Investigation of Monoclonal Antibodies Raised to Human Ovarian Carcinoma Cell Lines

by Allan Blacoe-Masterson (B Sc)

A thesis submitted for the degree of Ph D
Dublin City University

The research work described in this thesis was
carried out under the supervision of

Prof Martin Clynes and Dr Elizabeth Moran

National Cell and Tissue Culture Centre
School of Biotechnology

November 1998

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph D is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work

Signed Allan Blaxe-Watson ID No 9497 1285

Candidate

Date 16 November 1998

Abstract

Investigation of monoclonal antibodies raised to human ovarian carcinoma cell lines

Allan Blacoe-Masterson

Monoclonal antibodies (MAb) were generated to multidrug resistant (MDR) and sensitive variants of the ovarian carcinoma cell line OAW42 which over-express the MDR associated protein LRP/MVP MAb 3/B6 was raised to the intrinsically resistant variant OAW42-SR Immunofluorescence and immunocytochemical analysis indicated that the antigen detected by MAb 3/B6 was expressed primarily on the external surface of the plasma membrane but was also found to be expressed in the cytoplasm of a series of human MDR cell lines 3/B6 over-expression was associated primarily with cell lines which expressed the LRP/MVP
3/B6 expression was studied in paraffin-embedded normal and malignant adult and in foetal tumour tissue There was heterogeneous expression of the 3/B6 antigen and the LRP/MVP in normal adult and foetal kidney Low-level LRP/MVP expression was observed in 1/10 untreated malignant ovarian tumours while 3/B6 was absent In two paired pre- and post-chemotherapy breast tumours sections, 3/B6 expression was observed in the post-chemotherapy sections only LRP/MVP expression was also observed in these sections

A new commercially available immunoprecipitation protocol based on biotin labelling of cellular proteins was extensively modified and improved for this project The MAb 3/B6 was found to immunoprecipitate a 115 kDa un-glycosylated protein Immunoprecipitation experiments with anti-rat vault polyclonal serum, N2 and purified rat vault protems mdicated that MAb 3/B6 and LRP-56 (the standard MAb used to detect LRP/MVP) did not cross-react Competitive immunocytochemical studies confirmed these results Incubation of OAW42-SR cells with MAb 3/B6 did not have any effect on adriamycin drug accumulation or cellular proliferation

The anti-OAW42-SR MAb 5/C4 was also characterised by immunofluorescence and immunocytochemistry Results from these studies revealed that this MAb recognised a cytoplasmic antigen which migrated as 2 protein bands at 110 and 85 kDa by Western Blotting Further Western Blotting analysis indicated that MAb 5/C4 did not cross react with purified rat vault particles

The anti-OAW42-S MAb 3/E3 was partially characterised by immunofluorescence and immunocytochemistry There did not appear to be any significant difference in expression of this antigen m a panel of multidrug resistant cell lines It was not possible to determine the molecular weigh of the antigen by Western Blotting or immunoprecipitation This suggested that the epitope was destroyed during sample preparation

Dedication

This thesis is dedicated to the memory of my brother Robert who died tragically in a motor-rallying accident on the 10 March 1996. An individual strong in spirit, dedicated and loving, his memory will forever live in out hearts.

Time

Time is too slow for those who wait,
Too swift for those who fear,
Too long for those who grieve,
Too short for those who rejoice,
But for those who love, time is eternity
(Author-unknown)

Acknowledgements

"New opinions are always suspected and usually opposed, without any other reason but because they are not already common" –John Lock

I have a number of people to thank for helping me make sense of my "new opinions".

I would sincerely like to thank Prof. Martin Clynes for his assistance and encouragement during this project.

I would especially like to thank Dr. Liz Moran for her help and advice particularly with all those problems with cells, wells, eppies and flasks n 'stuff.

A big thanks to the crew in Immunology, Lisa, AnneMarie and Yizheng, for their constant friendship, help and advice, without which I would have never lasted the race! (particularly in '96 when life seemed so bleak).

Thanks to the "ladies who lunch", Aine, Helena and Maria, for good conversation and great craic. A big thanks to Rob (the red pen king) for solving all those computer problems.

To the "proofers and typers", AnneMarie, Lisa and Rob for being brave enough to deal with my bad spelling (even with spell check!).

I would also like to thank Prof. Rik Scheper and Prof. Leonard Rome for their kind gifts of antibodies and purified rat vault particles, Dr. Peter Kelehan and Joe Byrne, The National Maternity Hospital, for ovarian histological material and Eamonn Fitzpatrick in the Veterinary College ,UCD.

I would also like to thank everybody else at the NCTCC for their help over the past four years particularly Carol and Yvonne (for not making me give up my chocolate addiction!) and Mairead.

A big thanks to Kumiko, Susan, Thomas and Valerie P, Gayle, Mick, Mal and Vinne (Ossu!) and everybody else who have helped me get to this stage.

And last, but by no means least I would like to thank my family Dad and Mum, Doreen, Ray, Cian and Saoirse (and Mary) who have always supported me in all my endeavours. I am eternally grateful to them for their love and encouragement.

1 0 Introduction

1.1. General introduction

Recent reports have indicated that 7,306 deaths in Ireland could be attributed to cancer in 1997 (Irish Cancer Society, 1998). Although a small drop in cancer related deaths has been noted in the period from 1992-1994, there has been little change since then. Lung cancer accounts for the majority of cancer deaths (20%) while bowel and breast cancer were each responsible for about 10% of cancer deaths in 1994 (National Cancer Registry, 1997). Although not a significant figure, ovarian cancer accounted for 3% of all cancer deaths in Ireland in 1994. World-wide figures indicate an increasing prevalence of ovarian malignancies. It is now the fourth greatest cause of cancer death in women and the leading cause of gynaecological malignancy in the USA (Boring, 1993; Nahaas, 1997). Surgery and radiotherapy continue to be the first-line management for ovarian cancer, with chemotherapy being the chief follow-up treatment. However, the five year survival rate still remains at approximately 20% (Nahaas, 1997). This poor response rate is primarily due to the development of primary or recurrent tumours, which are either partially, or wholly non-responsive to a broad spectrum of structurally and functionally unrelated chemotherapeutic drugs i.e., multidrug resistance, MDR. A number of mechanisms of drug resistance have been characterised in multidrug resistant cell lines (Section 1.3.) which include the expression of energy dependant transmembrane drug efflux pumps such, as P-glycoprotein (Pgp) and multidrug resistance associated protein (MRP), (Bellamy, 1996; Clynes, 1993). The role of these proteins in multidrug resistance has been greatly enhanced by the use of monoclonal antibodies (MAb) in retrospective and functional studies. The recently discovered lung resistance related protein (LRP) has been shown to have a significant predictive value in the outcome of ovarian cancer in studies with the LRP specific MAb, LRP-56 (Izquierdo *et al.*, 1995) (Section 1.3.3.1.1). LRP is the human homologue of the major vault protein (MVP) a component of 13 MDa particles which are highly conserved in a wide variety of species from slime mould to man (Section 1.3.3.4.). Although the precise function of these particles has yet to be elucidated, they may play an important functional role in multidrug resistance (Section 1.3.3.13.). The recent isolation and characterisation of MAb to novel proteins expressed in multidrug resistant cell lines has revealed a myread of possible candidates for drug resistance associated markers (Flens *et al.*, 1997). Similarly the possibility of a non-functioning form of the LRP/MVP suggested by Moran *et al.*, (1997) indicates that

multiple forms of these drug resistance proteins may exist, as is illustrated by the recent discovery of a number of homologues of MRP (Kool *et al*, 1997) Clearly, MAb have a role to play in the identification and characterisation of potentially novel multidrug resistance mechanisms

1 2. Monoclonal antibodies

The first attempt to isolate homogenous antibodies was undertaken with B cell tumours from BALB/c mice where mineral oil was used to induce the tumour (Potter, 1972) The cell line derived from this tumour was cloned and found to secrete a homogeneous population of antibody, or monoclonal antibodies, (MAb, Horibata and Harris, 1972) There was still a major drawback however in that antibodies with a predefined specificity could not be produced This problem was solved by Kohler and Milstein (1975) when non-secreting B cell tumour cells were fused with splenocytes immunised with a specific protein In animals, antibodies are synthesised primarily by terminally differentiated B-lymphocytes but due to their limited capacity to grow in culture (not surviving for more that a few days) they could not be used as a source of antibody By fusing these cells with the immortalised myeloma cells, the antibody producing capabilities of the splenocytes could be extended indefinitely From these initial studies, MAb technologies have widened to include more sophiciated methods for MAb production These include electrofusion, where high voltages are used to physically fuse B-and myeloma cells, phage-display where short peptide sequences are expressed on the surface of M13 phage *In vitro* immunisation procedures have become popular over the past number of years This method allows the sensitisation of isolated lymphocytes/splenocytes to antigen *in vitro* The two major advantages for in vitro immunisation are the small amount of antigen required (as little a 1 ng) and the lack of cellular regulation on the developing immune response (Harlow and Lane, 1988) Other developments include advances in chimeric and bispecific MAb, which have useful applications in the area of immunotherapy and immunoscintigraphy

1 3 Multidrug resistance

Surgical excision has been the main mode of treatment of solid tumours The basis of conventional cancer therapy is the treatment of local or regionalised cancer with surgery and/or radiotherapy, and the administration of chemotherapy for disseminated disease (Nahaas 1997) With advances in the development of chemotherapeutic drugs and treatment regimes, a number of cancers such as acute leukaemias, lymphomas and selected solid tumours (including testicular carcinomas and certain childhood malignancies) have an increasingly positive prognosis The development of new combination therapies have increased the response of other malignancies such as ovarian, lung and advanced breast cancer although these cancers remain essentially incurable (de Vita, 1989, Izquierdo *et al* , 1996b)

One of the major limiting factors in chemotherapy is the existence of drug resistant cells which are intrinsically resistant or acquire resistance to chemotherapeutic drugs during treatment (Bellamy Dalton and Dor, 1990, Gottesman, 1993) Anticancer chemotherapy is essentially based on the capacity to combine a cytotoxic mechanism with a selective action of a drug in order to distinguish tumour cells from host cells (Coop, 1993) However during chemotherapy the cancer tissues, without modification of their anatomic and structural features, are able to change their functional characteristics, becoming rapidly resistant to many structurally and functionally unrelated cytotoxic drugs, in this way the selectivity of the drug completely vanishes (Clynes, 1993, Coop, 1993) This phenomenon is termed multidrug resistance It is not known whether selection of pre-existing subclones and/or mutation during chemotherapy has occurred in this acquired resistance but recent evidence suggests that both modes of development may be in operation in ovarian cancer (Moran *et al* , 1997, Coley, 1997, Izquierdo *et al* , 1996 a, b)

Three main changes have been observed in cells that develop the multidrug resistant phenotype,

(i) A decrease in cellular accumulation of cytotoxic drug

(ii) Changes in cellular physiology affecting the structure of the plasma membrane, cytosolic pH and the rates and extent of intracellular transport of membrane as well as lysosomal structure and function (Cleary, 1997, Izquierdo, 1998, Gottesman, 1993)

(iii) Changes in activity or expression of certain cellular proteins

The dominant feature of MDR cell lines is a decrease in accumulation of cytotoxic drugs (Gottesman, 1993) In the laboratory, tumour cell line models have added to the knowledge regarding the development of multidrug resistance These cell lines selected for resistance to a single cytotoxic drug by repeated exposure to increasing concentrations of drug often also display cross resistance to a range of structurally and functionally distinct cytotoxic drugs including anthracychnes, vinca alkaloids, epipodphylotoxins, taxanes and actynomycin D (Borst, 1991, Biedler, 1992, Clynes, 1993) Frequently the resistance observed in these cell lines is associated with the expression of one or more cellular proteins including P-glycoprotein (Pgp), the multidrug resistance related protein (MRP) and the lung resistance related human major vault protein (LRP/MVP) Over expression of other cellular proteins and mechanisms have also been observed such as alterations in topoisomerase II, (Reviewed by Hoffman and Mattern, 1993), alterations in the glutathionine system (Reviewed by Moscow and Dixon, 1993), and over expression of the T antigen presenting protein (TAP) (Izquierdo *et al*, 1997)

1.3.1 P-glycoprotein

Kessel *et al* (1968) first reported that anthracycline-drug selected cells appeared to accumulate less drug than their unselected parental cell line Further studies indicated that this reduction in cellular accumulation of cytotoxic drug was dependent on an ATP activated pump which was later identified and named P-glycoprotein (Pgp), (Dano, 1973, Juliano and Ling, 1976) Since these initial reports, a wide variety of multidrug resistant cell lines have been established in order to study this phenomenon (Clynes, 1993, Germann, 1996) It has been observed that many of these cell lines over-express Pgp (Beck and Danles, 1991, Hill, 1993, Germann, 1996)

P-glycoprotein (Pgp) has been extensively characterised It is a 170 kDa glycoprotein which is primarily associated with the plasma membrane It is comprised of 12 transmembrane domains and consists of a short highly charged cytoplasmic domain followed by 3 additional membrane loops and a second cytoplasmic region (Chen *et al*, 1986, Gros *et al* , 1986) It has been observed that P-glycoprotein comprises of 2 halves of similar structure, the 2 halves are connected by a linker region bridging the amino and carboxy halves (van der Blink *et al* , 1987) The linker region that conveys drug resistance contains sequences for c-AMP and c-GMP dependent protein kinase phosphorylation sites and appear to be important for Pgp function (Hsu *et al* , 1989)

The functional domains of the Pgp molecule have been analysed by a series of genetic and biochemical studies Photoaffinity labelling with drug and drug analogues have identified a number of binding sites (Germann *et al* , 1993 Germann *et al* , 1996) The results of these experiments also indicated a major role for the intracytoplasmic loop and transmembrane loop in substrate specificity of the transporter (Bruggerman *et al* , 1989, 1992, Safe *et al* , 1989, Busche *et al* , 1989)

In humans, 2 closely related genes, *mdr-1* and *mdr-3* located on chromosome 17q, encode highly homologous Pgp (Reviewed by Croop , 1993) A number of groups have observed that the *mdr-3* encoded gene product may not be associated with drug resistance (Chien *et al* , 1986, Ueda *et al* , 1987, van der Bliek *et al* , 1988) However,

recent evidence suggests that this may now be the case (Smit *et al*, 1993, Larkin *et al*, 1998a) The murine homologue, *mdr-2* does, however appear, to be active in transport and the secretion of phosphatidylcholine in bile (Smit *et al*, 1993, Borst and Schinkel, 1996, El Farink *et al*, 1997) A possible physiological role for Pgp has been demonstrated by Schinkel *et al* (1994, 1995, 1997) where *mdr-1* knock out mice which appeared phenotypically normal, displayed increased sensitivities to vinblastine, dexamethasone and cyclosporin A This sensitivity was attributed to the absence of Pgp in the blood brain barrier

Resistance to the vinca alkaloids, anthracyclines, epipodophyllotoxins and taxanes observed in human cancer cell lines is frequently caused by the over expression of Pgp (Flens, 1997 PhD thesis) Transfection of the *mdr-1* gene has shown that Pgp can mediate drug resistance directly (Higgins, 1992, Gottesmann *et al*, 1993, Endicott and Ling, 1989)

The discovery that Pgp belongs to a larger family of ATP-binding cassette transport proteins, many of which have clear transport functions has given a better insight into the normal physiological role of Pgp (Endicott and Ling, 1989, Gottesman, 1993) The mammalian MDR gene family is related to the ATP-binding cassette (ABC) superfamily of genes that encode a wide variety of proteins in various species This family of proteins is characterised by their 2 ATP-binding sites To date over 50 members have been identified (Higgins, 1992) Significant degrees of homology have been demonstrated between Pgp and the Hly B protein from *Escherichia coli* (Juranka *et al*, 1989) and the leukotoxin secretion protein Ikt in *Pasteurella Haemolytica* (Strathdee and Lo, 1989) Sequence homology has also been observed in humans with the cystic fibrosis trans membrane conductance regulator (CFTR) and the TAP 1, TAP 2 and PMP70 proteins (Higgins, 1992, Leveille-Webster and Arias, 1995)

Human Pgp is predominantly expressed in the apical membranes of organs with excretory functions (Georges *et al*, 1990, van der Valk *et al*, 1990) It is though that Pgp may play a role in the elimination of exogenous toxins or their toxic metabolites from the body (Horio *et al*, 1989) High levels of *mdr-1* mRNA expression has been observed in the adrenal glands, kidney, liver, small intestine and colon, whereas, low

levels have been observed in most other tissues (Fojo *et al*, 1987) Immunohistochemically, P-glycoprotein has been detected on the surface of cortical cells, the luminal surface of the brush border of the proximal renal tubule, the luminal surface of biliary hepatocytes and the mucosal surface of the columnar epithelial cells of the small intestine (Thiebaut *et al*, 1987, Moran *et al* 1997b) In human tumours, *mdr-1* expression has been observed in colon, renal carcinoma and adrenocortical heptoma, all of which naturally express *mdr-1* (Goldstein, 1989) Pgp expression has also been reported in endometrial and gastric cancers (Schnider *et al*, 1993, Robey-Cafferty *et al*, 1991), leukemias, lymphomas and some other tissues which do not normally express the *mdr-1* gene (Rischin and Ling, 1993, Goasguen *et al*, 1993) Researchers have also observed Pgp expression in breast and ovarian samples (Veneroni *et al*, 1994, Dixon *et al*, 1992) Pgp expression is most commonly found in samples from patients who have received chemotherapy than from untreated patients (Toffoli *et al*, 1992, Dalton *et al*, 1989, Goldstein *et al*, 1989) Opinions differ as to the significance of Pgp expression as a predictor of poor outcome and survival, many groups have used similar methods but have achieved significantly different results (Bater *et al*, 1991, Chan *et al*, 1991, Favrot *et al*, 1991) The outcome of the St Jude workshop has suggested the use of more than one antibody in an attempt to obtain more consistent results (Beck et al, 1996)

1.3.2. Multidrug resistance associated protein (MRP)

MRP mRNA was first isolated from H69AR cells by Cole *et al*, in 1992. Subsequent chracterisation revealed that the gene encoding MRP was located on chromosome 16 position 13.1 and encoded a 1531 amino acid, N-glycosylated integral membrane protein with a molecular weight of 190 kDa (Krishnamachary and Centre, 1993; Zaman *et al.*, 1994). Analysis of the MRP gene reveals that it has similar structural motifs to that of P-glycoprotein (Section 1.3.1.), however, it has only 15% homology with Pgp (Zaman *et al.*, 1994).

A number of models have been proposed for the membrane topology of MRP (Loe *et al.*, 1996) but it is now generally accepted that the transporter contains 12 transmembrane segments in the amino proximal half and 6 transmembrane segments in the carboxy proximal half. The topology of MRP allows it to be classified as a member of the ABC superfamily of genes (Section 1.3.1.). This membrane topology appears to be unique amongst the currently known ABC transporters (Bakos *et al.*, 1996; Muller *et al.*, 1996; Tusnady *et al.*, 1997). MRP appears, therefore, to be the first fully characterised member of a new sub-family of transporters distinctly related to *mdr-1*. Three other transporters have similar topology to MRP; β cell sulfonylurea receptor (SUR involved in insulin regulation), the cMOAT (multispecific organic anion transporter) and the yeast cadmium resistance factor (YCF1) (Lauther *et al*, 1996). Recently, a number of human homologues of MRP have been isolated (Kool *et al.*, 1997). Termed MRP-3-MRP-6; preliminary characterisation has revealed expression of the different homologues in human tissue. MRP-3 is mainly expressed in liver, MRP-4 exhibits low level expression in most tissues, while MRP-5 is expressed in almost all tissues assayed. Similarly, expression was determined in relation to drug resistance; findings suggest little correlation with resistance for the majority of these homologues (Kool *et al.*, 1997). Antibodies raised to MRP (Flens *et al.*, 1994), predominantly stain the plasma membrane in drug selected and in MRP cDNA transfected cells. Cytoplasmic staining has also been observed, usually as a densely stained spot close to the nucleus which may be related to the Golgi apparatus or to vesicular sequesterisation which has also been observed in some cells (Flens *et al.*, 1994; Hipfner *et al.*, 1994; Zaman *et al.*, 1994).

The physiological role of MRP has yet to be defined but it has been observed that inside out plasma-membrane vesicles isolated from cells over-expressing MRP have shown an increase in ATP dependant transport of Glutathione S conjugates including leukotriene C_4 (Jedhtschky *et al*, 1994, Leier *et al*, 1994) Photoaffinity labelling has confirmed MRP as a leukotriene C_4 binding protein (Jedelischly *et al*, 1994) Evidence that cancer cells require glutathione for MRP drug transport has been demonstrated by the cellular depletion of glutathione (Versanvoort *et al*, 1995, Zaman *et al*, 1995)

MRP mRNA has been detected in total RNA preparations of all human tissue and in cell types from peripheral blood (Cole *et al*, 1992, Zaman *et al*, 1993) Flens *et al*, (1994) have described the localisation of MRP in normal human tissues Staining (mainly cytoplasmic) has been observed in several types of epithelia particularly lung, skin, large and small intestine and heart muscle cells and macrophages This have concluded that the presence of this protein in these tissues suggests that MRP function in normal tissues is associated with the transport of natural xenobiotics (Flens 1994)

1.3.3. Lung resistance-related protein/human major vault protein (LRP/MVP)

1.3.3.1. LRP

Clinical drug resistance has been found more frequently in tumours where the patient has been previously treated with chemotherapy (Bellamy, 1990). Over the past few years, emphasis has shifted to the development of low level resistant cellular models which are thought to be more clinically relevant (Izquierdo *et al.*, 1998; Moran *et al.*, 1997a). To this end, a number of research groups initiated studies where human cancer cells were exposed to lower levels of drug, mainly doxorubicin, typically between 50 to 200 μ moles. (Kuiper *et al.*, 1990; Vesernvoot, 1995; Moran *et al.*, 1997a). Investigations by Kuiper *et al.*, and Flens *et al.*, in a series of sublines, revealed a typical multidrug phenotype but without the corresponding expression of Pgp. Subsequent investigation led to the discovery of a new multidrug resistant related protein, termed the lung resistance related protein LRP so named because it was first observed in the non-small lung carcinoma cell line SW1573/2R120 (Scheper *et al.*, 1993).

1.3.3.2. Molecular characterisation of LRP

Molecular characterisation of LRP has been undertaken by a number of researchers. Scheffer *et al.*, (1995) have mapped the gene encoding LRP to the short arm of chromosome 16 position 11.2. The proximity of the LRP gene to that of the other newly discovered MDR associated protein, MRP (Section 1.3.2.), posed the question whether these two genes were co-over expressed. Evidence from Slovak *et al.*, (1995) have shown that, although these genes are located within the same region of the chromosome, co-amplification rarely occurs and they are rarely found on the same amplicon. Only one incidence of co-amplification was observed, in the fibrosarcoma cell line HT1080/DR4. In seven other drug resistant cell lines (with doxorubicin resistance of between 20-300 fold), this co-amplification was not observed. In the same publication, Slovak *et al* have also demonstrated that over expression of the LRP is not associated with gene amplification in 2 LRP over expressing cell lines including the 2R120 subline. This

research group have concluded that LRP gene amplification is not a common occurrence and that other mechanisms such as transcriptional activation or mRNA stabilisation appear to be more likely to be responsible for the over expression of the LRP gene Recently Komarov *et al*, (1997) have mapped the gene encoding protein kinase C (to the short arm of chromosome 16) which has been shown to be involved in MDR by the activation of the *mdr-1* gene (Chambers *et al*, 1992) This mechanism may also be involved in LRP gene activation Beck *et al*, (1998), have recently demonstrated that the expression of the protein kinase C isozyme η (eta) correlates with *mdr-1* and LRP gene expression in blast cells from adult AML patients, ascites from aspirates from ovarian cancer patients and primary breast carcinoma

1 3 3 3 LRP is the human major vault protein (MVP)

Scheffer *et al*, (1995) have constructed an eukaryotic expression system for the isolation of the LRP cDNA Purified plasmids containing cDNA from a HT1080/DR4 cDNA library were transfected into MOP-8 mouse fibroblast cells Cells that transiently expressed the LRP protein were selected by immunocytochemistry Successive screenings produced a pure clone which was confirmed by immunoprecipitation experiments with LRP-56 Sequence analysis has revealed that the LRP gene has a single open reading frame of 2,688 base pairs coding for an 898 ammo acid protein with a calculated molecular weight of 100 kDa A computer-based search of the LRP cDNA versus the gene bank library has revealed that LRP is the human homologue of the rat major vault protein (MVP, Kedersha and Rome 1986 a, b) Alignment of the deduced amino acid sequence reveals a 57% homology with the major vault protein from the slime mould *Dictyostelium discoideum* and 87% homology with the MVP of *Rattus norvegicus*

1 3 3 4 Vault proteins

Vault particles were originally isolated by Kedersha and Rome (1986 (a) (b) as contaminants in rat coated vesicle preparations Vault particles appeared a ovoid shapes by scanning electron microscopy (EM), having a distinct morphology resembling the multiple arches of cathedral vaults, hence the name (Figure 1 3 3 4)

Vault particles are large ribonucleoproteins measuring 57 X 32 nM and have an estimated molecular mass of 13 MDa, composing of the largest ribonucleoprotein reported to date (being three times the size of a ribosome) Vault particles have a 2 fold octagonal symmetry, each half of the structure can open out into a flower like structure which contains eight petals surrounding a central barrel like ring (Kedersha and Rome, 1991) Vaults are composed (in rat liver) of a 104 kDa protein, the MVP, which accounts for 70% of the total weight of the vault particle, and 3 minor protein species, 210, 192 and 55 kDa along with a small RNA molecule

Polyclonal antisera prepared against whole vault particles is largely specific for the MVP (Kedersha *et al*, 1990), although there is an increased specificity for the minor species with repeated booster injections with purified rat vault preparations Mobility studies have shown similarities between coated vesicle components proteins and vault particles However, when purified coated vesicles are probed with anti-vault antisera no cross reactivity is observed

Vaults have been isolated from various species including the lower eukaryote *Dictyostelium*, amphibians, avians and mammalian (rat, rabbit and cow) (Kedersha *et al*, 1990) Vaults are most abundant in epithelial cells and macrophages EM reveals that vaults from these diverse species are similar both in dimension, morphology and polypeptide composition The MVP of 100 kDa predominated in most species This evolutionary conservation is highlighted by the observation that polyclonal antisera raised to rat vaults also recognise the MVP in these diverse species (Kedersha *et al*, 1990)

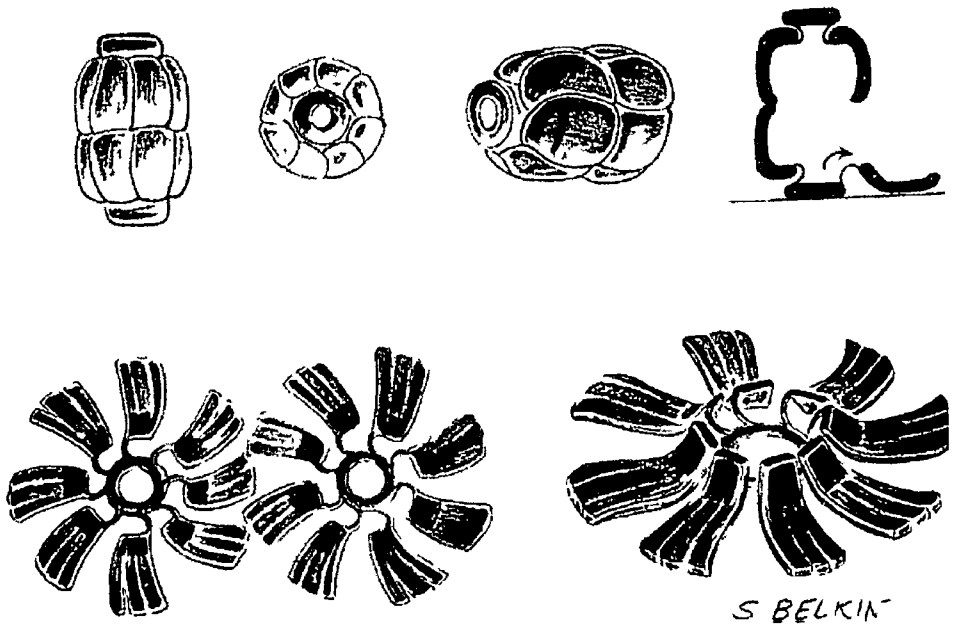


Figure 1 3 3.4.1 Proposed model for the folding of vault "flowers" Reproduced, with permission, from Kedersha *et al* , (1991)

1.3.3 5 Vault associated RNA

The vault associated RNA (vRNA) has an unusual base composition of adenosine (12%), guanosine (29 7%), uridine (30 9%) and cytidine (24 4%) (Kedersha and Rome, 1991) The vRNA constitutes approximately 4 6% of the entire structure, the large size of the vault particle requires that each one contains approximately 9 molecules of vRNA (a minimum of 55 copies of the MVP, (p104) are also present) A structural role for the vRNA seems unlikely, removal of the vRNA (by treatment of vaults with RNAse A) revealed no differences in mobility on sucrose gradients or on more sensitive agarose gels Similarly, there were no observed differences when vaults were viewed by EM (Chugani *et al*, 1993) vRNA has been found to vary in length and number between the species (Kickhoefer *et al*, 1993) Mouse and rat vRNA is 141 bases in length while bullfrog and humans have 2 forms, in bullfrog the sequences are 89 and 94 bases while in humans, the two forms are 80 and 95 bases long Despite the difference in length the secondary structure is highly conserved across the species suggesting an important role for vRNA in the function of vault particles (Kickhoefer *et al*, 1998) One of the genes coding for the human forms of vRNA, termed hgv 1 coding for the 95 base vRNA, has recently been cloned and has been shown to be a single copy gene Chromosome localisation is currently in progress (Kickhoefer *et al*, 1998, Rome, personal communication), hgv 2, which codes for the 80 base species of vRNA is currently being cloned

1 3 3 6 Characterisation of the MVP

Trypsin treatment of vault particles results in the exclusive cleavage of the MVP resulting in alterations in structure as revealed by EM This change in vault morphology is consistent with the localisation of the MVP in the petals of the particles (Kedersha and Rome, 1991) Two main changes are observed following trypsin treatment, vaults are either flattened and collapsed suggesting that the loss of the MVP results in the loss of radial symmetry or, less frequently observed, a reduction in the diameter of the barrel structure (Kedersha *et al*, 1990) Studies by Vasu *et al*, (1993, 1995) have shown that the MVP may be important for normal physiological growth in the slime mould *Dictyostelium Dictyostelium* contains 2 forms of the major vault protein, MVP A with a

molecular weight of 94 4 kDa and MVP B, 92 kDa MVP A was the first to be sequenced (Vasu *et al*, 1993) followed by MVP B (Vasu *et al*, 1995) Disruption of the MVP A gene results in a mould which retains normal function, it is thought that MVP B takes over the function of the A form However the morphology of these mutant vaults was not characteristic of normal vault particles Similar results were obtained with MVP B mutants Disruption of the MVP B gene in MVP A negative mutants results in impeded growth under conditions of nutritional stress Vault particles isolated from these mutants are truncated but are still functional under normal growth conditions These experiments have allowed the discovery of a third MVP protein in Dictyostelium, MVP C, which, like the A and B form in single mutation experiments, takes over the function of the other two, although not to the same extent Kickhoefer *et al*,(1994) have sequenced the cDNA encoding the MVP from rat vaults and shown that it has a57% homology with Dictyostelium vault particles

1 3 3 7 Cellular location of vault particles

The majority of vaults are located in the cytoplasm but a small fraction have been localised to the nuclear envelope, more specifically the nuclear pore complex, NPC (Chugani *et al*, 1993) There is evidence to suggest that vault particles may constitute the central plug of the NPC as vaults display structural similarities to the central plug of the NPC

The NPC are octagonal organelles which span the inner and outer membranes of the nuclear envelope providing a channel for nucleocytoplasmic transport (reviewed by Gerace and Bourke, 1988, Feldherr and Akin, 1990) Inserted into the centre of the channel is the central plug termed the NPC transporter consisting of 2 equivalent halves each with an 8 fold symmetry (Akey and Glodfarb, 1989, Akey, 1990) with an estimated molecular mass of 13 MDa (Reichelt *et al*, 1990) The dimensions, mass and geometry of the central plug of the NPC are comparable to those of the vault particle (Chugani *et al*, 1993) Western blotting and immunofluorescence studies have confirmed that vaults are located within the NPC Immunogold EM on isolated nuclei from rat fibroblasts in paraffin embedded rat liver has also demonstrated the presence of the vault particles in the region of the NPC

Vault particles have also been localised abundantly in cholinergic nerve terminals in close proximity to synaptic vesicles (Herrmann, 1996)

1 3 3 8 Vault function

As yet no precise function can be assigned to vault particles The similarities in molecular weight and the symmetry between rat vaults and the NPC (Section 1 3 3 7) suggest a function in bi-directional nucleocytoplasmic transport (Chugani *et al*, 1993) The association of vaults with coated pits indicates a possible role in vesicular transport of substrates (Kedersha and Rome, 1986, Section 1 3 3 4) Recently, Herrman *et al*, (1996) have isolated vault particles from the nerve terminals of *Torpedo* which also points to a role in vesicular transport Vault particles have also been found at the ruffling edges of rat fibroblasts and associated with components of the cytoskeletal system suggesting a possible role in motility (Rome *et al*, 1991) Vaults have also been located in the developing rat brain and in the microglia (Chugani *et al*, 1993) A possible role in the growth regulation of Dictyostelium is implied from studies by Vasu *et al*, (1993, 1995, Section 1 3 3 6)

Recently, Kickhofer *et al*, (1998), investigated the expression of vault particles in multidrug resistant cells It has been demonstrated that intact vault particles always associate with the pellet following centrifugation at 100,000g It has been shown previously that the human MVP was upregulated in multidrug resistant cells (Scheper *et al*, 1993) It was not certain whether this was an indication of total vault upregulation in these cells Scheffer *et al* (1996) demonstrated that MVP cannot confer drug resistance by itself) Kickhoefer *et al*, (1998) have clearly shown that there is a 15 % increase in vault synthesis in multidrug resistant cells Probes derived from hgv1 cDNA were used to quantify the level of vault synthesis It has been observed that the amount of vRNA associated with vault particles is upregulated in the LRP/MVP over-expressing cell line GLC4/ADR However, the total amount of vRNA is not increased There seems to a pool of vRNA, a certain fraction of which appears to be associated with the vault particle at any one time If the level of synthesis of intact vault particles is increased then there is

also an increase in the portion of the vRNA pool which is vault associated (figure 1 3 3 8 1) This not only suggests a functional role for vRNA but also suggests a role for vaults and therefore the MVP/LRP in drug resistance

Kickhoefer *et al* (1998)also observed that only hgv1 was associated with vault particles hgv2 is usually found in the supernatant of the 100,000g spin whereas hgv1, like LRP/MVP is associated with the pellet Hgv1 is only found in the supernatants of cells that do not express the LRP/MVP (Kickhoefer *et al*, 1998, Rome, personal communication)

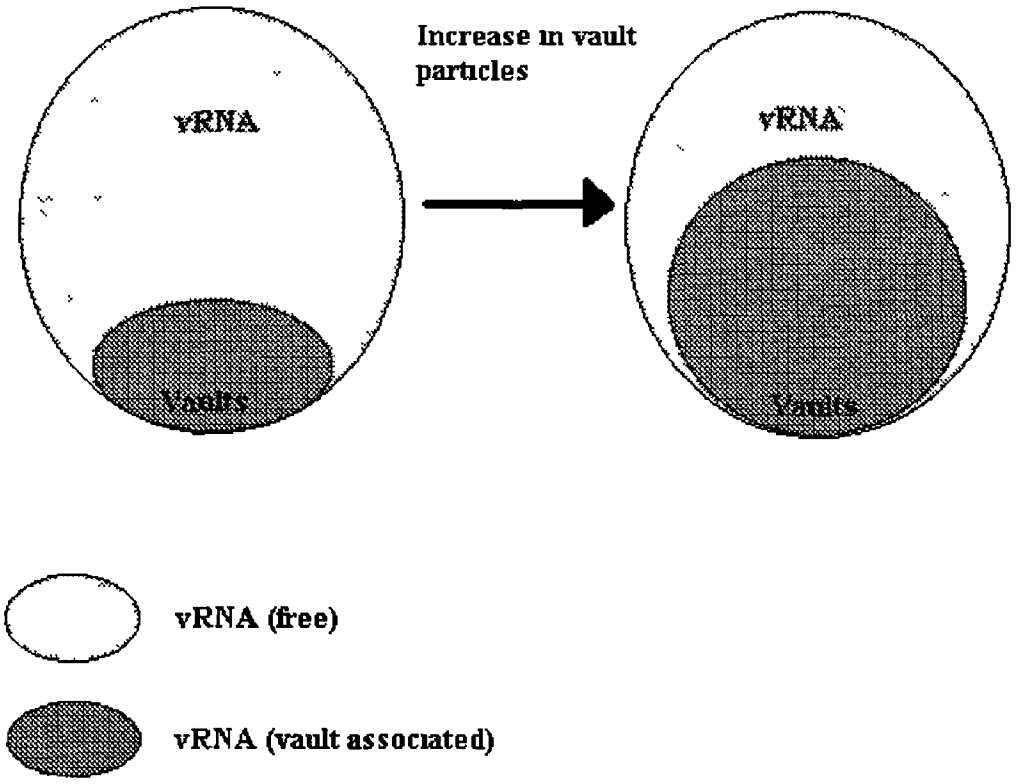


Figure 1 3 3 8 Location of cellular vRNA in MDR cell lines (adapted from Kickhoefer *et al*, 1998)

1 3 3 9 Monoclonal antibody LRP-56 recognises the LRP

Kuiper *et al*, (1990) derived a series of sublines of which the SW1573/2R120/ subline was characterised by moderate levels of resistance to doxorubicin, vincristine and etoposide, reduced intracellular accumulation of drug and the absence of p-glycoprotein expression (Kuiper *et al*, 1990) This cell line was used to immunise BALB/c mice the resulting hybridoma clones were screened against parental SW1573 (low expression of LRP) and SW1573/2R120 (high expression of LRP) cells Clone 56 was chosen for its strong immunoreactivity to the 2R120 subline (Scheper *et al*, 1993) Observations by Scheper *et al* demonstrated that the antigen detected by LRP-56 was predominantly located in the peripheral cytoplasm in a granular fashion indication that possible association with the endoplasmic reticulum This pattern of staining is characteristic of the LRP in a range of multidrug resistant cell lines and normal and malignant tissues (Section 1 3 11 3) A reduction in the level of expression was observed where P-glycoprotein expression was predominant which was observed by immunoprecipitation with MAb LRP-56 The molecular weight of the LRP has been determined by immunoprecipitation to be approximately 110 kDa Scheffer *et al* (1995) have shown that LRP mRNA is 8 times higher in the 2R120 subline than in the Pgp positive 2R160 subline and 4 times higher than the 2R120 reverent subline, grown in the absence of drug for 9 months

1 3 3 10 Expression of LRP/MVP m multidrug resistant cell lines

Over expression of the LRP/MVP has been observed in a number of drug selected cell lines (Moran *et al*, 1997a, Scheper *et al*, 1993, Versanvoort *et al*, 1995, Verovski *et al*, 1996, Wyler *et al*, 1997, Komarov *et al*, 1997, Futscher *et al*, 1994, Parker *et al*, 1997 and Ikeda *et al*, 1997)

Over expression of the LRP/MVP appears to be more closely associated with low level resistance where it is frequently observed at the early stages of resistance selection It has also been observed, though less often, in highly resistant cells, (Moran *et al*, 1997a, Scheper *et al*, 1993, Wyler *et al*, 1997, Vervoski *et al*, 1996, Versantvoort *et al*,

1995) The mechanisms of resistance that operate in the low-level resistant cell lines are thought to be more clinically relevant (Moran *et al*, 1997a, Izquierdo 1995, PhD thesis, Coley, 1997)

The expression of LRP/MVP and Pgp in drug resistant cell lines appears to be exclusive i e the majority of pgp over-expression cell lines are LRP negative and *vica versa* (Moran *et al*, 1997a, Versantvoort *et al*, 1995, Scheper *et al*, 1993) Moran *et al*, have demonstrated that, in a series of subhnes of the ovarian carcinoma cell line, OAW42, LRP/MVP over-expression is seen at the lower levels of resistance in OAW42-S, and OAW42-SR cell lines whereas a switch to pgp-over-expression is observed in the adriamycin selected OAW42-A1 and OAW42-A lines Similar over-expression at lower levels of resistance has been observed by Scheper *et al*, in their 1993 study The apparent association of LRP/MVP with low level resistance suggests that it could be a clinically relevant marker for drug resistance since low level resistance is considered to be more clinically relevant (Izquierdo *et al*, 1996 a, b, Moran *et al*, 1997) Concomitant over-expression of LRP/MVP and Pgp has been observed in very few cell lines namely the MCF7/D40 breast cancer cell line and certain 8266 myeloma cell lines (Scheper, 1996, Scheper *et al*,1993, Wyler *et al*, 1997, Shao *et al*, 1995) It has been suggested that in cell lines with over-expression of both pgp and LRP, the LRP associated mechanism of drug resistance may contribute to the MDR phenotype (Izquierdo *et al*, 1998) Evidence by Shao *et al*, has demonstrated that in 2 subhnes of the myeloma cell line 8266 where the Pgp content was similar, the greatest resistance was observed in the subline with the higher LRP/MVP content (Shao *et al*, 1995)

Many LRP/MVP over-expressing cell lines also appear to over-express MRP (Flens *et al*, 1994, Moran *et al*, 1997a) Whether the LRP/MVP or MRP contributes to MDR phenotype in these cell lines is unknown Preliminary evidence from LRP/MVP ribozyme and antisense transfected cells of OAW42-SR suggests that the LRP/MVP may play a significant role in drug resistance (Byrne, 1998) Moran *et al*, (1997a) have also observed a passage dependent increase in LRP/MVP expression in the OAW42-SR subline with a paralleled increase in drug resistance without any change in MRP expression It has also been shown that the non-small lung carcinoma cell line GLC4/ADR over-expresses of both LRP/MVP and MRP However, it has yet to be

established which drug resistance mechanism predominates, it has been suggested that resistance is due to the over-expression of LRP/MVP (Kickhoefer *et al*, 1998, Flens *et al*, 1997, Slovak *et al*, 1995) Despite the evidence to suggest that the drug resistance phenotype of many cell lines is due mainly to LRP/MVP expression, the observation that 2 and sometimes 3 MDR associated markers being expressed concomitantly suggests that several drug resistance mechanisms may be necessary to cause the phenotype of drug resistance observed in LRP/MVP positive drug-selected cell lines (Izquierdo *et al*, 1998 in press)

LRP/MVP expression does not appear to be associated with the so-called "classic" MDR drugs such as adriamycin A wide range of drugs have been used in the selection of LRP/MVP over-expressing drug resistant cell lines (including doxorubicin, mitoxantrone, etoposide, vincristine, cytavabine, methotrexate and cisplatin Moran *et al*, (1997), Wyler *et al*, (1997) and Scheper *et al*, (1993) have demonstrated that, despite the fact that the majority of LRP/MVP cell lines were selected by exposure to doxorubicin, they exhibit cross-reactivity to the majority of drugs listed above Studies with a number of drug resistant gastric cell lines have indicated correlation between LRP/MVP mRNA expression and resistance to etoposide and a topoisomerase inhibitor SN-38 (Ikeda *et al*, 1997)

Generally, the increase in LRP/MVP expression appears to be accompanied by a paralleled increase in drug resistance, however, recent observations have shown that in the OAW42-S cell line, cloned from the LRP/MVP over-expressing cell line OAW42-SR, LRP/MVP is also expressed in a passage dependant manner but without any increase in drug resistance (Moran *et al*, 1997a) This phenomenon will be discussed in more detail in Section 1 5 3 1

Over-expression of LRP/MVP is not limited to drug resistant cell lines selected with cytotoxic drugs In a study of a panel of 61 cell lines from the National Cancer Institute (NCI), many of which are intrinsically resistant (Izquierdo *et al*, 1996a), 24% of these cell lines exhibited Pgp over-expression while LRP (and MRP) were constitutively and heterogeneously expressed at varying levels in 87% (and 78% for MRP) of cell lines Statistical analysis indicated that pgp significantly correlated with MDR rank and

resistance to doxorubicin whereas MRP correlated with resistance to vincristine LRP expression correlated with resistance to melphalin, cisplatin vincristine and carboplatin Concomitant expression of different MDR mechanisms was also observed in 34% of cell lines 18% expressed pgp, MRP and LRP/MVP, 43% MRP and LRP/MVP and 3% Pgp and LRP/MVP In 8 of these cell lines, LRP/MVP mRNA expression, but not MRP mRNA expression was determined to be strongly correlated with drug sensitivity to both MDR and non-MDR related drugs LRP/MVP mRNA estimation appeared to be a slightly better indicator of drug sensitivity that LRP/MVP protein expression (Laurencot *et al*, 1997)

1 3.3 11 LRP/MVP expression in normal and malignant tissue

The information gained from *in vitro* drug selected and intrinsically resistant cell lines suggest a possible role for LRP/MVP in the MDR phenotype and as a marker for low-level resistance A number of groups have determined the expression of LRP/MVP in normal and malignant tissues in an attempt to determine distribution of expression of this antigen

