A new mdr-1 encoded P-170 specific monoclonal antibody: (6/1C) on paraffin wax embedded tissue without pretreatment of sections

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Abstract

***Aims*—The generation and characterisation of a monoclonal antibody that specifically recognises the mdr-1 encoded protein, P-glycoprotein (P-170), on routinely processed formalin fixed, paraffin wax embedded tissue sections.**

***Methods*—The monoclonal antibody, designated 6/1C, was produced following a combination of in vivo and in vitro immunisation regimens in Balb/c mice with a synthetic 12 amino acid peptide that corresponds to amino acids 21–32 (believed to be intracellularly located) of P-170 and has insignificant homology with the mdr-3 encoded P-170. Antibody 6/1C was characterised by western blotting and immunocytochemistry on cytospins of paired multidrug resistant or sensitive cell lines, including mdr-1 and mdr-3 transfected cells, and by immunohistochemistry on normal and malignant formalin fixed paraffin wax embedded tissue sections.**

***Results*—Antibody 6/1C showed a single band at 170 kDa on western blots of multidrug resistant cell lysates and mdr-1 transfected cell lysates that was absent on similar preparations of drug sensitive cells and mdr-3 transfected cells. Immunocytochemical studies on cytospins of multidrug resistant cells and mdr-1 transfected cells revealed strong inner plasma membrane/cytoplasmic staining. Staining was negligible on drug sensitive cells and cells transfected with the mdr-3 gene. Immunohistochemical studies on formalin fixed, paraffin wax embedded normal adult kidney, liver, and breast tissue and a range of fetal tissues exhibited staining patterns of a variety of secretory surfaces consistant with documented mdr-1 specific staining. Specific staining of malignant cells in similarly treated sections of breast tumours was seen also with antibody 6/1C. Staining on paraffin wax embedded tissue with this antibody did not require any pretreatment of tissue sections.**

***Conclusions*—This new monoclonal antibody, chosen for its specificity with the mdr-1 encoded P-170 and its reactivity on routinely fixed paraffin wax embedded tissue samples without pretreatment, appears to be useful for the investigation of P-170 in archival material. It is especially useful for retrospective studies on pre-treatment and post-treatment tissue sections, and could help establish when and how rapidly mdr-1 associated drug resistance develops during chemotherapeutic regimens. Immunohistochemical assessment of P-170 expression in many cancers has potential for diagnostic purposes and may influence the choice of chemotherapeutic drugs used in the treatment of refractory tumours.**

(*J Clin Pathol* 1997;50:465–471)

Keywords: monoclonal antibody; multidrug resistance; mdr-1; P-glycoprotein; P-170; paraffin wax embedded sections

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Accepted for publication
8 April 1997

A major problem encountered during the chemotherapy of many tumour types is the development of resistance by the target tumour cells to a broad spectrum of drugs, a phenomenon known as multidrug resistance. Multidrug resistance is characterised by resistance to a broad range of structurally and functionally unrelated chemotherapeutic drugs including antracyclines, vinca alkaloids, epipodophyllotoxins, and taxol. Several mechanisms of resistance have been identified including altered levels of multidrug resistance associated protein (an efflux pump),[1 2] lung resistance related protein (a major vault protein),[3 4] topoisomerase II,[5] and glutathione S transferase.[6] However, overexpression of the mdr-1 gene product, P-glycoprotein (P-170), an integral plasma membrane protein involved in the active efflux of cytotoxic materials from the cell, is consistently associated with multidrug resistance in cultured cell lines selected for multidrug resistance and in certain tumours.[7 8] P-170 is expressed in a large number of normal tissues including kidney, adrenal glands, large intestine, and liver indicating that it is involved in normal physiological functions including detoxification and transport of lipophilic molecules. Tumours arising from tissues that normally express P-170 may be intrinsically resistant to chemotherapeutic agents or, alternatively, tumours that were initially responsive to chemotherapy may develop multidrug resistance during the treatment regimen and subsequently not respond to therapy.[8 9]

