more malignant cell type (scale bar: 10 μm). *(b)* Peripheral blood from an AML stage M5a patient stained with antibody 6/1G showing MDR-3-positive staining in monoblasts (scale bar: 10 μm). *(c)* Peripheral blood from a follicle centre lymphoma (atypical type, $CD5^+$) patient stained with antibody 6/1G showing negative *MDR-3* Pgp expression (pre-treatment) (scale bar: 50 μm). *(d)* Peripheral blood from a follicle centre lymphoma (atypical type, $CD5^+$) patient stained with antibody 6/1G showing intense MDR-3-positive staining (post-treatment) (scale bar: 10 μm).

appears to specifically recognise the *MDR-3*-encoded gene product. Western blotting studies with antibody 6/1G identified a single band at 170 kDa on cell lysate preparations of the *MDR-3* transgenic cell lines V01-V01 and Vim 1. In their successful attempt to produce MDR-3-specific polyclonal antibodies, Smit *et al.* (1994) observed that the apparent m.w. of *MDR-3* Pgp in V01-V01 cells was approximately the same as for *MDR-1*-encoded Pgp. By comparison with MAb C219, this group showed that V01-V01 cells do not express *MDR-1* Pgp. We have previously shown by immuno-cytochemistry and Western blotting using BRI MAb MDR-1 6/1C that V01-V01 and Vim 1 cell lines do not express *MDR-1* Pgp (Moran *et al.,* 1997). It appeared therefore that antibody 6/1G specifically recognised the *MDR-3* gene product. The Western blotting results were confirmed by immuno-cytochemical studies, which revealed consistent cytoplasmic/plasma membranous staining on both V01-V01 and Vim 1 cells. Negligible staining was observed on preparations of FVB c cells. The C219 MAb, which recognises the *MDR-1*- and *MDR-3*-encoded gene products, gave a similar pattern of reactivity on the *MDR-3* transgenic cell lines. Two of the 3 polyclonal antibodies, anti-REG-1 and anti-REG-2, produced by Smit *et al.* (1994) showed a pattern of staining similar to that described here with antibody 6/1G, *i.e.,* specific staining of the plasma membrane and cytoplasm in *MDR-3* transgenic cells.

Antibody 6/1G was produced following immunisation with a peptide corresponding to amino acids 13–24 residing in the N-terminal part of the *MDR-3* gene product; this sequence has no significant homology with the *MDR-1*-encoded Pgp and is believed to be intracellularly located. The fusion protein used by Smit *et al.* (1994) to generate the REG-1 polyclonal antibody consisted of amino acids 2–58 from the N-terminal region of MDR-3 Pgp. The AVLCL2 polyclonal antibody was also produced by Smit *et al.* (1994), using a 13-amino acid synthetic peptide sharing 7 amino acids with the synthetic peptide used for the production of antibody 6/1G. However, this antibody appeared to work well on Western blotting only and did not appear suitable for immuno-histochemical studies.

Additional cell lines probed with antibody 6/1G showed MDR-3 Pgp reactivity, which reflected mRNA levels previously studied. Negligible staining was observed on the ovarian carcinoma cell line A2780; this cell line has previously been shown to not express the *MDR-3* gene (Herweijer *et al.,* 1990). The MDR non-small cell carcinoma cell line DLKP-A and the sensitive DLKP cell line

showed weak staining with antibody 6/1G on both immunocytochemical and Western blot analyses; DLKP-A and DLKP have previously been shown to have low-level *MDR-3* gene expression using RT-PCR (O'Driscoll, 1994).

On paraffin-embedded archival normal liver tissue we observed an overall high level of reactivity with antibody 6/1G. Positivity was observed in hepatocytes, with specific staining observed on canalicular membranes. We have previously shown low-level MDR-1 reactivity in liver (Moran *et al.,* 1997). Low-level reactivity with antibody JSB-1 (MDR-1-specific) and strong reactivity with MAb C219 (which recognises both the *MDR-1-* and *MDR-3-*encoded gene products) has also been observed in liver (Bittl *et al.,* 1993). These results reflect the documented distribution patterns of *MDR-1*/*MDR-3*.

As the human *MDR-3* gene is closely related to the murine *mdr-2* gene, we examined a selection of normal mouse kidney and liver cell lines as well as a small number of cell lines from different species available to us with antibody 6/1G. Antibody 6/1G identified a 170-kDa band on preparations of the Buffalo rat liver cell line BRL-3A but showed very low-level reactivity on the African green monkey kidney cell line BSC-1. A 170-kDa band was also identified on mouse liver and kidney preparations. These results reflect previously determined *mdr-2* mRNA expression patterns for these particular tissues (Croop *et al.,* 1989). This indicates that antibody 6/1G cross-reacts with the *mdr-2* gene. The antibody may therefore be useful for studies in several species.