Izquierdo *et al*, (1996b), found LRP/MVP to be widely expressed in normal tissue MAb LRP-56 showed strong immunoreactivity with epidermal keratinocytes and melanocytes with some staining of mesenchimal dermal cells Epithelial cells lining the bronchioles of the lung also stained strongly with MAb LRP-56 Type I and II pneumocytes exhibited low level staining while alveolar macrophages were also strongly positive for LRP/MVP expression In the urinary tract, strong staining was seen in proximal but not distal tubules of the kidney, while in the endocrine glands, the cortex of the adrenal gland gave the strongest staining In the male and female reproductive system and breast tissue, strongest staining was observed in germ cells of the seminferous tubules and leydig cells of the testes, in epithelial ovarian cells, placental trophblast and the duct lining cells of the breast Significant staining was also observed in cells of the immune system, particularly in macrophages Similar results were obtained by Sugawara *et al*, (1996) but with two notable exceptions, less staining was observed in epithelial cells of the ovary and in type I and II lung cells In a more detailed study of the over-

expression of LRP/MVP in normal lung, Dingemans *et al*, (1996) confirmed the observations of Izquierdo *et al* and Sugawara *et al*, regarding the general distribution of LRP/MVP in lung tissue This also demonstrated that the level of expression in smokers was not related to number of pack years smoked but there was a higher a trend for higher expression levels in patients who had smoked more that 10 pack years

The overall trend of LRP/MVP expression in normal tissues appears to suggest that it may play a role in defence against xenobiotics Highest LRP expression is seen in tissues chronically exposed to xenobiotics i e epithelia of the bronchus, digestive tract, keratinocytes and in metabolically active tissue such as the adrenal cortex and macrophages (Izquierdo *et al*, 1996b, 1998, Sugawara *et al*, 1996, Dingesman *et al*, 1996) Kedersha and Rome, (1990) have observed similar over-expression of rat vault particles in limited studies on animal tissues, particularly in epithelial cells and alveolar macrophages The distribution appears to resemble that of other drug resistance related proteins such as Pgp and MRP in normal tissue which have been implicated in protection against xenobiotics (Flens *et al*, 1996, van der Valk *et al*, 1990, Cordon-Cardo, 1991, Flens, 1997)

Izquierdo *et al*, (1996b) have also studied the expression of LRP/MVP in a panel of 174 tumour specimens comprising 27 tumour types Results from this study indicate that LRP/MVP expression appears to reflect the susceptibility to chemotherapy of different tumour types Highly chemosensitive cancers such as germ cell tumours and neuroblastoma expressed LRP/MVP in the minority of cases, partially chemosensitive cancers such as ovarian carcinoma expressed LRP/MVP in the majority of case while in highly chemoresistant cancers such colon, renal and pancreatic cancers, all samples expressed the LRP/MVP These initial studies were performed on frozen sections However Schroeijers *et al*, (1997) have recently modified and improved the staining protocol for MAb LRP-56 and a new rat MAb LMR-5 (Flens *et al*, 1997) for paraffin-embedded archival tissue Similarly, den Boer *et al*, (1998) have optimised flow cytometric analysis for LRP-56 in childhood acute lymphoblastic leukaemia

1 3 3 12. Clinical significance of LRP/MVP expression

A number of studies have been undertaken to determine the clinical significance of LRP/MVP in patient outcome and survival These studies are introduced according to tumour type

Neuroblastoma

Neuroblastoma is a relatively chemosensitive tumour type which does not appear to over-express Pgp in the MDR phenotype (Gottesman *et al*, 1989, Favrot *et al*, 1991) Neuroblastoma tumours investigated by Izquierdo *et al*, (1996b) demonstrated that LRP/MVP was not commonly expressed in this neoplasm Ramani *et al*, (1995), have also demonstrated that LRP/MVP expression was low in untreated tumours, 3 out of 10 tumours expressed low levels of LRP/MVP This figure increased following chemotherapy (9 of 11 samples) In 9 paired samples, 7 that were LRP/MVP negative, pre-chemotherapy, LRP immunorecativity was demonstrated post-chemotherapy However there was no correlation between survival and LRP/MVP expression

Melanoma

Schandendorf *et al*, (1995) have shown that Pgp is rarely expressed in malignant melanoma (1 out of 71 lesions), whereas MRP and (primarily) LRP/MVP appeared to be more clinically relevant LRP/MVP expression was mainly associated with a subset of nervi in nervus cells exhibiting up to 25% positive LRP reactivity In 13 out of 21 primary melanomas and 23 out of 37 metastases, greater that 25% of tumours cells stained with MAb LRP-56 In the metastases group, 50 % of positive samples came from patients previously treated with chemotherapy van der Pole *et al*, (1997) have also found LRP/MVP expression in a small panel of chronic melanoma tissue samples but were unable to determine if this was clinically significant Their results do however confirm the observations by Schadendorf *et al*, that the highest LRP/MVP expression was observed in tumour tissue previously treated by chemotherapy

Multiple myeloma

To date only 1 major retrospective study has been initiated to determine the significance of LRP/MVP expression in multiple myeloma (Raaijmakers *et al*, 1998) LRP/MVP expression was assayed in tumours from 70 patients who received varying degrees of treatment with Melphalan LRP/MVP expression was found in 47% of patients LRP/MVP appeared to be significant prognostic factor in Melphalan/Prednisone treated patients, but did not have a significant prognostic value in patients treated intermediate high dose melaphalan Results from this study also show that in a subset of untreated patients who may receive future treatment with Melphalan/Prednisone there was a poor probability of response to these drugs because of high LRP/MVP expression This research group has concluded that intensification of the Melphalan treatment could overcome LRP/MVP resistance

Breast cancer

Linn *et al*, (1997) have shown that 65% of breast tumours assayed expressed LRP/MVP but it appears that LRP/MVP has no predictive value in treatment outcome

Lung cancer

Dingesman *et al*, , (1996) have studied the expression of LRP/MVP in 36 non-small lung carcinoma (NSCLC) and 17 tumours (10 NSCLC and 7 small cell lung carcinoma, SCLC) derived from patients treated with chemotherapy LRP/MVP expression was found to be significantly higher in NSCLC than SCLC samples All SCLC displayed low level expression Within NSCLC, squamous cell and adenocarcinoma had higher expression of LRP/MVP than large cell, undifferentiated and mixed tumours In NSCLC patients, LRP/MVP expression was a prognostic factor for survival In a recent study be Volm *et al*, (1997a), of 87 NSCLC it was observed that there was a significant correlation between LRP/MVP expression and tumour resistance to doxorubicin There was no interrelationship between LRP expression and gender, age, stage, lymphnode

status, proliferation and survival time However, a correlation of borderline significance was noticed between LRP expression and patients smoking habits Carcinoma in heavy smokers (i e , more than 30 cigarettes per day) were more frequently LRP/MVP positive than carcinoma found in non-smokers

Childhood leukaemia

The molecular basis for drug resistance in childhood leukaemia, particularly acute lymphoblastic leukaemia (ALL) is still unclear (Peiters *et al*, , 1997) Multivariable analysis has shown that analysis of Pgp alone has a limited prognostic significance (Volm *et al*, , 1997b) It has also been observed that *in vitro* and *in vivo* resistance to daunorubicin of childhood ALL blast cells is related to prognosis and relapse prior to chemotherapy (Pieters *et al*, 1997)

Studies by Klumper *et al*, (1995) have shown that LRP/MVP but not Pgp was significantly associated with increased *in vitro* resistance of fresh leukaemia cells to daunorubicin Recently Veerman *et al*, (1997) have determined the LRP/MVP expression (as well as Pgp and MRP) in initial and relapse childhood ALL Results from this study showed that expression of MRP and Pgp did not correlate with accumulation or sensitivity to daunorubicin In contrast, expression of LRP/MVP was significantly higher in relapse samples and correlated weakly with *in vitro* resistance to daunorubicin

In a study of 38 children with initial and 25 children with relapsed lymphoblastic leukaemia, LRP/MVP expression was found in 18 out of 38 children with initial ALL and 17 out of 25 relapse ALL Children with initial ALL and without LRP/MVP expression had significantly longer relapse free intervals than patients with LRP/MVP expression (Volm *et al*, , 1997b) den Boer *et al*, (1998) have also determined the expression of LRP/MVP in childhood ALL In a study of 141 children, Pgp, MRP and LRP/MVP expression did not differ between 112 initial and 29 unrelated relapse samples or between paired initial and relapse samples from 9 patients In multiple relapse however, LRP/MVP expression was 1 6 fold higher compared with both initial and first relapse response which was not observed for Pgp or MRP LRP/MVP was found to be weakly

response which was not observed for Pgp or MRP. LRP/MVP was found to be weakly but significantly related to *in vitro* resistance to daunorubicin but not to vincristine, etoposide, and non-MDR-associated drugs, prednisone and L-asparaginase. The expression of Pgp, MRP and LRP/MVP was not higher in initial ALL patients with prognostically unfavourable immumotype, white cell count or age.

Adult leukaemia

Intrinsic or acquired resistance to antineoplastics limits the effectiveness of conventional treatment in acute myeloid leukaemia, AML (List *et al.*, 1996). Pgp has been implicated as a cellular mechanism contributing to chemotherapeutic resistance in this disease although it does not account for the majority of cases (List *et al.* 1993; Broxterman and Schuurhus, 1997). A number of studies have shown that, while pgp is over-expressed in AML, LRP/MVP appears to be more clinically relevant (List *et al.*, 1996; Borg *et al.*, 1996 unpublished; Hart *et al.*, 1997; Michieli *et al.*, 1997; Filipitus *et al.*, 1997; den Boer 1998).

List *et al.*, (1996) have demonstrated the prognostic significance of LRP/MVP expression in 87 patients with AML which included 21 of *de novo* AML, 27 secondary AML and 29 relapse AML and blast phase CML. LRP/MVP. Over-expression was observed in 37% of cases and was highest in secondary AML patients. It was noted that LRP/MVP expression was associated with an inferior response to chemotherapy, remissions were achieved in 35% of cases and appeared to be of independent prognostic significance. In this study, LRP/MVP also appeared to be linked with age and previous treatment with mitoxanthrone. Filipitus *et al.*, (1998) have observed complete remission rates in 80% versus 55% for LRP/MVP positive and negative groups. Overall survival rates were much lower for LRP/MVP patients; disease free survival was greatly reduced for LRP/MVP positive patients. Contrary to the apparent trend of LRP/MVP expression found in adult AML, Hart *et al.*, (1997) found that there is no significant difference between presentation and relapse in 8 patients studied sequentially at presentation and relapse. These researchers have concluded that LRP is not necessarily predictive of poor response to chemotherapy.

Ovarian cancer

To date, researchers have not agreed on the role of Pgp in ovarian cancer or its expression as a predictive marker Although some authors have found elevated levels of Pgp before and after chemotherapy, they differ as to the correlation if any with clinical resistance (Holzymer *et al*, 1992, Arao *et al*, 1994, Karvallis *et al*, 1996) Others have obtained clear results indicating no predictive value for pgp in ovarian cancer (Rubin *et al*, 1990, Veneroni *et al*, 1994)

Recently Izquierdo *et al*, (1995) determined the prognostic significance of LRP/MVP expression in a retrospective study of 57 women with FIGO stage III/IV ovarian cancer All patients in the study received platinum or alkylating-based chemotherapy following debulking surgery 77% of tumours expressed LRP/MVP This group has shown that only LRP/MVP expression (and not Pgp or MRP) correlated with survival and chemotherapy response, patients with LRP/MVP positive tumours exhibit poorer response to chemotherapy, shorter progression free and overall survival time Recent work has also shown a tendency for higher levels of LRP/MVP positivity in fresh tissue samples of patients treated with chemotherapy although there was no statistical significance associated with LRP/MVP expression (Coley, 1997)

1.3.3.13. Proposed function for LRP/MVP in MDR

From the large number of studies described, it is clear that LRP/MVP is associated with drug resistance in drug selected cell lines, inherently resistant cell lines, normal and malignant tissue (where it has been linked to poor prognosis). The expression of LRP/MVP in both normal and malignant tissues commonly associated with the transport of natural xenobiotics (Izquierdo *et al.,* 1996b) suggests that LRP/MVP, as a component of vault particle plays a functional role in transport. The localisation of rat vault particle to the nuclear pore complex appears to support this. Based on this evidence Izquierdo, constructed a hypothetical model for the functional role of vaults in MDR (Scheper, 1997; Izquierdo, 1996), Figure 1.3.3.13.

Vault particles have been found associated with components of intracellular transport (coated pits), (Kedersha and Rome, 1986 b; Rome *et al.,* 1990). MDR cells have been shown to redistribute daunorubicin into the perinuclear region and subsequently relocate the drug away from the nucleus in a granular cytoplasmic pattern reminiscent of LRP/MVP staining, as opposed to the diffuse nuclear and cytoplasmic localisation of drug sensitive parental cells (Gervasconi *et al.,* 1991). This phenomenon has been observed in LRP/MVP expressing cell lines notably in samples from AML patients (Michieli *et al.,* 1997; Schuurhuis *et al.* 1989). It has also been observed that in certain cell lines, drug redistribution is accompanied by the formation of vesicular structures (Dietal *et al.,* 1990). Cleary *et al.,* (1997) also developed and characterised a Pgp over-expressing cell line that also expresses LRP/MVP. Vesicular sequestration has been identified as an important characteristic of drug-resistant cells. Although only 10 % of cells express the LRP/MVP it may play a role in the drug resistance in this cell line since it has been clearly shown that these two MDR markers can be co-expressed (Moran *et al.,* 1997a; Wyler *et al.,* 1997; Shao *et al.,* 1995). The apparent association of vault particles with the nuclear pore complex suggests a functional role for LRP/MVP in nucleocytoplasmic transport (Chugai *et al.,* 1991). Vault particles have also been shown to associate with the synaptic vesicles in nerve endings but there is as yet no evidence that they have a functional role here (Herrmann *et al.,* 1996).

Although these studies strongly suggest a functional role for vault particles in drug resistance there is no direct evidence that they contribute to the MDR phenotype The only direct evidence comes from studies by Byrne, (as yet unpublished), which indicate that in LRP/MVP ribozyme and antisense transfected cells, down regulation of the LRP mRNA is accompanied by a concomitant reduction in drug resistance These observations by Byrne have been confirmed in part, in this thesis Transfection of the LRP/MVP into drug sensitive cell lines failed to confer drug resistance (Scheffer *et al*, 1995) suggests that all of the components of the vault particle are required for vault function This point was also demonstrated by Vasu *et al*, where the disruption of *Dictyostelium* MVP resulted in growth arrest (Vasu *et al*, 1993, 1995, section 1 5 3 6) The recent report that vault particles are up-regulated in MDR cell lines, has prompted further studies into the interdependency of the vault particle components (Kickhoefer *et al*, 1998, Scheper, Schroeijers and Rome, personal communication) Co-transfection of the cDNA encoding different vault proteins will give a clearer picture into the functioning of vault particles and may shed some light onto the possibility of non-functioning particles (Moran *et al*, 1997a, section 1 5 3 1)

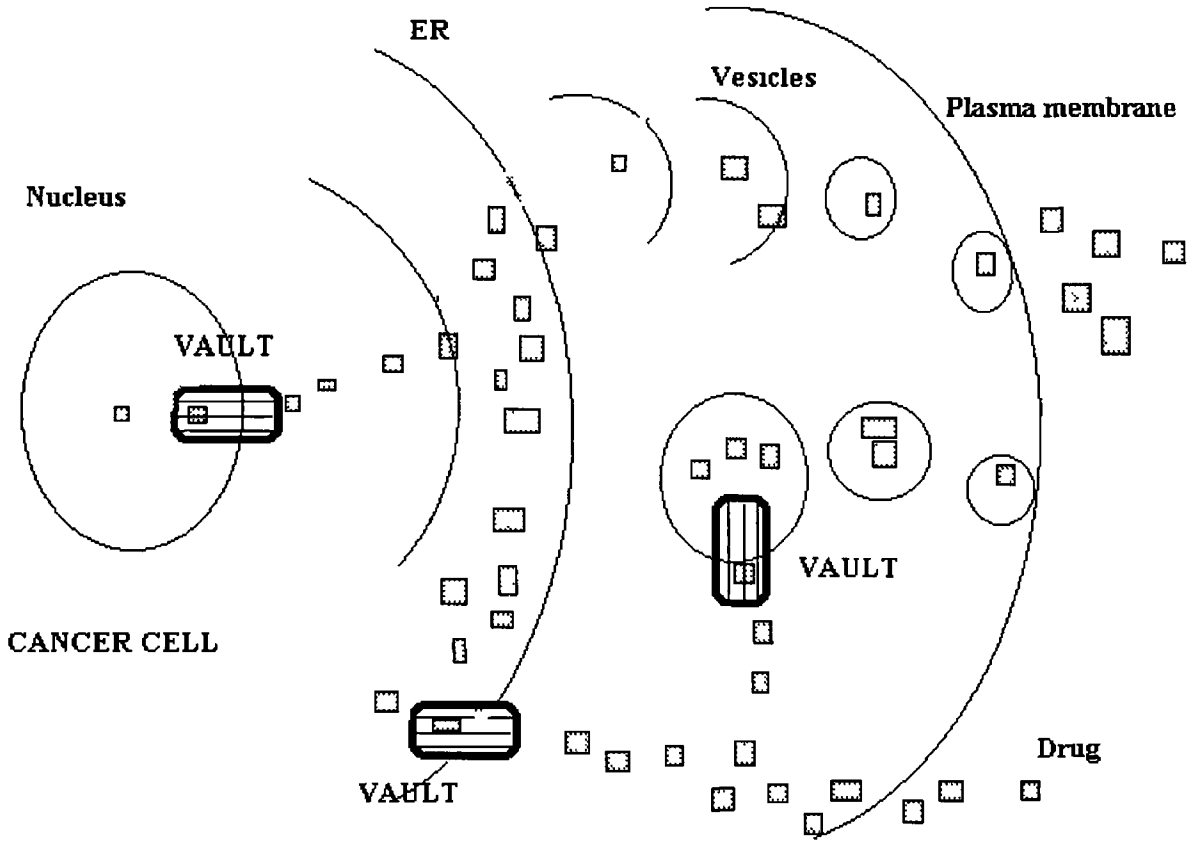


Figure 1 3 3 13 Proposed function for cellular vaults in multidrug resistant cells (adapter from Izquierdo, 1996 PhD thesis and Scheper, 1996)

1.4. Ovarian cancer

Ovarian cancer is the 5th leading cause of death in women in the USA and the 2nd most common gynaecological malignancy exceeded only by endometrial cancer (Runowicz, 1992; Naddas, 1997). Statistics for 1994 showed 24,000 newly diagnosed cases with 13,000 deaths from the disease in the same year. Estimates for 1997 indicate that as many as 26,800 women could have developed the disease with 14,200 deaths due by the end of the year (Parker, 1997). Unfortunately these neoplasms remain asymptomatic until massive ovarian enlargement causes compression of the pelvic structures, ascites, abdominal distension or distant metastasis (Merino and Jaffe, 1992). Only in a small percentage of patients will the tumours be found at an early stage, confined to the ovaries (Aure, 1971). The 5 year survival rate for patients with advanced disease FIGO stages I, III and IV (Bast and Berchuck, 1992) is 25% to 30% (Runowicz, 1992). This rate has changed little in the past 20 years. In a recent US statistical study the number of cancer deaths per 100,000 women increased by 8.9%, only lung cancer has surpassed this increase (Runowicz, 1992). Ovarian cancer develops in 1 woman in 70, with an expected-overall cure rate of 30% (Runowicz, 1992). Ovarian neoplasms are usually staged (i.e. description of appearance and malignancy) because of the late diagnosis involved. The International Federation of Gynaecology and Obstetrics (FIGO) have compiled a guide to the surgical staging ranging from Stage I where growth is limited to one ovary to stage IV where growth includes metastases, pleural effusion and associated problems (Nahaas, 1997).

Ovarian neoplasms are classified according to their histogenesis. Broadly speaking, they can be divided into two main groups; tumours occurring in pre and postmenopausal women (Merino and Jaffe, 1992). Germ cell and Sex cord tumours are primarily associated with premenopausal women. Marino and Jaffe (1992) have extensively reviewed the anatomy and incidence of the constituent tumours of these two groups. Neoplasms of the surface epithelium that covers the ovary are the commonest group of ovarian neoplasms (Godwin, 1993). Tumours affecting these two main age groups are outlined briefly below.

1 4 1 Anatomy of the normal ovary

Gross Anatomy

The ovaries are two nodular bodies located on either side of the uterus, attached to the broad ligament, and situated close to the pelvic wall in a shallow depression known as the ovarian fossa The anterior margin of the ovary is thin, straight and attached to the posterior surface of the broad ligament The posterior is rounded, convex and unattached Beside attachment to the broad ligament, the ovary is held in place by two ligaments one attached to the lateral angle of the uterus and the other, the suspensory ligament that extends form the rounded end of the ovary to the wall of the fallopian tube The ovary is covered by a layer of surface cells that are a modified form of peritoneal mesothelium It covers the whole surface but is separated form the rest of the pelvic peritoneum by a distance line of demarcation known as the Farre-Waldymer line (Blaustein, 1988) Surface cells are separated from the cortex by the tunica albuginea The cortex houses the functional components, namely the oocytes, follicles and corpra lutea, in varying stages of development and regression The stroma is composed of a surface layer of compact fibrous connective tissue

Surface epithelium

Epithelial cells that cover the surface of the ovary can vary from cubidol to columnar and squamoid although cuboidal type predominates It is not until the 3rd or early in the 4th month of gestation that they become separated from the cortical stroma by the basement membrane, until then they are indistinguishable (Blaustein, 1979) During the maturity of the foetus the epithelium become separated from the *tunica albuginea* Surface epithelia follow the contours of the ovary and extend into crypts Some of the crypts may be sealed off from the surface forming inclusion cysts The surface epithelia tend to have microvilli as well as cilia and are attached by desmosomes and lateral integration processes Nuceoli and mitocondria are abundant

Anatomy of the cortex

The appearance of the cortex is age dependant, in the new-born the cortex is packed with oocytes and primary follicles, during the reproductive years it contains follicles and corpora lutea in varying stages of maturation while in postmenopausal women the cortex is composed mainly of scarred remnants (Baker, 1963) The cells of the cortex are spindle-shaped and round fibroblast elements They form the matrix in which the structures mentioned above are found

1 4 2 Functions of the normal ovary

The surface epithelia of the adult human ovary has been described as a non-descript layer of cells that vary from squamous to cubidal and low columnar (Nicosia and Nicosia, 1988, Blaustein *et al*, 1979) The overall unremarkable appearance of this layer of cells suggests that it lacks any particular function, however this does not appear to be the case The ovary has two major functions, production of steroid hormones and the timed releases of ova Blaustein *et al*, (1979)believe that the surface epithelia play a significant role in the latter In the preparation for ovum release, production in lysosomal bodies has been observed in surface epithelial cells These lysosomal bodies are secreted/excreted from the cells' basal surface before ovulation These groups believe that the lysosomal bodies aid in the breakdown of the *tugesia albugenia* by exposure to specific lysosomal enzymes and thus aid in follicular rupture The resultant wound is repaired by growth of ovarian surface epithelia, a supposed source of mutation in epithelial ovarian cancer (Section 1 4 5 1)

1.4.3. Tumours occurring in young, pre-menopausal women

Germ cell neoplasms

Germ cell neoplasms account for 40% of ovarian lesions and 15-20% of all ovarian cancers (Merino and Jaffe, 1992). These neoplasms arise from germ cells that embryonically originate in the yolk sac. These neoplasms are more common in pre-pubertal children and young women accounting for 90% of tumours found in this age group and 60% of all tumours in women under the age of 20 years (Scully, 1979).

Dysgerminomas

These neoplasms arise from undifferentiated germ cells that have lost their capacity to differentiate. The average age of patients with this form of ovarian cancer is 20 years the majority of sufferers being under the age of 30 years. Tumours as large a 40-cm have been observed. Disgerminomas usually disseminate through the lymphatic system very early, to the lymph nodes, lung and bone. However the prognosis is excellent with a 5 year survival rate of 90 % (Merino and Jaffe, 1992).

Yolk sac tumours

These tumours are rare, constituting 1% of ovarian cancers, usually occurring in women under 20 years of age. They are usually highly malignant and have a poor prognosis. 5 year survival rates have yet to be established (Granai, *et al.*,., 1994).

Stromal tumours

These tumours are rare and usually secrete sex hormones. Associated neoplasms such as childhood granulosa cell tumour can cause precocious puberty although most stromal tumours are begin or of low grade malignancy. Of those neoplasms that are malignant, 10-15 % of cases present with metastasis. The 5 year survival rate has been calculated at 80 % (Granai *et al.*, 1994).

1 4 3 1 Tumours affecting post-menopausal women

Epithelial tumours are the most common histological types found in post-menopausal women Although tumours of the surface epithelium can occur in younger women, they are usually rare before menarche (Merino and Jaffe, 1992) Incidence of epithelial tumours increase after the age of 55 years, the most progressive forms predominating in elderly, postmenopausal women Younger women tend to have benign lesions, borderline tumours, tumours of low malignant potential or low-grade adenocarcinomas These neoplasms arise from the surface epithelia that cover the ovary and have the potential to undergo metaplastic changes to resemble to various types of epithelium, which cover the gynaecological tract The various histological types are briefly outlined below,

Serous tumours

Serous tumours account for 42% of all epithelial carcinomas (Blend and Ostrowski, 1994) Histologically, these neoplasms resemble the lining of the fallopian tube Serous cystadenocarcinoma account for approximately 65% of all serous neoplasms and is the most malignant histological type of epithelial ovarian cancer As many as two thirds of all neoplasms of this subtype present as bilateral at the time of diagnosis The overall prognosis for this type of tumour is poor with a 20-30% survival rate The prognosis depends heavly on the FIGO stage of the disease and the grade of the tumour Treatment for this tumour usually involves total abdominal hysterectomy and tumour debulking followed by chemotherapy (Merino and Jaffe, 1992)

Mucinous tumour

Mucinous tumours account for approximately 15% of all epithelial malignancies and 5% of all ovarian neoplasms Blend and Ostrowski, 1994) Tumours are characterised by tall, columnar mucin-producing cells which resemble the lining of the intestine or endocervex (Czarnobilsky, 1988) Mucinous adenocarcinoma accounts for 10% of all mucinous carcinoma These tumours are highly malignant and metastasise early As with the previous malignancy, the prognosis is heavily dependent on the FIGO stage of the lesion

but survival rates are still less that 60%, treatment is identical to serous carcinoma (Merino and Jaffe, 1992)

Endometroid tumour

These histological types are characterised by resemblance to cells of the endometrium Endometroid tumours are almost always malignant and account for 20% of all ovarian neoplasms (Blend and Ostrowiski, 1994) The mean age for patients with this form of epithelial ovarian cancer is 50 years and at the time of surgery, 30-50% of patients have bilateral disease Histologically, the tumours are composed of glands lined by columnar cells with large nuclei and scanty cytoplasm The tumours may produce mucin but the mucin is concentrated within the gland (Czarnoblisky, 1987, Merino and Jaffe, 1992) Prognosis is similar to that previously described

Clear cell carcinoma

These neoplasms comprise of 5% of all ovarian tumours They are rarely bilateral and mainly affect older post-menopausal women

Malignant Brenner tumours

One of the rarer histological types, Brenner tumour is also associated with older post-menopausal women Lesions are usually destructive to the surrounding stromal tissue and have a typical infiltrating pattern Metastasis usually occurs to the omentum, peritoneum and distant visera (Merino and Jaffe, 1992)

1 4 4 Epithelial ovarian cancer

In theory, all cells that comprise to human ovary have the potential for malignant transformation (Godwin *et al*, 1992) The vast majority of malignant ovarian tumours arise from the surface epithelium (Nahaas, 1997) The surface epithelia are thought to play an important function in follicular rupture and accompanying wound repair Epithelial tumours are known to disseminate primarily by surface shedding, lymphatic spread and occasionally hematogenous spread (Blend and Ostrowiski, 1994) Intraperitoneal spread is thought to occur when malignant cells are shed from the surface of primary tumour material These exfoliated tumour cells attach to peritoneal surface and form micrometastases (carcamatosis or peritoneal studding), which in turn shed cells These free-floating cells are thought to be captured by the lymphatic channels in the diaphragm It has been observed that clearance of cells occurs more extensively on the right side, overlying the liver (Blend and Ostrowiski, 1994) Obstruction of the lymphatics of the diaphragm then allows for the implantation of cells in the omentum and on other sites in the peritoneal area It has been shown that ovarian cancer can spread by direct extension and it has been found in the bladder, colon and pelvic peritoneum (Young *et al*, 1989)

1.4.4.1 Monoclonal nature of epithelial ovarian cancer

The colonality of disseminated ovarian cancer is controversial, histopathological examination alone cannot distinguish malignant material of monoclonal and multicentric origin (Kupryjanczyk *et al*, 1996) The question of monoclonality arises particularly where other anatomic sites are involved such as the omentum, peritonem endometrium etc In most tumours with identical bilateral disease there is no criteria to discriminative between a primary unilateral tumour and metastasis from bilateral primary carcinomas Previous cytogenetic and molecular genetic studies support the hypothesis of monoclonality in stage III ovarian carcinomas with bilateral involvement (Mok *et al*, 1992, Jacobs *et al*, 1992) These studies revealed that ovarian cancer may not be multifocal but that lesions identified in multiple sites were derived from a single origin

More recent evidence has supported the hypothesis of monoclonal origin of ovarian cancer Bilateral stage III ovarian cancer was assayed for known mutations in the p53 gene It was found that p53 patterns were identical In one patient, a p53 mutation in exon 5 was present in the malignancy in both of the ovaries and a metastatic tumour of the omentum (Kupryjanczyk *et al*, 1996) Pejoue *et al*, (1996) have found similar results in a study of 11 patients with Stage III bilateral carcinoma with identical genetic and cytogenetic abnormalities in each ovary

1 4 5 Causes of ovarian cancer

As epithelial cancer of the ovary accounts for the majority of ovarian neoplasms, discussion will be limited to this histological subtype Table 1 4 5 1 lists a number of possible causative factors that may be relevant in epithelial ovarian cancer

*Number of ovulation years
*First pregnancy>30 years
*Late onset of menopause
*First degree family member
*Exposure to asbestos and talc
*Diet high in red meat and animal fat
*Obesity
*Genotype changes

Table 1 4 5. Suggested causative factors of epithelial ovarian cancer

1 4 5.1 Multiple ovulation

Ovarian epithelia, unlike most epithelial cells, behave as generative stem cells, (Hamilton, 1992) Thus, the division of 1 epithelial cell is thought to yield two daughter cells with the equivalent potential for further growth This contrasts with replicative stem cell which yield two daughter cells, one of which has a loss of growth potential i e one cell will terminally differentiate In the case of ovarian surface epithelia, a single destructive mutation could easily be inherited by an exponentially expanding progeny (Godwin *et al*, , 1992) Therefore, the larger the number of cells that are available for additional mutations, the more likely it is that the mutation which may cause transformation will occur Thus, continual ovulation may play a role in the development of epithelial ovarian cancer Observations have shown that tumours in animals are rare This may be due to the fact that animals ovulate seasonally and usually become pregnant shortly after the onset of ovulation begins, therefore the number of ovulations is low The only animal

with a high incidence of epithelial ovarian cancer is the

development Studies have also shown oestrogen receptor expression in malignant surface epithelial cell lines and tissues to varying degrees There is limited evidence to suggest that oestrogen is a strong mytogen (Langdon *et al*, , 1990, Sawada *et al*, , 1990) These groups have also shown that oestrogen can influence substrate dependant growth, these findings suggest that the uncoupling of the control mechanisms involved in oestrogen stimulation of growth could have an effect in cancer

1 4 5 3 Peptide hormones

Transforming growth factors (TGFs) are potent mitogenic peptides (Chow *et al*, , 1996) Two classes of TGF have been isolated the alpha (α) and beta (β) forms (Anazno *et al*, , 1983) Mature TGF α is an acid and heat stable 50 amino acid peptide of 55 kDa (Derynck *et al*, , 1986) TGF α has been found to be secreted by a number of transformed cells and tumours (Todaro, 1983) TGF α has been shown to be an autocrine growth promoter by the incorporation of [^{3}H] Tymidine TGF α has been shown to share 80% homology with epidermal growth factor (EGF) and functions by binding to the EGF receptor (EGFR) TGF α was initially discovered in malignant cells as an EGF like factor binding to the EGFR It has been speculated that it may play a role in malignant transformation of epithelial ovarian cells, high levels have been reported in ascites and urine of patients with ovarian cancer (Arteaga *et al*, , 1988)

TGF β, on the other hand acts as an autocrine inhibitor of proliferation, being secreted as an inactive molecule bound to a precursor molecule which must be cleaved to release its activity (Chow, 1996) TGF β interacts with a cell surface receptor serine/threonine kinases (Berchuck and Carney, 1997) While normal cells produce active TGFβ and are growth inhibited by it, most ovarian cancer cell lines are not, having lost the ability to produce, activate or respond to TGF β (Berchuck and Rodriguez, 1992, Berchuck *et al*, 1990, Zhou and Leung, 1992) It is thought that the loss of TGFβ and the up-regulation of TGFα may play a role in ovarian cancer development (Berchuck and Carney, 1997)

1 4 5.4. Cellular oncogenes and tumour suppressor genes in ovarian cancer

The possible involvement of cellular oncogenes in ovarian cancer has been studied by a number of groups Chromosomal translocations during mitosis can lead to the relocation of cellular proto-oncogenes affecting the normal function of these genes and leading to changes in cellular proliferation (Chow, Chien and Chow, 1996) Similarly the loss of one allele of a tumour suppressor gene can lead to increased chances of the subsequent loss of the other allele resulting in the loss of control of proliferation To date, a number of oncogenes and tumour susppressor genes have been identified as being involved in the development of ovarian cancer

Mutations in the gene coding for the ras gene product leads to changes in the normal GTPase activity which may lead to alterations in control of cell growth Amplification and/or mutation/over-expression of the ras genes have been detected by a number of investigators Studies by Chien *et al*, have shown amplification of the K-ras gene in 33% of samples assayed Over-expression of the H-ras gene was detected in 12% of tumours The c-myc gene codes for a DNA -binding protein that appears to play an important role in cellular proliferation In a study by Zhou *et al*,, (1988) 25% of ovarian adenocarcinomas displayed c-myc gene amplification Baker *et al*, , (1990) have reported similar findings with 29% of ovarian samples assayed displaying c-myc over-expression as have Although c-myc over-expression has been detected in a number of studies, these does not appear to be any correlation between c-myc amplification, tumour grade and prognosis in ovarian cancer patients (Chow, Chien and Chow, 1996)

Over-expression of c-erb2 (a DNA binding protein) has been linked with poor survival in breast cancer patients (Salmon *et al*, , 1989) Slamon have found similar results in ovarian cancer patients, HER-2/neu the gene coding for c-erb2 was reported to be over-expressed in 33% of ovarian tumours in the study Expression also appeared to be linked to poor prognosis and survival Berchuck *et al*, (1990) have also reported expression of c-erb2 in a series of archival ovarian tissue specimens Patients with normal HER-2/neu expression were found to be less likely to require a second look laoparotomy compared to patients with amplification of the gene, those patients with HER-2/neu over-expression had a lower survival rate that those with normal expression

Tumour suppressor genes code for proteins that normally control proliferation at times when it is inappropriate The majority of tumour supressor genes encode nuclear proteins involved in cell cycle control It has been observed that in the majority of cancers, the incidence is due to the inheritance of a mutant copy of one of the tumour suppressor genes (Berchuck and Carney, 1997)

Loss of P53 function appears to be the most common genetic event described thus far in human cancers P53 usually acts as an inhibitor of proliferation by binding to transcriptional regulatory elements in DNA P53 function is thought play an important role in cancer prevention Mutation in the gene coding for P53 is usually accompanies by a deletion in the other allele resulting in mutant P53 Because mutant P53 is not degraded as rapidly as normal or "wild -type" P53, nuclear build up of the mutant form is seen Mutant p53 has been detected in half of advanced cancers, 15% of early stage and 4% of borderline tumours (Marks *et al*, , 1991, Koler *et al*, , 1993, Berchuck *et al*, , 1992)

Approximately 5-10% of epithelial ovarian cancers are thought to be due to inherited mutations in cancer susceptibility genes (Berchuck *et al*, , 1996) The BRCA1 gene is located on chromosome 17q and has been linked to the majority of inherited (familial) epithelial ovarian cancers It has been observed that the lifetime risk of the development of breast and/or ovarian cancer is approximately 90% in BACR carriers BACR1 is likely to be a tumour suppressor gene although its exact function remains unknown Although allelic deletion in the region 17q occurs frequently in sporadic ovarian cancer patients, acquired mutations in BACR1 appear to be relatively rare

1 4 5 Diagnosis of ovarian cancer

The advances in the diagnosis and screening methods have, unfortunately, only a small effect on the overall ability to predict incidence of this disease Patients are rarely diagnosed until they are in the late stages of the disease (FIGO stage III-IV, Nahaas, 1997) To date, three main screening/diagnostic methods are employed for the detection of ovarian neoplasms,

(i) bimanual recto-vaginal examination

(ii) trans-vaginal ultrasonography

(iii) radio-immunoassay for serum markers

Anatomical methods are far from ideal due to their inability to detect micro-metastases in the peritoneal cavity Serum markers associated with ovarian cancer have also found to be unregulated in other diseases particularly in the gastrointestinal tract (Nilof 1984) However, they are considered to be an integral part of ovarian cancer diagnosis and are certainly a good indicator of problems in the pelvic region Radioimmunoscintography has been used more extensively in the diagnosis of ovarian cancer (Section 1 6) This method of diagnosis appears to be very promising at catching malignancies at an early stage The major use of radioimmunoscintography in patients with ovarian cancer appears to be in the diagnosis of intra-abdominal and, particularly, peritoneal spread of the disease (Goldenberg and Larson, 1992)

1 4 6 Tumour markers in ovarian cancer

Primary ovarian cancer is usually treated by debulking surgery followed by chemotherapy The efficacy is routinely evaluated by repeated vaginal examination and trans abdominal ultrasonography However, these methods are unreliable for monitoring as aggressive and recurrent disease often remains hidden until the patient presents with a large tumour mass Over the last few decades, a variety of serological tumour markers have been proposed as a supplement to other non-aggressive diagnostic methods A number of monoclonal antibodies have been generated to ovarian and non-ovarian cells expressing potentially useful antigens Table 1 4 6 1 lists serological antigens that are now routinely used, in the diagnosis of these neoplasms

Table 1 4 6 Serological markers routinely used in ovarian cancer monitoring

Tumour marker	**Reference**
CA125	Bast *et al*,, 1981
CA72 4	Gadducci *et al*, 1989
CA15 3	Larbre *et al*, 1990De
CA195	de Bruijn *et al*, 1993
CA19 9	Larbre *et al*, 1993
CA50	Gadducci *et al*, 1990
OSA = Ovarain serum antigen	McGuckin *et al*, 1992
MSA = Mammary rerum antigen	McGuckin *et al*, 1992
90K	Panici *et al*, 1989
Sialyl-Tn	Inoue *et al*, 1992
Sialyl-Lewis Xi	Inoue *et al*, 1992
PLAP = Placental alkaline phosphatase	Epenetos *et al*, 1985
HCG= Human chorionic gonadotropin	Donaldson *et al*, 1980
AFP= Alpha-feto protein	Donaldson *et al*, 1980
LASA-P = lipid associated sialiac acid	Bernstein *et al*, 1991
Ferritin	Negishi *et al*, 1987
SP1	Pulay *et al*, 1987
IAP = immunosuppressive acidic protein	Negishi *et al*, 1987
PHI = phosphohexose isomerase	Paulick *et al*, 1985
$HMFG_2$ = Human milk fat globule membrane antigen	Taylor-Papadimitriou *et al*, 1981

1 4.6.1. CA125

CA125 has been the most widely used tumour marker for the investigation and management of ovarian cancer, therefore, the discussion on the uses of tumour markers will deal primarily with this antigen CA125 is an antigen expressed on many histological subtypes of ovarian carcinoma The antigen was first studied by Bast *et al*, (1981) who developed the first monoclonal against OVCA433 cells which over-expressed the CA125 antigen CA125 is a glycoprotein, aggregated to high molecular weight complexes larger that 200 kDa, but different subunit sizes have been reported (O'Brien *et al*, , 1991, Nagata *et al*, , 1991, Nustad *et al*, , 1994) The carbohydrate content is 24% lower than for typical mucins, sugar chains are both N-and O-linked (Nagate *et al*, 1991, Davis *et al*, 1986) CA125 appears to carry two major antigenic determinants, A, recognised by MAb OC125 (Bast *et al*, , 1981), and B, recognised by a new generation MAb MII (Nustad *Et al*, 1996) CA125 is primarily expressed on the cell surface which has been demonstrated by electron microscopy (Blaustein *et al*, 1984) while some intracytoplasmic staining has also been reported (Shishi *et al*, 1985, de Bruijn *et al*, 1997) In the original study by Kabawat *et al*, (1983), CA125 was found to be expressed in amnion and derivatives of foetal coelomic epithelium and in the epithelium of the fallopian tube, endometrium, endocervex, and in mesothelial cells of the adult pleura, pericardium and peritoneum, particularly in areas of inflammation and adhesion Initially these studies revealed that CA125 was not expressed in the adult normal ovary but on subsequent examination staining was seen on the normal ovary, colon, bladder, stomach, lung and kidney Radioimmunoassay has fixed the normal serum concentration at 35 units per ml (Kenemans *et al*, 1993) In healthy subjects, elevated CA125 levels have been seen during menstruation, early pregnancy in begin diseases of the urinogenital tract, and in non-gynaecological conditions such as peritonitis, liver cirrhosis, cancer of the breast, pancreas, liver and lung (de Bruijn *et al*, 1997) Serum levels of CA125 appear to correlate with FIGO staging of ovarian cancer (Tuxen *et al*, 1995) Markowska *et al*, (1990), have show elevated serum levels in 51% of Stage I and 98% of Stage IV CA125 appears to be expressed in all histological types but is higher in tumours classified as non-mucinous than in mucinous types Despite the large body of

knowledge which has been amassed about CA125, its exact function is still unknown as the gene or cDNA sequences have yet to be isolated (de Bruijn *et al*, , 1997)

1 4 6.2 Measurement of CA125 during chemotherapy

Measurement of CA125 levels is now widely applied to follow the response to chemotherapy It has been found that CA125 levels correlates with the course of the disease in about 90% of patients Regression gives declining levels in 97% of cases, progression gives increases in 83% of patients and in stable disease, the levels remain unchanged in 71% of patients (Kenemans *et al*, , 1993) Gallon *et al*, (1992) have shown that serum levels of 20-35% were related to residual disease in 12/13 patients This group has suggested resetting the upper limit of what is considered normal CA125 levels following hysterectomy to a lower level A number of trials have included CA125 levels in the evaluation of results from chemotherapy trials (Long *et al*, 1994, Rustin *et al*, 1996) However at least 1 study has reported the failure of CA125 to correlate with radiographically measurable disease in cases where patients received salvage chemotherapy (Morgan *et al*, 1995) Attention has also focused on CA125 as a prognostic predictor of remission and survival Following initial studies, CA125 half life measurements have now been documented as an important, independent indicator of survival (Buller, 1996, Gaddicci *et al*, 1992, Yedema *et al*, 1993) Values of less than 35 units/ml were associated with 50% survival at 24 months, 60% survival at 48 months (Gadducci *et al*, 1992, Marker *et al*, 1993) de Bruijn *et al*, (1996) have suggested that with elevated CA125 levels before the 3rd course of chemotherapy and a half life of more than 20 days, a change in the chemotherapy regime is warranted

1.5. Primary culture of ovarian tumour tissue and ascites

Primary culture or, the establishment of permanent human ovarian tumour cell lines in continuous culture, has proven to be one of the most useful techniques towards understanding these cells. The establishment of tumour cell lines serve as a valuable resource for multiple investigations. Ovarian cell lines have been used extensively in the identification of growth factors, receptors and oncogene expression. They have also been used to study the methods of steroid action, characterisation of tumour and the generation of specific monoclonal antibodies with the potential application in tumour diagnosis, monitoring disease progression and the development of treatment strategies (Hung, Sayaswaroop and Tabizadeh, 1994). These cell lines have also been useful in the development of experimental *in vitro* model systems for a range of disorders including MDR.

1.5.1. Sample preparation of ovarian tumour material

Since the mid-1970's, several permanent ovarian cancer cell lines have been established in culture. The literature contains descriptions of over 70 ovarian tumour cell lines the majority representing reports of one to three cell lines. However, a number of groups have reported the isolation of 5 or more cell lines (Fogh *et al.*, 1977, Wilson *et al.*, 1984, 1996; Hamalton *et al.*, 198;, Hill *et al.*, 1987, van Niekerk *et al.*, 1988 and Mobus *et al.*, 1992, 1994). More recent reports have detailed various techniques which my improve the success rate in cell line establishment such as transplantation to nude mice or isolation of cells from ascites (Hurteau *et al.*, 1994; Buller *et al.*, 1995; Gorai *et al.*, 1995 Gambona et al., 1995; Kim *et al.*, 1997 and Codegoni *et al.*, 1998).

A review of the literature in this area reveals a range of commonly used standard techniques for the desegregation of solid tumour material. In general, tumours are minced into 1-2mm^3 pieces and sieved to produce a cell suspension with relatively few cell clumps. Two researchers have reported the use of proteolytic enzymes (trypsin and collagenase) to aid dissociation of the tissue sample (Hill *et al.*, 1987; Gorai *et al.*, 1995).