Owing to the cellular heterogeneity of most tumours and the subsequent heterogeneity of P-170 expression (resistant cells may be present as clusters within a given tumour), it is important to use an investigational technique, such as immunohistochemistry, that can provide direct morphological confirmation of the pres-

ence of P-170 rather than "bulk" methods such as western blotting and some molecular biological techniques.[10]

To date, several monoclonal antibodies have been produced to different epitopes of P-170[11-15] and subsequently used in studies of various human tumour types.[10 16-18] However, many of these studies have been performed on frozen tissue sections (in which cellular architecture is not as well preserved as in paraffin wax embedded specimens) or paraffin wax embedded tissue sections following the use of aggressive antigen retrieval techniques (which may influence immunolocalisation of antigens or have adverse effects such as detachment of tissues),[19 20] long primary antibody incubation and/or the use of a high concentration of expensive primary antibody that may also influence end results.[21] On the other hand, there are several reports on the advantages of antigen retrieval—for example, the use of antibodies that were previously unreactive on routinly fixed paraffin wax embedded tissue.[20 22] The major disadvantage of having to use antigen retrieval methods appears to be the interlaboratory variability of techniques and equipment (for example, microwave ovens).[20 23] An antibody that does not require antigen retrieval methods has the advantage of potentially producing more consistent results. A recent report detailing a series of multi-institutional trials to assess sources of variability in assays to detect P-170 in tumour specimens recommended standardisation of approaches to the detection of P-170 in clinical specimens, including careful control of sample fixation and antigen preservation.[24] This same report encouraged the development of new high affinity antibodies to P-170. We have successfully generated a monoclonal antibody, designated 6/1C, which can be used to identify P-170 positive cells specifically without any pretreatment (with a two hour incubation at room temperature using ~ 6–8 μg IgG/ml) in normal and malignant tissue that has been routinely processed (formalin fixed, paraffin wax embedded) in the laboratory. This antibody is useful in retrospective studies of archival tissue sections taken pre- and postchemotherapy and possibly could determine when and to what extent P-170 is expressed following chemotherapy. It also has potential as a diagnostic tool for determining the presence of P-170 positive cells in tumour samples that could influence chemotherapeutic regimens.

Methods

CHOICE OF IMMUNOGEN FOR MONCLONAL ANTIBODY PRODUCTION

The peptide used for the production of antibody 6/1C was selected after alignment searches of the EMBL Swiss-Prot protein sequence database using the Mail-FASTA program. The entire amino acid sequence of the mdr-1 encoded P-170 (deduced from the known cDNA sequence)[25] was obtained from the data bank above using the NETSERV program. From this deduced sequence, a 12 amino acid peptide was chosen corresponding to the intracellularly located amino acids 21–32 of P-170. The peptide was synthesised by BioSyn Ltd (Belfast, Northern Ireland) and was purchased both in the free form and conjugated to bovine serum albumin (BSA) for immunisation and screening purposes. The chosen peptide had insignificant homology with the mdr-3 encoded P-170 that does not appear to be involved in drug resistance.[26]

IMMUNISATION REGIMENS

A Balb/c mouse was injected twice with the chosen peptide conjugated to BSA. Primary immunisation was with 100 μg conjugated peptide in complete Freund's adjuvant, and secondary immunisation (following a three week interval) was with 100 μg conjugated peptide in incomplete Freund's adjuvant. Four weeks after immunisation the spleen was removed and the spleen cells immunised with 60 μg unconjugated peptide following instructions provided with the in vitro immunisation kit (Cel-Prime; Immune Systems Ltd, Paignton, UK). Three days after in vitro immunisation, the spleen cells were fused with SP2 myeloma cells according to a modification of the method of Kohler and Milstein.[27] Initially, all clones produced were screened by enzyme linked immunosorbent assay (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. Clone 6/1C was cloned twice by limiting dilution and finally propagated as ascites in Balb/c mice. This ascites was used for characterisation of the antibody by western blotting and immunocytochemical methods.