To characterise antibody 6/1G further, a small number of B-cell leukaemias were examined as it has been indicated that *MDR-3* may be involved in drug resistance in certain B-cell lymphocytic leukaemias (Nooter *et al.,* 1990; Herweijer *et al.,* 1990).

For comparison, we also examined MDR-1 Pgp levels using BRI MDR-1 MAb 6/1C. MDR-1/ P170 has been implicated as a cause of clinical resistance in AML, multiple myeloma (MM) and possibly late stages of ALL and NHL. The significance of *MDR-1* /P-170 expression in other haematological malignancies remains to be clarified.

B-CLL has been previously shown to express *MDR-1* and *MDR-3* (Herweijer *et al.,* 1990; Sonneveld *et al.,* 1992; Nooter and Sonneveld 1993; Ludescher *et al.,* 1993). We found *MDR-1* and *MDR-3* Pgp expression in 2 post-treatment samples of B-CLL. In addition, we observed a differential pattern of MDR-3 staining with intense MDR-3 positivity, possibly indicating a more malignant phenotype in both cases. Prior treatment has been associated with higher MDR-3 levels (no influence on *MDR-1* levels) in one study (Herweijer *et al.,* 1990). Later work did not show such a correlation (Sonneveld *et al.,* 1992; Ludescher *et al.,* 1993). In patients with advanced B-CLL, MDR-3 mRNA expression (not *MDR-1* expression) was significantly higher than in early-stage disease (Sonneveld, 1992). Ludscher *et al.* (1993) also found a tendency towards higher MDR-3 mRNA expression in patients with advanced stages of CLL. Our observations together with these studies suggest that *MDR-3* over-expression (in our case as shown by intense MDR-3 positivity using antibody 6/1G) may be associated with a more malignant phenotype in this disease. We are currently examining more B-CLL cases with antibody 6/1G.

To date, MDR-3 expression has not been reported in AML. The *MDR-1* gene product has been shown to be frequently expressed in AML at diagnosis (*i.e.,* prior to treatment) and at relapse. Marie *et al.* (1996) reported a high correlation between P-170 over-expression and clinical drug resistance. In our study, 2 untreated cases of AML (FAB M0 and M1) showed MDR-1 positivity and were negative for MDR-3. A stage M5a untreated patient showed weak MDR-1 positivity but intense MDR-3 positivity. Since AML stage M5a has an undifferentiated monoblastic lineage, the possibility exists that *MDR-3* is not expressed in certain subtypes: M0, M1, M2 and M3, all of which have myeloblastic/myelocytic morphology. MDR-3 reactivity in a wider range of AML subtypes will be examined to investigate these observations further. Perhaps *MDR-3* expression is restricted to the M5a and M5b subtypes, which have a monocytic morphology. It has already been suggested that *MDR-3* expression may be restricted to certain B-cell developmental stages; no *MDR- 3* expression has been found in MM, which presents with end-stage B cells (Herweijer *et al.,* 1990). *MDR-3* expression appears to be restricted to those malignancies which represent intermediate and mature B-cell developmental stages; no MDR-3 was found in malignancies representing early B-cell development or in very mature B-cell tumours, such as Waldenström and MM, in a study carried out by Nooter and Sonneveld (1993). Interestingly, in one B-NHL we examined, the malignant cells comprised lymphoplasmacytoid cells, which are late-stage B cells; this sample was negative for both MDR-1 and MDR-3.

Most previous work on *MDR-1*-encoded Pgp in NHL has been performed on frozen lymph node sections. In general, more *MDR-1*/Pgp expression is seen in treated than untreated patients. There appears to be no difference in Pgp expression between high- and low-grade lymphomas and between B and T subtypes (Marie *et al.,* 1996). Adult T-cell lymphoma (ATL) has been shown to frequently express *MDR-1*/Pgp at presentation. As to whether *MDR-1*/Pgp is an indicator of better clinical response, 3 studies have shown a correlation. A larger study did not show such a correlation (for review: Marie *et al.,* 1996). To date, only one study has looked at *MDR-3* gene expression in B-NHL, where expression was observed in 9 of 21 B-NHL patients (Nooter and Sonneveld, 1993). As we have already outlined under ''Results'', the B-NHL cases in our study (mantle cell, follicle centre and unclassified B-NHL) showed varying levels of *MDR-1* and *MDR-3* Pgp expression. A high level of MDR-3 positivity appeared to develop following treatment in a case of follicle centre lymphoma (atypical, $CD5^+$). More work is obviously required to investigate whether *MDR-3* expression is significantly related to treatment in B-NHL and to assess the significance of the MDR-1/MDR-3 expression levels observed in other cases.

In summary, we have described a new MAb which appears to be specific for the *MDR-3*-encoded gene product. This novel antibody will be useful in the assessment of the *MDR-3* status of both normal and malignant cells/tissues. Antibody 6/1G may thus help in further defining a physiological role for *MDR-3* as well as establishing if this gene does indeed play a role in drug resistance, particularly with regard to haematological malignancies.

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