A number of researchers have transplanted solid tumours directly into nude mice in an attempt to improve the chances of establishing permanent cell cultures (Codegoni *et al*, 1998, Kim *et al*, 1997) However this technique does not appear to be any more successful than standard cell culture methods Tissue explants (i e small tissue pieces approximately $1mm^3$ are cultured until epithelial cells form outgrowths) have also been used as a source of epithelial cells however this method increases the risk of contamination and over growth by fibroblasts (Wilson *et al*, 1997)

Investigators have also established permanent cultures from ascites Due to the difficulty in diagnosis of ovarian malignancies, tumour growth is usually accompanied by considerable malignant ascites (Hung, Satyaswaroop and Tabibzadeh, 1994), section 1 4 1 The apparent easy with which researchers are able to successfully establish lines from ascites may be due to the culture conditions which the cells have become accustomed to i e growth in a free floating or partially attached state in a liquid environment (Wilson *et al*, 1997, O'Sullivan *et al*, 1998)

1 5 2 Isolation of pure epithelial cells for primary culture

Epithelial cells account for only a small percentage of the total cellular content of the human ovary As a result, contamination from other cell types is common in ovarian primary culture Fibroblast contamination appears to be a significant problem in most cultures derived from solid tumours and some ascitic samples (Wilson *et al*, 1997) Removal of fibroblasts is mainly achieved by differential trypsinization i e fibroblasts detach from cell culture flasks before epithelial cells on treatment with trypsin-versene therefore they can be completely removed from epithelial cultures over a number of days or weeks (O'Sullivan *et al*, 1998, Gorai *et al*, 1995, Gamboa *et al*, 1995, Mobus *et al*, 1994 and Hill *et al*, 1987)

Wilson *et al*, (1997) employed differential incubation with cell free ascites for the removal of mesothelial cells from epithelial cultures Cell free ascites was found to produce a fibrin mesh on exposed cells Brief exposure to trypsin-versene produced complete detachment of the mesothelial sheet of cells that attached specifically to the fibrin mesh without removing epithelial cells which do not attach to the fibrin

1 5 3 Studies with variants of the ovarian carcinoma cell line OAW42 as an *in vitro* model for MDR in ovarian cancer

Expression of Pgp, MRP and LRP/MVP in clinical samples has been discussed previously in section 1 3 3 12 A review of the literature suggests that clinical MDR is rarely associated with the expression of a single MDR-linked protein Instead, it is more likely that a number of MDR associated proteins may work in tandem giving rise to the MDR phenotype in ovarian cancer, (Izquierdo *et al*, 1995, Karvallis *et al*, 1996,Coley 1997) Analysis of the expression of these MDR markers in established ovarian cell cultures can mirror the *in vivo* case and therefore serve as suitable *in vitro* models (Moran *et al*, 1997a)

It has already been suggested that epithelial ovarian cancer is monoclonal in nature, that is, bilateral tumours arise from a common primary tumour (section 1 4 6) However, it has also been demonstrated that within monoclonal epithelial tumours there can exist a certain degree of heterogeneity (Bar *et al*, 1994) Wolf *et al*, (1987) has demonstrated that 2 cell lines derived from the same tumour sample had different degrees of drug sensitivity and growth characteristics This he assigned to the possible clonal selection of a drug resistant population of cells evolving from the minor to major population within a given tumour Similar observations have been made by Moran *et al*, (1997a) with respects to the expression of the LRP/MVP in variants of the ovarian carcinoma cell line OAW42

The OAW42 cell line was derived from ascites of a patient with serous cystadenocarcinoma of the ovary (Wilson, 1984) Moran *et al* (1997a) have derived a series of variants with exhibit increasing resistance to adriamycin OAW42-SR is a heterogeneous cell population that was derived by serial subculture of the OAW42 and appears to be intrinsically drug resistant i e exhibits resistance to adriamycin without to the drug *in vitro* OAW42-S is a drug-sensitive clonal sub-population of the OAW42-SR The OAW42-A1 and OAW42-A were derived by prolonged exposure to adriamycin Immunocytochemical analysis of these four variants revealed that a number of drug

resistance mechanisms were co-expressed in the OAW42-SR, Pgp, MRP and LRP/MVP. Further analysis of this variant revealed that following continuous subculture, LRP/MVP positive cells within the OAW42-SR population appeared to evolve form being a minor to the major cell population. This suggests clonal variation of this particular cell type may have occurred. This phenomenon is similar to observations by Izquierdo *et al.*, (1995), who observed distinct heterogeneity in LRP expression in frozen archival specimens. Mobus *et al.*, (1992) have also demonstrated clonal selection of CA125 negative cells *in vitro*.

In the drug selected variants, OAW42-A1 and OAW42-A, there appeared to be a shift in the mode of resistance with increased expression of Pgp and a reduction in LRP staining. This may also reflect the *in vivo* development of tumour resistance from low and possibly intrinsic resistance where pgp is undetectable, to higher levels where Pgp is detectable (e.g. in post chemotherapy situations). This phenomenon has also been observed in other cell line models such as the SW1573/2R series (Scheper *et al.*, 1993).

1.5.3.1. Expression of the LRP/MVP in OAW42-SR and OAW42-S

The increase in LRP/MVP over-expressing cells within the OAW42-SR was associated with an increase in drug resistance to adriamycin (Moran *et al.*, 1997a), similarly LRP/MVP over-expression has also been demonstrated in the OAW42-S, the sensitive clonal sub-population of the OAW42-SR. The OAW42-SR variant displayed a passage dependant increase in the expression of the LRP/ MVP with an associated increase in drug resistance (an increase in IC_{50} values). This observation suggested a direct role for the LRP/MVP in drug resistance in the OAW42-SR variant (see section 1.3.3.12.). The increase in LRP/MVP expression in the OAW42-S variant was not accompanied by an increase in drug resistance. Moran *et al.*, (1997a) have postulated that the LRP/MVP present in the OAW42-S may have a non-functioning component.

Another alternative may also be possible. Rome *et al.*, (personal communication) have failed to isolate vault particle from cells in culture. This group suggest that these changes in vault conformation may be due to the vault particles being in a state of "flux" i.e. continually changing shape to suit its proposed function Therefore alternative

conformational states may account for the lack of vault function in the OAW42-S cell line

The development of ovarian cancer is therefore, a complex group if interactions between a wide variety of cellular antigens In order to understand the mechanisms by which these neoplasms form and develop resistance to chemotherapeutic drugs it is necessary to fully understand the cellular processes and identify the antigens involved in these mechanisms

1.6. Aims of thesis

The aims of this thesis were to raise monoclonal antibodies to a sensitive OAW42-S and a resistant OAW42-SR variant of the ovarian carcinoma cell line OAW42, and to use these monoclonal antibodies to identify antigens which may play a role in resistance.

Work undertaken previously in this laboratory (discussed below) had indicated that the LRP/MVP may play a role in chemo-resistance in these cell lines and might exist in multiple alternative active or inactive forms. It was hope that some of these anti-OAW42SR and OAW42-S MAbs might recognise antigens related to LRP/MVP, or other resistance-related proteins.

Following isolation these antibodies could be characterised by a range of immuno-analytical methods including ELISA, immunocytochemistry, immunofluorescence and Western Blotting.

In the characterisation of monoclonal antibodies, not all antibodies recognise their target antigens by conventional Western Blotting procedures. Other methods such as immunoprecipitation can be used in their characterisation.

In order to characterise the antigens detected by such antibodies, the objective of the work in the next phase was to modify a pre-existing non-radioactive method for labelling of cellular proteins with biotin derivatives. This would allow estimation of molecular weight and confirmation of distribution of the antigen in cell lines by immunoprecipitation coupled to Western Blotting.

The results from these studies revealed similar but not identical patterns of expression and molecular weight between the antigen detected by the anti-OAW42-SR MAb 3B6 and the LRP/MVP.

The aim of the final part of the project was to determine whether,

(i) these two antigens differed in glycosylation, and/or,

(ii) shared sequence homology.

This latter question was approached by amino sequencing of internal peptides of the 3/B6 antigen and subsequent protein data base searches to determine the degree of homology, if any, between these two proteins

Two other monoclonal antibodies were also chosen for further study The aim in this part of the study was to partially characterise these antibodies to determine whether there were any similarities in the staining characteristics between these antibodies and the anti-LRP/MVP monoclonal antibodies LRP-56 and LMR-5 This could be undertaken in cross-reactivity experiments with purified rat vault particles

2 0 Materials and Methods

2 1 Water

Ultrapure water was used in the preparation of all media and solutions This water was purified by reverse osmosis system (Millipore Milli-RO 10 Plus, Elgastat UHP) to a standard of 12-18 megaohms/cm resistance

2 2 Glassware

Most solutions pertaining to cell culture and maintenance were prepared and stored in sterile glass bottles All glassware including these bottles, used for cell culture work were soaked in a 2% (v/v) solution of RBS (AGB Scientific) fort at least one hour after which, they were scrubbed and rinsed several times in tap water They were then washed by machine using Neodisher detergent (an organic, phosphate-based acid detergent) rinsed twice in distilled water, once in ultrapure water and sterilised by autoclaving

2 3. Sterilisation

Water, glassware and all thermostable solutions were sterilised by autoclaving at 120^0C for 20 min under pressure of 1 bar Thermolabile solutions were filtered through a 0 22 μm sterile, low protein binding filter (Millipore)

2.4 Media preparation

The base media used during routine cell culture were prepared according to the formulations shown in table 2 4 1 10X media were added to sterile ultrapure water, buffered with HEPES and $NaHCO_3$ and adjusted to a pH of 7 45-7 55 using sterile 1 5M NaOH and 1 5M HCl The media was then filtered through a sterile 0 22 μm bell filter (Gelman G1423S) and stored in 500 ml bottles at 4^0C up to the expiry date specific for each individual 10X medium container Prior to use, 100 ml aliquots of basal media were supplemented with 2mM L-glutamine (Gibco 043-0503) and 5-15% foetal calf serum This was then used as routine culture medium This was stored for up to 2 weeks at 4^0C after which time fresh culture medium was prepared Hybridoma and Sp/2 myeloma cells

were grown in a basic basal media of commercially available DMEM, DMEM with Glutamax I (Glutamax I is L-Alanyl-Lglutamine Gibco BRL, Pasiley, Scotland, 61965-026) supplemented with 10% heat inactivated FCS (Myclone, Gibco 10082-147) This was further supplemented for the hybridoma growth and cloning (section 2 8 2 and 2 8 5)

Table 2 4 1 Preparation of basal media

	DMEM (Gibco 042-0250M)	**Hams-F12** (Gibco04201430M)
10X Medium	500 ml	500 ml
Ultrapure H_2O	4300 ml	4700 ml
1M HEPES (Sigma H9136)	100 ml	100 ml
7.5 % $NaHCO_3$ (BDH 30151)	45 ml	45 ml

2 5 Sterility checks

Sterility checks were routinely carried out on all media, media supplements and reagents used in cell culture The various solutions were inoculated onto Columbia (Oxid CM331) blood agar plates, Saburaud (Oxid CM217), Dextrose and Thioglycollate (Oxid CM173) broths and incubated for 2 days to 3 weeks Media was sterility checked for at least 2 days prior to use by incubating samples at 37^0C

2.6. Cell lines

All cell culture work was carried out in a class II down-flow re-circulating laminar flow cabinet (Nuaire Biological Cabinet) and any work which involving toxic compounds was carried out in a cytoguard (Gelman) Strict aseptic technique was adhered to at all times The laminar flow cabinet was swabbed with 70% industrial methylated spirits (IMS) before and after use, as were all items used in the cabinet Each cell line was assigned specific media and waste bottles and only one cell line was worked with at a time in the cabinet which was allowed to clear for 15 min between cell lines The cabinet itself was cleaned weekly with industrial disinfectants (Viron, Antec International TEGO, TH Goldschmidt) as were all incubators used in the culture of cell lines and hybridomas Cell lines were maintained in 25 cm^2 (Coster 3035) 75 cm^2 (Coster 3075) or 175 cm^2 (Nunclon, NUNC) tissue culture flasks at 37^0C and fed every 2-3 days Cell lines were cultured through 7-10 passaged before they were discarded and new cultures grown from frozen stocks Section 2 5 4 The cell lines used during the course of this study, their sources and growth media requirements are listed in Table 2 6 1 1 and 2 6 1 2

2 6.1 Subculture of adherent cells

During routine subculturing or harvesting of adherent cell lines, cells were removed from their flasks by enzymatic detachment Waste medium was removed from the cells which were then rinsed with pre-warmed trypsin-versene/EDTA (TV) solution (0 25% trypsin (Gibco 043-05090), 0 01% EDTA (Sigma) solution in PBS (Oxoid BR14A) This ensured that any naturally occurring trypsin inhibitor in residual serum was deactivated Fresh TV was then placed in the flask and incubated until the cells were seen to have detached (2-10 min) The TV solution was deactivated by the addition of pre-warmed basal medium containing serum The entire solution was then transferred to a 30 ml sterile universal tube (Sterilin 128a) and centrifuged at 1,000 rpm for 5 min The resulting pellet was then resuspended in pre-warmed growth medium, cells counted and tissue culture flasks re-seeded at the required density

2 6 2. Subculture of cells in suspension

Hybridoma and Sp/2 cells are partially adherent and mainly grow in suspension Cells were passaged by tapping the flask lightly and/or gentle pipetting with a 9 ml sterile pipette (Elkay, Ireland 100str) Cell suspensions were pooled and centrifuged at 1,000 rpm for 5 min Cell pellet was then resuspended in culture medium and a cell count performed (section 2 6 3) and the cells resuspended at the desired density Cells were grown in 5% CO_2

2 6 3 Cell counting

Cell counting and viability were carried using trypan blue (Gibco, 15250) dye exclusion technique An aliquot of trypan blue was added to a single cell suspension ant a ration of 1 2 After 3 min incubation at room temperature, a sample of the mixture was applied to the chamber of a haemocytometer over which a glass cover slip had been placed Cells in the 4 outer corner grids of the chamber were counted microscopically, an average per corner grid was calculated with the dilution factor taken into account and the final number multiplied by 10^4 to determine the number of cells per ml Non-viable cells were those which stained blue while viable cells excluded the trypan blue dye and remained unstained

Table 2.6.1.1. Cell line used in this study

Cell line	Source	Growth medium
Ovarian Carcinoma		ATC[1]
OAW42-SR	ETCC[3]	ATC
OAW42-S	U Gilvarry, NCTCC[4]	ATC
OAW42-A1	Dr A Redmond, NCTCC	ATC
OAW42-A	Dr I Cleary, NCTCC	ATC
OAW42-SR RZ2	D Byrne, NCTCC	ATC
OAW42-SR RZ4	D Byrne, NCTCC	ATC
OAW42-SR AS1	D Byrne, NCTCC	ATC
A2780	Prof R Scheper, Amsterdam	DMEM
A2780/AC12	Prof R Scheper, Amsterdam	DMEM
A2780/AC16	Prof R Scheper, Amsterdam	DMEM
Lung carcinoma		
DLKP	Dr G Grant, NCTCC	ATC
DLKP-A	Dr G Grant, NCTCC	ATC
DLKP-A10	Dr I Cleary, NCTCC	ATC
COR-L23S	Dr P Twentyman	RPMI 1640
COR-L23R	Dr P Twentyman	RPMI 1640
Nasal carcinoma		
RPMI 2650	ATCC[5]	MEM[2]

[1]ATC consists of 1 1 mixture of DMEM and HAMS F12, this was supplemented with 2mM l-glutamine, FCS and 1 mM sodium pyruvate for OAW42 cell lines
[2]MEM was supplemented with 1% (v/v) MEM Non-essential amino acids (NEAA) (Gibco, 043-01140) 2 mM l-glutamine, FCS and 1 mM sodium pyruvate
[3]ETCC European Tissue Culture Collection
[4]NCTCC National Cell and Tissue Culture Centre
[5]ATCC American Type Culture Collection

Table 2 6 1 2. Animal cells used in this study

Cell line	Source	Growth medium
Rat kidney		
NRK	[3]ATCC	[1]ACT
African Green Monkey Kidney		
BS-C-1	ATCC	[2]MEM
Hamster kidney		
BHK	ATCC	MEM
Bovine Kidney		
MDBK	ATCC	MEM
Canine kidney		
MDCK	ATCC	MEM
Mouse fibroblast		
MOP-8	Prof R Scheper Amsterdam	ATC

[1]ATC consists of 1 1 mixture of DMEM and HAMS F12, this was supplemented with 2mM l-glutamine, FCS and 1 mM sodium pyruvate for OAW42 cell lines
[2]MEM was supplemented with 1% (v/v) MEM Non-essential amino acids (NEAA) (Gibco, 043-01140) 2 mM l-glutamine, FCS and 1 mM sodium pyruvate
[3]ATCC American Type Culture Collection

2 6 4 Cell freezing

Cells of various passage numbers were frozen and cryo-preserved to serve as master stocks Vials could then be thawed and cultured for study

Cells to be frozen were harvested in the log phase of growth and counted as described in Section 2 6 3 The pellets were re-suspended in foetal calf serum (pre-cooled to 4^0C) and equal volumes of freezing medium (DMSO/serum 1 9 (v/v) was added dropwise to the cell suspension to give a final concentration of at least $5x10^6$ cells per ml 1 5 ml of the cell suspension was quickly placed in a cryovial (Greiner 122278) which was placed in the vapour phase of liquid nitrogen container for 2 5-3 5 hr After this, the cryovials were stored in liquid nitrogen until required

2 6 5 Cell thawing

Immediately prior to removal of a cryovial from the liquid nitrogen, a sterile universal tube containing growth medium was prepared for the rapid transfer and dilution of thawed cells (thus reducing the exposure time to DMSO which is toxic at room temp) Following the addition of the thawed cell suspension to the growth medium, the suspension was centrifuged at 1,000 rpm for 3 min after which the pellet was re-suspended in fresh growth medium A viability count was carried out (Section 2 6 3) and the thawed cells were placed in tissue culture flasks and allowed to attach over night, The following morning the cultures were re-fed with growth medium to remove any residual DMSO

2 7 *Mycoplasma* analysis

Mycoplasma examinations were carried out routinely (every 3 months) on all cell lines used in this study

2 7 1 Indirect staining procedure

Mycoplasma negative NRK cells (Normal Rat Kidney fibroblasts) were used as the indicator cells These cells were cultured with supernatant from test cell lines , fixed and stained with Hoescht fluorescent stain which binds specifically to DNA, thus it would stain the nucleus of the cell and any *Mycoplasma* infection which can be seen as small fluorescent bodied outside the cell

NRK cells were seeded onto sterile coverslips at a density of $2x10^3$ cells per ml and allowed to attach over night at 37^0C and 5 % CO_2 1 ml of cell free test supernatant was then inoculated onto each coverslip and the cells incubated as before until the culture reached 30-50% confluency Following this, the medium was removed and the coverslips rinsed twice in PBS and once in PBS/Carnoys (50 50) and fixed in Carnoys solution (acetic acid, methanol 1 3) for 10 min After the solution was removed the coverslips were allowed to air dry for 10 min The coverslips were then washed with twice with sterile water and stained with Hoechst 33258 stain (BDH) for 10 min Following 3 rinses with PBS the coverslips were mounted in 50% (v/v) glycerol in 0 05 M citric acid, 0 1 M disodium phosphate and examined using a Nikon phase contrast microscope fitted with a UV filter Positive controls consisted of NRK cells infected with *Micoplasma* enriched supernatants

2 7.2. Direct staining

The direct stain for *Mycoplasma* involved inoculating test samples onto an enriched *Mycoplasma* culture broth to optimise the growth of any contaminants and incubated at 37^0C for 48h Samples of this broth were then streaked onto plates of *Mycoplasma* agar base and incubated for 3 weeks at 37^0C and 5% CO_2 Plates were checked microscopically every 7 days for the growth of "fried egg" like colonies, which was indicative of *Mycoplasma* infection

2 8 Monoclonal antibody production

2 8 1 Immunisation procedure

2 Balb/C mice were injected 3 times in 6 weeks with $2x10^6$ OAW42-SR cells A further pair of Balb/C mice was injected with $2x10^6$ OAW42-S cells

2 8 2 Fusion Procedure

The fusion procedure was a modification of the protocol outlined by Koler and Milstein (1974) Prior to the removal the spleen from the sacrificed mouse, Sp/2 myeloma cells were prepared for cell fusion by harvesting from 75 cm^2 flasks and centrifuging at 1,000 rpm for 5 min in HEPES free serum free medium This step was repeated twice A cell count was then performed (Section 2 6 3) and the cells kept at 37^0C Balb/C mice were then sacrificed by cervical dislocation The animal was swabbed with 70% IMS and the spleen removed in a laminar flow cabinet with sterile dissection instruments Single cells were obtained by forcing the spleen through a sterile falcon cell strainer (Becton Dickinson 2360) using the plunger from a 10 ml syringe into serum free DMEM (the DMEM referred in this section is DMEM with Glutamax I (Gibco 61965-026) containing pyridoxine and without sodium pyruvate or HEPES, Glutamax is L-Amyl-L-Glutamine) This cell suspension was placed in a 50 ml centrifuge tube and the volume adjusted to 20 ml Large clumps of cells were allowed to pellet by standing at room temperature for 2-3 min The supernatant was then transferred to a fresh centrifuge tube and centrifuged at 1,000 rpm for 5 min A cell count was performed as before (Section 2 6 3) Splenocyte and Sp/2 myeloma cells were mixed in a 50 ml universal tube at a ratio of 10 1 (a minimum of $1x10^7$ Sp/2 are required for this procedure), centrifuged at 1,000 rpm for 5 min and resuspended in serum free medium This step was repeated twice Following the final washing step, 1 ml of PEG (polyethylene glycol, Boehringer Mannheim, 783641 pre-warmed to 37^0C) was added to the cell pellet with a Pasteur pipette using a gentle swirling and aspirating action for 30 sec After 30 sec the aspiration was discontinued After 75 seconds 0 5 ml of plating medium (DMEM with Glutamax I, 10 % heat inactivated FCS, 5 % Briclone and 1% HAT (Hypoxanthine,

Aminopterin, Thymidine, Boehringer Mannheim, 644579) was added slowly down the side of the 50 ml centrifuge tube while continuing to swirl gently 8 ml of plating medium was added over the next 5 min (3 ml at 1 min intervals followed by the addition of 5 ml) Following this step, the cell suspension was centrifuged at 500 rpm for 5 min The supernatant was removed and the cells were resuspended in 10 ml of plating medium and incubated at room temperature for 15 min 0 5 ml of plating medium was dispensed into each well into each well of 8x48 well plates (Coster, 3548) 1 drop of fused cells was added to each well and the plates were incubated for 12 days at 37^{0}C, 5% CO_2 for 12 days

2 8 3 Screening of hybridomas

OAW42-SR and OAW42-S cells were seeded at 3x10^4 cells per 100 µl of ATCC growth medium per well in 96 well tissue culture treated microtitre plates (Falcon, 3072, Becton Dickinson) and allowed to grow for 48 hour or until confluent The culture medium was discarded and the wells were washed with PBS Cells were then fixed with 0 5% glutaraldehyde (v/v), 150 µl per well, (Sigma, G6257) and incubated for 10 min at 4^{0}C to fix the cells The glutaraldehyde was then discarded and the wells were washed 3 times with PBS If the plates were not required immediately, they were stored in blocking buffer (1% w/v BSA, Sigma 3803, 0 1% sodium azide, BDH, in PBS, 150 µl per well) at 4^{0}C otherwise, plates were incubated at 37^{0}C for 1 5 hr in 0 1% (w/v) gelatine in PBS When large hybridoma colonies were observed in the 48 well plates 100µl of supernatant was aseptically removed, added to the cell coated plated and incubated at 37^{0}C for 1 hr This solution was then discarded and the plates washed with wash buffer (0 1% (v/v) Tween 20 (Merk) in PBS) 50 µl of secondary antibody, alkaline -phosphatase-linked rabbit anti-mouse immunoglobulins, IgG, IgM (Dako, diluted 1/10,000 in PBS 0 1% (v/v) Tween 20) was added to each well and incubated at 37^{0}C for 1 hr The secondary antibody was removed and the plated washed with wash buffer as before Plates were then incubated with the substrate solution (1 mg/ml p-nitrophenyl phosphate (PNPP, Sigma 104-0) in 0 1M glycine, 0 001% $MgCl_2$, 0 001 M $ZnCl_2$, pH 10 4) at 37^{0}C for 0 5-1 hr or until a yellow colour appeared in the wells The reaction was stopped by the addition of 1M NaOH which also enhanced to colour Absorbencies

were read on an Titerex ELISA plate reader at 405 nm Positive reactivity was determined by comparing supernatant containing wells with those which had been incubated with PBS instead of supernatant

2 8 4 Subculture of Hybridomas

Positive clones were further cultured to 6 well plates (Costar) and gradually transferred into medium containing HT (Hypoxanthine, Thymidine, Boehringer Mannheim, 623091) Eventually hybridoma clones were weaned off HT containing medium into DMEM supplemented with 5% Briclone and 10 % heat inactivated FCS

2.8 5 Single cell cloning by limiting dilution

Using a mulit-channel pipette (eppendorff) 100 µl of DMEM growth medium was pipetted into each well of a sterile 96 well tissue culture treated plate 100 µl of cell suspension from rapidly growing hybridomas at a concentration of $1x10^4$ cells was added to the top left hand well and mixed by pipetting 1 in 2 doubling dilutions were performed down the left hand row of the plate (8 wells, 7 dilution steps) and mixed by pipetting ensuring to change t pipette tip each time 1 in 2 dilutions were also performed across the plate using a multi-channel pipette Plates were then incubated for 7-10 days at 37^0C, 5 CO_2 Wells with a single colony were chosen Hybridomas were cultured to 25 cm^2 flasks and the procedure repeated The selected clones were screened by immunocytochemistry (Section 2 9 5) and frozen stocks made of positive clones

2 8 6 Propagation of hybridomas by ascitic tumour production

Prior to introducing the hybridoma cells into the peritoneal cavity, Balb/C mice were primed with 0 5 ml of Freunds incomplete adjuvant (Sigma P5506) into the peritoneum 24 hours later, $5x10^5$ hybridoma cells were aseptically resuspended in PBS (Oxide, 1 tabled dissolved in 100ml dH_2O yielding PBS pH 7 4), and injected inraperitoneally Tumour growth was evident 7-10 days post-injection The mice were sacrificed and the ascitic tumour fluid drained under sterile conditions by insertion of a sterile 21-gauge

needle into the peritoneal cavity The ascitic fluid was centrifuged at 3,400 rpm for 10 minutes and the supernatant carefully removed, aliquoted and stored at -20^{O}C Hybridoma cells were re-cultured in DMEM growth medium

2 8 7 Isotype analysis

Isotype analysis was carried out using the ELISA Isotyping Kit (Biorad) and the Isostrip mouse monoclonal antibody isotyping kit (Boehringer Mannheim, 1493027)
For the ELISA method, ascites was diluted 1/100 and 1/1000 in carbonate buffer pH 9 5 100µl of the ascites solution was added to each well of a chosen column and plates were incubated for 1 hr at 37^{0}C and then over night at 4^{0}C Mouse immunoglobulins were included as a positive control Plates were washed with wash buffer (0 1% (v/v) Tween 20 in PBS Non-specific sites were blocked with 1% (w/v) BSA in PBS for 1 hr at 37^{0}C The blocking buffer was then discarded and washed as before 100 µl of appropriate rabbit-anti-mouse immunoglobulin (i e anti-I_gG 1, 2a, 2b, 3, anti-IgM, anti-kappa, and anti-lambda chain etc) from the kit was added to each row (each column containing immobilised test antibody or standards) Plates were incubated at 37^{0}C for 1 hr Plates were then washed as previously described 100 µl of goat anti-rabbit immunoglobulin-alkaline phosphatase-conjugated antibody diluted in blocking buffer was then added to each well and incubated at 37^{0}C for 1 5 hr Following the washing step, PNPP in substrate solution was added to each well and incubated for 5-1 5 hr at 37^{0}C Colour enhancement was achieved by the addition of 0 2 M NaOH Plates were read o an ELISA plate reader at 405 nm

Isotype analysis was also carried out using the Isostrip method The isotyping strip bears immobilised bands of goat anti-mouse antibodies corresponding to the common mouse antibody isotypes and to kappa and lambda light chain The development tube also contains anti-mouse lambda and kappa light chain antibodies immobilised on latex beads which bind to all mouse monoclonal antibodies regardless of isotype Upon addition of the test strip to the development tube containing the teat antibody/latex bead complex, the complex travels up the strip until it binds to the goat anti-mouse antibody specific for the monoclonal isotype Results appear as a blue band in two sections corresponding to

the subclass and light chain type.

Hybridoma supernatants were diluted 1/100 and ascites 1/20,000 in PBS and incubated for up to 10 min at room temperature. Once the positive control bands were suitably developed, the black strip ant the base of the isotyping strip was removed to prevent any further band development.

2.8.8. Antibody purification

Monoclonal antibody was purified from ascitic fluid using the Immunopure (A/G) IgG Purification Kit (Pierce 44902). The protein A/G column was first equilibrated with 10 ml of binding buffer. Ascites was diluted 1:1 in binding buffer. The diluted sample was applied to the column and allowed to flow completely through the column and washed with 20 ml of binding buffer. The bound IgG was the eluted by the addition of 10 ml of elution buffer. The elute was collected as a 7 ml fraction. The column was regenerated by running thorough 8 ml of elution buffer followed by 10 ml of dH_2O with 0.02% (w/v) sodium azide and the column stored in this way. The pH of the elution buffer was raised to approximately 7.5 with the addition 3 ml of binding buffer. Elutes were then transferred to Slid-a-lyzer dialysis cassettes (Pierce 66406) and dialysed against 2 changes of PBS overnight on a stirring platform at 4^0C.

2.8.9. Determination of Protein concentration

Protein concentration was determined by the BCA method (Pierce, 23225). Protein samples (cell lysates or purified IgG) were diluted to a final volume of 100 µl in PBS. BSA protein standards of known concentration were also prepared from a 2mg/ml stock solution in PBS in borosicilliate test tubes. Negative controls consisted of PBS. 100 µl of the BCA reagent (prepared according to the manufacturers instructions) was added to each protein sample, mixed and incubated at 60^0C for 30 min. Following a brief cooling period, samples were transferred to plastic cuvettes (Elkay) and the absorbencies read on spectrophotometer at 540 nm. A standard curve of absorbance vs protein concentration

of the BSA standards was constructed The protein concentration of the test samples was then estimated

2 8 10 Determination of IgG concentration

IgG concentration was determined by radial immuno-diffusion RID (The Binding Site)

2 9 Immunological studies

2 9.1. Coating slides with poly-L-lysine

Washed microscope slides were coated with poly-L-Lysine hydrobromide (Sigma P1274) following the procedure of Huang *et al* (1983) Slides were stored at room temperature for up to 1 month

2 9 2 Preparation of cytospins

Cells from actively growing cultures were trypsinised (Section 2 6 1), washed 3 times in 10 min in PBS and diluted to a final concentration of $1x10^6$ cells/ml in PBS 100 µl of the cell suspension was then applied to modified eppindorf component of a cytofuge and spun onto poly-L-lysine coated glass slides at 400 rpm for 4 min Cytospins, consisting of 2 "sections" were allowed to air dry over night Slides were dipped in ice cold acetone at -20^0 C, allowed to air dry and stored wrapped in tin foil at -20^0 C until required

2 9.3 Immunofluorescence analysis on live cells

When immunofluorescence is carried out on live cells only cell surface components are detected All reagents were kept at 4^0C Cell suspensions at $1x10^6$ cells/ml were prepared in PBS 100 µl of cell suspensions was placed in an eppindorf tube mixed and incubated at 4^0C for 30 min Negative controls consisted of cell suspension incubated with mouse immunoglobulins (Vector Labs, I-2000), diluted 1/10,000 in PBS Cells were washed 3 times in 12 min in PBS by centrifuging at 2,000 rpm in a microfuge Cell pellets were resuspended in 100 µl of anti-mouse IgG FITC-labelled secondary antibody, diluted 1/50 in PBS, (Boehringer Mannheim 814385) and incubated in the dark for 30 min at 4^OC Cell suspensions were washed as described and following the final washing step the supernatant was removed Cells were then aspirated in 1 drop of Vectashield Mounting Medium (Vector Labs Ltd UK H1000) 20 µl of the suspension was placed on a microscope slide, covered with a coverslip and the edges sealed with nail polish Slides were viewed using a Nikon phase contrast microscope fitted with an FITC filter

For cytoplasmic antigens, cells were fixed with Facslyse (Becton-Dickinson) diluted according to the manufacturers instructions Cells were incubated for 1 min in the Facslyse solution

2 9 4 Immunofluorescence analysis on fixed cells

Indirect immunofluorescence on fixed cells was carried out on cytospin preparations (Section 2 9 2) Cytospins were fixed in ice-cold acetone (BDH) for 1,2,4,7 and 9 min and allowed to air dry at room temperature The test antibody was applied to one cytospin section on the cytospin while mouse IgG diluted 1/10,000 in PBS was applied to the other section The cytospins were incubated for 30 min at room temperature in a humidified chamber Slides were then washed in PBS 0 1% (v/v) Tween 20 3 times in 10 min 50 µl of an anti-mouse FITC-labelled secondary antibody diluted 1/50 in PBS was applied to each section and incubated at room temperature for 30 min Slides were washed as before 1 drop of Vecta Shield mounting medium was applied to the centre of the slide and mounted as described in Section 3 9 3

2 9 5 Immunocytochemical analysis using the StrepAB complex/HRP method

All immunocytochemical studies on cytospins or formalin fixed paraffin-embedded archival material were performed following the method of Hsu *et al*, (1981) using an avidin-biotin (ABC) horseradish peroxidase (HRP) conjugated kit The use of diaminobenzidine (DAB) as a substrate for the peroxidase enzyme produced an insoluble brown precipitate, which was indicative of primary antibody activity

Frozen cytospin preparations were allowed to equilibrate to room temperature (approximately 10-15 min) Slides were then fixed in ice cold acetone for 4 to 10 min, depending on the antibody, and allowed to air dry A wax pen (Dako S2002) was used to

encircle each "section" to contain the various solutions involved Cytospins were then incubated with 0 3% (v/v) hydrogen peroxide (BDH) in methanol (BDH) to quench endogenous peroxidase activity followed by a brief rinse in dH_2O and incubation in 1xTBS for 5 min Following this cytospins were incubated in 20% (v/v) normal rabbit serum (Dako X0902), in 1xTBS for 20 min at room temperature to block any non-specific binding of the secondary antibody (a rabbit host) The excess serum was tapped off and the optimally diluted primary antibody was applied to one of the cytospin sections while mouse immunoglobulins diluted 1/10,000 in 1xTBS served as a negative control Slides were placed in a humidified atmosphere and incubated at room temperature for 2 hr or at 4^0 C over night Following primary antibody incubation, cytospins were washed 3 times in 15 min in 1xTBS, 0 1% (v/v) Tween 20 (TBS/Tween) and then incubated with a biotinylated rabbit anti-mouse IgG secondary antibody (Dako E345) diluted 1/300 in TBS/Tween, for 30 mm at room temp Following this cytospins were washed as before in TBS/Tween The Strep AB complex (Dako K337 prepared according to manufacturers instructions) was applied to the cytospin and incubated at room temperature for 30 min Following the washing steps, liquid DAB (Dako K3565 prepared according to the manufacturers instructions) was applied to each "section" of the cytospin for 7-10 min at room temp Cytospins were rinsed in tap water, terminating the reaction, counterstained in Coles haemotoxylin (Sigma HHS-1S, 15 sec), differentiated in 1% (v/v) hydrochloric acid in methanol (15 sec) and differentiated in Scotts tap water (10 sec) rinsing in water between each step Cytospins were then dehydrated in graded alcohols (2 x 3 min in 70 %, 90% and 1005 (v/v) IMS in dH_2O and cleared in xylene (BDH, 2 changes of 5 min each) Cytospins were mounted in DPX mounting medium (BDH) and allowed to dry over night Cytospins were viewed under a light microscope

For 3/B6/LMR-5 competition experiments, cytospins were incubated with either 3/B5, LMR-5 or the anti-vault polyclonal antibody N2 for 2 hours at room temperature followed by 3 washed in 1 x TBS/Tween and a second with MAb 3/B6 LMR-5 or N2 polyclonal antibody overnight at 4^0C To determine the specificity of species-specific secondary antibodies, the mouse primary antibody, 3/B6 was incubated with the rabbit anti-rat specific (Dako E0468) and the goat anti-rabbit specific (E0432) specific biotinylated secondary antibodies This experiment was repeated for the rat primary antibody LMR-5 (cytospins were probed with an anti-mouse secondary (Dako E0464)

and the anti-rabbit secondary biotinylated antibody) and the rabbit polyclonal antibody N2 (cytospins were probed with the anti-rat and anti-mouse specific biotinylated secondary antibodies) Cytospins were then processed as outlined above

A modified protocol was use for formalin-fixed paraffin-embedded histological material, slides were de-waxed in 2 changes of xylene (4 min each, BDH) and cleared in graded alcohols (IMS, 100%, 90% and 70%, 2 X 4 min each), washed briefly in ultrapure and then in 1 X TBS for 5 mm Slides were then incubated for 5 min in 0 3% (v/v) hydrogen peroxide in ultrapure water to quench endogenous peroxidase activity Following all antibody incubations, sections were first washed gently with 1 X TBS 0 1% (v/v) Tween 20 from a wash bottle before standard wash procedures Slides were then viewed by Dr Peter Kelehan, Dept of Pathology, The National Maternity Hospital, Holles St Dublin)

Table 2 9 5 Antibodies used in immunocytochemical analysis

Antibody	Source	Concentration	
		Cytospins	Paraffin-embedded tissue sections
3/B6		4-6 µg/ml	10-15µg/ml
LRP-56	Prof Rik Scheper	5µg/ml	10µg/ml
LMR-5	Prof Rik Scheper	2µg/ml	4-6µg/ml
MRP-r1	Prof Rik Scheper	2µg/ml	5µg/ml
UIC-2	commercial	ND	2µg/ml
MDR-1 (clone 6/1C)	BioResearch Ireland	ND	1/50 dilution of mouse ascites
Anti-vault poly clonal antibody	Prof L Rome	1/100 dilution of rabbit serum	ND

2 10 Protein analysis by Western Blotting and immunoprecipitation

2 10 1 Sample preparation for gel electrophoresis

Cells were prepared in PBS as outlined in section 2 9 3 Protein inhibitors were also added to the cell suspension (protease inhibitor cocktail tablets, Boehringer Mannheim 1697498) Cells were lysed by sonication Complete lysis was confirmed by viewing under an inverted microscope Protein concentration was determined by the BCA method (Section 2 8 9) Protein samples were prepared in loading buffer (2 5 ml 1 25M-Tris/HCL pH 6 8, 1 0g SDS, 2 5 ml mercaptoethanol (Sigma M6250), 5 8 ml glycerol and 0 1% bromophenol blue (Sigma B8026) and boiled for 3 min Samples were stored at -20 until required for a maximum of 1 month

2 10.2 Gel electrophoresis

Proteins for Western blotting were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, Laemmli *et al*, 1970) Gels were prepared as outlined in table 2 10 1 and poured into clean 10cm x 8cm gel casting cassettes (Mighty Small II, Hoefer) which consisted of 1 glass plate and 1 aluminium plate separated by 0 75cm^2 plastic spacers The resolving gel was poured flat and allowed to set A layer of saturated isopropyl alcohol was gentle layered over the resolving to prevent drying out The stacking gel was then poured and a comb was fitted allowing the formation of wells for sample loading Once the gels had set (at room temperature), they were immediately wrapped in tinfoil and stored at room temperature or at 4^{0}C if not used immediately 10-15 µg of protein was applied to each well of the polyacrylamide gel Empty wells were loaded with 1 x loading buffer to ensure even running of the gel Pre-stained molecular weight markers (Sigma, SDS-6H) were also loaded onto the gel for he determination of the molecular weight of unknown protein samples Gels were run at 250 volts and 45 mih-amps (Atto power pack, Atto Corp , Japan) for 1-1 5 hr (values were halved if only one gel was being run) When the bromophenol dye front had reached the end of the gel,

electrophoresis was stopped, the gel removed and equilibrated in transfer buffer (Tris-Glycine, 10X, BDH) for 15 min

Table 2 10 2 Preparation of electrophoresis gels

Stock solutions	Resolving gel (7 5% acrylamide	Stacking gel (5% acrylamide)
[a]Acrylamide/ bis-Acrylamide stock	3 8 ml	0 8 ml
dH_20	9 1 ml	4 0 ml
[b]8X Tris-HCl pH 8 8	1 88 ml	
[c]16X Tris-HCl pH 6 8		0 3 ml
[d]10% SDS	150 µl	50 µl
[e]10% Ammonium persulphate	60 µl	17 µl
[f]TEMED	10 µl	5 µl

a 30% Acrylamide/ 1 034% bis-Acrylamide (Scotlab, Anachem SL-9232)

b PAGE resolving gel buffer (BDH 444092L)

c PAGE stacking gel buffer (BDH 444102T)

d 10% SDS (BDH) (W/V) in DH_2O

e 10% Ammonium persulphate (Sigma A-3678) (w/v) in dH_2O

f TEMED [N,N,N,N-Tetramethylethylenediamine] (Sigma T-9281)

2 10 3. Western Blotting

Western Blotting was carried out according to the protocol of Towbin *et al* (1979) 8-10 sheets of Watman filter paper (Grade I) were soaked in transfer buffer (Tris-glycine, 10X BDH 444145H diluted 1 in 10 in dH_2O) and placed on the anode of a semi-dry blotting apparatus (Biorad Transblot SD) taking care to remove all bubbles by rolling a glass pasteur pipette across the sheets PVDF membrane (Boehringer Mannheim, 972206, pre-soaked according to the manufacturers instructions) was placed over the filter paper and the gel laid on top of this A further 8-10 sheet of filter paper were paced over the gel and any air bubbles removed The cathode was carefully laid on top of the stack and the proteins transferred form gel to PVDF membrane at 15 volts and 34 mili-amps for 25 min

2 10 4 Development of western blots by enhanced chemiluminescence (ECL)

Following Western Blotting, the filter paper was removed and the membrane blocked for non-specific binding by incubating for 2 hr on a rocking platform with non-fat milk (Marvel, Cadbury) After the blocking step was complete, the blot was rinsed once in TBS (0 05 M Tris-HCl pH 7 5), cut into strips (if required) and incubated with the primary antibody optimally diluted in TBS/Tween over night at 4^0 C on a rocking platform Mouse IgG diluted 1/5,000 in TBS/Tween served as a negative control The following day blots were washed 3 times within 30 min with TBS 0 5% (v/v) Tween 20 Blots were then incubated with a rabbit anti-mouse HRP-conjugated secondary antibody (Dako, P0447) diluted 1/4,000 in TBS/Tween for 1 hr at room temperature on a rocking platform The Blots were washed as before, laid out on a glass plate covered in parafilm and incubated with ECL reagents (Amersham RPN 2109) for 1 min at room temp Super Signal Ultra (Pierce) was mixed according to the instructions and applied to the blots for 5 min at room temperature Following this the solution was tapped off, the blot laid between two sheets of cling film and exposed to LUMI film, chemiluminescence film (Boehringer Mannheim 166657) for various time periods and processed using standard x-ray developing procedures Dried film was aligned with prestained molecular markers for molecular weight determination

This protocol was modified for biotin-labelled immunoprecipitates PVDF were incubated in 1 5% BSA as it had been demonstrated previously that non-fat milk contains endogenous biotin, which caused non-specific bands to appear, (Section 2 10 5) Following the blocking step, blots were rinsed once in 1xTBS and placed in an anti-biotin secondary antibody diluted 1/4,000 in TBS/Tween for 1 hr at room temperature on a rocking platform and processed as above

2.10 5. Immunoprecipitation

Immunoprecipitation of specific proteins was carried out using a new cellular labelling and immunoprecipitation kit (Boehringer Mannheim 1647652) The protocol was extensively modified for the purposes of this study (Masterson, Moran, Scheper and Clynes, in press)

The principle of this procedure is based non-radioactive labelling of proteins or glycoproteins on intact cells (or in cell lysates) with D-biotinoyl-ε-aminocarporic acid-N-hydroxysuccinimide ester (biotin-7 NSH) which covalently binds free amino groups (mainly lysine residues) These labelled proteins can then be immunoprecipitated and detected using a biotin specific antibody

Unless stated otherwise, all reagents were supplied in the immunoprecipitation kit The modified procedure was carried out as follows, whole cell lysates were prepared from cell pellets containing a maximum of $5x10^7$ cells/ml Cell lysates were prepared in lysis buffer and sonicated as outlined in Section 2 10 1 Following a 20 min incubation period in lysis buffer (to solubilise membrane protein), cell lysates were labelled with biotin-7-NHS for 15 min at 4^0 C This reaction was stopped by the addition of stop reagent for a further 15 min at 4^0 C Cell lysates were then diluted 1 1 with dilution buffer and pre-cleared by incubating with 2 changes of Protein A-linked Agarose beads (2x4 hours) following the final incubation step, the primary antibody was optimally diluted in the cell lysates (anti-OAW42-SR MAb 3/B6 final concentration of 40 µg/ml, LRP-56, 15 µg/ml, mouse immunoglobulins at a concentration of 15 µg/ml served a negative control) and incubated at 4^0 C for 1 hour to over night on a rocking platform These incubation times

were based on the protocol of Scheper *et al* (1993) The following day 50 µl of protein A/G-linked agarose beads were added to the cell lysates and incubated for various time periods at 4^0 C on a rocking platform to precipitate the antibody-antigen complex Pelleted beads were then washed with two changes of wash buffer 1, one changes of wash buffer 2 or 2 changes of a low salt buffer (100 mM NaCl) and two changes of wash buffer 3 Following the removal of any remaining supernatant, pellets were resuspended in 60 µl or loading buffer and prepared for gel electrophoresis as outlined in section 2 10 2 followed by western blotting, section 2 10 3 and blot development using ECL section 2 10 4

For competitive immunoprecipitation experiments, supernatants from over-night precipitations were carefully removed concentrated to 1/5 of their original volume (section 2 10 6), and subjected to SDS-PAGE (section 2 10 2) and Western Blotting (2 10 4)

For glycosylation studies, OAW42-SR and SW1573/2R120 cells were seeded in 75 cm^2 tissue culture flasks and allowed to attach for 4 hours at 37^0C Following this incubation period, Tunicamycin (preventing N-glycosylation Sigma T3843) was added to the flasks at a final concentration of 8 µg/ml of growth medium PNAG (preventing O-glycosylation was added to different flasks at a final concentration of 2 5 mM/ml of growth medium Negative controls consisted of OAW42-SR and SW1573/2R120 cells grown without glycosylation inhibitors Flasks were incubated for 16 hours at 37^0C Following this incubation period, the growth medium containing the glycosylation inhibitors was removed and the monolayers washed three times with sterile PBS Cells were then prepared for immunoprecipitation as outlined above