CONTROL CELL LINES

Western blotting studies and initial immunocytochemical studies were performed on a multidrug resistant non-small cell lung carcinoma cell line, DLKP-A (made resistant by exposure to increasing concentrations of adriamycin) and its drug sensitive counterpart, DLKP.[28] Overexpression of P-170 in DLKP-A cells compared with the parental DLKP cells had been confirmed in previous studies using other commercially available antibodies raised against P-170 (C219, Centocor, France; and JSB-1, Serotec, UK). Control cells were used to test antibody 6/1C for mdr-1 specificity. The mdr-3 transgenic fibroblast cell line, VO1 VO1 was kindly provided by Professor Piet Borst (department of molecular biology, Netherlands Cancer Institute, Amsterdam, Netherland) and mdr-1 transfected DLKP cells were obtained from Dr Seamus Coyle in our laboratory. Cytospin preparations were made on poly-L-lysine coated slides, air dried overnight, wrapped in foil, and stored at −20°C until required.

PEPTIDE NEUTRALISATION STUDIES

In addition to the control cells used to determine the specificity of antibody 6/1C for the mdr-1 encoded P-170, competitive inhibition studies with the peptide used as the immunogen were performed. Both purified 6/1C antibody (ascites purified using an ImmunoPure (A/G) IgG purification kit from

Pierce and Warriner, UK) and unpurified supernatant from the hybridoma secreting 6/1C antibody were preincubated with a 10-fold (by weight) excess of the peptide (unconjugated form) in phosphate buffered saline (PBS, Dulbecco A, 10 mM, pH 7:4) overnight at 4°C. To reduce background (caused by "sticky" aggregates) the antibody–peptide complex was centrifuged at full speed (15 000 ×*g*) in a microcentrifuge (Heraeus Instruments, Germany) and the supernatant removed carefully before use in immunocytochemical studies on cytospins of control cells.

TISSUE SECTIONS

Formalin fixed, paraffin wax embedded tissue was donated by the histopathology departments of St Vincent's Hospital and The National Maternity Hospital, Holles St, Dublin, Ireland. Sections (5 μm) of tissue blocks were cut, mounted on to poly-L-lysine coated slides, and dried overnight at 37°C. Slides were stored at room temperature until required.

IMMUNOFLUORESCENCE STUDIES ON LIVE CELLS

Paired resistant and sensitive viable cells were tested for reactivity with antibody 6/1C by indirect immunfluorescence studies. When immunofluorescence studies are performed on viable cells only cell surface components are recognised.[29] Briefly, test cells were adjusted to a concentration of 1×10^6 cells/ml in PBS and 100 μl of the cell suspension was aliquoted into each of two Eppendorf tubes. A volume of 100 μl antibody 6/1C (1 in 100 dilution of ascitic fluid in PBS) was added to one tube and 100 μl of control irrelevant mouse ascites (diluted 1 in 100 in PBS) was added to the other. The tubes were mixed and incubated for 30 minutes at 4°C. The primary antibody was removed by centrifugation of cells at 1000 rpm for five minutes. The cells were washed three times with PBS using the same procedure and 100 μl of fluorescein isothicyanate linked (FITC) sheep antimouse IgG (Boehringer Mannheim, Germany) diluted 1 in 50 in PBS was added. The tubes were mixed and incubated for 30 minutes at 4°C after which the secondary antibody was removed and the cells were washed as above. Each cell pellet was resuspended in a minimum amount of Vectashield (Vector Laboratories, UK) mounting medium and the cells were viewed using a Nikon phase contrast microscope fitted with an FITC filter.