2 10 6 Immunoprecipitation experiments with OAW42-SR culture medium

To determine whether the 3/B6 antigen or the MVP/LRP was shed into culture medium, 10 ml of spent medium was taken from cell cultured for 6 days in 75 cm^2 tissue culture flasks Samples were concentrated 1/10 in Ultrafree 15 concentrators (Milipore UFV2BGC10,) MAb 3/B6 and LRP-56 were added at a concentration of 40 µg/ml and 15 µg/ml respectively and incubated overnight at 4^0C on a rocking platform Samples were then processed as previously described (Section 2 10 5)

2 10 7 Micro-sequencing of 3/B6 protein samples

3/B6 immunoprecipitates of OAW42-SR cells were prepared as previously described (Section 2 10 5) and applied to polyacrylamide gels (Section 2 10 2) Following this procedure, the gel was halved and one half was stained with Instaview SDS rapid stain (BDH 444313F) When the staining procedure was complete, the 2 halves of the gel were re-aligned The area of unstained gel corresponding to the stained 3/B6 band was excised, minced, placed in 0 5 ml of elution buffer (2 38% (w/v) Hepes, 0 1% (w/v) SDS pH 8 0, adjust pH with 1 N NaOH) and incubated over night at room temperature The following day, the gel pieces were centrifuged in a microfuge at 13000 rpm for 5 min The supernatant was saved and a further 0 25 ml of elution buffer added to the gel pieces, and microfuged as before The protein concentration of the sample was determined as previously described (Section 2 8 9) From this method, excised bands consistently yielded a minimum of 15µg of the 115 kDa 3/B6 protein

Gel fragments were prepared for sequencing in the following manner Following gel electrophoresis, gels were stained with Coomassie Brilliant-Blue solution [0 025% (w/v) Coomassie Brilliant Blue (Sigma), 40% (v/v) Methanol (BDH), 7% (v/v) acetic acid (BDH)] for 10 min at room temperature on a stirring platform The gel was then destained (2 changes) to remove background staining with destains solution (10% (v/v) acetic acid 30% (v/v) ethanol (BDH) for 30 min The 3/B6 band was removed and stored at sent to Eurosequence qv (Groningen, The Netherlands) for internal sequencing

Following trypsin protein digestion of the eluted band, peptides were purified be high performance liquid chromatography (HPLC) Purified peptides were then subjected to Edman degradation (Edman 1956 Ilse and Edman 1963) Phenylisocyanate (Edman's reagent) reacts with the N-terminal amino acid groups of proteins under alkaline conditions to form their phenylthiocarbamyl adduct This produce was then treated with anhydrous hydrofluoric acid which cleaves the N-terminal residue as its thiazolinone derivative but does not hydrolyse other peptide bonds These residues were then selectively derivatived to more stable phenylthiohydantion derivatives and sequenced in an automated pulsed-liquid sequencer (Applied Biosystems model 477A) Stepwise released phenylthiohydantion amino acids were identified by on-line RP-HPLC on the basis of their elution times Calibrations were in the form of known derivatised amino acids

2.11 Effect of MAb on cultured cells

This experiment was carried out according to the protocol of Yang and Page (1995) Heat inactivated FCS was used in the culture medium (heat inactivation was achieved by incubating aliquots of FCS at 50 ^{0}C for 30 min) OAW42-SR cells were seeded at densities of 1 X 10^4/well and cultured overnight at 37^{0}C, 5% CO_2 in 96 well tissue culture plates The following day, growth medium was removed and fresh growth medium containing varying concentrations of MAb 3/B6 were added to each column of the plate Controls consisted of OAW42-SR cells grown in the absence of MAb and in the presence of mouse IgG control antibody (Pierce) diluted over the same range of concentrations as MAb 3/B6 Plates were prepared in duplicate and incubated for 6 days as before Cell density was determined using acid phosphatase end-point assay (Martin and Clynes, 1991) 100 µl of assay substrate (10 mM p-nitrophenyl phosphate (Sigma p104) in 0 1 M sodium acetate (Sigma S-2889), 0 1% Triton X-100 (Sigma X100) was added to each well The assay substrate was prepared just before use with the pH adjusted to pH 5 5 The plates were incubated for 2 hours at 37^{0}C in 5 % CO_2 and read in a dual beam ELISA plate reader at a wavelength of 405 nm (reference wavelength 620

nm) Colour enhancement was achieved by the addition of 50µl of 1 5 N NaOH

2 12 Drug accumulation studies with MAb 3/B6 and LRP-56

The accumulation of adriamycin in the presence of monoclonal antibodies was measured by a modification of Ganapathi and Grabowski (1983) and Versantvoort *et al* (1992) OAW42-SR cells were grown in 75cm^2 flasks until approximately 80% confluency was achieved Cells were then trypsinised and 1 ml was added at a concentration of 4 X 10^5 cells/ml to each well of a 6 well cluster plates (Costar 3616) A further 3 ml of complete medium was added to each well and the cells incubated at 37^0 C, 5 % CO_2 over night The following day the medium was decanted and 1 ml of DMEM containing 100µg/ml of MAb 3/B6 or LRP-56 for 30 min at 37^0 C This medium was then decanted and 4 ml of complete medium containing 10µM adriamycin or adriamycin/cyclosporin A, 10µg/ml was added to the plate and incubated at 37^0 C in 5 % CO_2 A series of controls were used where cells were grown in the absence or presence of monoclonal antibody, adriamycin or the circumvention agent, cyclosporin A or combinations of these reagents After specific time intervals, the drug-containing medium was decanted and the cells were rinsed twice in ice-cold PBS 2 ml of ice-cold ultrapure water was added to the wells and left for approximately 5 min to facilitate lysis of the cells The adriamycin was the extracted from the cells by the addition of 2 ml of 0 6 N HCL-Methanol solution for approximately 5 min The resulting solution was transferred to a universal container and centrifuged at 4,000 rpm for 10 min at 4^{0}C The supernatant was collected and the fluorescence of each solution determined using a Perkin-Elmer LC50 luminescence spectrometer with an excitation wavelength of 470 nm and an emission wavelength of 585 nm Slit widths for excitation and emission were 10 nm and 15 nm respectively The concentration of adriamycin present in each sample was quantified from a linear standard curve prepared form the fluorescence of known adriamycin concentrations

3 0 Results

3 1 Production and purification of monoclonal antibodies

3 1.1 Production of monoclonal antibodies

Using whole cell preparations of the resistant cell line OAW42-SR and its resistance clone OAW42-S, an immunisation schedule was carried out as described in section 2 8 2 of Materials and Methods The fusion rate for both immunisations was approximately 95%

Following three preliminary screenings of hybridoma supernatants 6 appeared to consistently reacted with the OAW42-SR cells while 5 reacted with the OAW42-S cells Of the 6 anti-OAW42-SR clones, 2 clones designated 3/B6 and 5/C4 were chosen for further characterisation while 1 anti-OAW42-S clone, designated 3/E3 was also chosen for characterisation In initial experiments MAb 3/B6 did not cross react with OAW42-S cells, subsequent experiments showed that it did cross react This suggests that there may have been low concentrations of MAb in the initial test supernatants Longer culture times would have increased the amount of MAb being secreted by the hybridoma clones

3 1 2 Isotyping of monoclonal antibodies

Supernatants from hybridoma clones 3/B6 5/C4 and 3/E3 were used to determine the class and subclass of the MAb using an antibody isotyping kits as described in section 2 8 7 of Materials and Methods Results indicated that the isotype of the of the 3 MAb was I_gG class, subclass 1

3 1 3 Cloning of Hybridomas by limiting dilution

Following cloning by limiting dilution, four 5/C4 clones were isolated and further characterised by immunocytochemistry Each clone gave similar granular outer cytoplasmic staining therefor it was decided to choose clone 5/C4 E9B for further characterisation based on absorbance results by ELISA, (immunocytochemistry results not shown)

Nine 3/B6 clones were isolated and characterised in the same manner Clone 3/B6 1E3 was chosen for further study and recloned, (immunocytochemistry results not shown)

3 1 4 Propagation of hybridomas by ascitic tumour production

Ascitic fluid and hybridoma cells were removed from the peritoneal cavity of Balb/C mice after 7 days as outlined in section 2 8 6 of Materials and Methods Hybridoma cells were successfully separated from the ascitic fluid and re-cultured Approximately 5 ml of ascites was obtained from clones 3/B6 and 3/E3

This technique proved unsuccessful for clone 5/C4 Approximately 4 days after the I P injection the mice became unhealthy and were sacrificed immediately 2 ml of ascitic fluid was obtained from the mouse which reacted weakly with OAW42-SR cell by ELISA This may have been a problem with the mice however mice are generally in a suitably healthy state prior to ascites production Hybridoma cells may have been carrying bacterial or fungal infections which may have affected the health of the mice Cells recovered from the ascitic fluid did not survive in culture It was decided therefore to use culture supernatant from the 5/C4 hybridoma in subsequent experiments There appeared to be sufficient quantities of MAb in these culture supernatants and it was not necessary to concentrate them

3 1 5 Determination of antibody concentration

Antibody concentration in the ascites was determined using RID plates (section 2 8 10 materials and methods) Results showed that 3/B6 ascites contained approximately 2 5 mg/ml I_gG while clone 3/E3 contained an antibody concentration of 2 7 mg/ml Culture supernatant from clone 5/C4 was not assayed by this method

3 1 6 Purification of ascites from hybridoma clone 3/B6

MAb was purified on protein A/G sepharose mini-columns as outlined in section 2 8 8

Initial purification steps resulted in a low yield of I_gG concentration, approximately 80 µg/ml Yields were increased in subsequent purification procedures by omitting the desalting column and dialysing the buffered antibody solution against 1 X TBS (0 5 M pH 7 5) This resulted in yields of between 200 and 210µg/ml I_gG concentration The loss of MAb could have been due to incorrect elution from the desalting column

3 2 Characterisation of MAb 3/B6

3 2.1. Indirect immunofluorescence on live cells with MAb 3/B6

Immunofluorescence studies were carried out on live cell preparations of OAW42-SR with MAb 3/B6 and LRP-56 (section 2 9 3 materials and methods) Results are illustrated in Figure 3 2 1 1

MAb 3/B6 recognised a cell surface antigen, indicated by a fluorescent ring around the cell No staining was observed when OAW42-SR cells were incubated with the anti-LRP/MVP antibody LRP-56 Similarly, no plasma membrane staining was observed in the negative control (OAW42-SR cells incubated with mouse control IgG) These results indicated that MAb 3/B6 recognised a surface antigen while confirming previous observations that the LRP/MVP is not expressed on the cell surface

A series of multidrug resistant/sensitive cell lines were screened with MAb 3/B6 in order to further examine the staining pattern of this antibody, results are outlined in figures 3 2 1 2 and 3 2 1 3 and table 3 1 2

It appeared from these studies that MAb 3/B6 detected a cell surface antigen which was expressed primarily in low level resistant cell lines of different histological origins Heterogeneous staining was observed on the OAW42-SR, DLKP, DLKP-A, A2780, SW1573/2R120, OAW42-A1 and OAW42-A cell lines Approximately 80% of OAW42-SR cells had strong plasma membrane staining while a greater degree of heterogeneity in staining was observed on the DLKP and DLKP-A cell lines with 30% and 10% of cells stained Similar results were observed with the SW1573-2R120 cell line The A2780 line exhibited weak membranous staining on approximately 20% of cells The OAW42-A1 and OAW42-A cell lines also had mainly weak staining with approximately 10% of cells staining intensely Examination of the COR-L23R cell line revealed intense membranous fluorescent staining on all cells This was also observed on the negative control All other negative controls had negligible staining

Table 3 2 1 Expression of the antigen detected by anti-OAW42-SR MAb 3/B6 on a panel of human MDR human sensitive and resistant cell lines by indirect immunofluorescence on live cells

Cell line	% of positive cells
OAW42-SR	80% of cells stained positive, 60% of cells with strong plasma membrane staining ~
OAW42-S	90% of cells with weak plasma membrane staining
OAW42-A1	10% with weak plasma membrane staining
OAW42-A	10% have weak plasma membrane staining
DLKP	30% of cells had plasma membrane staining
DLKP-A	10% of cells stained, 5% had strong plasma membrane staining
A2780	20% of cells were stained with weak plasma membrane staining
SW1573/2R120	30% of cells stained, 10% have strong plasma membrane staining
COR-L23R	all cells had strong plasma membrane staining including the negative control

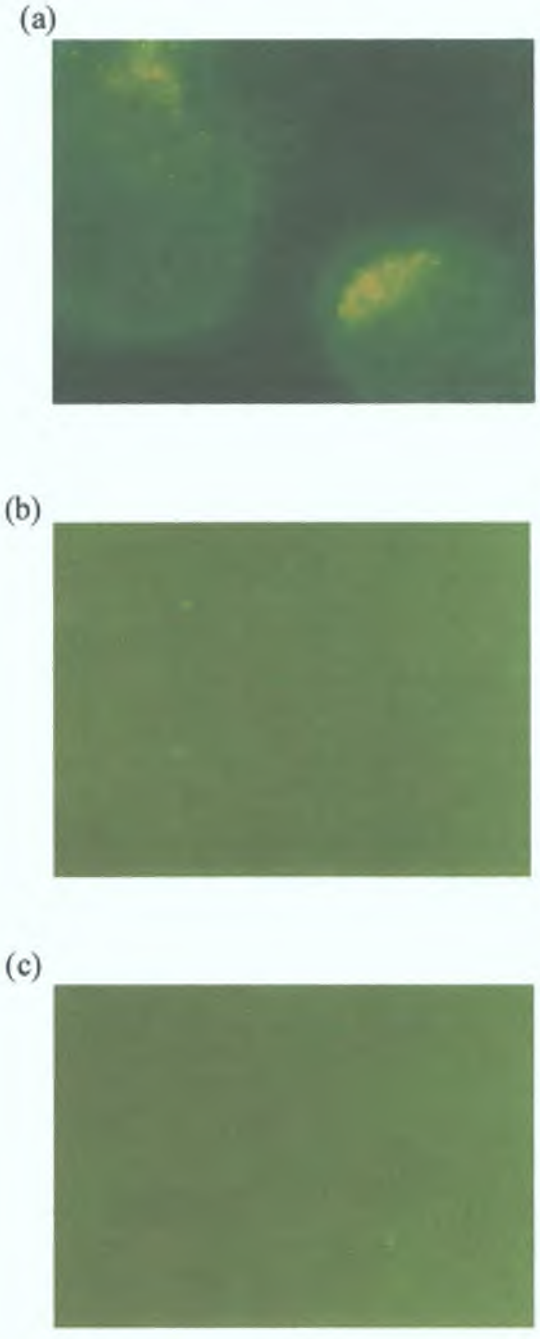


Figure **3.2.1.1**. Immunofluorescence studies on live cells (section 3.2.1.). OAW42-SR cells probed with MAb 3/B6 and LRP-56 detected using an FITC-linked secondary antibody, (a) OAW42-SR probed with MAb 3/B6, (b) OAW42-SR probed with MAb LRP-56, (c) negative control, OAW42-SR probed with mouse IgG. Photographs taken at 40X.

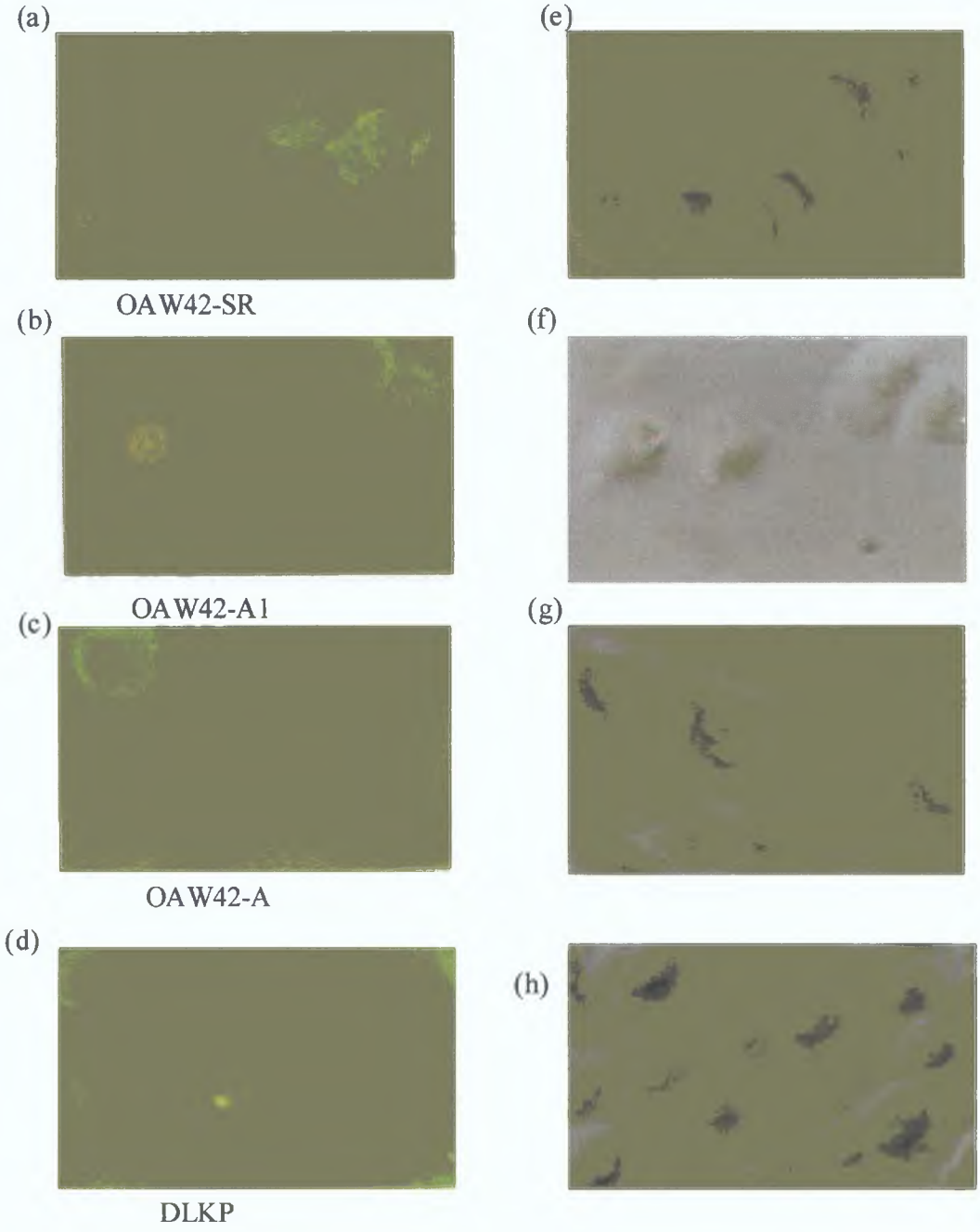


Figure 3.2.1.2. Indirect immunofluorescence studies on live cells with MAb 3/B6 (section 3.2.1.). MAb 3/B6 was detected with a FITC-labelled secondary antibody. (a)-(d) were taken with FITC filter, (g)-(h), were taken under normal light (40X).

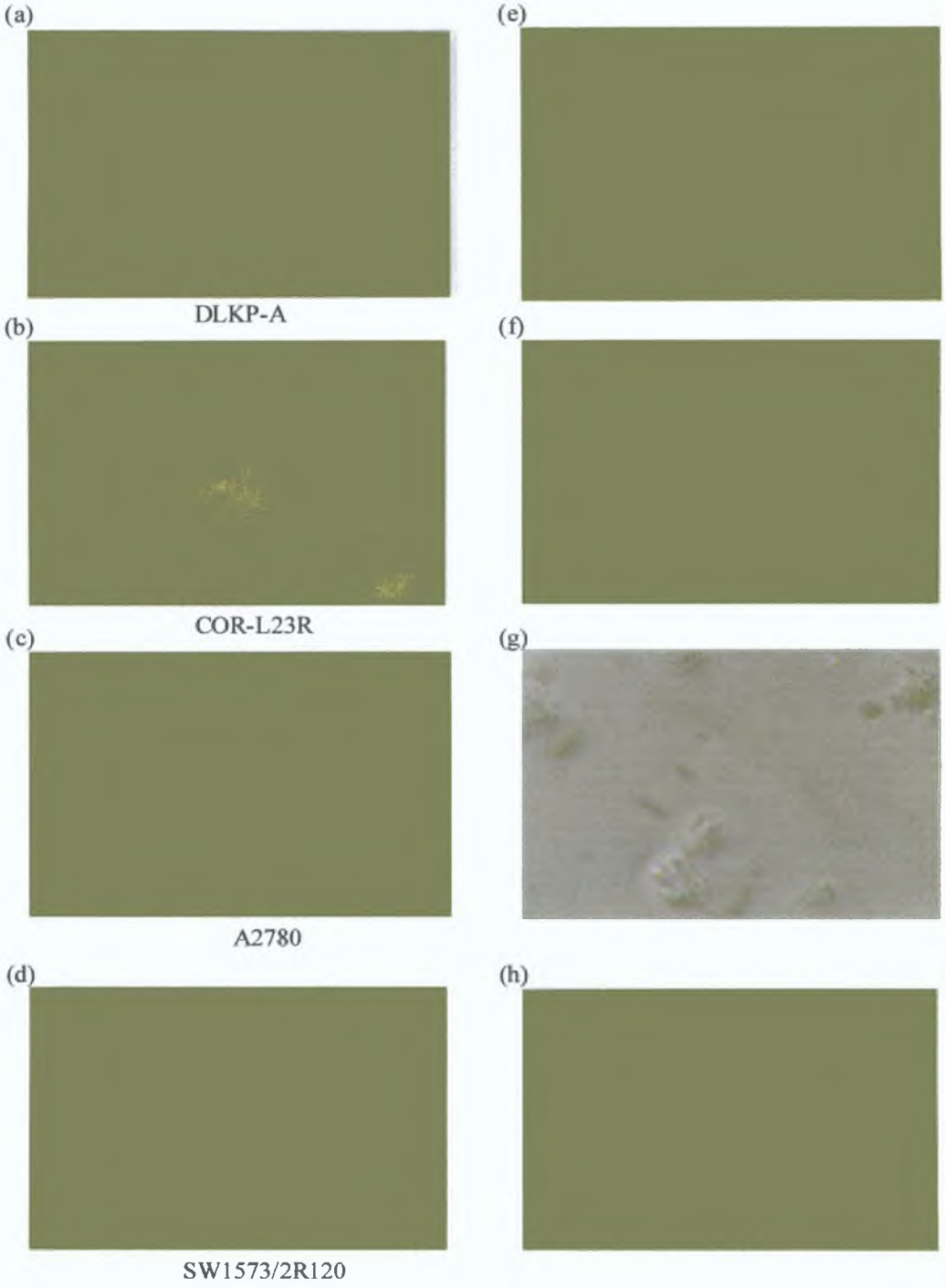


Figure 3.2.1.3. Indirect immunofluorescence studies on live cells with MAb 3/B6 (section 3.2.1.). MAb 3/B6 was detected with a FITC-labelled secondary antibody. (a)-(f) taken with FITC filter, (e), (f) and (h) are negative controls, cells probed with mouse IgG (g) was taken under normal light, (40X).

3 2.2 Indirect immunofluorescence on fixed cells

Acetone fixed cytospins of OAW42-SR and OAW42-S cells were probed with MAb 3/B6 to investigate if the antigen recognised by this antibody was also expressed intracellularly MAb LRP-56 was included as a control (section 2 9 4 materials and methods) The optimum fixation time was found to be 4 min in ice cold acetone

Granular cytoplasmic and plasma membrane staining was observed when OAW42-SR cells were probed with MAb 3/B6, Figure 3 2 2 The staining appeared to be evenly distributed throughout the cytoplasm No staining was observed on the negative control In contrast, there appeared to be a greater degree of plasma membrane staining when this antibody was used to probe the OAW42-S cells but similar fluorescent staining was also observed on the OAW42-S negative control The anti-LRP MAb LRP-56 stained OAW42-SR and OAW42-S cells, with fluorescent staining localised mainly to the peripheral cytoplasm These results appeared to correlate with those observed in section 3 2 1 for live cell preparations of OAW42-SR and OAW42-S Similarly, it was also confirmed that MAb LRP-56 did not stain the plasma membrane of the OAW42 variants

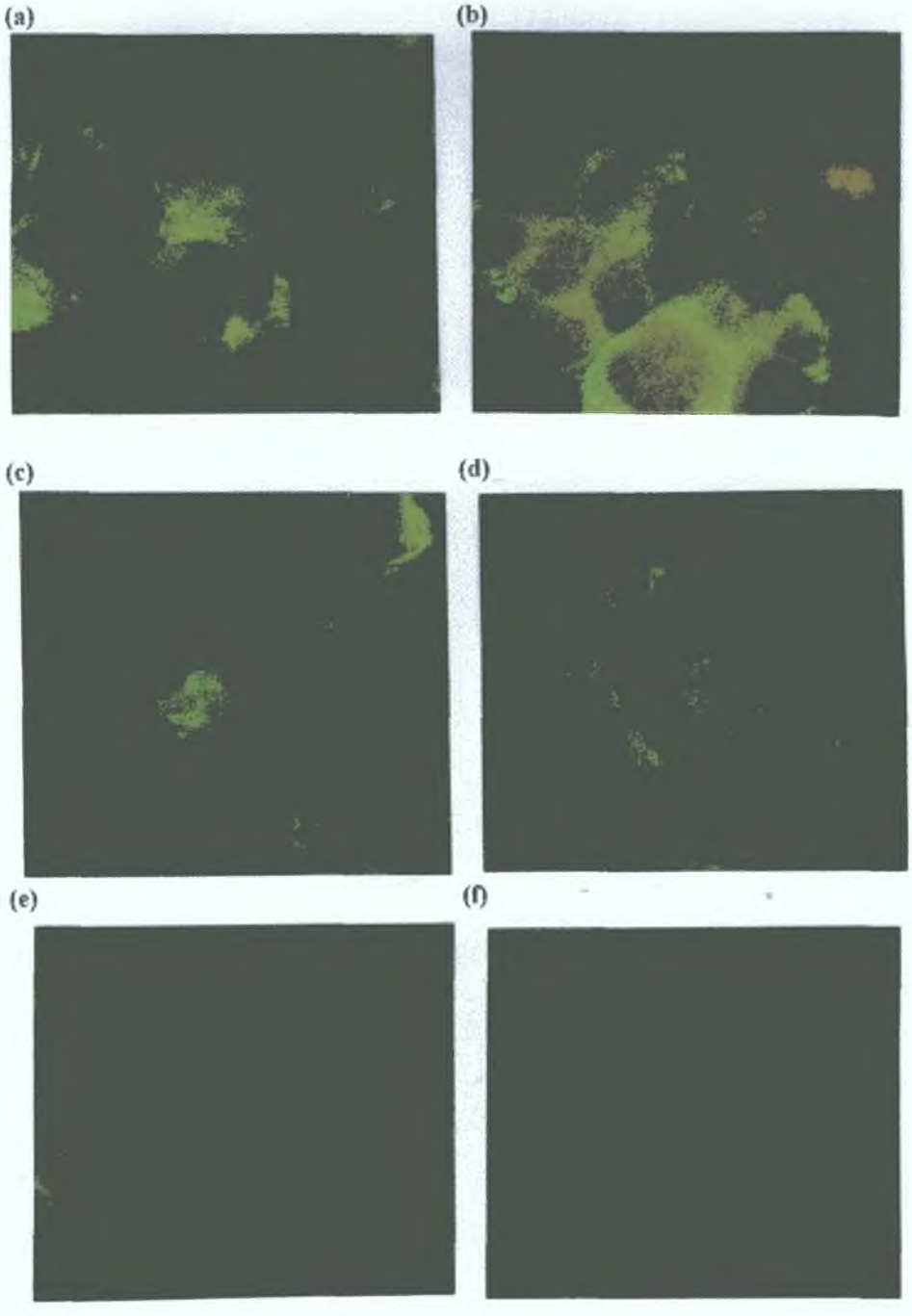


Figure 3.2.2. Immunofluorescence studies on OAW42 variants with MAb 3/B6 and LRP-56 (section 3.3.2.). (a) OAW42-S probed with 3/B6, (b) OAW42-S probed with LRP-56, (c) OAW42-SR probe with 3/B6, (d) OAW42-SR probed with LRP-56, (e) and (f) are negative controls, OAW42-S and OAW42-SR respectively probed with mouse IgG. Photographs were taken at 40X.

3.2.3. Immunocytochemical analysis of MDR cell lines using the ABC method

3.2.3.1. OAW42 variants

OAW42-SR and OAW42-S cell lines were re examined with MAb 3/B6 and LRP-56 using the ABC/HRP method (outlined in section 2.9.5.). Results are illustrated in Figure 3.2.3.1

Approximately 80% of OAW42-SR cells stained positively with MAb 3/B6. The majority of cells exhibited granular cytoplasmic staining which appeared to be evenly distributed in the cytoplasm, plasma membrane staining was also observed on these cells. MAb 3/B6 also stained approximately 80% of OAW42-S cells. Clear plasma membrane staining was observed in this cell line with diffuse granular cytoplasmic staining in some cells.

MAb LRP-56 stained the cytoplasm of OAW42-SR and OAW42-S cells. In OAW42-SR cells, the staining was mainly located in the periphery whereas in the OAW42-S cells, the staining was more diffuse. No plasma membrane staining was observed with this antibody. There was no significant staining observed in the negative controls. These results were similar to those observed in the previous section.

3.2.3.2. Screening of other cell types

To further investigate the expression of the antigen recognised by MAb 3/B6, a panel of multi-drug resistant human and murine tumour cell lines were examined with MAb 3/B6 and LRP-56 by immunocytochemistry (which was included for comparison). Results are outlined in table 3.2.3.2. and Figures 3.2.3.2.1-3.2.3.2.4. MAb 3/B6 reacted positively with the majority of cell lines in this panel but with different degrees of intensity.

Strong granular cytoplasmic and plasma membrane staining was observed in the LRP positive cell line SW1573/2R120 with 90% of cells showing positive staining, while the parental SW1573 and 2R160 variant were stained to a lesser degree when these cell lines were probed with 3/B6. In the A2780 series of variants, 3/B6 stained 90% of the LRP cDNA transfected line AC16 whereas the AC12 variant (transfected with a partial cDNA

sequence) only 30% of cells were stained The staining was predominantly located in the plasma membrane which differed from the parental (untransfected) A2780 cell line where 10% of cells showed low level granular cytoplasmic staining GLC4/S and GLC4/ADR cells also displayed predominantly plasma membrane staining with 30% of GLC4/ADR and 5% of GLC4/S cells staining positively with 3/B6 No 3/B6 reactivity was observed in the MRP over-expressing cell line COR-L23R but some , mainly cytoplasmic, staining was seen in the sensitive COR-L23S variant 3/B6 staining was also absent from the LRP negative MOP-8 mouse fibroblast line Low level cytoplasmic staining was observed in DLKP and DLKP-A10 cells with 20% of cells staining positively with 3/B6 Similar staining was also seen in the RPMI 2650 line but to a lesser degree,(approximately 5% of cells)

A reduction in staining was seen when one LRP antisense and two LRP transfected clones of OAW42-SR were probed with this antibody The greatest reduction was observed in the ribozyme clone, OAW42-SR RZ4 where 5% of cells had faint, mainly, cytoplasmic staining A reduction in LRP staining was also seen in the A/S1 and RZ2 (Antisense 1 and Ribozyme 2 clones) but not to the same extent The staining pattern in these two clones was more heterogeneous with a large variation in staining

The LRP staining pattern was generally similar to that observed with MAb 3/B6 A lower level of staining was observed in the SW1573, SW1573/2R160,A2780/AC12 and OAW42-SR RZ2 cell lines Similar levels were seen on the SW1573/2R120, COR-L23S, DLKP, and A2780 cell lines No plasma membrane staining was observed with LRP-56 Staining was primarily localised to the peripheral cytoplasm and was granular in nature In certain cell lines namely, COR-L23S and RPMI 2650, the staining was more diffuse in the cytoplasm There was no significant staining on the negative controls

These results illustrated the general pattern of co-expression of the 3/B6 and LRP/MVP antigens in these cell lines which was evident in the LRP/MVP cDNA transfected A2780/AC12 and AC16 clones which over-express the LRP/MVP compared to the parental A2780 cell line Whereas, there was less co-expression observed in the SW1573, SW1573/2R160, GLC4 and GLC4/ADR Significant reduction in the LRP/MVP ribozyme transfected OAW42-SR cells suggested that the expression of these

proteins were closely linked Subsequent experiments were undertaken to determine the relationship between these two proteins

Table 3 2 3 2. Immunocytochemical analysis of a panel of human and murine multidrug resistant/sensitive cell lines with MAb 3/B6 and LRP-56

Cell line	Antibody	
	3/B6	LRP-56
OAW42-SR	+++	+++
OAW42-S	+++	+++
SW1573	++	+
SW1573/2R120	+++	+++
SW1573/2R160	++	+
A2780	+	+
A2780-AC12	++	+
A2780-AC16	+++	+++
MOP-8	-	-
GLC4/S	+	+
GLC4/ADR	++	+++
OAW42-SR/RZ2	++	+
OAW42-SR/RZ4	+	++
OAW42-SR/AS1	++	+
COR-L23R	-	-
COR-L23S	+	+
DLKP	++	++
DLKP-A	+	+/-
DLKP-A10	+/-	+/-
RPMI 2650	+	+

This staining index is based on the number of cells showing positive staining for each particular antibody ranging from +, approximately 5% of cells to +++, 90-100% of cells

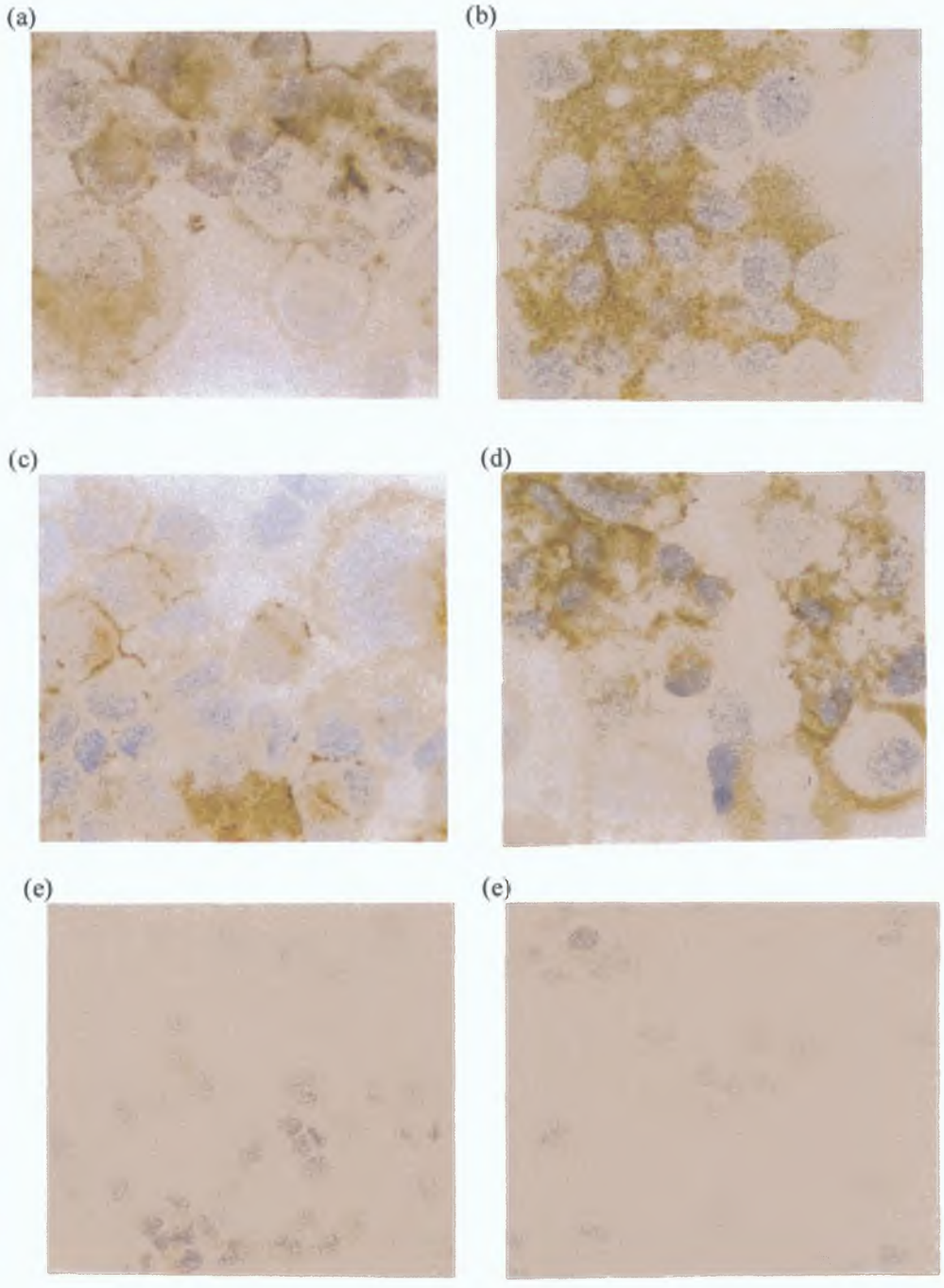
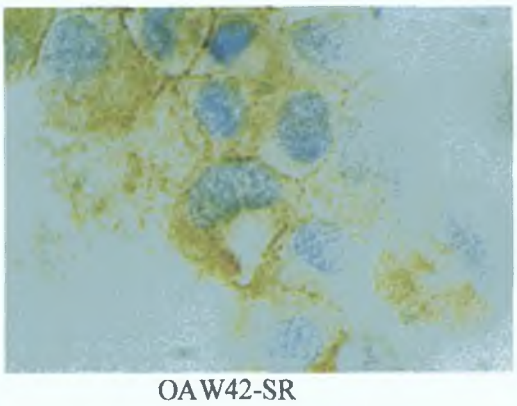
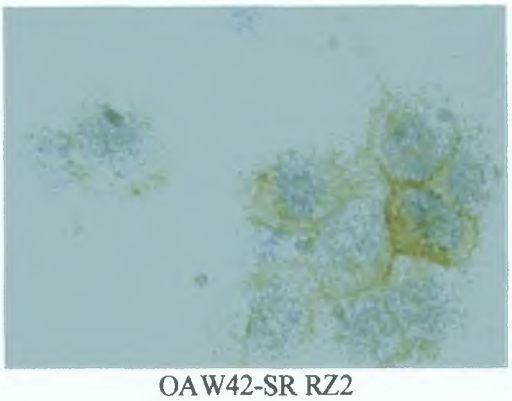


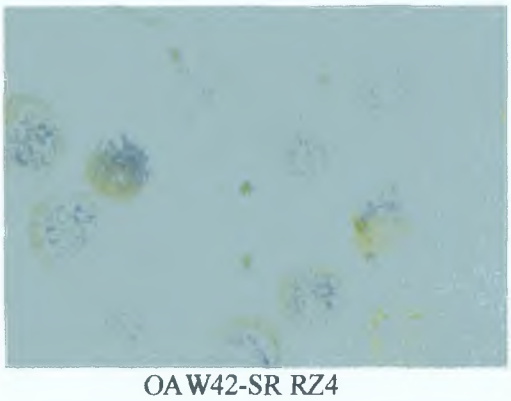
Figure 3.2.3.1. Immunocytochemical analysis of OAW42 variants with MAb 3/B6 and L5P-56 (section 3.2.3.1.) (a) OAW42-S probed with 3/B6, (b) OAW42-S probed with LRP-56, (c) OAW42-SR probed with 3/B6, (d), OAW42-SR probed with LRP-56, (40X). (e) and (f) are negative controls, OAW42-S and OAW42-SR respectively probed with mouse IgG. Photographs were taken at 20X.



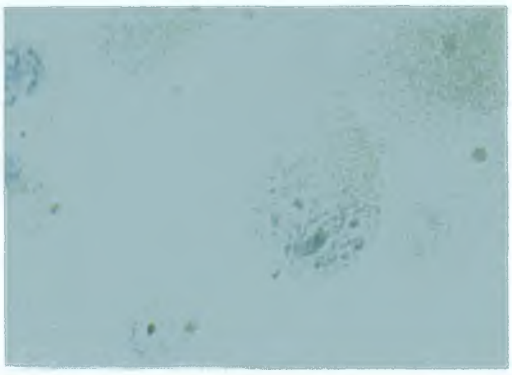
OAW42-SR



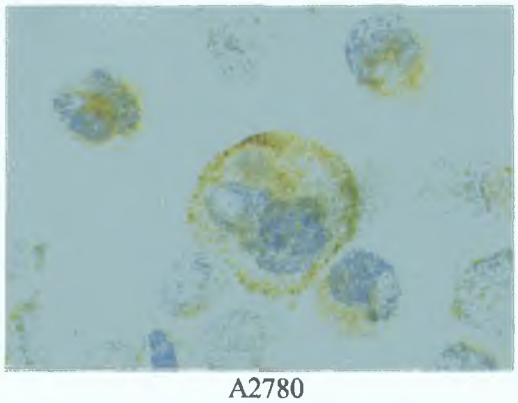
OAW42-SR RZ2



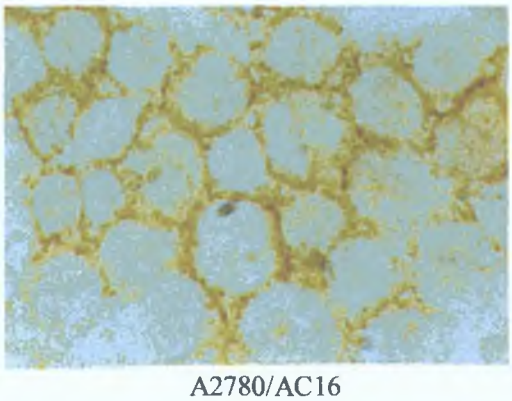
OAW42-SR RZ4



MOP-8



A2780



A2780/AC16

Figure 3.2.3.2.1. Immunocytochemical analysis of MDR cell lines with MAb 3/B6 (section 3.2.3.2.) using the DAB/ABC method (section 2.9.5. materials and methods). Photographs were taken at 40X.

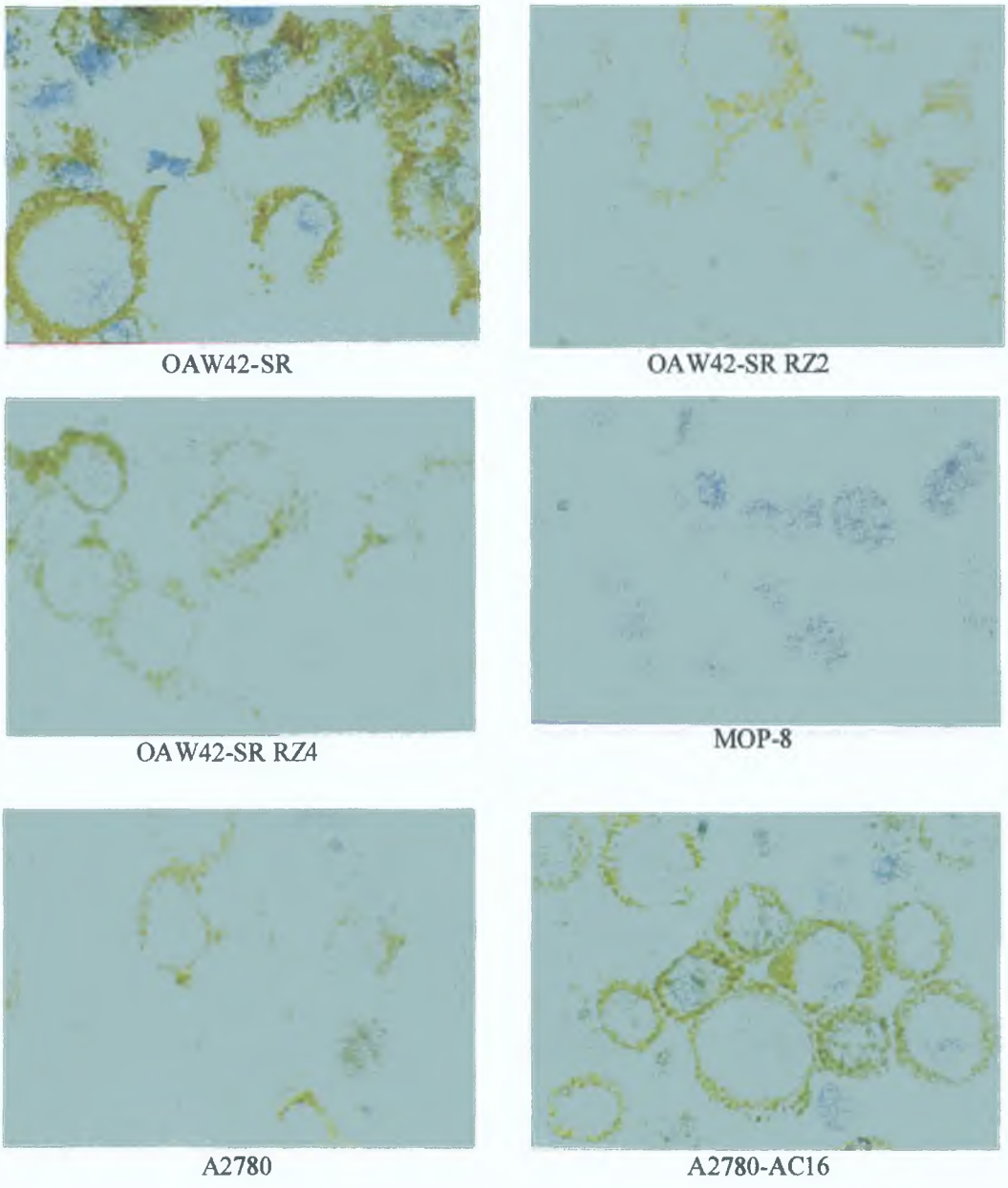


Figure 3.2.3.2.2. Immunocytochemical analysis of MDR cell lines with MAb LRP-56 (section 3.2.3.2.) using the DAB/ABC method (section 2.9.5. materials and methods). Photographs were taken at 40X.