IMMUNOHISTOCHEMISTRY

All immunocytochemical studies on cytospins of cell lines or on formalin fixed paraffin wax embedded tissue sections were performed following the method of Hsu *et al*,[30] using an avidin-biotin horseradish peroxidase (HRP) conjugated kit (ABC) plus an appropriate secondary antibody from Dako, UK. Briefly, cytospin preparations were fixed for one minute in ice cold acetone and allowed to air dry for at least 15 minutes before immunostaining. Paraffin wax embedded tissue sections were dewaxed in xylene (2 × 5 minutes), 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 five minutes at room temperature. All slides were blocked for non-specific staining with 20% (vol/vol) normal rabbit serum/TBS for 20 minutes at room temperature. Antibody 6/1C was applied to each sample (1 in 100 dilution in TBS/0.05% (vol/vol) Tween 20 for tissue sections and 1 in 40 dilution in the same diluent for cytospin preparations) for two hours at room temperature. This was followed by a 30 minute incubation with biotinylated rabbit antimouse IgG (1 in 300 dilution in TBS/0.05% (vol/vol) Tween 20) at room temperature. Finally, ABC (HRP conjugated) was applied for 25 minutes at room temperature. The peroxidase substrate, 3',3-daminobenzidine tetrahydrochloride (DAB; Dako) containing 0.02% (vol/vol) H_2O_2, was applied for 10–15 minutes at room temperature. All slides were washed after each incubation in three changes of TBS/0.05% (vol/vol) Tween 20 over 15 minutes. Tissue sections and cells were lightly counterstained with Cole's 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, UK). Negative control slides in which antibody 6/1C was replaced by control mouse ascites (Sigma, UK) at the same dilution were included in all experiments. Antibody 6/1C has also been used in a separate study of ocular melanoma using the alkaline phosphatase anti-alkaline phosphatase technique on cryostat sections.[31]

WESTERN BLOTTING

Denaturing polyacrylamide gel electophoresis (SDS-PAGE) followed by western blotting for the detection of P-170 were performed by the methods of Laemmli[32] and Towbin *et al*,[33] respectively. Briefly, whole cell lysates of control cells were prepared by a modification of the method of Gerlach *et al*[34] and separated on 7.5% polyacrylamide gels followed by electroblotting on to polyvinylidene difluoride support material. Following incubation of blots with antibody 6/1C, diluted 1 in 200 to 1 in 300 in TBS overnight at 4°C, blots were incubated with biotinylated rabbit antimouse IgG, diluted 1 in 2000 in TBS for one hour at room temperature. Blots were then incubated with ABC, HRP conjugated, diluted according to the manufacturer's instructions, for 30 minutes at room temperature before being developed using the enhanced chemiluminesence method (Amersham, UK). In all cases negative control blots were included in which antibody 6/1C was substituted with diluent or control mouse ascites.

Results

WESTERN BLOTTING

Recognition by antibody 6/1C (IgG_1 isotype) of a single band at 170 kDa (associated with the presence of P-170) on preparations from drug resistant cells and cells transfected with the

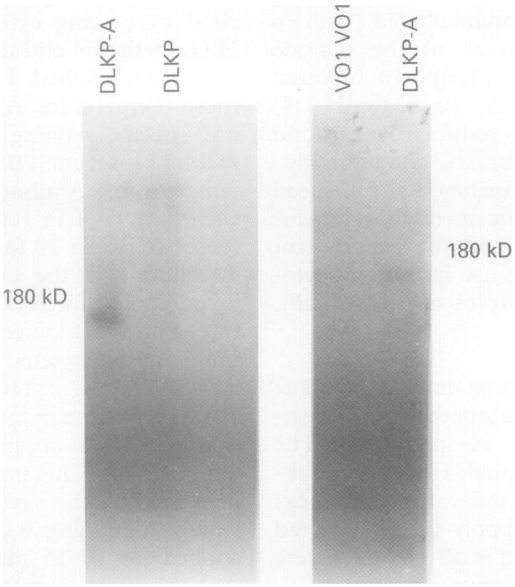

Figure 1 Recognition by antibody 6/1C (IgG_1 isotype) of a single band at 170 kDa (associated with the presence of P-170) on preparations from drug resistant cells (DLKP-A) plus the lack of reactivity on preparations of drug sensitive cells (DLKP) and cells transfected with the mdr-3 gene (VO1 VO1) confirming the specificity of 6/1C for mdr-1 encoded P-170.

mdr-1 gene plus the lack of reactivity on preparations of drug sensitive cells and cells transfected with the mdr-3 gene (VO1 VO1 cells) confirmed the specificity of this antibody for mdr-1 encoded P-170 (fig 1).