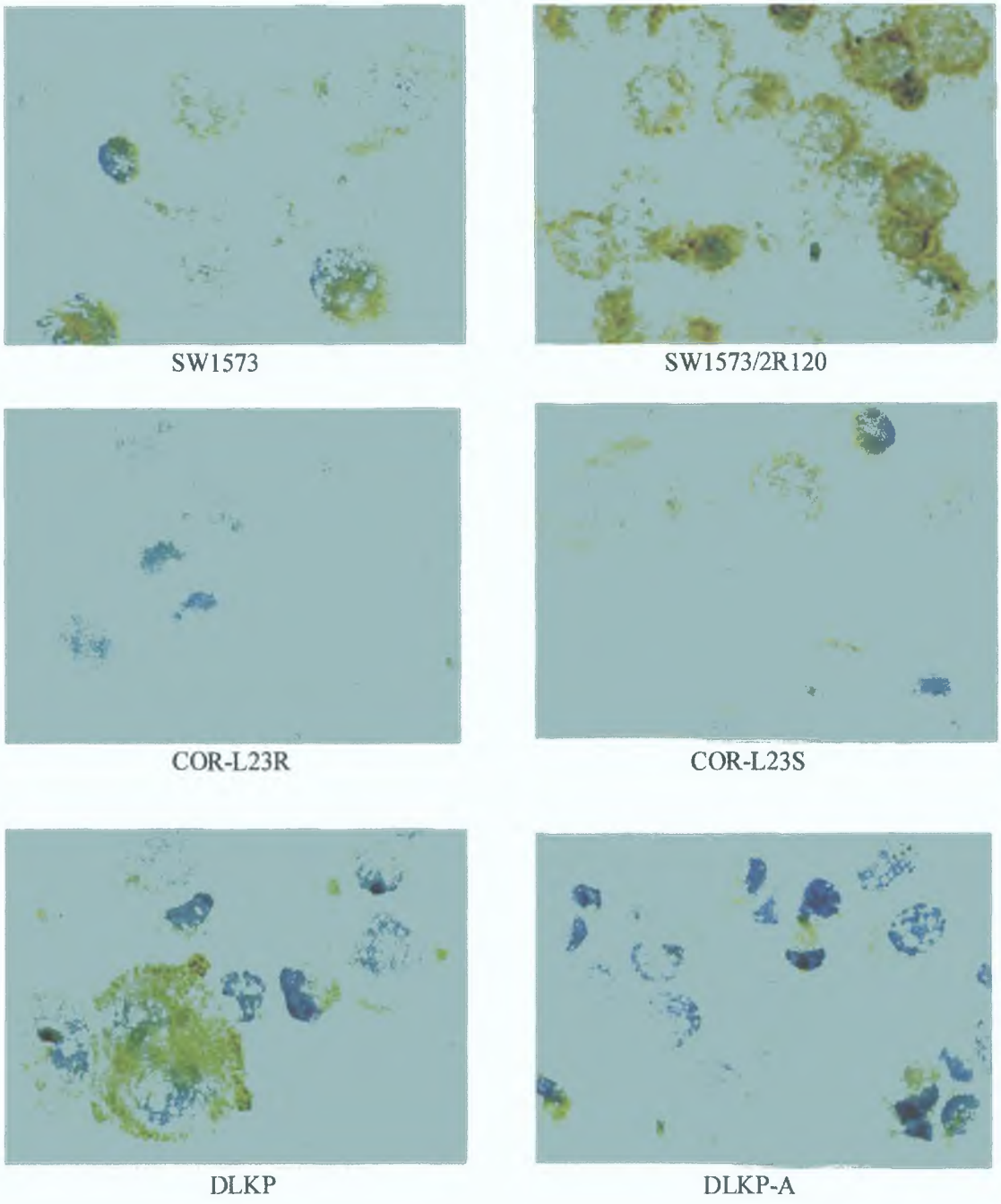
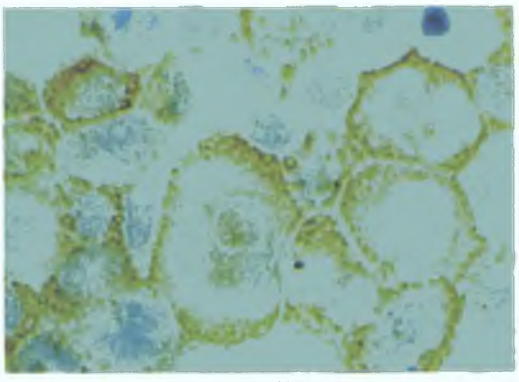
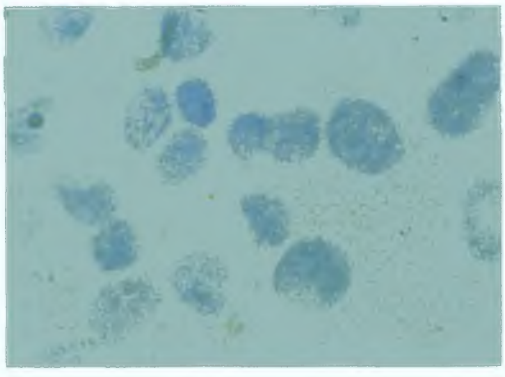


Figure 3.2.3.2.3. Immunocytochemical analysis of MDR cells lines with MAb 3/B6 (section 3.2.3.2.) using the DAB/ABC method (section 2.9.5. materials and methods). Photographs were taken at 40X.

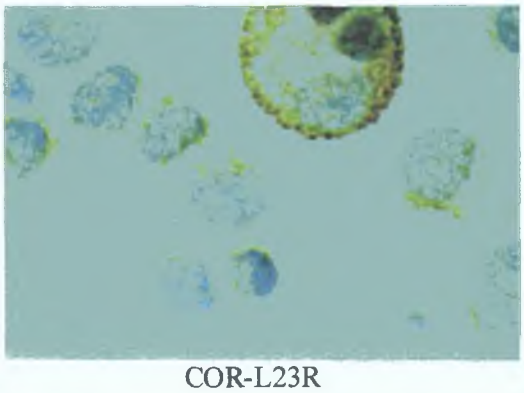
SW1573



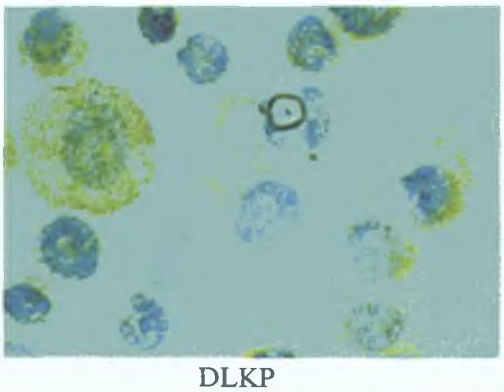
SW1573/2R120



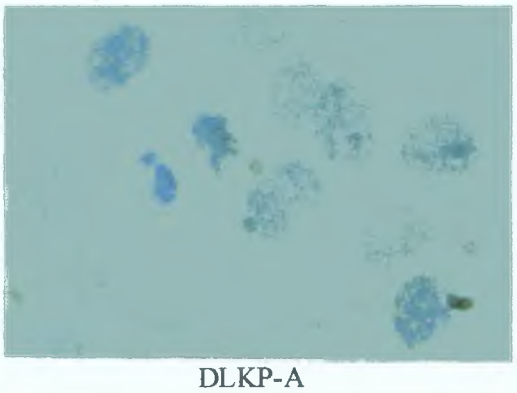
COR-L23R



COR-L23R



DLKP



DLKP-A

Figure 3.2.3.2.4. Immunocytochemical analysis of MDR cell lines with MAb LRP-56 (section 3.2.3.2.) using the DAB/ABC method (section 2.9.5. materials and methods). Photographs were taken at 40X.

3 2 3 3. Competition studies with MAb 3/B6 and LMR-5 and the anti-vault polyclonal antiserum N2

To determine whether MAb 3/B6 cross reacted with the MVP/LRP cytospins of OAW42-SR were probed with anti-LRP/MVP/ MVP and anti-OAW42-SR antibodies (section 2 9 5 materials and methods) A series of controls were designed to determine whether species specific biotinylated secondary antibodies recognised their target primary antibodies OAW42-SR cytospins were probed with (a) MAb 3/B6 and incubated with the anti-rat and anti-rabbit-specific secondary antibodies, (b) MAb LMR-5 (a rat primary antibody) and probed with the anti-mouse and anti-rabbit-specific secondary antibodies and (c) N2 (a rabbit primary antibody) and probed with the anti-mouse and anti-rat–specific secondary antibody Results are illustrated in figure 3 2 3 3 1 c, d No staining was observed when MAb 3/B6 was incubated with the anti-rat and anti-rabbit specific secondary antibodies compared to the positive control, OAW42-SR probed with MAb 3/B6 and detected with the anti-mouse specific secondary antibody, 3 2 3 3 1 b Similarly, there was no significant staining observed on cytospins probed with MAb LMR-5 and incubated with the anti-mouse and anti-rabbit specific secondary antibodies There was also a lack of significant staining on cytospins probed with the polyclonal antibody N2 and incubated with the anti-mouse and anti-rat-specific secondary antibodies

Cytospins of OAW42-SR were pre-incubated with MAb 3/B6 and probed with MAb LMR-5 to determine if MAb 3/B6 blocked the epitope recognised by MAb LMR-5 Similarly, cytospins of OAW42-SR were also pre-incubated with MAb LMR-5 and probed with MAb 3/B6 There did not appear to be any reduction in 3/B6 staining on OAW42-SR cytospins pre-incubated with LMR-5 when compared to the positive control, OAW42-SR cytospins probed with MAb 3/B6 alone figures 3 2 3 3 1 b and 3 2 3 3 2 b Similarly, there was no significant reduction in the levels of staining with MAb LMR-5 when OAW42-SR cytospins were pre-incubated with MAb 3/B6 and probed with MAb LMR-5 (figures 3 2 3 3 1 a and 3 2 3 3 2 a) These experiments were repeated with the anti-vault polyclonal antibody N2 There did not appear to be any reduction in staining when OAW42-SR cytospins were pre-incubated with MAb 3/B6

and probed with the N2 polyclonal antibody compared to the positive control (figures 3 2 3 3 1 c and 3 2 3 3 3 a), levels of staining were unchanged when cytospins were pre-incubated with N2 and probed with MAb 3/B6 (figure 3 2 3 3 3 b) Reduced levels of LMR-5 staining were observed when cytospins were pre-incubated with the N2 polyclonal antibody and probed with MAb LMR-5 (figure 3 2 3 3 4 a), this reduction in staining was not observed when cytospins were pre-incubated with MAb LMR-5 and probed with N2 (figure 3 2 3 3 4 b)

These studies indicated that MAb LMR-5 and the polyclonal antibody N2 did not recognise the same epitope and possible the same protein as MAb 3/B6 Further biochemical characterisation of the 3/B6 antigen were undertaken to clarify this point

Primary antibody 1	Primary antibody 2	Secondary antibody	Staining intensity
LMR-5	3/B6	anti-mouse	+++
3/B6	LMR-5	anti-rat	+++
N2	3/B6	anti-mouse	+++
3/B6	N2	anti-rabbit	+++
LMR-5	N2	anti-rabbit	+++
N2	LMR-5	anti-rat	+
3/B6	_	anti-rat	_
3/B6	_	anti-rabbit	_
LMR-5	_	anti-mouse	_
LMR-5	_	anti-rabbit	_
N2	_	anti-mouse	_
N2	_	anti-rat	_

Table 3 2 3 3 Results from competition and secondary antibody species specificity experiments with MAb 3/B6, LMR-5, the anti-vault polyclonal antibody N2 and biotinylated anti-mouse, -rat and –rabbit specific secondary antibodies.

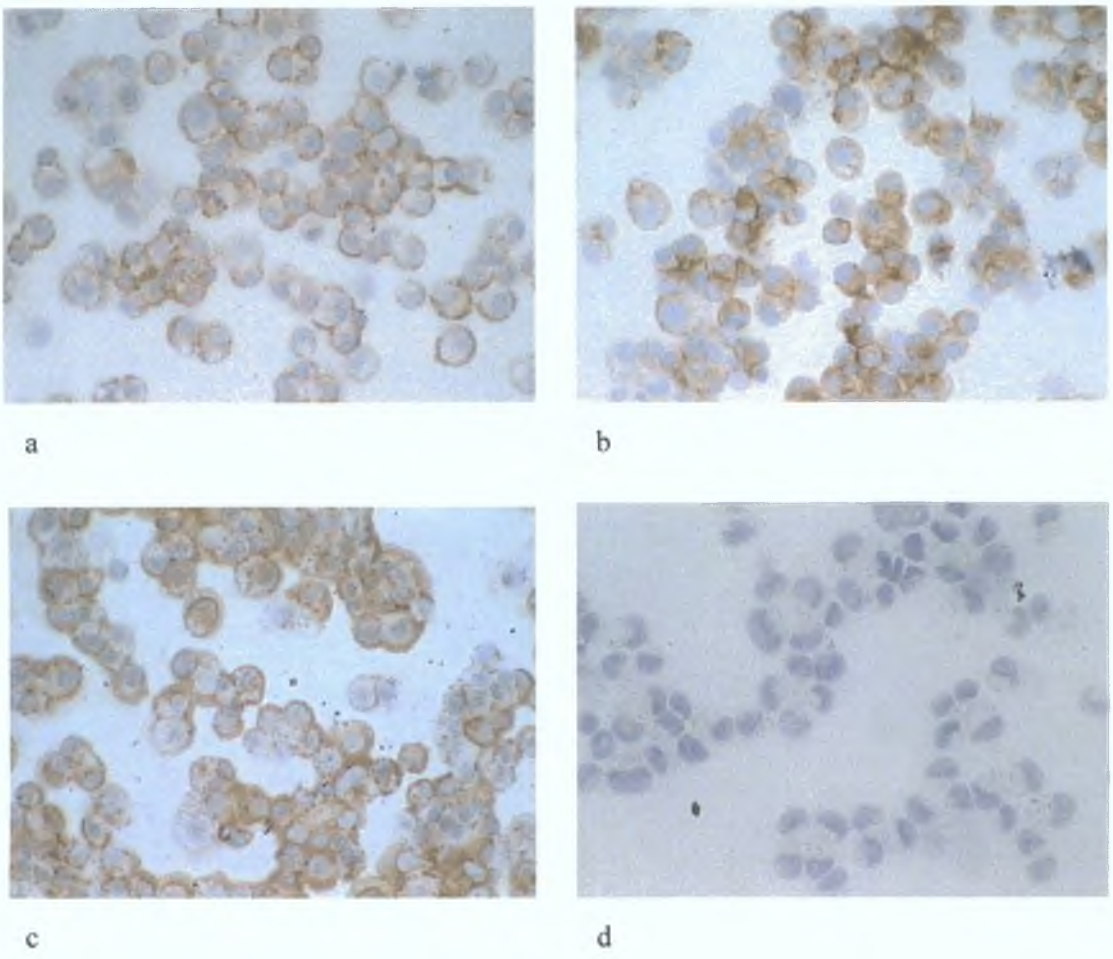
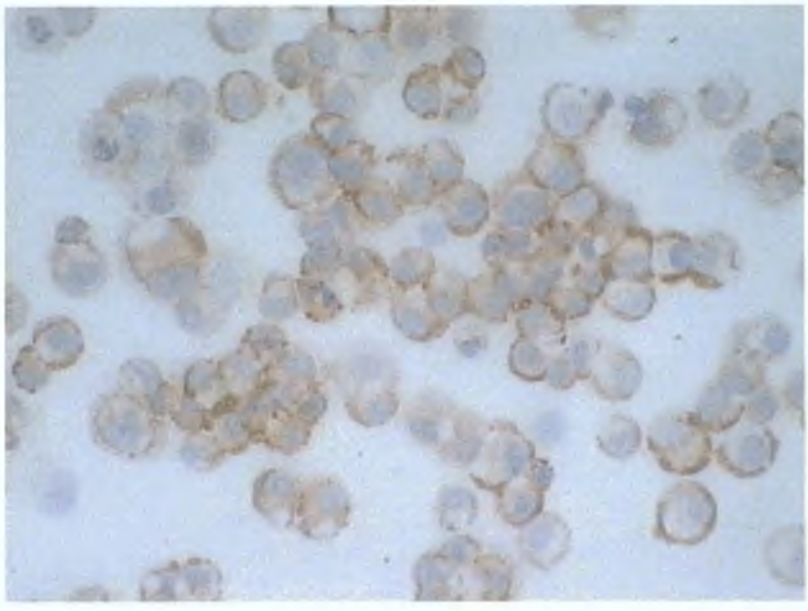
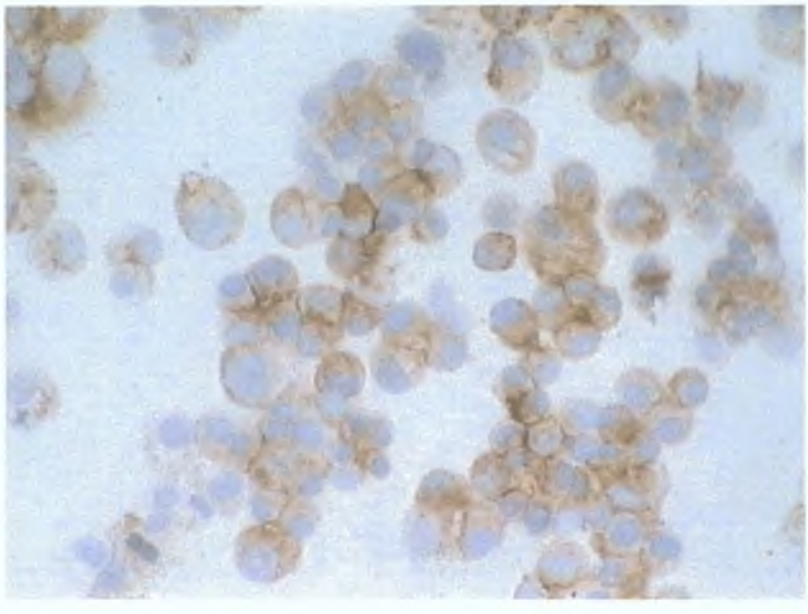


Figure 3.2.3.3.1. Competition studies with MAbs 3/B6 and LMR-5 and the anti-rat vault polyclonal antibody N2 using the DAB/ABC method (section 2.9.5. materials and methods). Positive control slides. OAW4-SR cytospins probed with, **a**. LMR-5, **b**. 3/B6, **c**. N2 and **d**. mouse IgG (negative control). Photographs were taken at 20X.



a



b

Figure 3.2.3.3.2. Competition studies with MAbs 3/B6 and LMR-5 using the DAB/ABC method (section 2.9.5. materials and methods). OAW42-SR cytospins probed with **a**. 2 hour incubation with MAb 3/B6 followed by an overnight incubation with MAb LMR-5 and **b**. 2 hour incubation with MAb LMR-5 followed by an overnight incubation with MAb 3/B6. Photographs were taken at 20X.

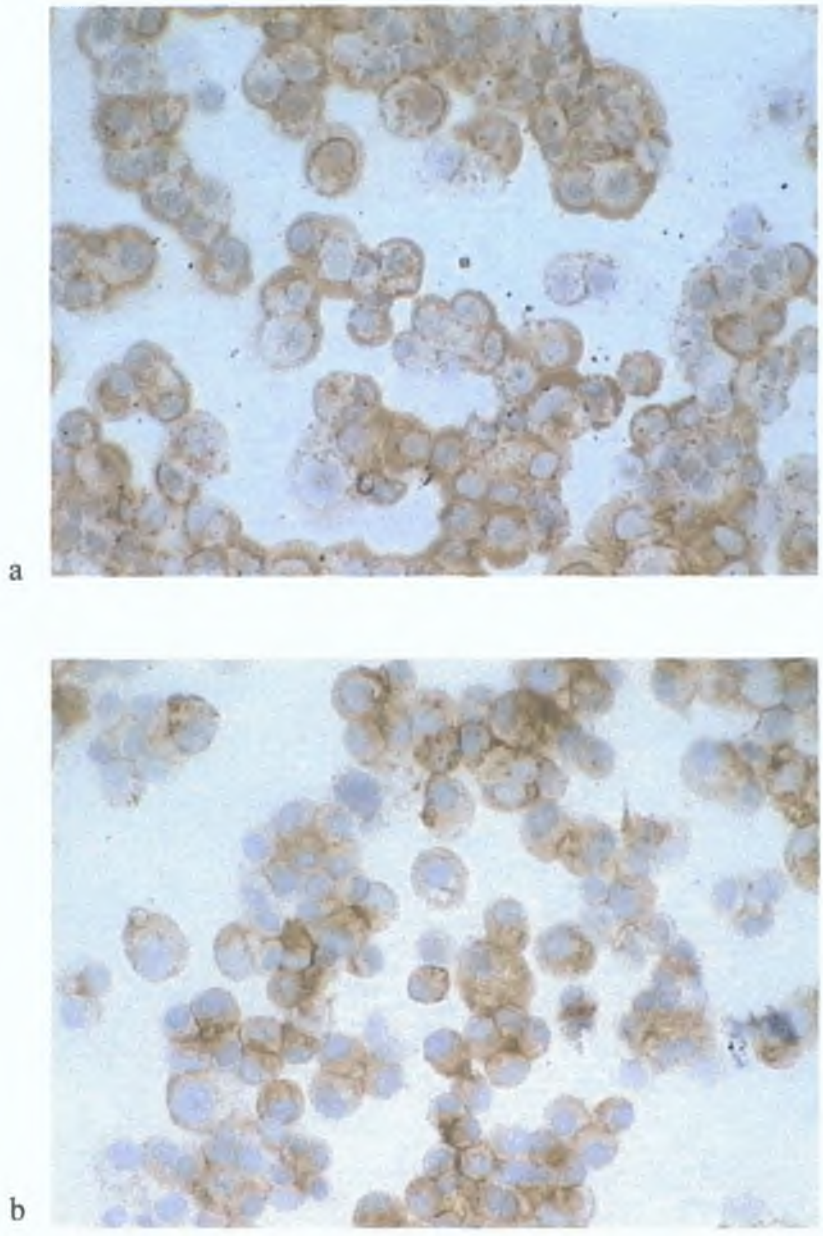


Figure 3.2.3.3.3. Competition studies with MAb 3/B6 and the anti-vault polyclonal antibody N2 using the DAB/ABC method (section 2.9.5. materials and methods). OAW42-SR cytospins probed with **a**. 2 hour incubation with MAb 3/B6 followed by an overnight incubation with polyclonal antibody N2 and **b**. 2 hour incubation with polyclonal antibody N2 followed by an overnight incubation with MAb 3/B6. Photographs were taken at 20X.

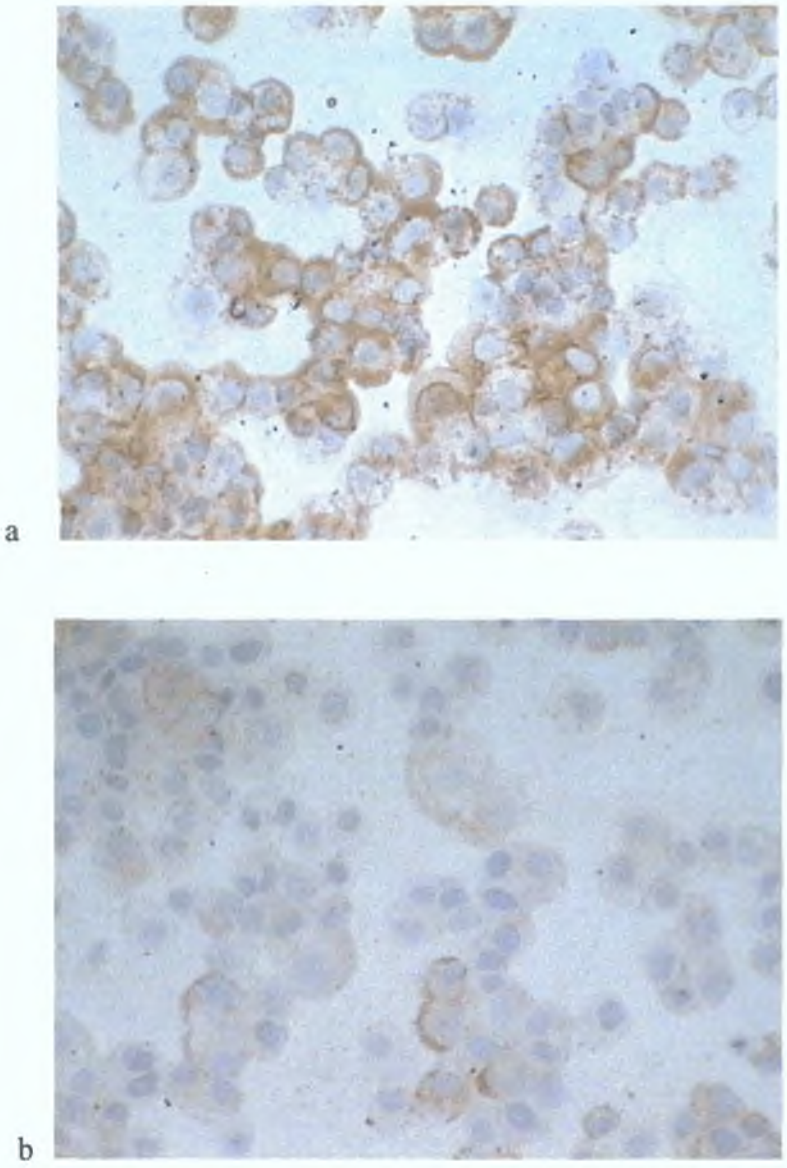


Figure 3.2.3.3.4. Competition studies with MAb LMR-5 and the anti-vault polyclonal antibody N2 using the DAB/ABC method (section 2.9.5. materials and methods). OAW42-SR cytospins probed with **a**. 2 hour incubation with MAb LMR-5 followed by an overnight incubation with polyclonal antibody N2, and **b**. 2 hour incubation with polyclonal antibody N2 followed by an overnight incubation with MAb LMR-5. Photographs were taken at 20X.

3.2.4.1. Optimisation of Immunoprecipitation protocol

A new cellular labelling and immunoprecipitation kit based on biotin labelling of cellular protein was extensively modified in the development of a non-radioactive method for the characterisation of the 3/B6 antigen (section 2.10.5 materials and methods).

Cell lysates of OAW42-SR were incubated with MAb 3/B6 for various time periods ranging from 1 hour to over night. Antibody-antigen complexes were immunoprecipitated with protein G coated sepharose beads for time periods ranging from 1 to 6 hours at 4^0C. These initial incubation times were chosen on the basis of work carried out previously with MAb LRP-56 by other researchers (Scheper *et al*, 1993). Results of these experiments are illustrated in Figure 3.2.4.1.1.

There appeared to be low level immunoprecipitation with antibody and immunoprecipitation incubation times of 1 hour Figure 3.2.4.1.1. The quantity of 3/B6 antigen appeared to increase with extended incubation times with the primary antibody . There was a second reduction in immunoprecipitates when the antibody antigen complex was washed with a high salt buffer. Levels were restored when the washing buffer was changed to either (a) a low salt buffer or (b) 1 wash with the high salt buffer. These results indicated that extended incubation times were more suitable for this antibody.

To determine the most suitable method for blocking non-specific binding and detection of biotin labelled proteins, Western Blots of 3/B6 immunoprecipitates were initially developed using Strepavidin/biotin HRP in conjunction with non-fat milk for the detection of biotin labelled proteins which was suggested in the kit protocol. It was not possible to clearly distinguish immunoprecipitate bands when blots were blocked with non-fat milk and developed using ABC/HRP reagents (figure 3.2.4.1.2.) There appeared to be less background staining when blots were blocked with biotin free BSA and probed with ABC/HRP (b) It has been demonstrated that non-fat milk can contain high levels of endogenous biotin therefore causing high background in this experiment. It was still difficult to interpret the blots with this method therefore an anti-biotin secondary

antibody coupled with biotin-free BSA was eventually chosen as the reagents which produced the cleanest blots

3 2 4 2 Immunoprecipitation and Western Blotting studies with MAb 3/B6

Initial attempts to determine the molecular weight of the antigen recognised by MAb 3/B6 by western blotting were unsuccessful The antibody was then used to immunoprecipitate cell lysate preparations labelled with D-biotinoyl-ε-aminocarproic acid-N-hydroxysuccinimide ester (biotin-7-NHS)

Results indicated that the antigen detected by 3/B6 has a molecular weight of approx 115 kDa, figure 3 2 4 2 The secondary antibody also appeared to detect a range of bands in the negative control lane The molecular weight assigned to the 3/B6 antigen was similar to that determined for the LRP/MVP (110 kDa) which prompted further investigation

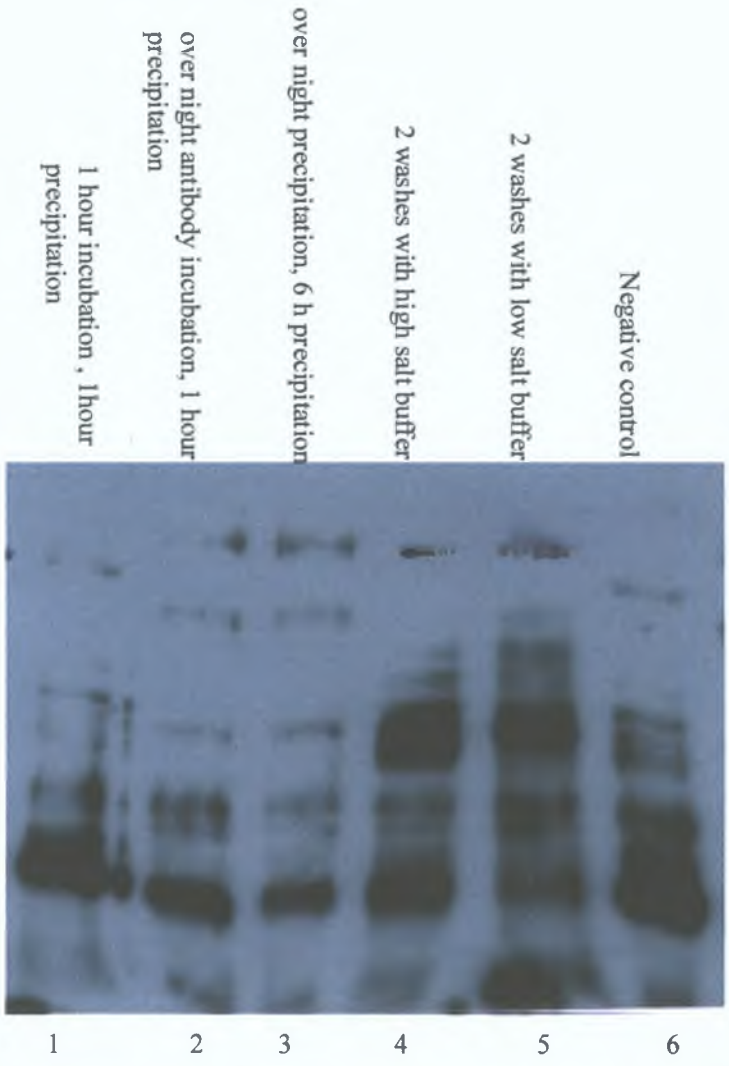


Figure 3.2.4.1.1. Optimisation of immunoprecipitation protocol (section 3.2.4.1. and section 2.10.5. materials and methods). All lanes are OAW42-SR cells immunoprecipitated with MAb 3/B6. Negative control is OAW42-SR immunoprecipitated with mouse IgG detected with a horseradish peroxidase-labelled secondary antibody and enhanced chemiluminescence (section 2.10.4. materials and methods).

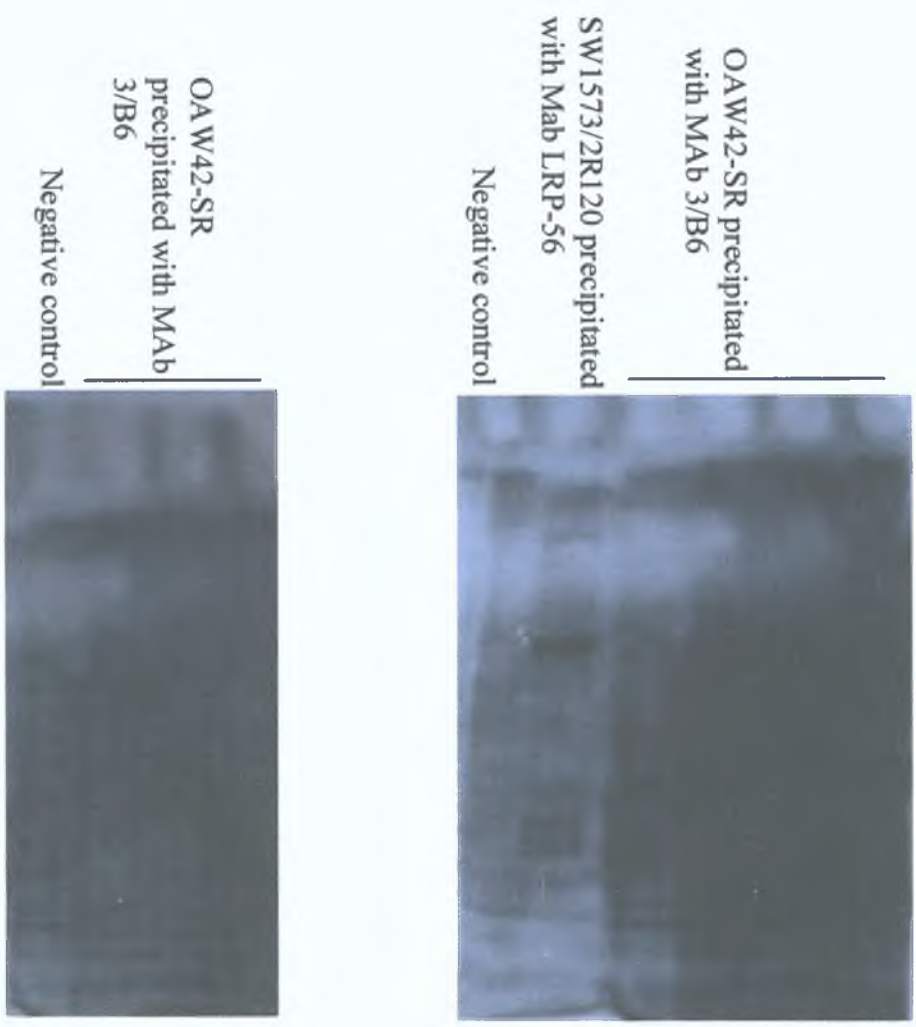


Figure 3.2.4.1.2. Optimisation of biotin labelling and immunoprecipitation protocol (section 3.2.1.). Blots were blocked for non-specific background with (a), 5% non-fat milk. (b) biotin free BSA. Biotin labelled proteins were detected with ABC/HRP and enhanced chemiluminescence (2.10.4. materials and methods).

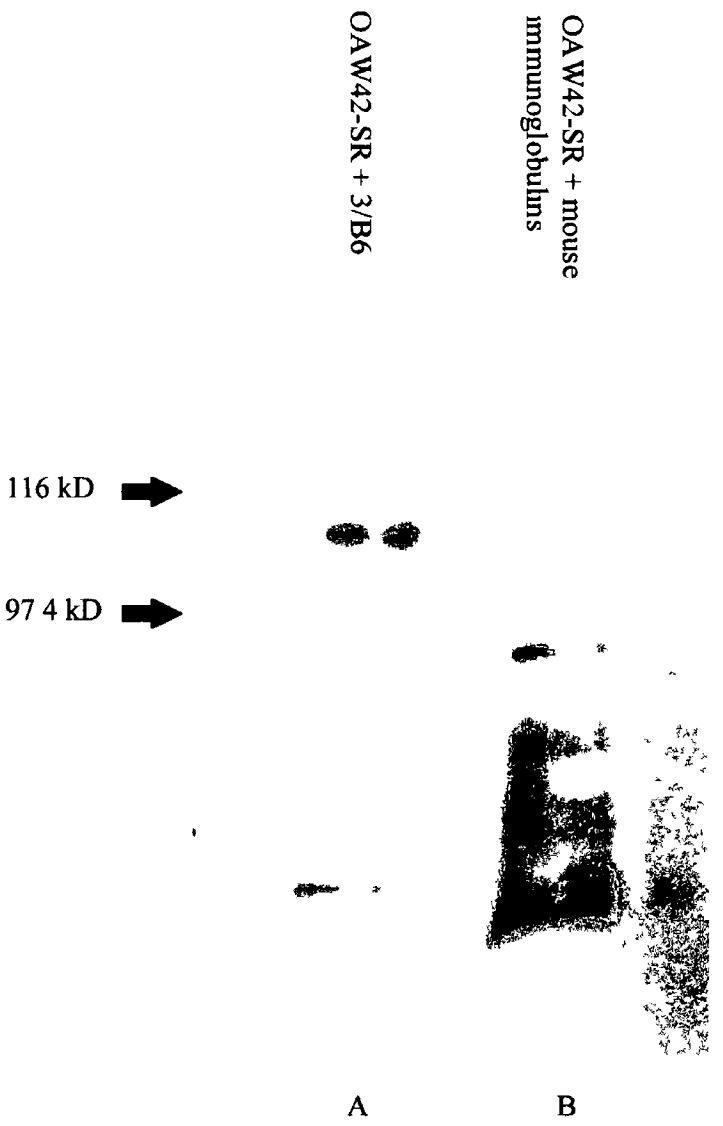


Figure 3 2.4.2 Immunoprecipitation experiments with MAb 3/B6 (section 3 2 4) Immunoprecipitates were detected by an anti-biotin horseradish peroxidase-labelled secondary antibody and enhanced chemiluminescence (section 2 10 4 materials and methods) (A), test lane, (B), negative control, OAW4-SR cell lysates precipitated with mouse IgG

3 2 5 Determination of protein glycosylation

To determine if the 115 kDa protein recognised by MAb 3/B6 was glycosylated, OAW42-SR cells were grown in the presence of tunicamycin (preventing N-glycosylation) and phenyl-acetyl-α-D-Galactosaminide (PNAG, preventing O-glycosylation) LRP immunoprecipitates from cells grown in glycosylation inhibitors were included as a control Results are illustrated in figure 3 2 5 (a) Lanes 1 and 2 contain OAW42-SR immunoprecipitates precipitated with 3/B6 and grown in the presence of tunicamycin (8μg/ml) and Phenyl-N-acetyl α D Galactosaminide (PNAG) respectively There was no detectable shift in the molecular weight of the 115 kDa band (or in the band size) when compared to precipitates of OAW42-SR grown without glycosylation inhibitors, lane 3 This suggested that N or O linked carbohydrates are not attached to this protein A second band was detected at 60 kDa but since this band is also present in the LRP and negative controls it was considered to be background due to mouse IgG and therefore not significant

There was no shift in the band at 110 kDa i e the LRP immunoprecipitate, in OAW42-SR cells (lanes 4 and 5) or in LRP precipitates of SW1573/2R120 cells (lanes 6 and 7) when these cells were grown in the presence of the same glycosylation inhibitors Lanes 8 and 9 contain SW1573/2R120 and OAW42-SR controls i e , cells which were grown without glycosylation inhibitors

There was a detectable shift in the positive control DLKP-A (a Pgp over-expressing cell line, Pgp is a known glycosylated protein), cell line when these cells were grown in the presence of tunicamycin, figure 3 2 5 (b) MAb C219 (which recognises the MDR-1 gene product, p-glycoprotein) detected 2 bands at 170 and approximately 140 kDa

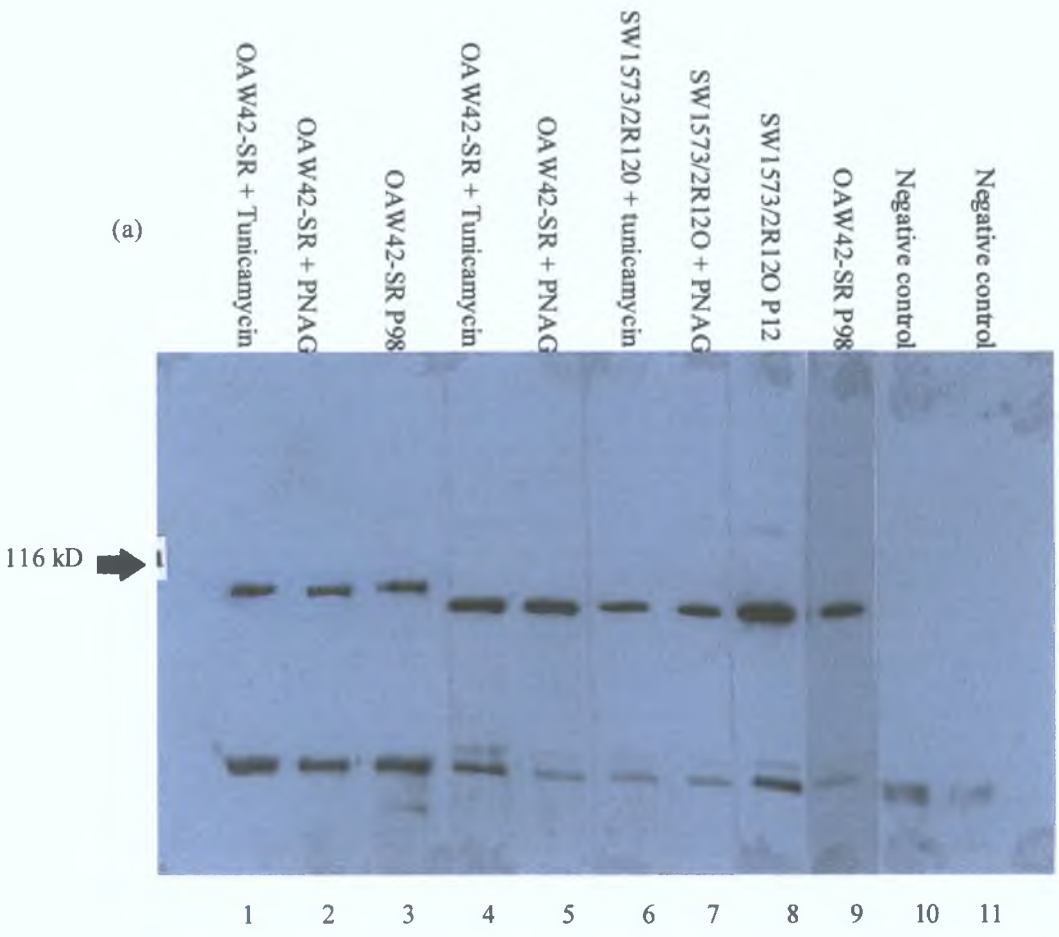


Figure 3.2.5. Glycosylation studies with MAb 3/B6, LRP-56 and C219. (**a**) biotin labelled protein samples detected with an anti-biotin horseradish peroxidase labelled secondary antibody and enhanced chemiluminescence (section 2.10.5.materials and methods), lanes 1, 2 and 3 were precipitated with 3/B6, lanes 4-9 were precipitated with LRP-56, lanes 10-11 were precipitated with mouse IgG. (**b**), Crude cell lysates of DLKP-A probed with MAb C219, visualised with an anti-mouse house horseradish peroxidase labelled secondary antibody and enhanced chemiluminescence.