IMMUNOFLUORESCENCE STUDIES ON LIVE CELLS

There was no detectable staining of live resistant cells, indicating that the antigen recognised by antibody 6/1C is not expressed on the cell surface of these cells. In addition, staining of sensitive cells was not seen with this antibody.

IMMUNOCYTOCHEMICAL STUDIES ON FIXED CELLS

Strong staining of the plasma membrane/cytoplasm was seen with antibody 6/1C on resistant DLKP-A cells and cells transfected with the mdr-1 gene, whereas negligible staining was detected on drug sensitive and mdr-3 transfected cells (fig 2). Other paired sets of drug resistant/drug sensitive cell lines were examined with antibody 6/1C and similar results were obtained.

PEPTIDE NEUTRALISATION STUDIES

Immunocytochemical studies on fixed control cell lines showed that the reactivity of antibody 6/1C (both in its purified form and in hybridoma supernatants) was obliterated following incubation with the peptide used as the immunogen for production of the antibody.

IMMUNOHISTOCHEMICAL STUDIES ON FORMALIN FIXED, PARAFFIN WAX EMBEDDED TISSUE

The suitability of antibody 6/1C for use on routinely fixed, paraffin wax embedded tissue without pretreatment of the tissue was demonstrated initally on normal adult tissue. Under the aforementioned conditions antibody 6/1C gave more intense staining of cells than either antibodies C219 or JSB-1 although the overall percentage of staining was similar. Normal adult kidney showed specific mdr-1 staining in collecting and proximal tubules (fig 3A). In normal adult liver the overall mdr-1 specific staining was low compared with the kidney, and low levels of cytoplasmic staining were seen in hepatocytes. In normal breast tissue, some ducts showed low mdr-1 positivity and some non-specific staining of adipose tissue was seen (similar staining was seen with the antibody JSB-1). A number of normal fetal tissues were also investigated with antibody 6/1C including lung, small intestine, adrenal gland, liver, and placenta.

Lung—Epithelial cells lining the alveoli, epithelial cells, and cilia of the bronchus, macrophages, and some interstitial cells (probably erythroid precursor cells) showed specific mdr-1 staining (fig 3B).

Small intestine—Enterocytes, the absorptive cells of the small intestine, showed intense mdr-1 staining. In general, cells at the bottom of crypts stained much less than those in the villi. The goblet cells were negative. The muscularis propria also stained intensely (fig 3C).

Adrenal gland—A low level of staining was present in the medulla (but neuroblast cells