PNAG is Phenyl-N-Acetyl-α-δ-Galactosanimide

3 2 6 Immunoprecipitation studied on purified rat vault proteins

3/B6 and LRP-56 immunoprecipitates of OAW42-SR and SW1273/2R120 cells were probed with an anti-biotin secondary antibody to detect biotin-labelled protein in the immunoprecipitates These precipitates were also probed with the polyclonal serum N2 which was raised to rat vaults and detects the MVP Supernatants from overnight precipitation experiments were also probed for the presence of LRP That is, following the final incubation step where protein G coated spharose beads were used to precipitate the antibody-antigen complex, the supernatant which is usually discarded was retained, concentrated (section 2 10 5) and applied to the SDS-PAGE gel, any remaining un-precipitated LRP would be detected by Western Blotting using the anti-MVP antibody N2 If 3/B6 was an anti-vault antibody then there would be a reduction in the amount of LRP detected in 3/B6 supernatants

In figure 3 2 6 1 (A), the molecular weight of the antigen detected by MAb 3/B6 (lane a) is slightly higher than the LRP/MVP precipitated by MAb LRP-56 The p115 band is expressed at much lower amounts in the SW1573/2R120 cell line, lane 3 MAb 3/B6 and MAb LRP-56 both appeared to detect a protein at a molecular weight of 192 kDa and a range of bands between approximately, 50-30 kDa

Figure 3 2 6 1 (b) shows the same immunoprecipitates probed with the anti-vault polyclonal antibody, N2 There is no corresponding band at 110 or 115 kDa in immunoprecipitates precipitated with 3/B6, lanes a and c When the supernatants of these immunoprecipitates were probed with the N2 polyclonal antibody there was no significant reduction in the level of LRP, in those cells lines precipitated with LRP-56 there was a significant reduction in LRP levels, Figure 3 2 6 2

These results indicated that the antigen detected by the MAb 3/B6 was not the LRP/MVP This study also suggested that the 3/B6 antigen was probably not a modified form of the LRP/MVP This conclusion agreed with the results of the competition studies already discussed in section 3 2 3 3

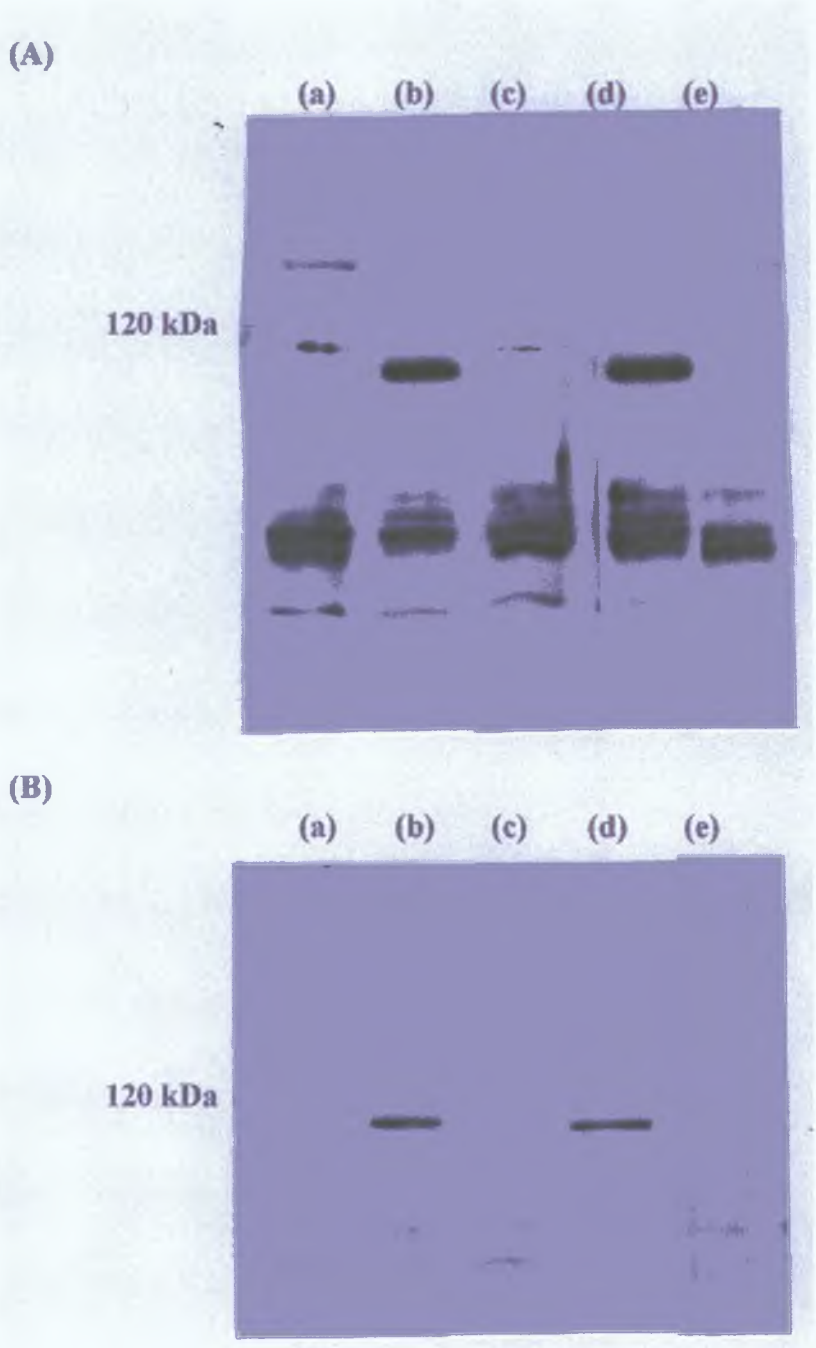


Figure 3.2.6.1. Immunoprecipitates of OAW42-SR and SW1573/2R120 cells probed for the MVP/LRP (section 3.2.6.). (**A**), immunoprecipitates were probed with an anti-biotin horseradish peroxidase labelled secondary antibody and enhanced chemiluminescence, (**a**) OAW42-SR precipitated with 3/B6, (**b**) OAW42-SR precipitated with LRP-56, (**c**) SW1573/2R120 precipitated with 3/B6, (**d**) SW1573/2R120 precipitated with LRP-56 and (**e**) negative control, OAW42-SR cells precipitated with mouse IgG. (**B**) the same immunoprecipitates probed with the anti-rat vault polyclonal antibody N2 detected using a horseradish labelled anti-rabbit secondary antibody and enhanced chemiluminescence (section 2.10.4. materials and methods).

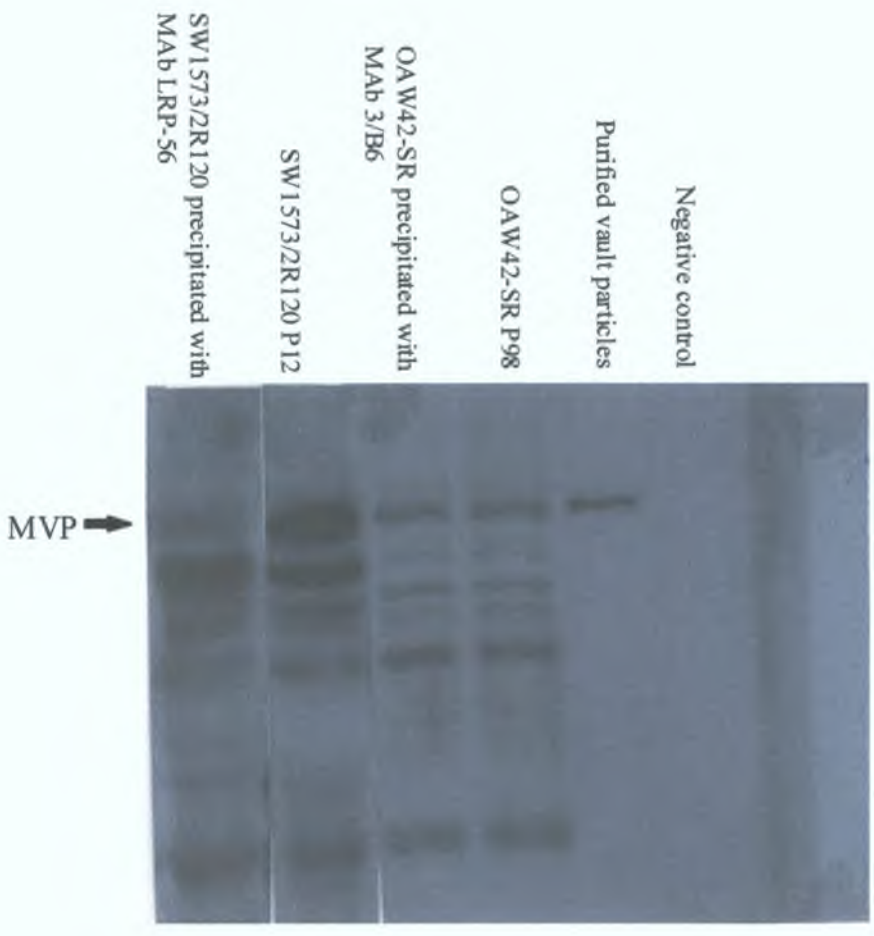


Figure 3.2.6.2. Supernatants from immunoprecipitation studies with MAb 3/B6 and LRP-56 probed with an anti-rat vault polyclonal antibody N2 detected using a horseradish labelled anti-rabbit secondary antibody and enhanced chemiluminescence (section 3.2.6 and section 2.10.5.materials and methods).

3 2 7. Immunoprecipitation studies on a panel of multidrug resistant/sensitive cell lines

A panel of multidrug resistant and sensitive cell lines were assayed to confirm the expression of the antigen detected by MAb 3/B6 observed immunocytochemical analysis of MDR cell lines The results are illustrated in Figure 3 2 7 1 , Levels of expression were compared with the positive control, OAW42-SR cells DLKP cells exhibited low level expression as did the COR-L23R cells (lanes 1 and 5 Low level expression was observed in the Pgp over-expressing cell line DLKP-A and the COR-L23S (lanes 2 and 6) Expression of the antigen detected by MAb 3/B6 was also seen in the LRP transfected cell line, A2780/AC16 while it was absent from the parental, A2780 cell line A band at approximately 50 kDa which appears in all immunoprecipitates including the negative control (OAW42-SR cells precipitated with control mouse IgG) was also observed Figure 3 2 7 2 illustrates the results of studies with MAb LRP-56 which was used in immunoprecipitation studies with the same panel of cell lines Low levels of LRP/MVP expression were observed in the DLKP and A2780 cell lines Increased levels of expression were observed in the COR-L23R , COR-L23S cell lines and in the SW1573/2R120 cell line, the LRP/MVP positive control, LRP/MVP expression was absent from the DLKP-A

There appeared to be some unequal loading of lanes in the DLKP and DLKP-A cell lines This may account for differences in the levels observed however these results appear to correlate with those obtained by immunofluorescence and immunocytochemistry Results with MAb LRP-56 did not correlate with immunocytochemical studies in the COR-L23 variants These differences may have been due to the fixation of the antibody in the immunocytochemical analysis

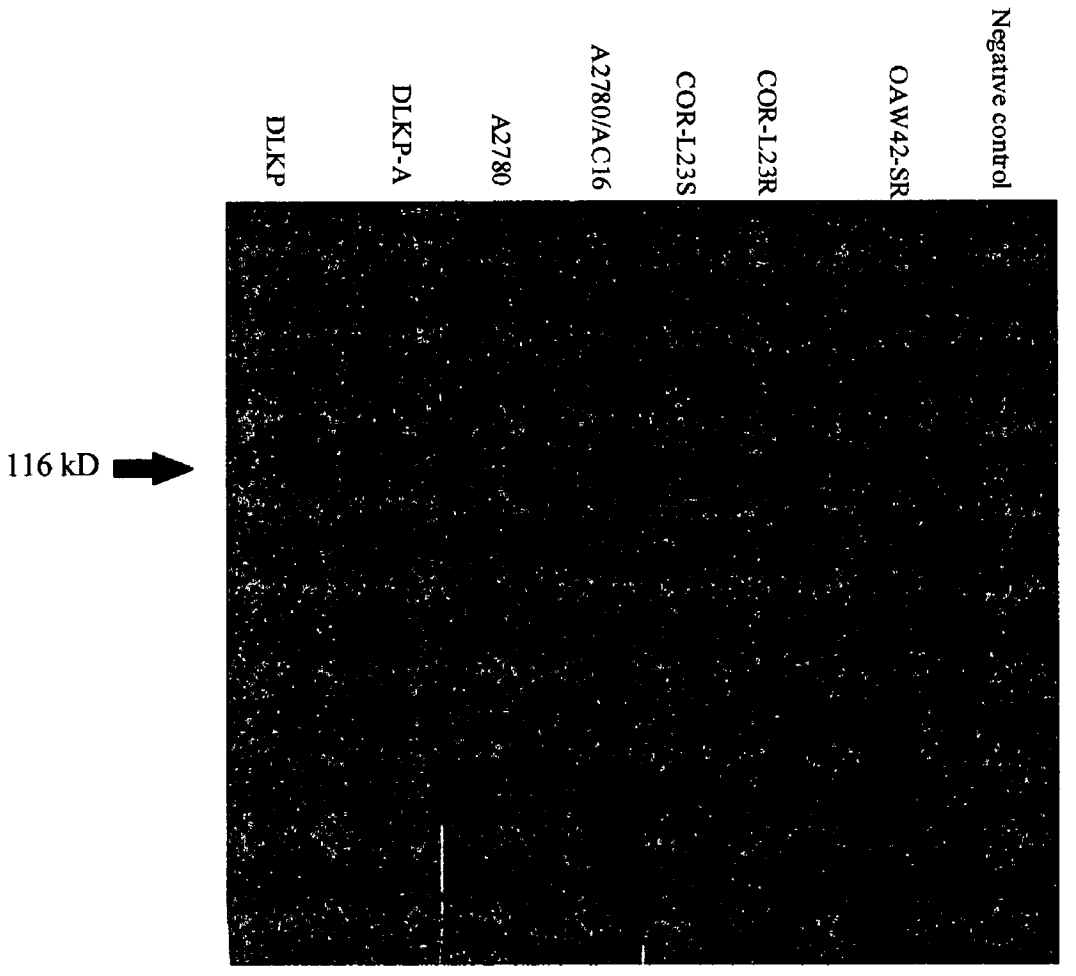


Figure 3 2 7 1. Immunoprecipitation studies with MAb 3/B6 on drug resistant and sensitive cell lines (section 3 2 7) Immunoprecipitates were probed with an anti-biotin horseradish peroxidase labelled secondary antibody and enhanced chemiluminescence (section 2 10 4 materials and methods)

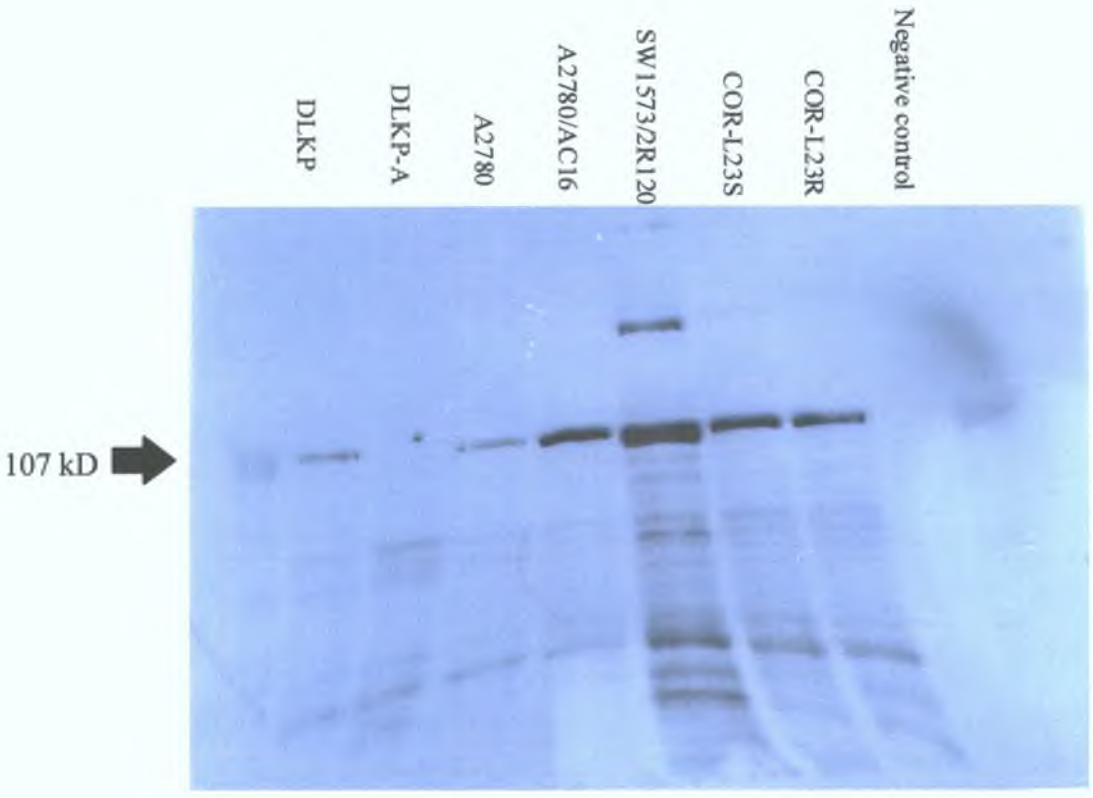


Figure3.2.7.2. Immunoprecipitation studies with MAb LRP-56 (section 3.2.7.). Immunoprecipitates were probed with an anti-biotin horseradish labelled secondary antibody and enhanced chemiluminescence (section 2.10.4. materials and methods).

3 2 8 Immunoprecipitation studies with LRP ribozyme and antisense transfected OAW42-SR cells

Immunoprecipitation of LRP ribozyme and antisense transfected OAW42-SR cells was carried out in order to confirm observations of reduced 3/B6 staining by immunocytochemistry Results are illustrated in figure 3 2 8

There did not appear to be a significant reduction in the LRP/MVP expression in the antisense 1 clone (lane 1) Similarly, there did not appear to be any reduction in the levels of the 3/B6 antigen (lane 5), when compared to the control, OAW42-SR precipitated with MAb 3/B6 (lane 8) In the ribozyme clone 2, (lane 3), there was a significant reduction in LRP expression compared to the positive control OAW42-SR cells precipitated with MAb LRP-56 whereas only s small reduction in the band of the antigen detected by MAb 3/B6 was observed (lane 6) In the ribozyme 4 clone (lane 7), levels of the antigen detected by MAb 3/B6 appeared to be greatly reduced, only a slight reduction in LRP/MVP levels was observed in this ribozyme treated clone (lane 2) Non-specific binding of the secondary antibody detecting bands at 90 and 50 kDa was observed in all immunoprecipitates including the negative control

Although it appeared from previous studies that the LRP/MVP and the 3/B6 were different antigens, these studies indicated that LRP/MVP may contribute to the control of the expression of 3/B6

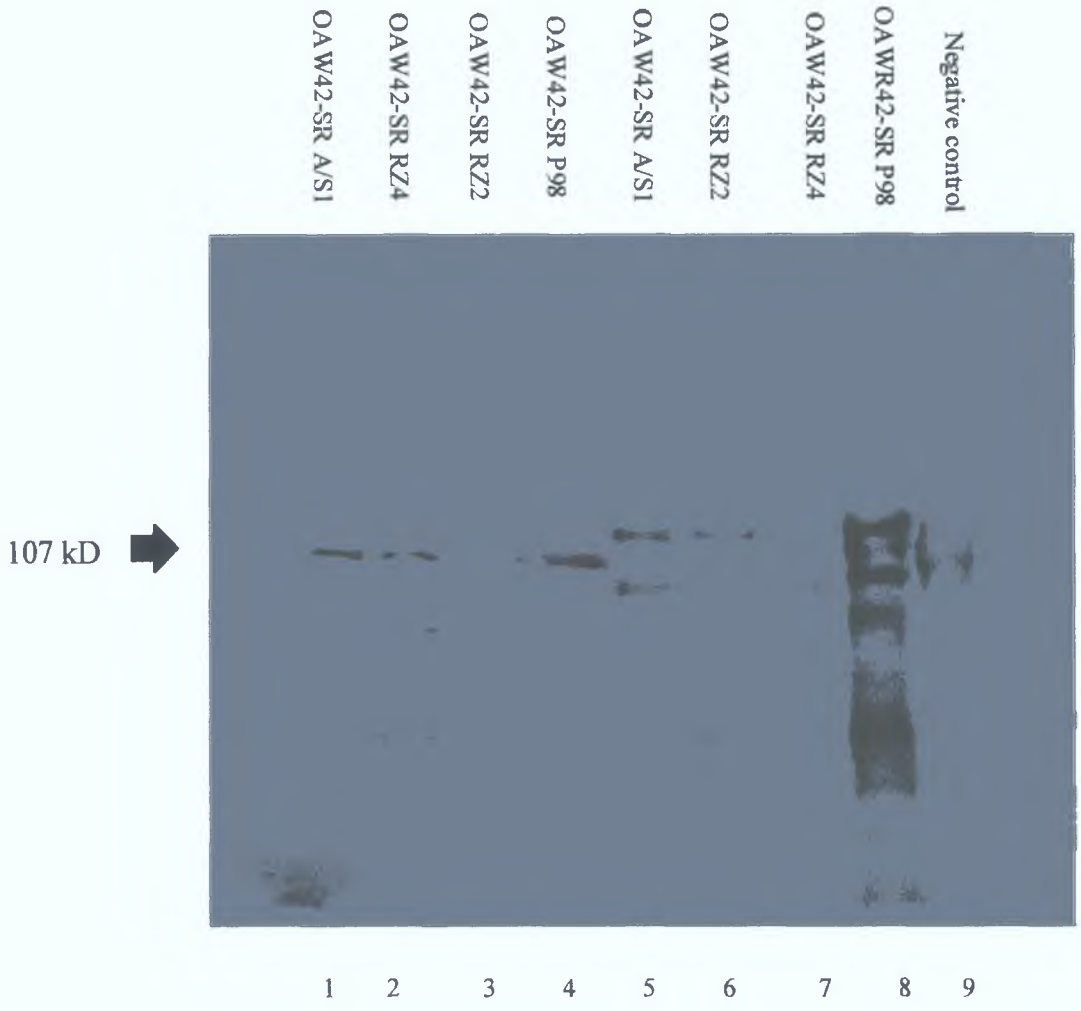


Figure 3.2.8. Immunoprecipitation experiments on ribozyme and antisense transfected OAW42-SR cells (section 3.2.8.). Immunoprecipitates were probed with an anti-biotin horseradish peroxidase labelled secondary antibody and enhanced chemiluminescence (section 2.10.4. materials and methods). Lane 9 is OAW42-SR cells precipitated with mouse IgG.

3 2 9 Immunoprecipitation experiments with OAW42-SR spent culture medium

Conditioned medium from OAW42-SR cells grown for 6 days was immunoprecipitated with MAb 3/B6 and LRP-56 to determine whether these antigens were shed into the growth medium (section 2 10 6 materials and methods) The results are illustrated in figure 3 2 9 There was no detectable band at 115 kDa in OAW42-SR conditioned medium immunoprecipitates (lane 1) The 115 kDa band was detected in immunoprecipitated of OAW42-SR whole cell lysates Similarly the 110 kDa LRP/MVP band was absent in the conditioned medium immunoprecipitates compared to whole cell lysates (lanes 3 and 4) 2 Bands at approximately 85 and 55 kDa were observed in the negative control (OAW42-SR conditioned medium immunoprecipitated with mouse IgG, lane 5) These results confirm the cytoplasmic location of the LRP/MVP however the results for 3/B6 are more surprising in that many antigens expressed on the cells surface are excreted into the growth medium including Pgp and many ovarian associated antigens

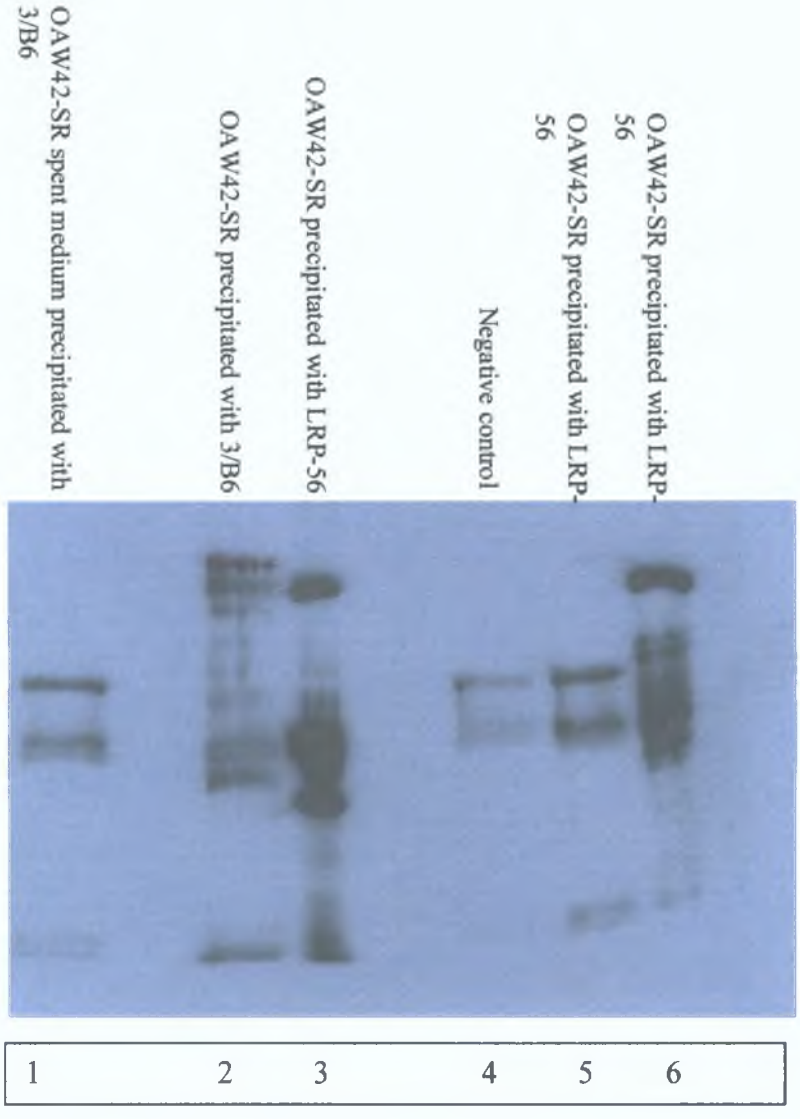


Figure 3.2.9. Immunoprecipitation of spent medium from OAW42-SR cells (section 2.10.5. materials and methods). Lanes 1-2, cells were precipitated with MAb 3/B6, lanes 3, 5-6, cells were precipitated with MAb LRP-56. Lane 4, cells was precipitated with mouse IgG. The blot was probed with an anti-biotin horseradish peroxidase-labelled secondary antibody and enhanced chemiluminescence (section 2.10.4. materials and methods).

3 2 10 Immunohistochemical studies in normal human and foetal tissues

The distribution of the 3/B6 antigen was investigated in a series of formalin fixed paraffin-embedded normal adult and foetal tissues (section 2 9 5 materials and methods)

In adult kidney, 3/B6 staining which was cytoplasmic in nature, was predominantly localised in the distal collecting tubules with some low level staining in the proximal collecting tubules MAb LRP-56 stained the proximal tubules with characteristic cytoplasmic reactivity No staining was observed in the distal tubules with this antibody, (Figure 3 2 10 1 (a) (b) There was no significant staining on thyroid tissue sections with MAbs 3/B6 or LRP-56 (Figure 3 2 10 1 (c) (d)

A series of foetal formalin fixed paraffin-embedded foetal tissues were also probed with the MAb 3/B6 and LRP-56 Results are illustrated in figure 3 2 10 2 and 3 2 10 3
3/B6 staining, which was cytoplasmic in nature, was observed primarily in the distal tubules of the kidney There did not appear to be 3/B6 expression in the proximal tubules Cytoplasmic staining was observed in the trophoblasts of the placenta and in the enterocytes of the small intestine No staining was observed in the foetal liver or adrenal gland Low level LRP-56 staining was observed in the distal but primarily in the proximal tubules where strong cytoplasmic staining was observed Staining was also seen in the cytoplasm of placental trophoblasts and enterocytes of the small intestine In the foetal liver, bile ducts exhibited low level staining while the adrenal gland did not exhibit any reactivity when probed with MAb LRP-56 There appeared to be some co-expression of the LRP/MVP and 3/B6 antigens in foetal tissues excluding the foetal liver however this co-expression was absent from the adult tissues surveyed The difference in expression amongst normal tissues indicated that 3/B6 and LRP/MVP may have independent control mechanisms in theses tissues The shift in the expression of LRP/MVP in foetal kidney (distal and proximal tubules) to distal tubules only in the adult kidney might indicate a change in function in human adult kindey

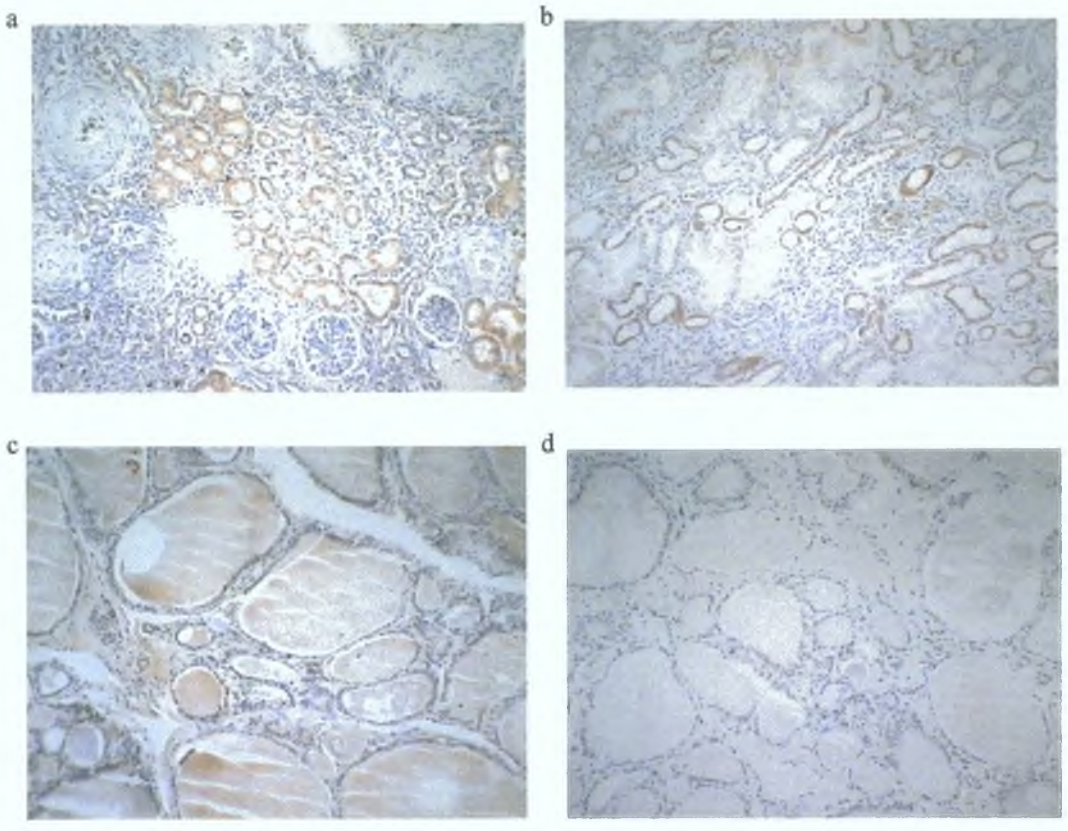


Figure 3.2.10.1. Immunohistochemical studies on normal tissue sections with MAb 3/B6 and LRP-56 (section 2.9.5. materials and methods), (a) and (b), kidney section stained with 3/B6 and LRP-56 respectively. (c) and (d), thyroid section stained with 3/B6 and LRP-56 respectively. Photographs were taken at 10X.

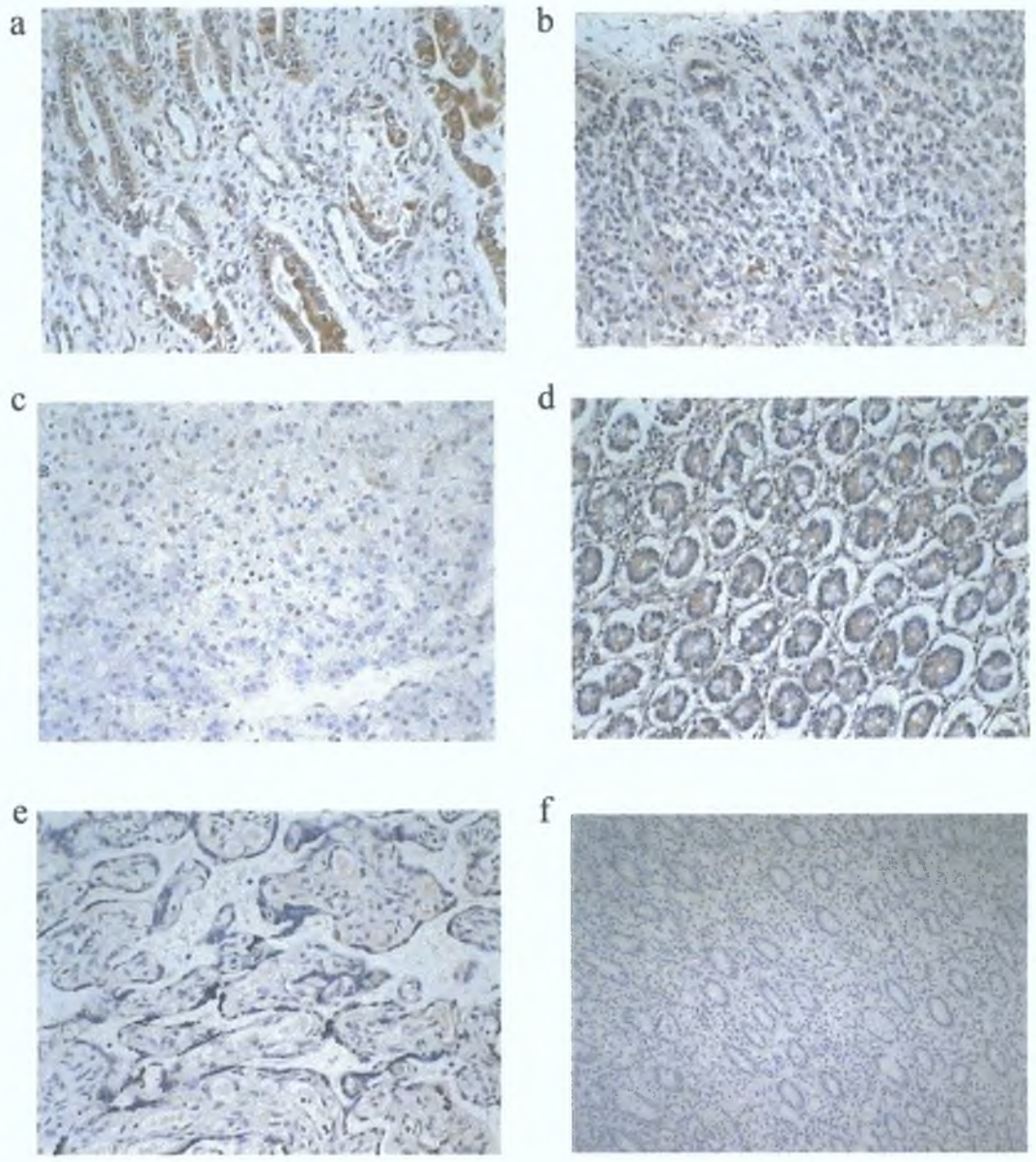


Figure 3.2.10.2. Immunohistochemical studies on human foetal tissue sections with MAb 3/B6 (section 3.2.10.), (a) foetal kidney, (b) foetal liver, (c) foetal adrenal, (d) foetal small intestine, (e) placenta. Photographs were taken at 20X. (f) negative control, foetal kidney probed with mouse IgG, photograph taken at 10X.

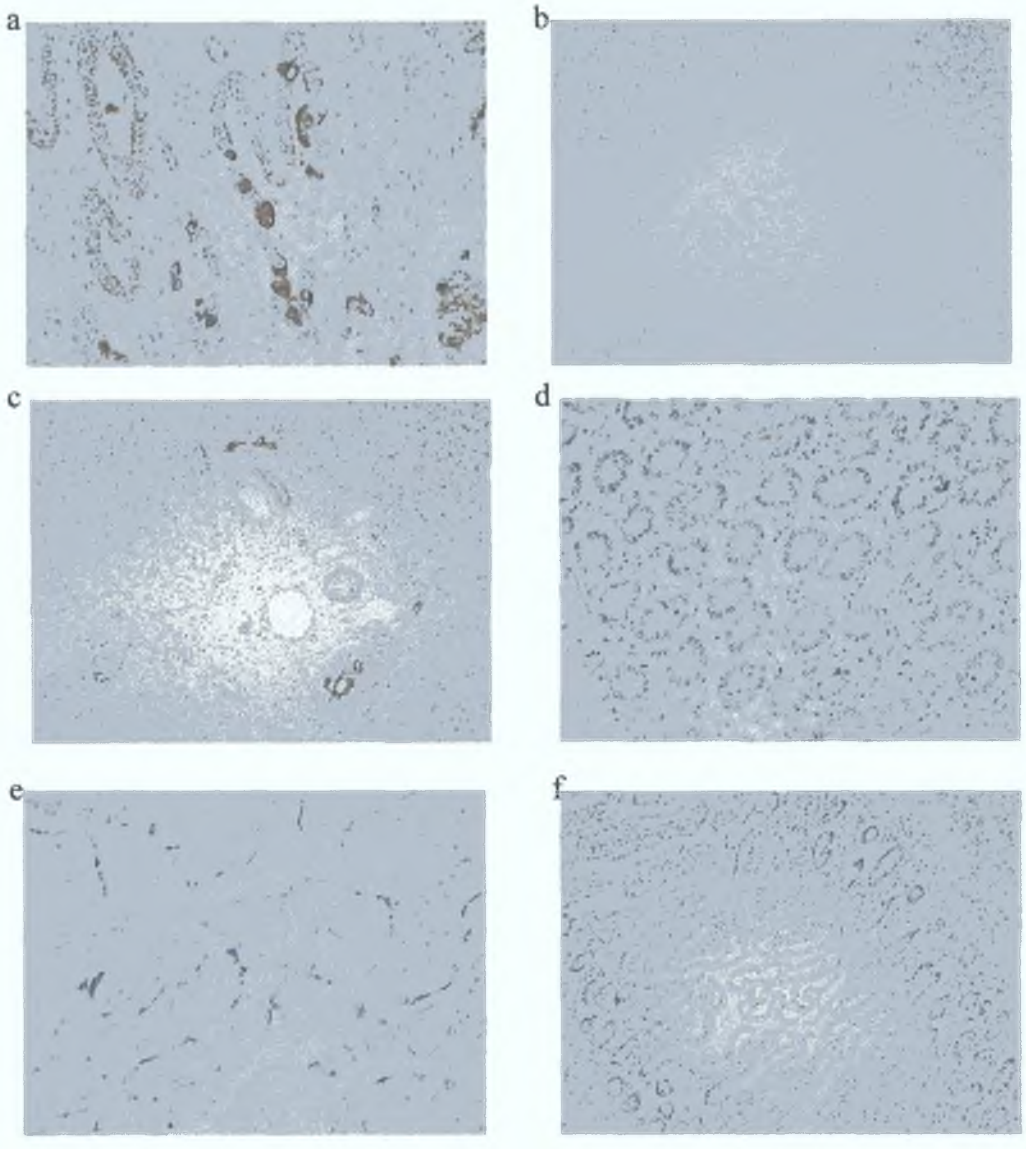


Figure 3.2.10.3. Immunohistochemical studies on human foetal tissue with MAb LRP-56 (section 3.2.10.), (a) foetal kidney, (b) foetal liver, (c) foetal adrenal, (d) foetal small intestine, (e) placenta, (f) negative control, small intestine probed with mouse IgG. (a), (d) and (e) were taken at 20X, (b), (c) and (f) at 10X.

3 2 11 Immunohistochemical investigation of ovarian and breast tumour tissue sections

Two normal ovary and ten pre-chemotherapy ovarian tumour blocks were probed with MAb raised to Pgp, MRP and LRP Two paired pre- and post-chemotherapy tissue blocks were also screened with MAb 3/B6 (section 2 9 5 materials and methods)

LRP/MVP was expressed at low levels in both normal ovary blocks (1604/97 and 1313/98) exhibiting diffuse cytoplasmic staining Some staining of blood vessels was also observed 3/B6 staining was absent on both of these tumour blocks (figure 3 2 11 1) LRP/MVP appeared to be expressed at low levels in 1/10 ovarian tumour blocks, block 1793/95 (figure 3 2 11 1) 3/B6 staining showed no reactivity on all pre-chemotherapy tumour blocks screened Some non-specific binding of both LRP-56 and LMR-5 was observed in the majority of tumour sections

The anti-Pgp MAb MDR-1 clone 6/1G and JSB-1 did not react with any of the tumour blocks assayed Low level MRP positivity was observed in 1/10 tumour blocks, block 2079/93 (figure 3 2 11 1) One post-chemotherapy ovarian tissue block was probed with MAb 3/B6 and LRP-56 to determine whether the 3/B6 antigen or the LRP/MVP were over-expressed in this tumour type Results from this experiment proved inconclusive in that there did not appear to be any areas of specific staining

The expression of the 3/B6 antigen was also examined in 2 ductal breast tumours as outlined above MAb 3/B6 did not appear to stain the pre-chemotherapy blocks (figure 3 2 11 2 (a) and (c) Whereas, in the post chemotherapy blocks, distinct cytoplasmic staining was evident in the infiltrating cells of the tumour This was particularly evident in block 1644/94 (d) Some low level background straining was also observed

Table 3 2.11 Immunohistochemistry results for normal, pre- and post chemotherapy ovarian tissue samples and pre- and post-chemotherapy breast tissue sections

Block Number	Antibody staining					
	3/B6	LRP-56	LMR-5	MDR-1	UIC-2	MRP r1
Normal Ovary			ND	ND	ND	ND
1604/97	-	+/-				
1313/98	-	+/-	ND	ND	ND	ND
Pre-chemotherapy						
2966/93	-	-	-	-	-	-
1797/93	-	+	+	-	-	-
2079/93	-	-	-	-	-	+
2122/93	-	-	-	-	-	-
2527/93	-	-	-	-	-	-
1994/94	-	-	-	-	-	-
2472/94	-	-	-	-	-	-
2664/94	-	-	-	-	-	-
1/95	-	-	-	-	-	-
5/97	-	-	-	-	-	-
Post-chemotherapy						
3069/92	inconclusive	inconclusive	ND	ND	ND	ND
Breast tumour Pre-chemotherapy						
[a]1423/92	-	- (Larkin *et al* 1998b)	ND	ND	ND	ND
[b]1418/86	-	-(Larkin *et al* 1998b	ND	ND	ND	ND
Breast tumour Post-chemotherapy				ND		ND
[a]1664/94	++	+(Larkin *et al* 1998b	ND	ND		ND
[b]9987/86	++	-(Larkin *et al* 1998b	ND	ND		ND

This staining index is based on the number of cells staining positive for 5/C4 ranging from approximately 5-10% of cells (+), to 90% of cells (+++) (a) and (b) are paired pre-and post-chemotherapy samples

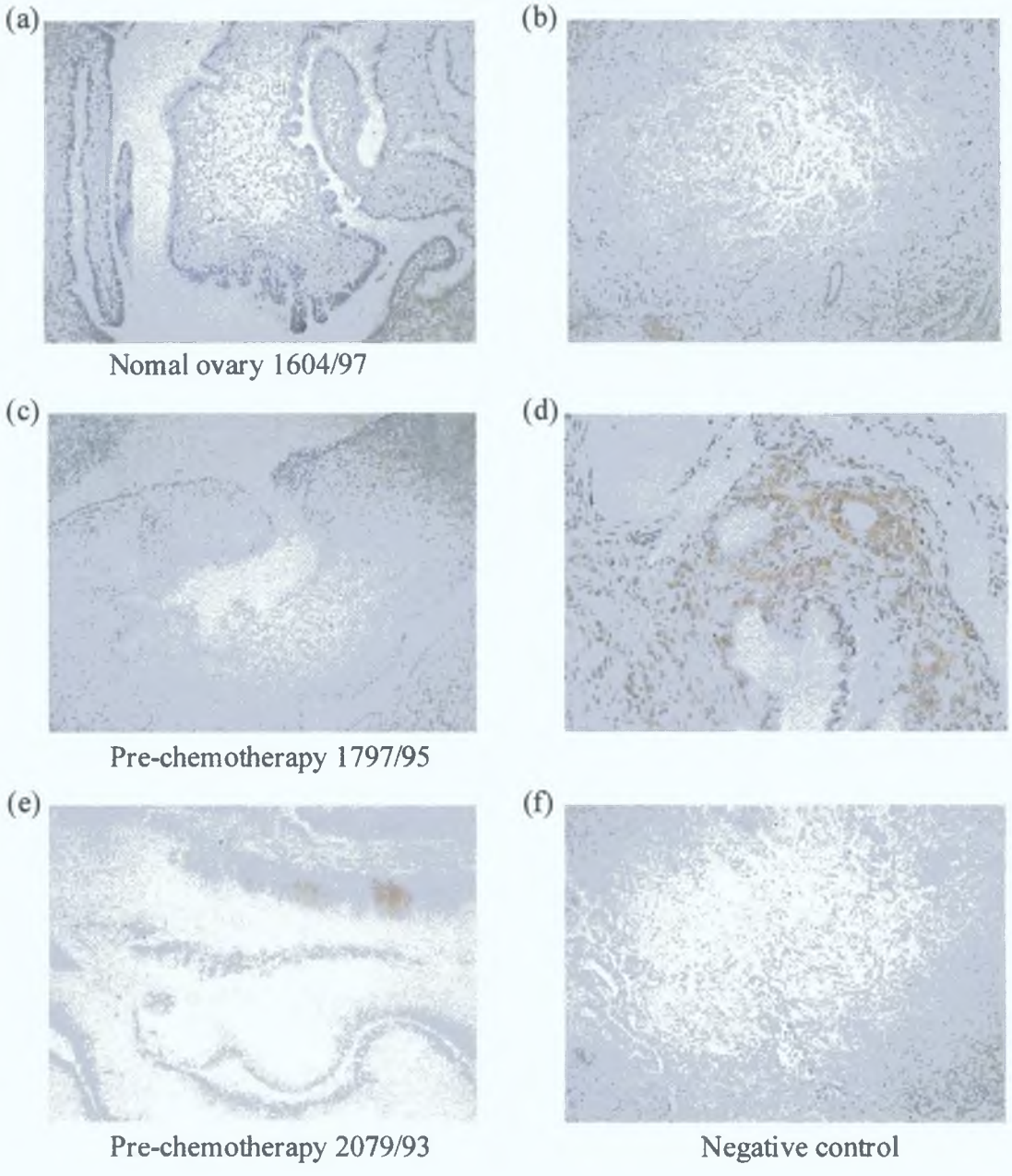


Figure 3.2.11.1. Immunohistochemical analysis of normal and pre-chemotherapy ovarian histological tissue sections (section 3.2.11.) (a) and (c) were probed with MAb 3/B6, (b) and (d) were probed with MAb LRP-56. (e) was probed with MAb MRPr1. (f) was probed with mouse IgG. Photographs were taken at 10X

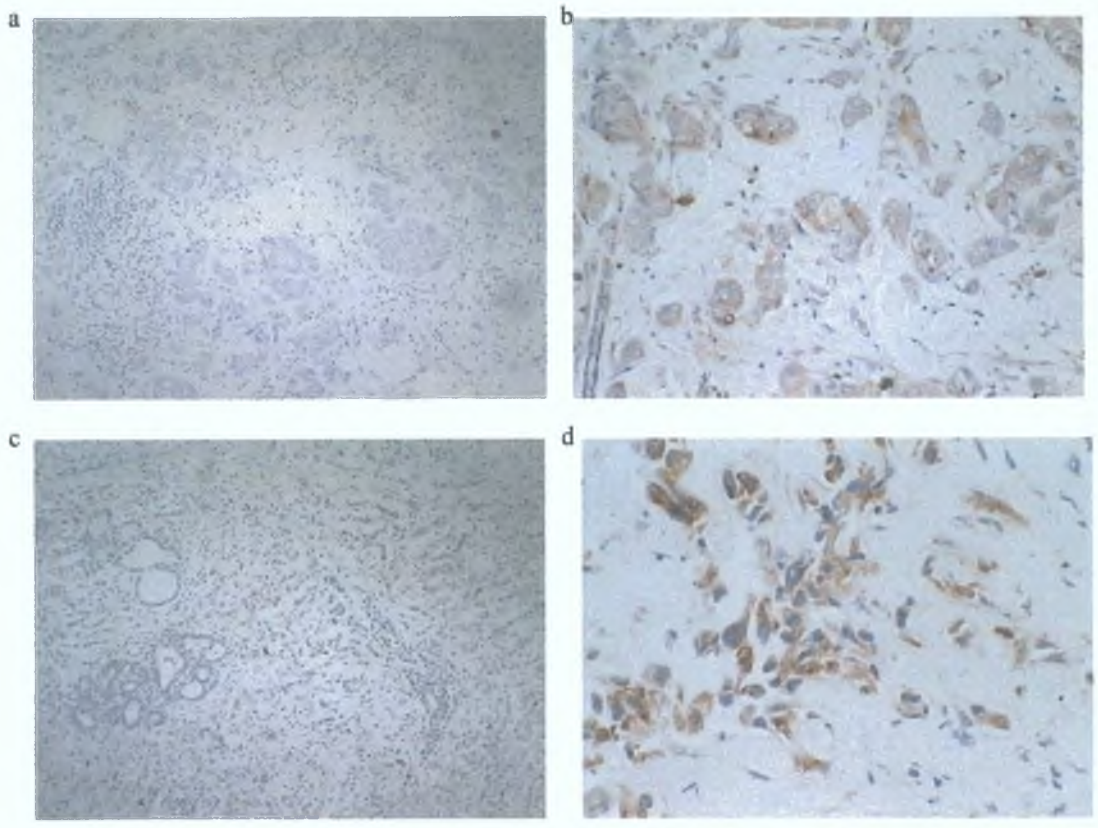


Figure 3.2.11.2. Immunohistochemical studies with MAb 3/B6 on paired pre- and post-chemotherapy breast tumour sections (section 2.9.5. materials and methods). (a) and (c), pre-chemotherapy treatment, (b) and (d), post-chemotherapy treatment (a) and (c) were taken at 10X, (b) and (d) were taken at 20X.

3.2 12 Investigation of species cross-reactivity with MAb 3/B6 and LRP-56

A series of rodent and primate kidney cell lines were investigated to determine the expression of the 3/B6 antigen by immunoprecipitation Results indicate that 3/B6 expression was confined to the African Green Monkey kidney cell line B-SC-1, figure 3 2 12 1 lane 5 These results suggested that 3/B6 may be a human protein homologue which was not expressed in the lower mammals

These cell lines were also assayed for the expression of the LRP/MVP Results from these experiments indicated that the LRP/MVP was expressed in the bovine kidney cell line MDBK and at very low levels in the green African monkey cell line BS-C-1, figure 3 2 12 2 This suggests that the 3/B6 antigen and the LRP/MVP are differentially expressed in these animal cell lines

3 2 13 Addition of MAb 3/B6 to OAW42-SR cells in culture

OAW42-SR cells were grown in the presence of MAb 3/B6 and LRP/MVP to determine whether these antibodies had any effect on cellular proliferation (section 2 11) Results from this experiment indicated that the addition of MAb 3/B6 and LRP-56 did not have any significant effect on the proliferation of OAW42-SR cells These results suggested that the 3/B6 antigen was not involved in cellular proliferation

3 2 14 Drug accumulation studies with MAb 3/B6

OAW42-SR cells were incubated with adrimycin in the presence of MAb 3/B6 and or cyclosporin A (an agent which is known to enhance drug accumulation by inhibiting Pgp) This experiment was carried out to determine whether 3/B6 had any effect in blocking drug efflux and thereby imply a role for the 3/B6 antigen in drug resistance and transport (section 2 12)

Incubation of OAW42-SR cells with MAb did not appear to have any effect on the accumulation of adriamycin over a 3 hour time period (Table 3 2 14 and Figure 3 2 14) A maximum of 375 n moles of adriamycin was accumulated after a 3 hours Similar

accumulation values were observed in the negative control (OAW42-SR cells incubated with adriamycin but without MAb 3/B6 Increased adriamycin accumulation (to a maximum level of 995 pmoles/million of cells) was observed in the positive control sample, OAW42-SR cells incubated with 10 mM adriamycin and 10 μg/ml cyclosporin A Despite this lack of effect by the MAb 3/B6 , it did not imply that 3/B6 did not have a direct role in drug resistance, simply that this MAb had no direct effect on the function of the protein

Table 3 2 14 Drug accumulation in OAW42-SR with MAb 3/B6

	Time points (hours)	Drug accumulation (pmoles/million cells)
OAW42-SR + Adr	**0**	0
	1	295
	2	320
	3	375
OAW42-SR + Adr + 3/B6	**0**	0
	1	290
	2	305
	3	370
OAW42-SR + Adr + cyclosporin A	**0**	0
	1	576
	2	895
	3	995

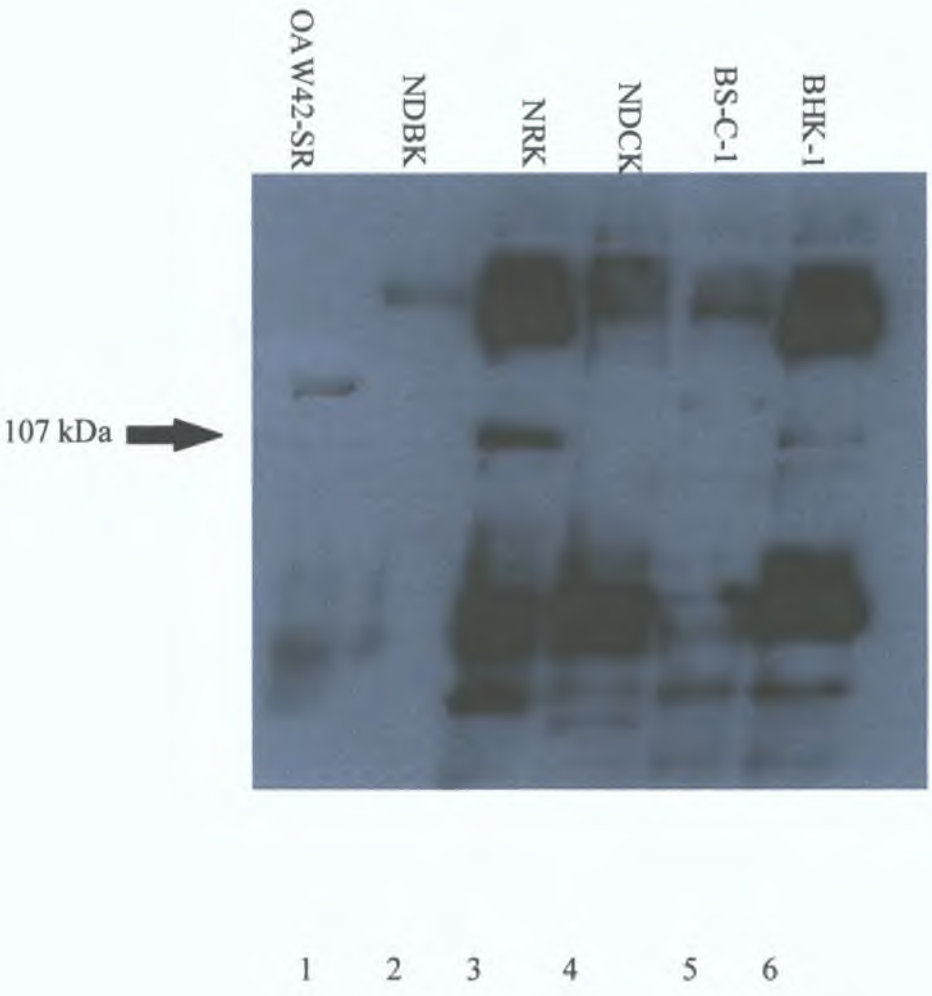


Figure 3.2.12.1. Species cross reactivity studies with MAb 3/B6. Blots were probed with an anti-biotin horseradish labelled secondary antibody and enhanced chemiluminescence (section 2.10.4. materials and methods).

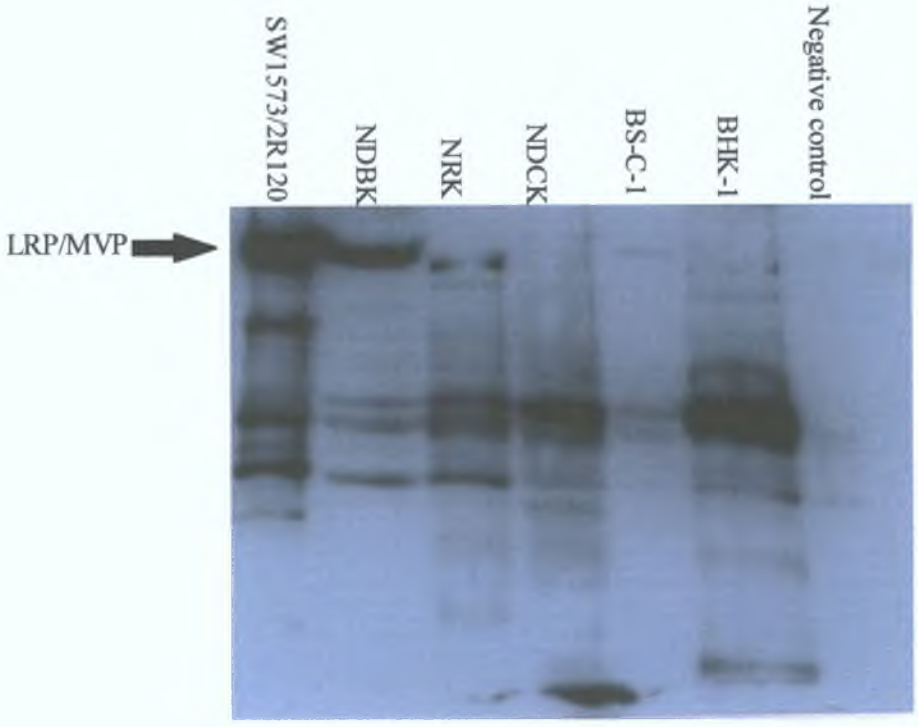


Figure 3.2.12.2. Species cross-reactivity studies with MAb LRP-56. All cell lines were immunoprecipitated with MAb LRP-56 (section 3.2.12.). Blots were probed with an anti-biotin horseradish labelled secondary and enhanced chemiluminescence (2.10.4. materials and methods).

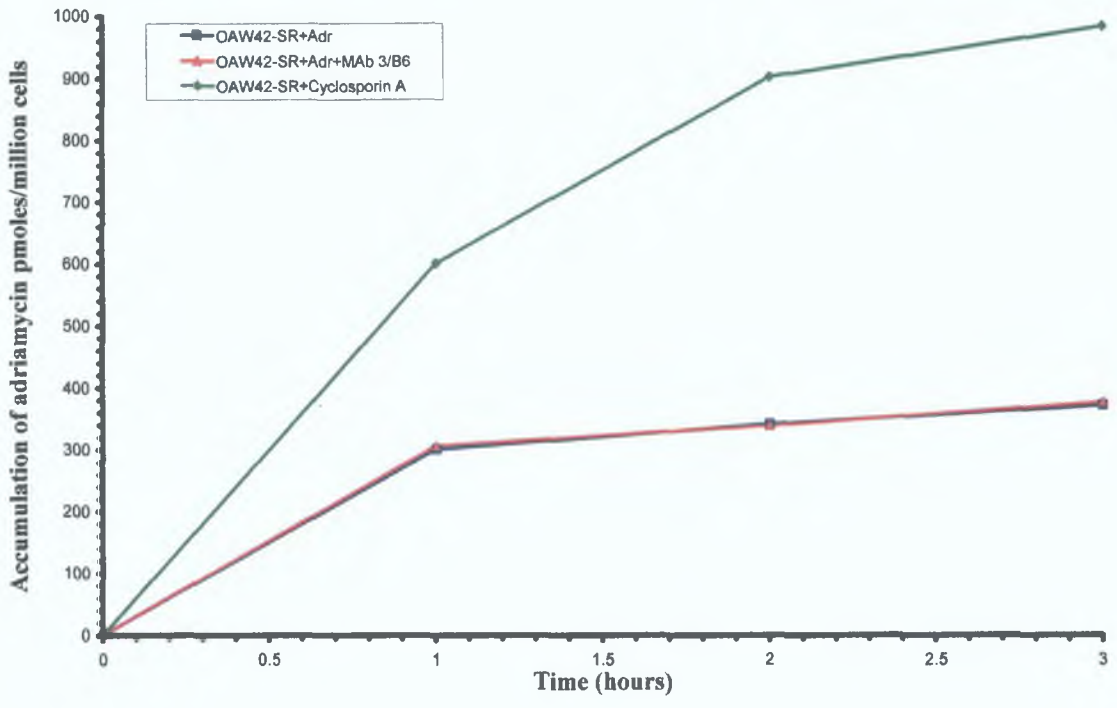


Figure 3.2.14. Drug accumulation studies with MAb 3/B6 (section 2.12. materials and methods). Time course of adriamycin drug accumulation in OAW42-SR cells, section 3.2.14.

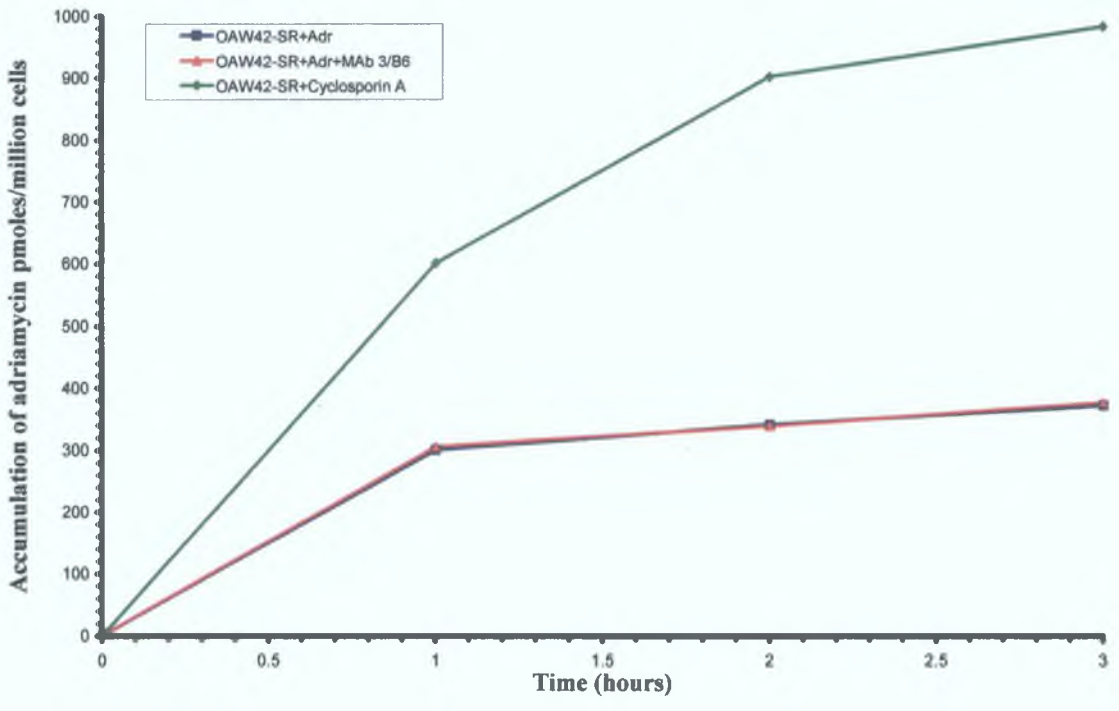


Figure 3.2.14. Drug accumulation studies with MAb 3/B6 (section 2.12. materials and methods). Time course of adriamycin drug accumulation in OAW42-SR cells, section 3.2.14.

3 2 15 Micro-sequencing of 3/B6 peptides

3/B6 immunoprecipitates were separated by SDS-PAGE (section 2 10 2 materials and methods) and the 3/B6 band excised from the gel Gel fragments were then sent to Eurosequence (Groningen, The Netherlands) for internal peptide sequencing Following elution, protein samples were subjected to trypsin digestion The resultant peptides were purified by reverse phase HPLC (RP HPLC) These peptides derived from the 3/B6 protein were then subjected to N-terminal sequencing by Edman degradation and sequencing on a pulsed liquid sequencer (section 2 10 7 materials and methods) Sequence analysis of the 3/B6 protein yielded a 14 amino acid peptide with the following amino acid sequence,

S-T-V-S-L-T-C?-M-V-T-S-F-W-P

5 10 14
Ser-Thr-Val-Ser-Leu-Thr-(Cys?)-Met-Val-Thr-Ser-Phen-Tyr-Pro

There was a tentative assignment of a cysteine residue at position 7 Cystine residues form disulfide bonds which must be cleaved before the proteins can be sequenced Unless specifically requested from the company, cysteine residues are not derivativised and therefore cannot be sequenced In this case, this option was not requested, (cysteine is usually converted to a stable derivative by 4-vinylpyridine) Processing of the peptide for sequencing can lead to modification of the cysteine residues e g carboxy-methylcystine HPLC analysis identified a peak at an elution time point which was similar to cystine and therefore, there was a tentative assignment of a cysteine residue at position 7 A protein database search revealed homology with the following proteins,

Table 3 2.15 Sequence homology results for 3/B6 antigen

Protein	% Homology
Blood Coagulation Factor XII a Inhibitor	57%
MHC class I alpha fragment	47%
Rat IG gamma-1-chain C region	47%
MHC class I alpha 2 fragment[a]	47%
MHC class I alpha 2 fragment[b]	46%

a and b are fragments of the same protein and constitute individual entries to the data base

Appendix 2 contains the high performance liquid chromatographs for the 14 cycles which yielded the peptide illustrated above The aspartic acid residue at time point 3 91 minutes was incorrectly assigned, this peak originates from ammonia which is a by-product of phenylthiocarbamyl derivitisation of amino acids and is present in all peptide sequencing results Dmp, dptu, dpt and mptc are also by-products of the Edman process, dptu and mptc are always present at these quantities A number of amino acids appeared at low levels in the chromatograph The relatively low level amounts of these residues present in each cycle originated from a general background present during the sequence analysis Glycine was found to be present at higher amounts in the early cycles, reducing in concentration in later cycles Glycine is generally found at these levels in internal peptide sequencing When glycine is an amino acid in the sequence, a substantially larger peak is observed

These results implied sequence homology with these proteins however 3/B6 may not necessarily be a member of these protein families particularly since it has been demonstrated that 3/B6 recognises an un-glycosylated protein whereas both of these protein families are glycosylated

3 3 Characterisation of MAb 5/C4

3 3 1 Immunofluorescence on live cells with MAb 5/C4

MAb 5/C4 was raised to the OAW42-SR cell line Immunofluorescence studies were carried out on live cell preparations of OAW42-SR and OAW42-S, (section 2 9 3 materials and methods) Results are illustrated in Figure 3 3 1

MAb 5/C4 did not detect its antigen on the surface of OAW42-SR or OAW42-S cells i e there was no fluorescent ring observed around the cells When these cells were incubated with a glutaraldehyde based fixative (FacsLyse solution), for 1 minute, faint cytoplasmic staining was observed in OAW42-SR cells only The negative controls, consisting of cells incubated with mouse control IgG, did not exhibit plasma membrane or cytoplasmic staining These results indicated that the antigen recognised by MAb 5/C4 was expressed exclusively in the cytoplasm of OAW42 variants

3 3 2 Immunocytochemical analysis of a panel of multidrug resistant/sensitive cell lines

This panel of cell lines contained a series of cell lines with varying degrees of drug resistance and were assayed using the AB/HRP complex method (section 2 9 5 materials and methods), results are outlined in table 3 3 1 and illustrated in Figure 3 3 2

Immuno-reactivity was observed in all of these cell lines There appeared to be little difference in the intensity of staining although differences in the number of cells stained was observed Approximately 90% of OAW42-SR and OAW42-S cells were stained positive with MAb 5/C4 There was no variation in the intensity of staining in the cell lines Approximately 50% of A2780/AC12 and A2780/AC16 exhibited immuno-reactivity with MAb 5/C4 while 10% of A2780 cells were positive Low level staining was also observed in the MOP-8, COR-L23R and COR-L23S cell lines Negligible staining was seen in the DLKP, DLKP-A and DLKP-A 10 lines Some variation in staining was observed in the LRP ribozyme and antisense transfected OAW42-SR cells

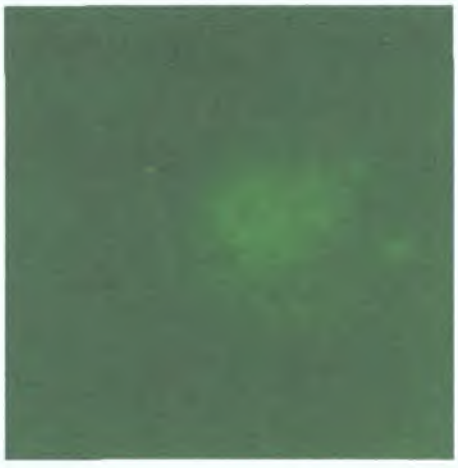
with a reduction in staining in the RZ2 and A/S1 clones These results suggested that, unlike the MAb 3/B6, MAb 5/C4 did not appear to be associated with any particular resistance associated mechanism

Table 3 3 2 Immunocytochemical analysis of a panel of drug resistant/sensitive cell lines with MAb 5/C5

Cell line	Antibody positivity
OAW42-S	+++
OAW42-SR	+++
SW1573	+
SW1573/2R120	+
SW1573/2R160	+
A2780	+
A2780/AC12	++
A2780/AC16	++
MOP-8	+
GLC4	+
GLC4/ADR	+
OAW42-SR A/S1	++
OAW42-SR RZ2	++
OAW42-SR RZ4	+++
COR-L23R	+
COR-L23S	+
DLKP	+/-
DLKP-A	+/-
DLKP-A10	+/-

This staining index is based on the number of cells stained positive for 5/C4 ranging from approximately 5-10% of cells (+), to 90% of cells (+++)

(a)



(b)

Figure 3.3.1. Indirect immunofluorescence on live cells with MAb 5/C4 (section 3.3.1.), (a) OAW42-SR cells treated with facs-lyse fixative for 1 min. at room temperature, (b) OAW42-SR cells untreated and probed with MAb 5/C4 for 0.5 hours at 4^0C. MAb 5/C4 was detected with a FITC-labelled secondary antibody (section 2.9.4. materials and methods). Photographs were taken at 40X.

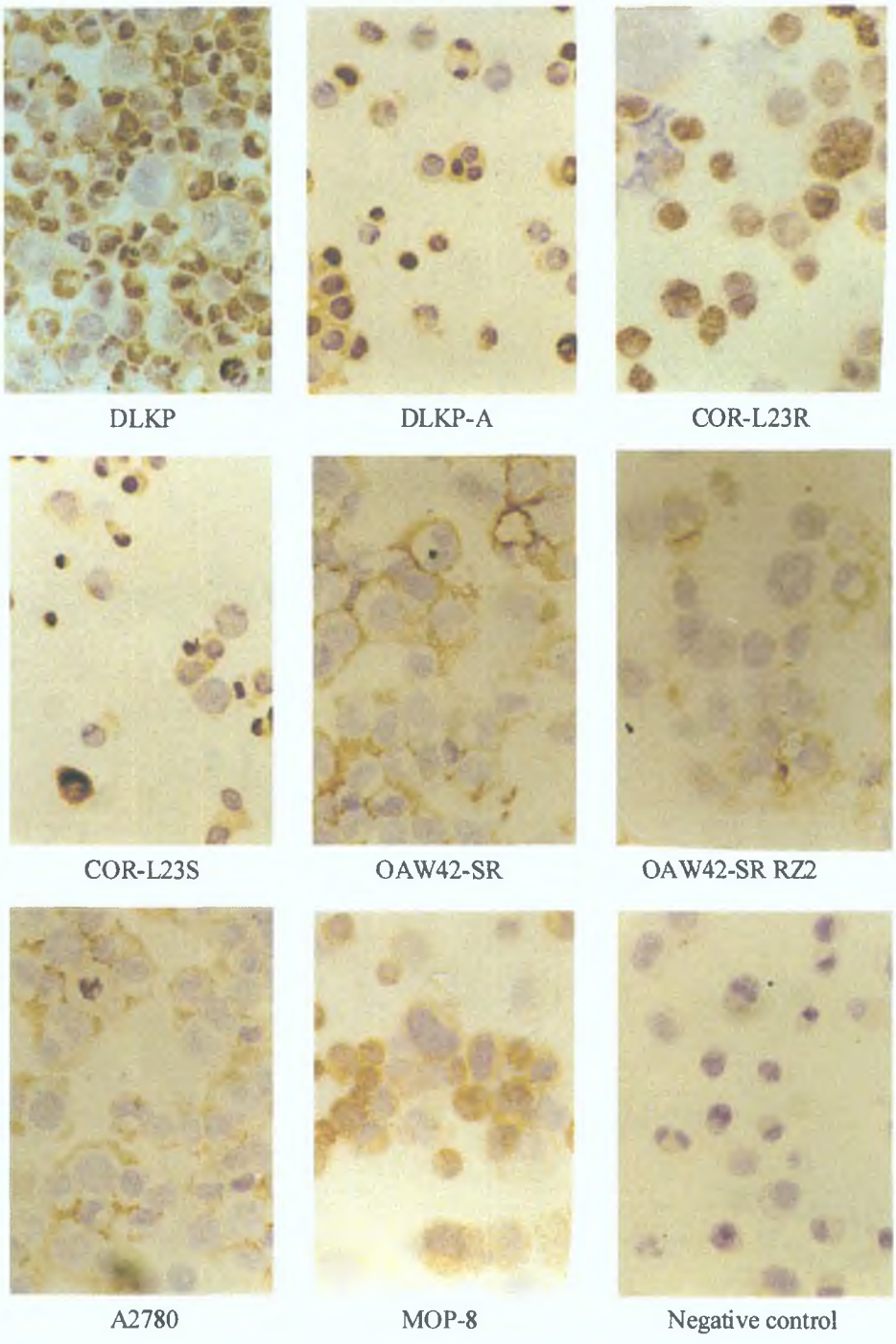


Figure 3.3.2. Immunocytochemical analysis of MDR cell lines with MAb 5/C4 (section 3.2.2.) using the DAB/ABC method (section 2.9.5.). Negative control cytospin was probed with mouse IgG. Photographs were taken at 20X.

3 3 3 Determination of molecular weight by Western Blot analysis

The molecular weight of the antigen detected by MAb 5/C4 was determined by Western Blotting (section 2 10 3 materials and methods)
Analysis of OAW42-SR and OAW42-S cell lysated indicate that the anti-OAW42-SR antibody 5/C4 recognised 2 bands with molecular weights of approximately 110 kDa and 85 kDa, figure 3 3 3 The 2 bands appeared to be of equal intensity An anti-MVP MAb , 1032 was included for comparison, it also detected 2 bands at 110 kDa and 85 kDa, figure 3 3 3 The 110 kDa band detected by MAb 1032 was more intense in the LRP positive control cell line, SW1573/2R120 whereas the lower 85 kDa band was more intense in OAW42-SR cells These results implied that the MAb 5/C4 may recognise an alternative form of the LRP/MVP Detection of a modified form was more likely because of the differences in the staining pattern between MAb 5/C4 and results previously observed with LRP-56 (section 3 2 3 2)

3.3 4 Western blotting studies in a panel of multidrug resistant/sensitive cell lines

A series of paired drug resistant cell lines and their sensitive counterparts were examined by Western Blotting with MAb 5/C4, results are illustrated in figure 3 3 4 Low expression of the antigen detected by MAb 5/C5 was observed in DLKP and the Pgp over-expression DLKP-A cell lines with 2 faint bands at 110 kDa and 85 kDa (lanes 1 and 2) Significantly low levels were also detected in the COR-L23R cell line (lane 6) There did not appear to be any significant expression in the sensitive COR-L23S cell line (lane 7) A2780 and the 5/C4 positive control cell line OAW42-SR appeared to strongly over-express the 2 bands recognised by MAb 5/C4, in the LRP/MVP transfected A2780/AC16 cell line, only the 110 kDa appeared to be strongly expressed (lanes 3, 4 and 5) Expression of the 110 kDa or 85 kDa band may have some significance for the function of the 5/C4 and or the LRP/MVP In order to determine the specificity of the MAb 5/C4 further experiments were carried out

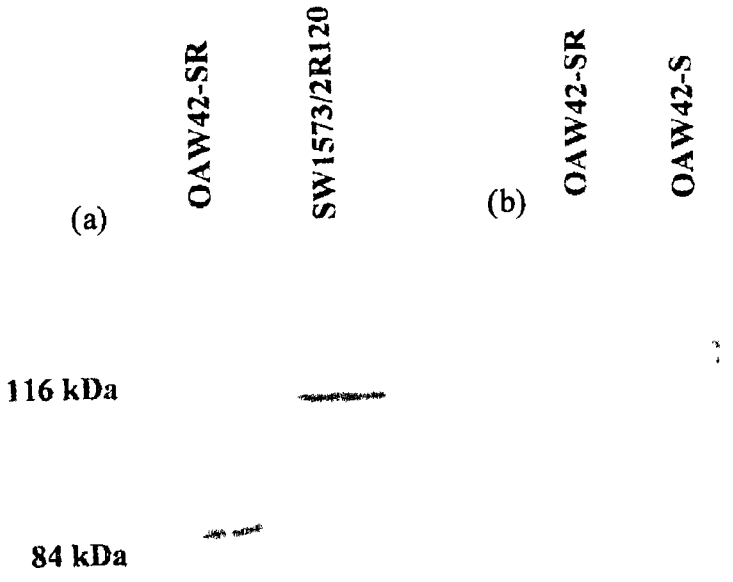


Figure 3 3 3 Western blotting results of crude cell lysates of OAW42-SR, OAW42-S and SW1573/2R120 (section 3 3 3 and section 2 10 3 materials and methods) Blots were probed with (a) anti-MVP antibody 1032 and (b) MAb 5/C4 visualised with a horseradish peroxidase labelled anti-mouse secondary antibody and enhanced chemiluminescence (section 2 10 4 materials and methods)

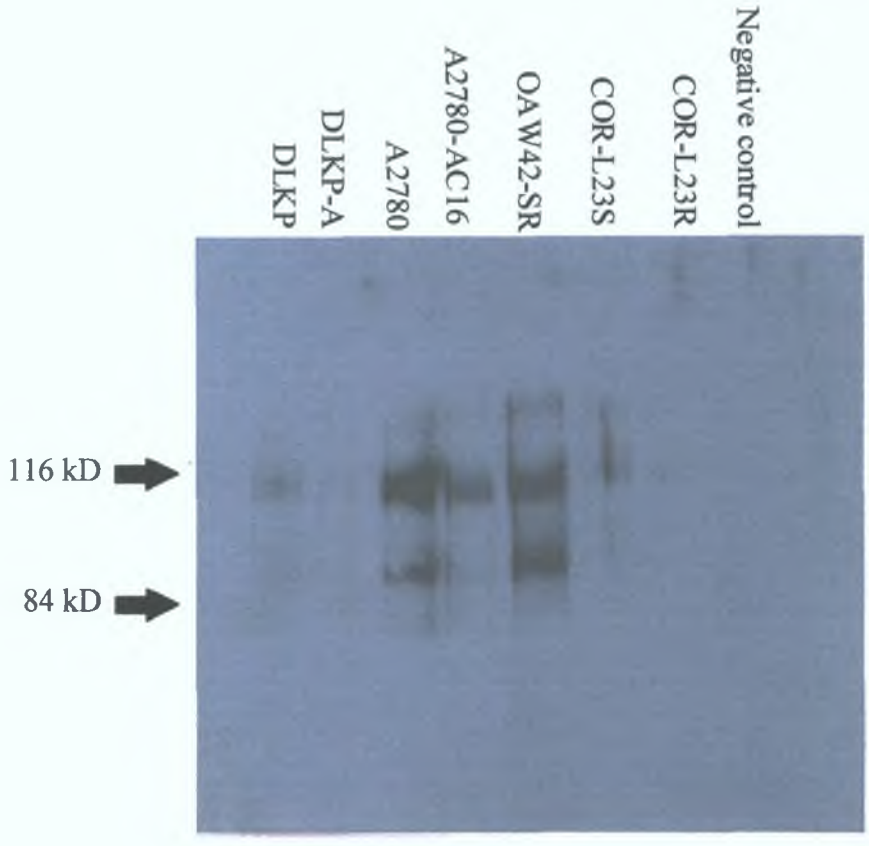


Figure 3.3.4. Western blots of crude whole cell lysates of MDR cell lines probed with MAb 5/C4 (section 3.3.4.). Blots were visualised with an anti-mouse horseradish peroxidase-labelled secondary antibody and enhanced chemiluminescence (section 2.10.4. materials and methods).

3 3 5 Western Blotting studies with purified rat vaults

In this study purified rat vaults (Kedersha and Rome 1986) were probed by Western Blotting (section 2 10 3 materials and methods) with MAb to determine if cross reactivity occurred Results indicate that MAb 5/C4 does not recognise the any of the constituent proteins of rat vault particles The MAb 1032 (which was raised to purified MVP) does detect a band at 104 kDa but not at 84 kDa (Figure 3 3 5)

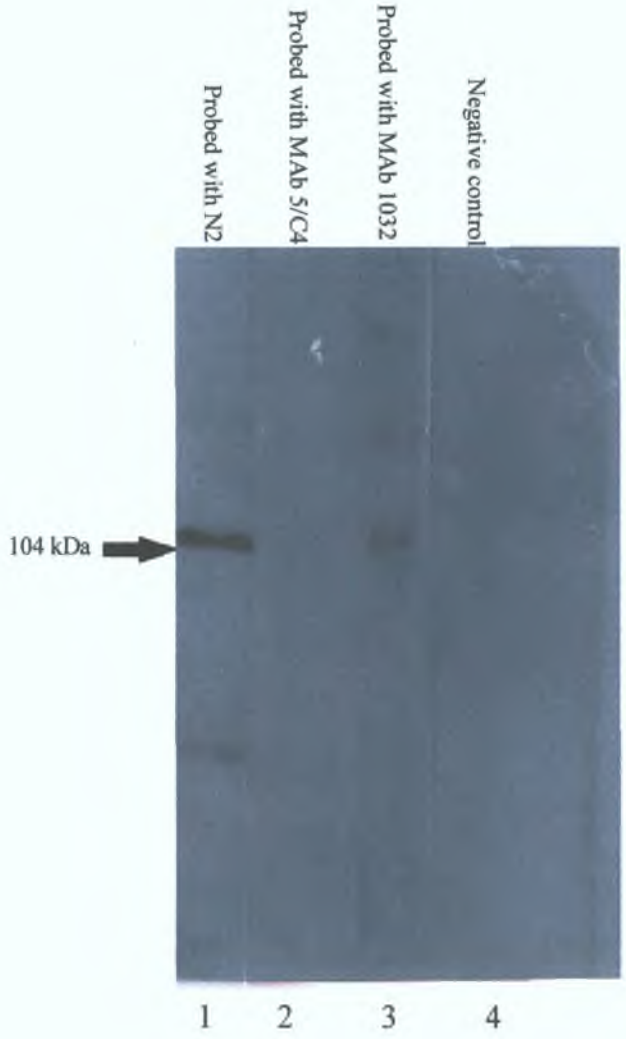


Figure 3.3.5. Western Blotting studies with purified rat vault particles (section 2.10.3. materials and methods). Blots were probed with **1**, anti-rabbit horseradish peroxidase-, **2-4**, anti-mouse horseradish peroxidase-labelled secondary antibodies and enhanced chemiluminescence (section 2.10.4. materials and methods).

3 4. Characterisation of MAb 3/E3

3 4 1 Indirect immunofluorescence studies on live cells with the anti-OAW42-S MAb 3/E3

Live cell preparations of OAW42-S were incubated with MAb 3/E3 to determine whether it detected a plasma membrane associated antigen (section 2 9 3 materials and methods) Results show that OAW42-S cells exhibited plasma membrane staining characterised by a fluorescent ring around the cells which was absent from the negative control Results are outlined in figure 3 4 1

3 4 2 Indirect immunofluorescence on fixed cells with MAb 3/E3

Cytospins of OAW42-SR and OAW42-S cells probed with MAb 3/E3 to investigate if the antigen recognised by this antibody was also expressed intracellularly Results are illustrated in figure 3 4 2

Staining in OAW42-S cells was predominantly plasma membrane with some diffuse faint cytoplasmic staining In OAW42-SR cells, the staining mainly cytoplasmic and granular in nature In some cells, the antibody appeared to stain structures associated with the nuclear envelope Some plasma membrane staining was also observed, no staining was observed in the negative control

3 4 3 Immunocytochemical analysis of a panel of paired drug resistant/ sensitive cell lines

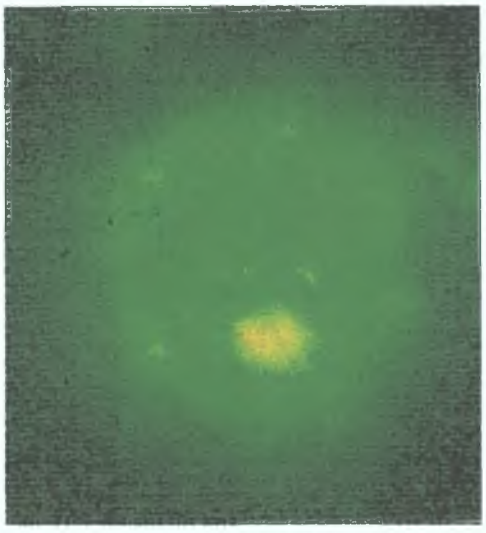
A series of multidrug resistant and sensitive cell lines were examined to determine the expression of the antigen detected by MAb 3/E3 Immuno-reactivity was observed on all cell lines tested There was no apparent differences in the number and intensity of cells stained There was significant difference in the cellular location of the antigen detected by MAb 3/E3 Typical results are illustrated in figure 3 4 3 These results suggested that

the antigen recognised by MAb 3/E3 was a constituent plasma membrane protein in human cells

3 4 4 Determination of molecular weight or MAb 3/E3

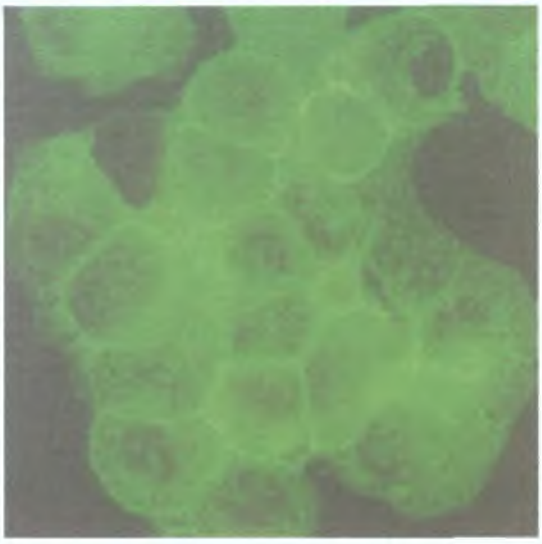
MAb 3/E3 failed to recognise its antigen by Western Blotting and immunoprecipitation techniques It is therefore not possible to assign a molecular weight to the antigen detected by MAb 3/E3 This suggested that this antibody may recognise a conformational epitope which was destroyed by Western Blotting The inability of the antibody to immunoprecipitate the 3/E3 antigen suggested that the buffer constituents of the lysis buffer may have contributed to the destruction of the epitope

(a)

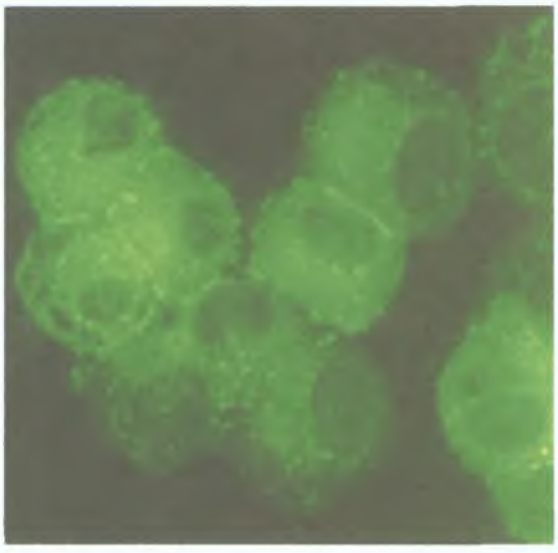


(b)

Figure 3.4.1. Immunofluorescence studies on live OAW42-S cells. Cells were probed with MAb 3/E3 and detected with FITC-linked secondary antibody, (a). (b) is a negative control, OAW42-S cells probed with mouse IgG. All photographs were taken at 40X.



OAW42-S cells probed with MAb 3/E3



OAW42-SR cells probed with MAb 3/E3

Figure 3.4.2. Immunofluorescence studies on fixed cells with MAb 3/E3 (section 2.9.4. materials and methods). MAb 3/E3 was detected with a FITC-linked secondary antibody. Photographs were taken at 40X.

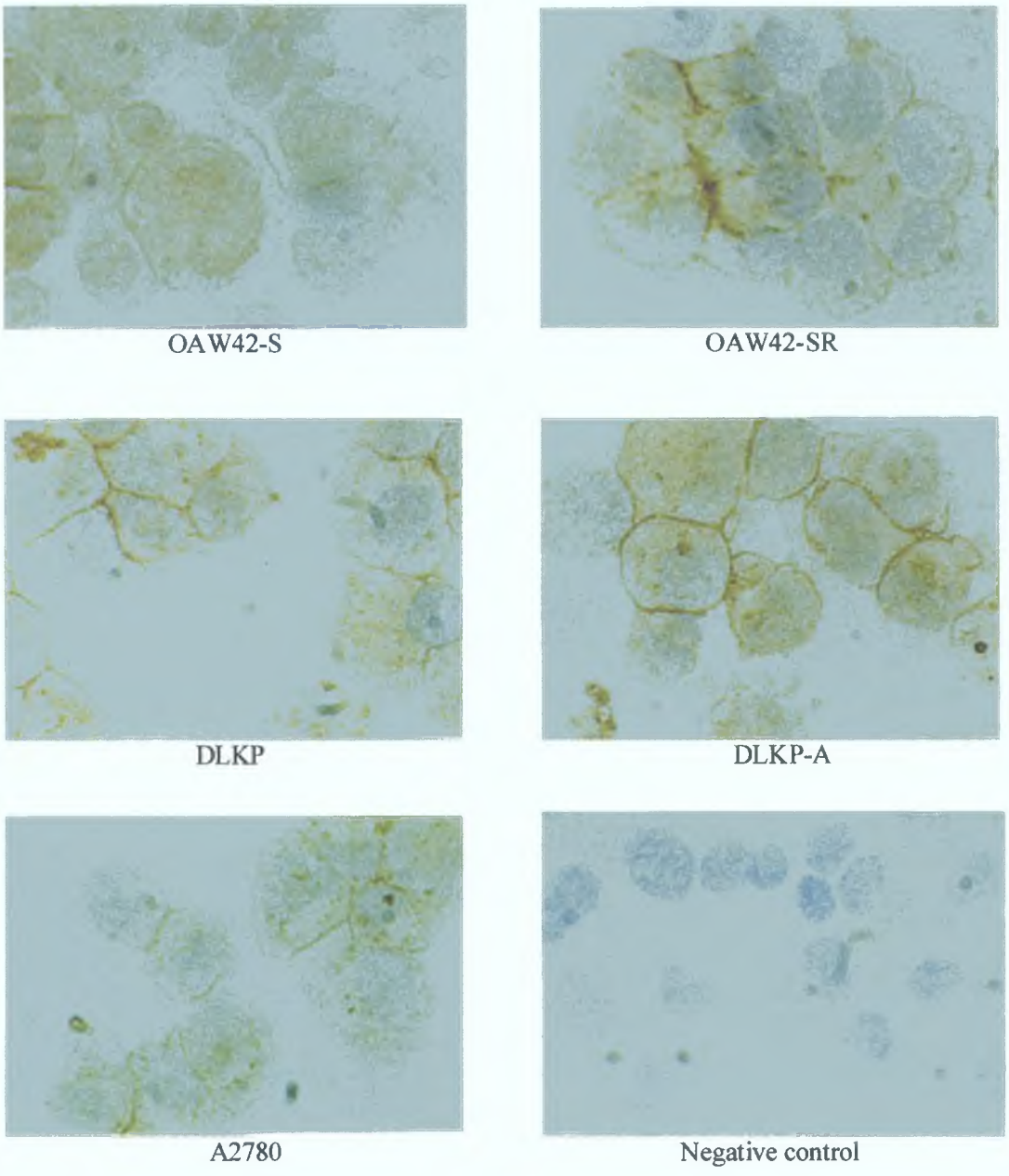


Figure 3.4.3. Immunocytochemical analysis of MDR cell lines with MAb 3/E3 (section 3.4.3.). Negative control was probed with mouse IgG. Photographs were taken at 40X.