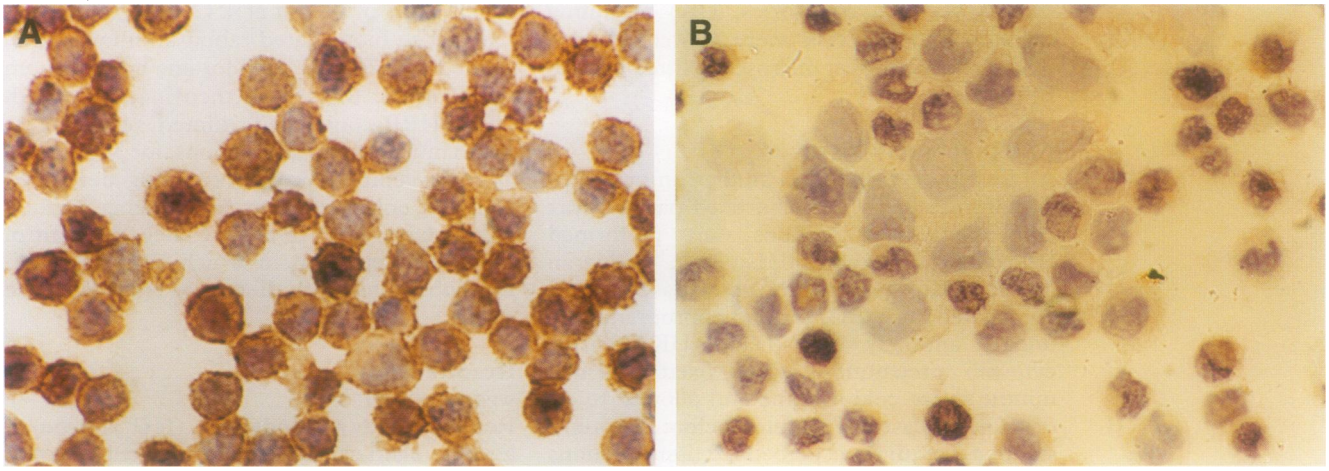

Figure 2 (A) Strong staining of the plasma membrane/cytoplasm with 6/1C on multidrug resistant cells (DLKP-A). (B) Negligible staining detected on drug sensitive (DLKP) cells. (Original magnification ×40).

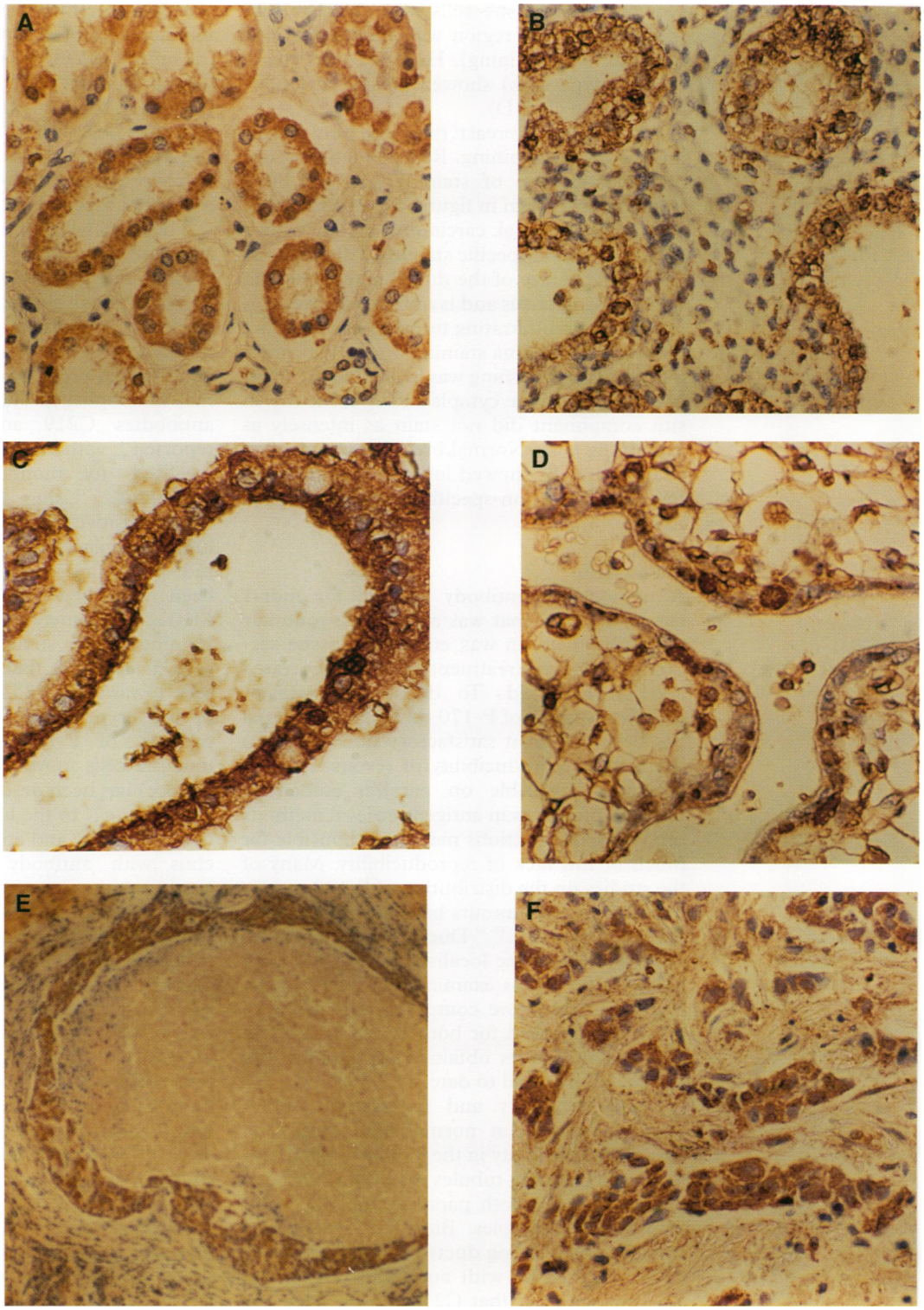

Figure 3 (A) Normal adult kidney showing specific mdr-1 staining in collecting and proximal tubules (original magnification ×40). (B) Epithelial cells lining the alveoli, epithelial cells, and cilia of the bronchus, macrophages, and some interstitial cells (probably erythroid precursor cells) showing specific mdr-1 staining (original magnification ×40). (C) Enterocytes of small intestine showing intense mdr-1 staining. In general, cells at the bottom of crypts stained much less than those in the villi; the goblet cells were negative. The muscularis propria also stained intensely (original magnification ×60). (D) Tropoblasts in placental villi showing mdr-1 positive staining. This was localised to the inner layer of cytotrophoblastic cells. The syncytiotrophoblastic cells were negative, but the brush border region was positive. Hoffbaur cells (macrophage type cells) showed positive cytoplasmic staining (original magnification ×40). (E) Ductal breast carcinoma in situ showing strong specific 6/1C staining. The centre of the ductal carcinoma had undergone necrosis and is mdr-1 negative (original magnification ×10). (F) Infiltrating tumour cells in a ductal breast carcinoma staining intensely positive for mdr-1, this staining was located in the inner plasma membrane/cytoplasm (original magnification ×40).

within the medulla were negative) but the cortex region was mdr-1 negative.

Liver—The liver had low levels of overall mdr-1 positivity; hepatocytes exhibited faint cytoplasmic staining. Erythroid precursor cells showed very intense cytoplasmic staining as did myeloid precursor cells. Bile duct epithelial cells in portal tracts appeared to be negative for mdr-1.

Placenta—Trophoblasts in placental villi were mdr-1 positive. This positivity was localised to the inner layer of cytotrophoblastic cells. The

syncytiotrophoblastic cells were negative, but the brush border region was positive (plasma membraneous staining). Hoffbaur cells (macrophage type cells) showed positive cytoplasmic staining (fig 3D).

Six malignant breast tumours were examined for mdr-1 staining. Representative examples of the type of staining seen in these tumours are shown in figures 3E and F. Figure 3E shows a ductal carcinoma in situ which exhibited strong specific staining with antibody 6/1C. The centre of the ductal carcinoma had undergone necrosis and is mdr-1 negative. Figure 3F shows infiltrating tumour cells in a ductal breast carcinoma staining intensely positive for mdr-1, this staining was located in the inner plasma membrane/cytoplasm. Overall, the in situ component did not stain as intensely as infiltrating cells. Normal breast ducts adjacant to tumour cells showed low level staining and there was some non-specific staining of adipose tissue.

Discussion

A monoclonal antibody specific for mdr-1 encoded P-170 that was reactive on routinely processed, paraffin wax embedded tissue sections without pretreatment has been generated and characterised. To date, immunohistochemical studies of P-170 on archival material has been less than satisfactory due mainly to the lack of reproducibility of results with the antibodies available on paraffin embedded tissue. Differences in antigen retrieval methods used on tissue sections may be responsible for much of this lack of reproducibility. Many of the studies on the distribution of P-170 in normal tissues and tumours have been performed on frozen sections.[35-39] During characterisation studies in which the localisation of P-170 with antibody 6/1C was examined on paraffin wax embedded tissue we compared our results to those documented for both frozen tissue and the variable results obtained on paraffin wax embedded material to date.

Initial specificity and localisation studies were performed on normal adult and fetal tissue. The positivity in the kidney of the proximal and collecting tubules for mdr-1 has been described using both paraffin embedded and frozen tissue samples. Bittl *et al*[40] described staining of collecting ducts in paraffin embedded kidney tissue with antibodies C494 and 4E3 but reported that C219 did not give any conclusive staining pattern. Sugawara *et al*[16] reported staining of proximal tubules with antibody MRK16 on frozen sections of kidney. Staining patterns on normal liver using various antibodies against P-170 are inconsistent. The low level mdr-1 positivity that we found localised to the cytoplasm of hepatocyes was in agreement with a study performed by Bittl *et al*[41] on frozen tissue using antibody JSB-1. In this same study, positive staining on bile ducts and bile canaliculi was seen with antibody C219. These differences in staining patterns may be accounted for by the reported differences in mdr-1 and mdr-3 distribution in some tissues. The P-170 encoded by mdr-3 is reported to be more abundantly expressed in liver than the mdr-1 encoded protein,[42] which may account for the low level staining seen with the mdr-1 specific 6/1C antibody; C219 recognises both mdr-1 and mdr-3 encoded P-170.[26] In normal breast tissue the low level of mdr-1 staining observed with antibody 6/1C is consistent with documented data,[43] although there are also reports of mdr-1 negativity found in normal breast tissue.[40]

The expression of mdr-1 in the fetal tissues surveyed was similar to the patterns seen in the corresponding adult tissue with the exception of the adrenal gland. Fetal liver showed low level mdr-1 positivity, however, intense mdr-1 positivity was observed in erythroid precursor and myeloid precursor cells. Reactivity of stromal macrophages and endothelial cells with antibodies C219 and MRK16 has been reported[44]; with antibody C219, positivity was localised to monocytes, histocytes, and fibroblasts.[45] Using antibody 6/1C, we observed positive staining of macrophages in fetal lung and placenta (Hoffbaur cells). Positive mdr-1 staining of placental trophoblasts has been described previously using antibody MRK16.[16] Generally, the pattern of mdr-1 staining in the small intestine reflected that seen in the adult tissue with greater positivity seen towards the villi compared to the bottom of the crypt; this differentiation dependent pathway of P-170 expression (as normal mucosal cells move up the crypt towards the surface) has been described in the colon by De Angelis *et al*[46] In the lung, the positive staining of alveoli, cilia, and epithelial cells of the bronchus with antibody 6/1C corresponds to similar results observed in the epithelium of the bronchus and digestive tract.[47 48] It has been shown that mdr-1 encoded P-170 is expressed in the medulla and cortex of the adult adrenal gland,[47 49] and it has been suggested that P-170 plays a particularly important physiological role in the adult adrenal and that this role may increase with age as P-170 was not found in the fetal adrenal gland.[16] Binding studies with antibody 6/1C on fetal adrenal tissue showed no mdr-1 positivity in the cortex and a very low level of staining in the medulla region. Initial studies on malignant breast tumours show that antibody 6/1C recognised mdr-1-positive cells specifically within these tumours, indicating that this novel antibody may prove clinically useful for the detection of P-170 positive cells within a tumour mass. The reactivity of this antibody on routinely fixed tissue could enable larger studies on archival material to be carried out in an attempt to establish a prognostic role for P-170.

The authors acknowledge the expert advice and technical assistance with animal handling of Mr Nick O'Connor and staff at the Biomedical Research Facility, Beaumont Hospital, Dublin, Ireland.

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