4 0 Discussion

4 1 General discussion

Over the past two decades, extensive laboratory studies on the mechanisms of MDR have focused on drug-selected resistance cancer cell lines where a number of genetic, molecular and biochemical changes have been observed compared to parental cell lines In a number of these *in vitro* models, the expression of the MDR-1 gene product P-glycoprotein (Pgp), and the MRP gene product (MRP) appear to play a direct role in cytotoxic drug transport (Coley *et al*, 1989 Goldstein 1992, Zaman *et al*, 1994) The search for alternative mechanisms of drug resistance led to the discovery of the 110 kDa protein LRP which did not appear to be co-over-expressed with Pgp (Scheper *et al*, 1993)

The subsequent discovery by Scheffer *et al*, (1995) that LRP was the human homologue of rat and slime mould, major vault protein (MVP) and the subsequent (apparent) association of vault proteins with the central plug of the nuclear pore complex and clathrin-coated vesicles suggested some important physiological role for vault proteins and possibly LRP/MVP

Immunocytochemical analysis of OAW42-SR cells, the intrinsically resistant variant of the ovarian cell line OAW42, revealed up-regulation of the LRP/MVP associated with an increase in resistance to adriamycin (Moran *et al*, 1997) which suggested a role in drug resistance A similar increase in LRP/MVP expression in the OAW42-S cell line, a drug sensitive clonal sub-population of the OAW42-SR, without an associated increase in drug resistance suggested the existence of multiple forms of the LRP/MVP These observations prompted the research detailed in preceding chapters and discussed below

The initial aim of this project was the isolation of monoclonal antibodies raised to the OAW42-SR and OAW42-S variants In achieving this aim, a total of 11 clones were isolated from two fusions, 3 or which were chosen for further characterisation (see section 4 2)

Comprehensive characterisation of theses MAbs was the next major goal of the thesis Immunofluorescent and immunocytochemical analysis revealed that the anti-OAW42-SR

MAb 3/B6 displayed significant similarities in staining pattern with the anti-LRP/MVP monoclonal antibody LRP-56 while illustrating differences in cellular location of these antigens (Section 4 4 and 4 5)

These characterisation studies also suggested that 3/B6 recognised a epitope whose confirmation was destroyed in the course of sample preparation and Western Blotting in that the molecular weight could not be determined by conventional Western Blotting techniques (section 4 6) Immunoprecipitation of cellular antigens allows detection of intact antigens by a range of MAbs, which are unable to detect these antigens by conventional Western Blotting techniques The major goal in this phase of the project was to establish a reliable non-radioactive method for labelling and detecting the 3/B6 antigen This was achieved by the modification and further development of a new cellular labelling and immunoprecipitation kit based on labelling protein with biotin derivatives (section 4 6) This improved method allowed the characterisation of the 3/B6 antigen as a non-glycosylated protein with an apparent molecular weight of 115 kDa Further immunoprecipitation studies revealed that the expression of the 3/B6 antigen appeared to be closely linked with that of the LRP/MVP (section 4 6 1 4 6 2 and 4 6 4) confirming the pattern of staining observed for these two MAbs by immunocytochemistry

LRP/MVP vs 3/B6 antibody competition experiments (outlined in sections 3 2 3 3 and 3 2 6) were undertaken to determine if the antigen detected by 3/B6 was closely related to the LRP/MVP These experiments indicated that, although the 3/B6 antigen and the LRP/MVP had similar molecular weights and expression patterns in MDR cell lines, MAb 3/B6, LRP-56 and LMR-5 do not appear to recognise the same antigen (section 4 7)

Protein sequence data indicated some homology with constituent proteins of the MHC and blood coagulation cascade the percentage homology was low within a short (14 amino acid) peptide (section 4 12) This observation suggested that MAb 3/B6 may recognise a novel antigen which might be associated with drug resistance A tissue survey was undertaken to establish the tissue distribution of the antigen and to determine whether the 3/B6 protein was associated with drug resistance *in vivo* (section 4 8-4 9)

Analysis of the results indicated that the 3/B6 antigen might be an indicator of drug resistance in ovarian and breast cancer (section 4 8 1) rather than playing a direct role in drug resistance This tissue survey data correlated with the distribution in of the 3/B6 protein MDR cell lines which also pointed to an association with the drug resistance phenotype (section 4 4 and 4 5) Drug accumulation studies (section 4 10), data indicated that 3/B6 had no effect on drug accumulation

Isolation of the second anti-OAW42-SR MAb 5/C4 also revealed similarities between the antigen detected by this MAb and the LRP/MVP Immunocytochemical analysis revealed that MAb 5/C4 also stained the cytoplasm in a granular cytoplasmic fashion However, there was significant differences in their patterns of expression in MDR cell lines (4 13 1)

Western Blotting studies revealed that the 5/C4 antigen consisted of 2 protein bands which migrated as 110 kDa and 85 kDa which was similar to the anti MVP monoclonal antibody 1032 (4 13 2) However no cross-reactivity was observed with purified rat vault particles (4 13 4) The role, if any in drug resistance for this antigen has yet to be established

Increased expression of the LRP/MVP was observed in the OAW42-S without an associated increase in drug resistance (Moran *et al*, 1997a) This observation suggests the existence of alternative forms of the LRP/MVP, which may also have been present in the OAW42-SR The final aim of the project was to raise MAbs to the OAW42-S in an attempt to isolate antibodies to these possible alternative forms of LRP/MVP The anti-OAW42-SR MAb 3/E3 did not display any characteristics in staining consistent with known anti-LRP/MVP antibodies (section 4 14) It was not possible to determine the molecular weight of this antigen by Western Blotting or immunoprecipitation suggesting that the 3/E3 epitope may be conformational

4 2 Production of monoclonal antibodies

From a total of 370 clones isolated by cell fusion, supernatants from six of these clones consistently reacted with OAW42-SR cells while supernatants taken from five clones reacted with the OAW42-S cell line (Section 2 8 2) Initially the anti-OAW42-SR MAb 3/B6 did not cross-react with OAW42-S cells but in subsequent ELISA assays cross reactivity was observed Similarly the anti-OAW42-S MAb 3/E3 appeared to recognise a sub-population of the OAW42-SR, however this was not consistently observed In order to ensure that the results of these studies were consistent, OAW42-SR were used between passage 95 and 100 and OAW42-S between 1-6 only The different expression patterns observed in these early studies could have been due perhaps, to different clonal populations within the OAW42-SR however, it is more likely that these differences were due to an artifact of the immunocytochemistry procedure

4.3 Purification of ascites from hybridoma clone 3/B6

The concentration of IgG (as determined by the RID method, 2 8 1) was found to be approximately 2 5 mg/ml, while protein concentration for purified 3/B6 MAb was between 190-200 µg/ml which is a significant drop from the previous figure The purification columns used to purify the antibody should theoretically bind all IgG sub-classes as sepharose beds are coated in a fusion protein of protein A and G It is therefore assumed that the remaining substantial quantity of IgG was lost on the column due to precipitation of the antibody or simply that it did not bind to the column, the effluent was not assayed for IgG content to confirm this

4 4 Indirect immunofluorescence on live and fixed cells with MAb 3/B6

The initial characterisation of the antigen recognised by the anti-OAW42-SR MAb 3/B6 was carried out by immunofluorescence on live cells A fluorescent ring around the cell indicates specificity for an external epitope whereas The absence of cell surface fluorescence indicates a cytoplasmic epitope When OAW42-SR cells were probed with the MAb 3/B6 a bright ring was observed around the cell indicating that the epitope recognised by this antibody was expressed on the cell surface MAb LRP-56, which recognises the LRP/MVP, (a cytoplasmic protein), gave not staining The negative control for all these experiments was purified mouse IgG (which also gave a negative result, no staining was observed, Figure 3 2 1 1)

The expression of the 3/B6 antigen was then assayed in a panel of MDR drug resistant and sensitive paired cell lines including OAW42 variants Moran *et al*, (1997a) have investigated the expression of a number of MDR associated antigens in OAW42 patients Their results show the over-expression of the LRP/MVP in the OAW42-S and OAW42-SR with reduced expression in the OAW42-A1 and OAW42-A These variants were also examined during the characterisation of MAb 3/B6 (Figure 3 2 1 2, 3 2 1 3)
The highest level of staining was observed in the OAW42-SR (the cell line which the antibody was raised to table 3 2 1) Not all of the cells were stained with equal intensity Similar levels of staining were observed in the OAW42-S cell line The OAW42-S variant was cloned from the OAW42-SR, therefore some degree of positivity would be expected 3/B6 staining was less intense in other OAW42 variants, the OAW42-A1 and OAW42-A

Low level expression of the 3/B6 antigen was also detected in the DLKP-A, a Pgp over-expressing cell line whereas in the parental drug sensitive DLKP line 30% of cells were stained intensely with MAb 3/B6 The 3/B6 antigen appeared to be over-expressed primarily on low level resistant cell lines including the SW1573/2R120 and the sensitive A2780 DLKP has been shown to express low levels of MRP but little or no Pgp (Duffy *et al*, 1998) Although the OAW42-S have been shown to over-express the LRP/MVP, no change in resistance was observed (Moran *et al*, 1997a) LRP/MVP has been shown

to be primarily associated with low level resistant drug selected and intrinsically resistant cell lines (Moran *et al*, 1997a, Izquierdo *et al*, 1996a) The MRP over-expressing cell line COR-L23R did not appear to be specifically stained with MAb 3/B6 in that both the negative control (which was probed with mouse IgG) also displayed intense plasma membrane staining (figure 3 2 1 3) This suggests that the staining observed in the COR-L23R was probably non-specific

Immunofluorescence on fixed cells indicated that the antigen detected by MAb 3/B6 was also expressed in the cytoplasm of OAW42-SR cells,(Figure 3 2 2 1) Plasma membrane staining was also evident from this experiment, the granular cytoplasmic staining observed did not appear to be localised to any particular region of the cytoplasm Flens *et al*, (1997) have reported that a novel MAb, LMR-94 exhibits strong cytoplasmic staining close to the nucleus This group believe that this antibody may detect a component of the Golgi apparatus The 3/B6 antigen was located mainly on the plasma membrane of OAW42-S cells with some diffuse cytoplasmic staining The anti-LRP/MVP MAb LRP-56 was used for comparison in this study There was clear peripheral cytoplasmic staining with the absence of plasma membrane staining which is consistent with previous observations (Moran *et al*, 1997a, Izquierdo *et al*, 1996a, Scheper *et al*, 1993) This differed from the 3/B6 staining pattern where the cytoplasmic staining was more diffuse

The optimum fixation for MAb 3/B6 was determined for this experiment It was found that the best results were achieved when cytospins of cells were fixed in ice cold acetone for 4 min A high level of background was observed in negative controls of OAW42-S cells This may have contributed to some of the staining with these antibodies Results from these initial experiments were conflicting, MAb 3/B6 recognised an antigen on a number of LRP/MVP over-expressing cell lines but staining was primarily plasma membrane in nature LRP/MVP extracellular staining has not been reported Similarly, vault particles appear to be confined exclusively to the cytoplasm and nuclear membrane (Kedersha and Rome, 1986a, Chugani *et al*, 1992) This observation warranted further investigation

4 5 Immunocytochemical analysis with MAb 3/B6 and LRP-56

OAW42-S and OAW42-SR cells were re-examined using the ABC/ HRP method (as outlined in section 2 2 3) Results from this experiment agreed with immunofluorescence studies on fixed cells in that MAb 3/B6 stained both the cytoplasm and plasma membrane of OAW42-S and OAW42-SR cells with MAb 3/B6, (figure 3 2 3 1) There appeared to be a greater expression of the 3/B6 antigen in the cytoplasm The staining also appeared to be more granular in nature rather than the diffuse staining observed in the previous experiment

A number of paired drug resistant and drug sensitive cell lines (with different expression levels of the LRP/MVP) were probed with MAb 3/B6 and LRP-56 to determine if the pattern of staining for MAb 3/B6 in this expanded panel of cell lines was similar to the pattern observed with immunofluorescence on live cells, (Table 3 2 2 , figures 3 2 3 2 1 to 3 2 3 2 4) As with the previous experiments, MAb 3/B6 and LRP-56 appeared to stain the low level resistant cells with a greater degree of intensity The SW1573/2R120 drug selected cell lines are part of a larger series of variants isolated by Scheper *et al* (1993) Intense plasma membrane and cytoplasmic staining was observed in the LRP/MVP over-expressing 2R120 variant with less staining on the parental SW1573 However, the level of staining in the SW1573 cell line was higher than that observed with MAb LRP-56 where approximately 5-10% of cells displayed LRP/MVP positivity Similar levels of staining were observed in the Pgp positive 2R160 variant with both antibodies The drug resistant GLC4/ADR cell line over-expressed both the LRP/MVP and MRP (Flens *et al* , 1998) Staining was mainly confined to the plasma membrane of GLC4/ADR cells with only a small percentage of cells displaying cytoplasmic staining 3/B6 staining (in general) appeared to be consistent with that observed for LRP-56

Drug resistant variants of the non-small cell lung carcinoma cell line DLKP (Law *et al* , 1992) were established by exposure to adriamycin resulting in the isolation of the DLKP-A, (Clynes *et al* 1992) and DLKP-A10 cell lines (Cleary *et al* , 1997) The latter cell line over-expresses Pgp and exhibits low level MRP (Cleary 1995, PhD thesis) It was observed that the staining patterns of 3/B6 and LRP-56 in these variants were in general agreement i e the highest level of staining was observed in the DLKP and DLKP-A10

cell lines LRP/MVP and the 3/B6 over-expression was observed in a sub-population of the Pgp over-expressing cell line DLKP-A10 Co-expression of Pgp and LRP/MVP has been observed in some low level drug resistant sublines of the myeloma cell line 8226, the breast carcinoma cell line MCF7 and in variants of the OAW42 which have been described here In each case it was observed that LRP/MVP was expressed at higher levels (Moran *et al*, 1997a, Wyler *et al*, 1997, Cleary *et al*, 1997, Shao *et al*, 1995, Scheper *et al*, 1993,) Cleary *et al*, (1997) observed novel vesicular transport mechanisms in this cell line by confocal laser microscopy There also appears to be a significant level of LRP/MVP expression which suggests that LRP/MVP may play a role in the observed (novel) vesicular transport mechanism These immunocytochemistry results did not correlate with those obtained from immunofluorescence experiments discussed previously Higher levels of 3/B6 expression were observed by immunofluorescence in the DLKP-A and A2780 cell lines(figures 3 2 1 2 and 3 2 1 3) The difference in results could be due to the acetone fixation method used in the permeabilisation procedure

There was apparent up-regulation of the 3/B6 antigen in the LRP-cDNA transfected A2780-AC16 variant compared to the untransfected parental cell line A2780, similar results were obtained with MAb LRP-56 However, 3/B6 staining was absent from other cDNA transfected cells (Scheffer and Scheper, personal communication, results not shown), whereas, these cells displayed strong cytoplasmic staining when probed with MAb LRP-56 and the anti-vault polyclonal antibody N2 Byrne (1998) observed increased levels of LRP/MVP expression in certain LRP/MVP ribozyme and antisense transfected OAW42-SR clones) The increase in 3/B6 expression in the AC16 variant may be due simpley to the transfection procedure However, another LRP/MVP cDNA transfected clone, the AC12 variant displays less staining with MAb 3/B6 and LRP-56 It has been suggested that this clone did not receive the full length LRP/MVP cDNA resulting in the reduced LRP-56 staining (Scheffer, personal communication) This implies that the staining observed in both the AC12 and AC16 with MAb 3/B6 is specific The 3/B6 antigen appeared to be expressed primarily in the plasma membrane of the transfected variants whereas in the parental A2780 cell line, the staining was predominantly cytoplasmic The apparent shift in the location of the 3/B6 antigen has been observed with another anti-OAW42 MAb, Mc Garry *et al*, (1996) observed a

switch in the expression of a 48 kDa antigen detected by MAb P1H10 in OAW42 variants In OAW42-S cells staining was located mainly in the plasma membrane with no cytoplasmic staining while in the OAW42-SR, immunoreactivity with MAb P1H10 was seen mainly on the plasma membrane with some cytoplasmic/ nuclear staining

Byrne (1998) has isolated a series of stable LRP/MVP ribozyme and antisense transfected OAW42-SR clones Evidence from these immunocytochemical studies on LRP/MVP ribozyme transfectants suggests that the co-expression of LRP/MVP and the antigen detected by MAb 3/B6 may be influenced by LRP/MVP expression Immunocytochemical analysis reveals reduced expression of LRP/MVP in both LRP ribozyme transfectants with a significant reduction of LRP/MVP expression in the ribozyme 2 subline (section 3 2 3 2) A similar reduction in 3/B6 staining (compared to the untransfected parent) was also observed but with a greater reduction in the ribozyme 4 transfectant (figure 3 2 3 2 1)

In this study, LRP/MVP was absent from the MRP expressing COR-L23R cell lines and expressed at low levels in the COR-L23S, the sensitive counterpart MAb 3/B6 also stained these cell lines in a similar manner indicating that their expression maybe linked

Low expression of both LRP/MVP and the antigen detected by MAb 3/B6 was observed in the sensitive nasal carcinoma cell line RPMI 2650 which has been confirmed by others (Liang, unpublished) Staining was absent from the mouse fibroblast cell line MOP-8 which has been used for transfection and transient expression of LRP/MVP cDNA with no associated change in resistance (Scheffer *et al* 1995) Results for the OAW42 variants, OAW42-A1 and OAW42-A are absent due to the "sticky" nature of these cells This problem was also encountered by Moran *et al*, (1997a), with MAb s to MRP Moran *et al* have observed that an increase in resistance is accompanied by an increase in the expression of cytoskeletal elements, this may have some baring on the lack of clear result from these sublines The increased expression of vimentin and cytokeratin may be accompanied by increased expression of cell adhesion molecule causing non-specific binding of MAb

The staining pattern of 3/B6 did not appear to correlate with resistance to any particular

cytotoxic drug Moran *et al* (1997a) determined the cross-resistance profile of the OAW42 variants Generally, the OAW42 variants display the highest degree of resistance for vincristine, Adriamycin and VP-16 with lowest resistance for Cisplatin and 5-FU DLKP-A and DLKP-A10 had a similar pattern (Cleary, 1995 PhD thesis) The majority of other cell lines tested had similar drug resistance profiles Flens *et al*, (1998) have demonstrated that within a range of rat MAb raised to the SW1573/2R120 cell line, some antibodies have a greater affinity for vincristine selected cell lines Staining was absent from single step, low dose doxorubicin-selected SW1573 sublines

4 6 Development of an immunoprecipitation protocol for the characterisation of the 3/B6 antigen

In order to characterise and quantitate cellular proteins, the molecules are usually labelled with radioisotopes, immunoprecipitated and analysed by SDS-PAGE and autoradiography Membrane proteins are most often labelled by iodmation while intracellular antigens are usually labelled metabolically with radiolabelled amino acids such as [^{35}S] Methionine (Harlow and Lane, 1988)

Attempts have been made to use biotin labelling, initially for the labelling of cell surface proteins (Meier *et al* , 1992), coupled with enhanced chemiluminescence (Nesbitt *et al* , 1992) As described in section 2 10 5 , biotin labelling is based on the principle of covalent binding of biotin-7-NSH to amino groups, mainly on lysine residues Proteins are then immunoprecipitated Biotin labelling does not appear to interfere significantly with antibody binding (Levy-Toledano *et al* , 1993)

Conventional Western Blotting techniques failed to allow the estimation of the molecular weight of the 3/B6 antigen Furthermore, non-reducing and non-denaturing loading buffers failed to give a positive result This suggested that the 3/B6 MAb recognised an epitope which was destroyed by conditions for preparation for Western Blotting

A biotin labelling and precipitation kit was extensively modified in order to allow successful immunoprecipitation of the 3/B6 antigen and subsequent determination of the molecular weight (Masterson, Moran, Scheper and Clynes, 1998) The detergents provided (Nonidet-P40 and sodium deoxycholate), were gentle enough to allow detection of the antigen by MAb 3/B6 antigen but not to solubilise the background protein to too high a degree This was particularly important since 3/B6 is expressed in the cytoplasm and the plasma membrane Preclearing (incubation of the cell lysate with protein A or G labelled sepharose to remove any protein which may non-specifically bind to them) and test antibody incubation times were increased in order to improve immunoprecipitation Scheper *et al* (1993) and Flens *et al* (1998) have reported preclearing and antibody incubation times in the range of 1-2 hours Observations in this

laboratory for a number of non-MDR related MAb found that pre-clearing steps of up to 8 hours were required to reduce non-specific background in the development of Western Blots by ECL Test antibody incubation times were carried out overnight on a belly dancer which appeared to improve the results (Figure 3 2 4 1 1) A minimum of 4 hours immunoprecipitation with Protein-G coated sepharose beads gave optimal results for this these antibodies Other researchers in this laboratory have used longer incubation times with other monoclonal antibodies (Walsh and Meledy, personal communication) It was found that repeated washing steps with high salt buffers reduced the quantity of protein obtained from immunoprecipitation experiments with a range of antibodies Antibodies with low affinities for their target proteins tend to dissociate in high salt buffers (Harlow and Lane, 1988)

Procedure guidelines included with the kit suggested streptavidin-biotin/HRP complex as method for the detection of biotin labelled protein bands which was based on the protocols of Levy-Toledano *et al*, 1993, and Meier *et al*, 1992) However, significant background staining was observed when Western Blots of 3/B6 immunoprecipitates were developed by ECL (Figure 3 2 4 1 2) This high degree of background staining was due to endogenous biotin present in non-fat milk used as blocking buffer Chu *et al*, (1989) reported similar background in blot development with ABC/HRP complex Further experimentation revealed that the combination of an anti-biotin secondary labelled coupled with biotin free BSA significantly reduced the background staining levels It was found that antibody dilutions of the secondary antibody of 1/4000 to 1/6000 in TBS 0 1% (v/v) Tween 20 reduced the levels of background staining without compromising antigen detection

4 6 1 Determination of the molecular weight of the 3/B6 antigen

A biotin labelled band at approximately 115 kDa was detected in 3/B6 immunoprecipitates of OAW42-SR cell lysates This band was absent from the negative control lane consisting of OAW42-SR cell lysate precipitated with mouse IgG1 antibody (Figure 3 2 4 2) A series of bands at 95 kDa and 55-45 kDa were detected in the majority of immunoprecipitation samples These bands were detected non-specifically by the secondary antibody Izquierdo *et al* (1996b) observed similar bands in immunoprecipitation preparations from fresh tissue samples They have assigned these bands to (a) precipitation of mouse IgG at 90 kDa and (b) breakdown of IgG molecules at 55-45 kDa Mouse IgG was subjected to SDS-PAGE and formed a similar pattern of bands

4 6.2. Determination of protein glycosylation of the 3/B6 antigen

Proteins destined for secretion, incorporation into membranes or localisation inside membranous organelles, usually contain carbohydrate moities (Lodish *et al*, 1988) Glycosylation appears to play an important role in the sorting and distribution of these proteins to their correct cellular destinations (Pfeffer *et al*, 1987) The oligosaccharide portion is usually classified as being N-linked where sugar groups are linked by a β-N-glycoside bond to an Asn residue in the sequence Asn-X-Ser or Asn-XThr where X may be any amino acid except Pro or Asp They may also be classified as O-linked where oligosaccharides are attached to their polypeptide chain through an α-O- glycoside bond to Ser or Thr (Voet and Voet, 1990) Inhibitors of N-glycosylation include tunicamycin which specifically inhibits synthesis of intermediaries in N-linked synthesis by binding irraversably to glycosyl transferase Similarly PNAG binds O glycosyl trnsferase Therefore the presence of these compounds in the growth medium of cultured cells should inhibit the glycosylation in a population of cells

Analysis of the results from this experiment reveals that there were no extraneous bands visible in Western Blots of 3B6 immunoprecipitates from OAW42-SR when treated with

tunicamycin or PNAG (Figure 3 2 5 1 (a) Scheper *et al*, (1993) have established that the LRP/MVP is unglycosylated therefore this protein was used a negative control, no extra bands were visible on Western Blots of LRP/MVP immunoprecipitates The lower band visible in immunoprecipitation preparations is due to non-specific binding of the secondary antibody to IgG A clear shift in the molecular weight and the appearance of a second band at approximately 140 kDa is apparent in the positive control, DLKP-A cell lysated treated with tunicamycin and detected using the anti-Pgp MAb C219 (Figure 3 2 5 1 (b)

The ovarian carcinoma-associated antigens listed in section 1 4 6 have been characterised as glycoproteins therefore it is possible to conclude that 3/B6 is probably not one of these proteins or a segment thereof

4 6 3 Immunoprecipitation of MDR cell lines

Analysis of a series of drug resistant and sensitive cell lines was undertaken to confirm the levels of expression of the 3/B6 antigen and LRP/MVP observed previously by immunocytochemistry

A faint band was detected in DLKP, compared to the positive control, OAW42-SR cells precipitated with MAb 3/B6 (figure 3 2 7 1) There was no band detected in preparations of DLKP-A cells suggesting that the staining detected by immuno-analysis may have been non-specific A large band at 115 kDa was observed in A2780/AC16 cells confirming the immunocytochemical results discussed earlier This also indicates that the 3/B6 staining observed previously was specific

A 110 kDa band (LRP/MVP) was also absent in the DLKP-A LRP-56 immunoprecipitates (Figure 3 2 7 2) Surprisingly, high levels of LRP/MVP expression was observed in the A2780, COR-L23S and COR-L23R cell lines These results do not correlate with those obtained by immunocytochemistry The lack of correlation may be due to fixation of the protein which may destroy some of the epitope sites whereas immunoprecipitation theoretically allows detection of the entire protein although this has not been observed in this laboratory before

4 6.4. Analysis of LRP ribozyme-transfected OAW42-SR sublines by immunoprecipitation

Byrne (1998) have isolated a number of LRP/MVP cDNA ribozyme and antisense transfected sublines of OAW42-SR to determine whether this protein plays a direct role in the MDR phenotype In the course of characterising the MAb 3/B6 the three selected clones, one antisense and two ribozyme transfectants were investigated further in order to determine if LRP/MVP expression influences that of the 115 kDa antigen recognised by 3/B6

Reduced LRP/MVP expression was observed in all 3 clones compared to the positive control, untransfected OAW42-SR cells (figure 3 2 8 1) Immunocytochemical analysis had shown that the expression of LRP/MVP was down regulated in the ribozyme 2 and 4 sublines with the greatest reduction observed in the ribozyme 2 line A faint band at 110 kDa was observed for OAW42-SR RZ2 compared to the positive control, OAW42-SR cells precipitated with LRP-56 Densitometry analysis by Byrne (1998) has indicated a 10 fold reduction in the expression of LRP/MVP in the ribozyme 2 clone compared to the untransfected control with no significant change in cells transfected with the pHβ plasmid This indicates that these results are not simply an artefact of the transfection procedure Reduced 3/B6 antigen expression was observed in 1 clone only, the ribozyme 4 clone (when compared to the untransfected control) These results are consistent with the immunocytochemical results discussed earlier

Drug toxicity profiles of these transfected OAW42-SR clones show a marked decrease in resistance to adriamycin compared to the parental untransfected OAW42-SR cells with the ribozyme 2 transfectant showing the greatest reduction in resistance (10 fold) This clone also showed a 70 fold decrease in resistance to vincristine and 71 fold reduction in Taxol resistance (Byrne 1998, PhD thesis) Byrne did not observe a significant reduction in LRP/MVP mRNA in the ribozyme 4 clone whereas in the ribozyme 2 clone, significant reduction was observed

It appears from these results that 3/B6 expression in the OAW42-SR is closely linked to that of the LRP/MVP This observation can be made from both the immunocytochemistry and immunoprecipitation results which appear to be consistent for 3/B6 antigen expression

4 7 Cross-reactivity studies with anti-MVP and anti-LRP/MVP antibodies

Scheffer *et al*, (1995) demonstrated that the LRP is the human homologue of the rat major vault protein (MVP) Flens *et al* (1997) also confirmed that the rat MAb LMR-5 recognises the LRP/MVP Evidence from immunofluorescence and immunocytochemical studies have indicated a distinct similarity in the staining patterns of MAbs 3/B6 and LRP-56 Immunoprecipitation of OAW42-SR cell lysates have shown similarities in molecular weight and glycosylation pattern (Section 3 2 4 and 3 2 5)

These studies were concerned with determining whether the MAb 3/B6 cross-reacted with the LRP/MVP Two experiments were undertaken In the first experiment 3/B6 and LRP-56 immunoprecipitates from OAW42-SR and SW1573/2R120 cells were probed by Western Blotting with the anti-rat vault polyclonal antiserum Supernatants from these overnight precipitations (i e following the addition of protein A or G coated sepharose beads to cell lysates containing the antibody antigen complex, the suspension is spun down and supernatant concentrated) were subjected to SDS PAGE If 3/B6 is an anti vault antibody then there would be a significant reduction in the levels of MVP detected in the supernatants of these immunoprecipitates The second part of the experiment involved competition studies with the anti-vault MAb LMR-5, the anti-vault polyclonal antibody N2 and MAb 3/B6

Results indicate that although there are similarities between the two proteins, MAb 3/B6 does not recognise the MVP (Figure 3 2 6 1) The N2 polyclonal antibody detected a 104-110 kDa band in LRP-56 immunoprecipitates OAW42-SR and SW1573/2R120 only In 3/B6 immunoprecipitates this band was absent There appeared to be a reduction in the level of 3/B6 expression in the 2R120 variant compared to immunocytochemical results (Section 3 2 6 2) There is as yet no explanation for these results The supernatants taken from the previous immunoprecipitation experiments were also probed with the anti-rat vault polyclonal antibody to determine the specificity of MAb 3/B6 Immunoprecipitation effectively clears most or all of the target antigen from the supernatant (cell lysates) Probing the supernatants from 3/B6 immunoprecipitates with the N2 antibody determines whether the LRP/MVP expression is affected (Figure

3 2 6 2) There was a significant reduction in LRP/MVP expression in supernatants form LRP-56 immunoprecipitates compared to the control, SW1573/2R120 whole cell lysates No such reduction was observed in the 3/B6 immunoprecipitates

The second part of this experiment involved competition experiments with anti-vault antibodies and the MAb 3/B6 (as outlined in materials and methods section 2 9 5) No apparent reduction in 3/B6 MAb staining was observed when OAW42-SR cells were pre-incubated with LMR-5 or N2 (Figure 3 2 3 3 2 and 3 2 3 3 3) As a polyclonal antibody, N2 would normally contain antibodies that which bind to multiple sites on the antigen thereby blocking more binding sites than a MAb Whereas, pre-incubation of OAW42-SR cells with N2 resulted in significant reduction in LMR-5 staining (Figure 3 2 3 3 4) Taken with the immunoprecipitation results it is possible to conclude that the MAb 3/B6 probably does not cross react with the LRP/MVP It is also possible to conclude that MAb 3/B6 probably does not detect an alternative form of the LRP/MVP

4.8. Studies on the expression of the 3/B6 antigen in normal adult and foetal tissue

Antigen expression *in vitro* is usually a good indicator of expression *in vivo*. However in some cases, continuous culture can cause changes in the expression of some antigens therefore it is important to determine the expression *in vivo*. A limited panel of normal adult and foetal tissue of different histological origins were probed with MAb 3/B6 and LRP-56.

The 3/B6 antigen was found to have high expression in the adult kidney, localised to the distal collecting tubules (Figure 3.2.10.1.). The staining appeared to cytoplasmic in nature; no plasma membrane staining was evident. No specific staining was observed in the adult thyroid. Izquierdo *et al.*, (1996b) have reported absence of LRP-56 staining in the adult thyroid and granular cytoplasmic staining in the adult kidney. LRP/MVP appears to be primarily expressed in the proximal tubules differing from that of 3/B6. Scheper *et al*, (1993) also observed this pattern of LRP-56 staining. Thiebault *et al.*, (1987) have reported that Pgp expression is localised to the proximal tubules of the adult kidney. Flens *et al*, (1996) reported positive MRP staining in both the distal and proximal tubules of the adult kidney whereas no staining was observed in adult thyroid. The expression of the LRP/MVP and the 3/B6 antigen in the adult kidney may reflect differences in the function of these proteins. The primary function of the distal tubules is to aid the reabsorption (into the blood) of sodium and calcium ions. Similar functions are carried out in the proximal tubules however, their functions also include reabsorption of amino acids and glucose (Tortora and Reynolds-Grabowski 1996). These results may give some insight into the functions of the LRP/MVP (and possibly 3/B6) *in vivo*.

3/B6 expression was also screened in a series of foetal tissues (Figure 3.2.10.2.). 3/B6 staining was similar to that observed in adult tissues; primarily localised in the distal tubules of the foetal kidney. Some low level staining was also observed in the proximal tubules. Low level staining was also observed in the trophoblasts of the placenta and enterocytes of the small intestine. Staining was found to be absent from the liver and adrenal gland. LRP/MVP expression was found primarily in the proximal tubules of the kidney with some low level staining in the distal tubules (Figure 3.2.10.3. The staining

pattern of LRP-56 was similar to that observed with of 3/B6, low level staining was observed in the trophoblasts of the placenta and entreocytes of the small intestine No staining was observed in the adrenal gland Low level staining was observed in the bile ducts of foetal liver with MAb LRP-56 which differed from the results observed with MAb 3/B6 There did not appear to be the same level of correlation in LRP-56 and 3/B6 staining which was previously observed in cultures cell lines Mc Garry *et al* (1996) have report absence of P1H10 (an anti-OAW42 MAb) staining in normal lymphocytes and low level staining in normal buccal cells, other human tissues were not screened therefore it is not possible to draw comparisons between these two anti-OAW42 MAbs

4 8 1. Studies on the expression of the 3/B6 antigen in archival breast and ovarian material

Two normal ovary, ten pre-chemotherapy tissue blocks and one post-chemotherapy tissue block were probed with MAb 3/B6 and LRP-56 to compare the staining patterns in these tissue types and to determine the association, if any with drug resistance in ovarian carcinoma The 10 pre-chemotherapy blocks were also examined for Pgp, and MRP expression using a number of MAbs (section 2 9 2 materials and methods, 3 2 11 results)

Analysis of the results from these studies revealed that the 3/B6 antigen was not expressed in normal ovarian tissue or in pre-chemotherapy tissue blocks (figure 3 11 1) These results indicated that the 3/B6 antigen may only be associated with drug resistance phenotype in this tissues Analysis of the expression of the LRP/MVP in normal and pre-chemotherapy ovarian tissue revealed that there was low-level expression in normal tissue but an absence of expression in the pre-chemotherapy tissue sections (figure 3 2 11 1) Izquierdo *et al*, (1996b) reported LRP/MVP expression in normal ovary They also reported high-level expression of the LRP/MVP in ovarian carcinoma but they did not indicate whether these tumour samples were from pre- or post-chemotherapy patients It would appear from the results discussed here, that there does not appear to be a significant association of the LRP/MVP with untreated ovarian carcinoma

Thiebault *et al* (1987) did not find any significant Pgp expression in untreated ovarian

tumour tissue Similarly, Flens *et al* (1994) observed only low level expression of MRP in ovarian neoplasms These findings appear to be consistent with the results outlined here There did not appear to be any significant Pgp expression and only low level MRP expression (1/10 tumours, figure 3 11 1) These results indicated that the 3/B6 antigen was not co-expressed with other MDR associated markers which was observed in immunocytochemical analysis of MDR cell lines (section 4 5)

Izquierdo *et al* (1995) have shown that, in tumours treated with platinum- or alkylating-based treatment LRP/MVP expression was significant and this expression correlated with poor prognosis and reduced disease free period One post-chemotherapy tumour sample (from a patient who received 3 cycles of cisplatinum and adriamycin treatments) was investigated with MAb 3/B6 and LRP-56 The results from this study were inconclusive There appeared to be a high level of (apparently non-specific) nuclear staining, this type of staining has not been observed with these MAbs in previous studies Therefore it was not possible to draw any conclusions from this study Repeating this experiment with a larger number of samples may give a better indication of the association of the 3/B6 antigen ,if any, with drug resistance in ovarian cancer

Two paired pre- and post-chemotherapy breast tumour samples (infiltrating and ductal carcinoma *in situ*) were investigated with MAb 3/B6 These tissue blocks had been previously examined with MAb LRP-56 (Larkin *et al*, 1998b), therefore this experiment was undertaken to compare 3/B6 staining with that of LRP-56 to determine if there was any association in expression between these two antigens

There did not appear to be any significant staining with MAb 3/B6 in the pre-chemotherapy tissue blocks (1423/92 and 1418/86) 3/B6 staining was observed in the tumour cells of both of the post-chemotherapy tissue blocks (1664/94 and 9987/86) Results from the study by Larkin *et al* , (1998b) showed low level LRP/MVP expression in 1 of this set of tumour blocks (the post-chemotherapy block 1664/94) Although this was a limited study, these results indicated that the 3/B6 antigen was associated primarily with the MDR phenotype in breast cancer whereas, LRP/MVP expression was not significant which appears to concur with findings by Linn *et al* (1997) The treatment regimes for the two patients examined in this study consisted of 9 cycles of CMF

(cyclophosphomide, methotrexate and 5-fluoruracil) adriamycin and tamoxifen Patient 1418/86 also received CMF and tamoxifen The expression of the 3/B6 antigen may be linked to the administration of one or more of these drugs although there did not appear to be any association between 3/B6 expression and the choice of drug used in the selection procedure in *in vitro* cell lines (section 4 5) There appeared to be correlation with the expression of the 3/B6 antigen expression in both breast and ovarian cancer in that 3/B6 staining was absent from untreated/pre-chemotherapy tumour tissue The apparent co-expression of the 3/B6 antigen and LRP/MVP was not evident in this study A larger survey of breast tissue samples may show an association does exist between the expression of the 3/B6 antigen and the MDR phenotype

4 9 Detection of the 3/B6 antigen in spent medium

Ovarian cancer-associated antigens have been used as markers for the progression of ovarian cancer for a number of years Table 1 4 6 lists a number of common tumour markers which have been used in the management of patients with ovarian cancer These antigens share a common feature in that they are shed into serum Quantification of these proteins allow an estimation tumour progression

In these studies, growth medium from cultures of OAW42-SR were concentrated (section 2 10 6 materials and methods) and immunoprecipitated with MAb 3/B6 Analysis of these results show that the 3/B6 antigen was not secreted from OAW42-SR cells when compared to immunoprecipitates of whole cell lysates of the same cell line(Figure 3 2 9 1) The negative control consisted of media from OAW42-SR cells immunoprecipitated with LRP-56 As expected, there was no band at 110 kDa

Since it was first reported by Bast *et al*, (1981), CA125 (recognised by MAb OC125 and OC125 MII) has been one of the primary tools in the diagnosis and monitoring of epithelial ovarian cancer The secretion of this molecule into the serum has allowed the development of a range of sensitive radioimmunoassay kits which can readily detect serum levels of CA125 The MDR associated marker, P-glycoprotein has also been detected in spent medium or MDR cell lines Chu and Kawinski (1994) detected soluble Pgp in the spent medium drug resistant A2780AD cells while Pgp was absent from the drug sensitive parental A2780 cells This specificity was mirrored *in vivo*, soluble Pgp was observed in the extracellular fluids of cancer patients such as ascites and serum),but not in the serum of healthy individuals There did not appear to be any difference in the molecular weight or other characteristics of this soluble Pgp McGarry *et al*, (1997) have also demonstrated that the cell surface associated P1H10 antigen (recognised by MAb P1H10) was absent from spent medium of the HepG2 cell line In this study, HMFG2 was used as positive control was detected in the spent medium by ELISA This suggests that, although the 3/B6 antigen may be associated with cellular organelles which merge with the plasma membrane but do not shed its components It has been demonstrated that elements of clathrin coated pits are expressed in the cell surface during

vesicular transport (Goldstein *et al*, 1985) Further investigation possibly with fluorescent labelled antibody fragments may give some insight into the function and of the 3/B6 protein

4.10 Drug accumulation studies with MAb 3/B6

Evidence from immunocytochemical and immunoprecipitation experiments in this study have indicated that the 3/B6 antigen and the LRP/MVP may be co-expressed It has also been suggested that the LRP/MVP may play a direct role in drug resistance, possibly as a transport molecule (Section 1 3 3 13) In an effort to determine whether the 3/B6 antigen influenced the accumulation of adriamycin , OAW42-SR cells were incubated with this MAb prior to exposure to adriamycin Drug accumulation was then measured at a number of time points to determine the cellular concentration for the cytotoxic drug

There was no observable effect on drug accumulation when OAW42-SR cells were incubated with MAb 3/B6 compared to cells incubated in adriamycin only (Figure 3 2 14) In the positive control samples (OAW42-SR cells incubated with adriamycin and the Pgp circumvention agent cyclosporin A) there was a significant increase in drug accumulation to a maximum concentration of 995 pmoles Moran *et al*, (1997) have shown previously that cyclosporin A is a potent circumvention agent in the OAW42-SR cell line The ability of MAbs to modulate to accumulation of cytotoxic drugs has been demonstrated previously for Pgp MAb MRK-16 raised to the external portion of Pgp increased the accumulation of vincristine in K562/ADM cells (Hamada and Tsuro, 1986) Merchetner and Rominson (1992) demonstrated a similar effect on vincristine, vinblastine, VP-16 and taxol with MAb UIC-2 as have Shi *et al*, (1995) with 2 MAbs raised to an external epitope of adriamycin resistant cell lines

4 11. Studies on the possible anti-proliferative effects of MAb 3/B6

Yang and Page (1997) have reported on the anti-proliferative effects of a MAb raised to an MDR ovarian cell line A MAb raised to a 7 kDa membrane protein specifically inhibited the proliferation of drug resistant variants of the SKOV3 and OVCAR4 cell lines but did not inhibit the drug sensitive parental cell line In the process of characterising the effects if any 3/B6 exerted on the cellular processes of OAW42-SR cells

No anti-proliferative effects were observed when these cells were incubated with MAb 3/B6 when these readings were compared to (a) cells grown in the presence of mouse IgG1 or (b) cells grown in the absence of MAb The negative control consisted of OAW42-SR cells grown in the presence of MAb LRP-56

4 12 Studies on distribution and species cross reactivity with MAb 3/B6 and LRP-56

A series of rodent, primate, canine and bovine cell lines were investigated for 3/B6 and LRP-56 expression by immunoprecipitation (section 2 10 5 materials and methods, 3 2 12 results) This was carried out to determine the distribution and species cross-reactivity of MAbs 3/B6 LRP-56

Results indicated that the 3/B6 antigen was not widely expressed amongst the cell lines tested Low level expression was confined to green African monkey kidney cells LRP/MVP expression was limited to two cell lines High level expression was observed in the bovine kidney cell line NDBK and also in the African monkey cell line BS-C-1 Analysis of these results indicated that the MAb 3/B6 did not cross react significantly with kidney cells from other species It also indicated limited distribution of the antigen amongst other mammals This suggested that the 3/B6 antigen may be a human homologue of a protein, sharing homology with the primate protein but differing significantly from antigens which may be expressed other mammals and lower eukaryotes Alternatively, it may also suggest that the epitope recognised by MAb 3/B6 is not found in the 3/B6 protein expressed in the lower mammals

The limited expression of the LRP/MVP amongst these cell lines is unusual Kedersha *et al* (1990) demonstrated that vault particles (recognised by an anti-vault polyclonal antibody) are wide spread amongst a wide variety of prokaryotic and eukaryotic cell types These results indicate that there is limited antibody cross reactivity with MAb LRP-56 This suggests that this MAb recognises an epitope which is found in the major vault protein on the higher mammals only

4 13 Micro-sequencing of 3/B6 protein sample

The isolation and subsequent sequencing of short peptides by proteolytic cleavage are important in the characterisation of unknown/novel proteins In this experiment, 3/B6 immunoprecipitates of OAW42-SR were separated on polyacrylamide gels and the band of interest at 115 kDa was excised, exposed to trypsin digestion and sequenced on a pulsed liquid sequencer (section 2 10 7)

Results from this experiment indicated that the 14 amino acid peptide sequence (which had a high serine and threonine content) had 67% homology with blood coagulation factor XIIa inhibitor It also shared 43% with peptide sequences derived from MHC class I after 2 peptide (section 3 2 15 , table 3 2 15)

Isolation of proteins by immunoprecipitation and Western Blotting can lead to problems of purity Multiple proteins may have similar molecular weights and therefore migrate on polyacrylamide gels at similar positions In this case, protein coated sepharose beads were added to cell lysates to remove any proteins which may have bound non-specifically to this protein (discussed in section 4 6) SDS-PAGE of 3/B6 immunoprecipitates produced a single band at 115 kDa when gels were stained with Coomassie Brilliant Blue (section 210 7) which was consistent with biotin labelled bands detected by enhanced chemiluminescence of Western Blots (section 3 2 4 2) Finally, if the 115 kDa band contained more than 1 protein, there have been a series of variables (amino acids) assigned to each position of the peptide Analysis of the chromatographs in Appendix 2 reveals a series of peaks corresponding to all major amino acids During the sequencing procedure, residues from proteolytic cleavage invariably lead to some background however, as in this case, they rarely contribute significantly to the results of each cycle Background levels of Glycine appear to contribute significantly to the results in the first 3-5 cycles These levels are reduced in subsequent cycles Similarly, levels of break down products from reagents used in the formation of amino acid derivatives (Dmp, dptu, dpu,and mptc) and ammonia (which is used in the reagent buffers)are apparent in the chromatographs but do not interfere with the overall result (Appendix 2)

The homology of the 115 kDa 3/B6 protein with elements of such diverse proteins from the coagulation and immune system suggested possible functions for the 3/B6 protein The blood coagulation factor XII (Hageman) is an 80 kDa involved in a complex series of reactions which leads to the eventual conversion of fibrinogen to fibrin (Voet Voet 1990) Bovine Factor XIIa inhibitor is one of a number of control mechanisms employed in the control of blood coagulation The inhibitor has been isolated and characterised as an 88Kda glycoprotein which is expressed exclusively in the cytoplasm (Thorton and Krkby 1987) N-terminal sequencing (14 a a) was completed by Muldbjerg *et al* , 1993 and the sequence deposited in the protein bank

MHC (Major Histocompatability Complex) proteins are expressed on the cell surface of nearly all nucleated invertebrate cells (Voet and Voet 1990) The MHC class I consist of polypeptide chains, the α or heavy chain of about 44 kDa and a 12 kDa polypeptide forming the core of the β chain Both chains have a series of N-linked polysaccharides (Alberts *et al* , 1994) Analysis of these two proteins suggest that the 3/B6 protein is not a constituent peptide of either of these proteins In both cases the 3/B6 protein has a greater molecular weight 3/B6 has been shown to be un-glycosylated (section 3 2 5) Analysis of evidence from this experiment although encouraging, suggests that 3/B6 proteins is not MHC or Factor XII

Therefore, the evidence from protein sequencing indicates that the 3/B6 antigen may be a novel drug resistance associated protein Analysis of the results from this project appear to confirm this observation Immunocytochemical studies on a panel of drug resistant cells lines indicated that, in general, the 3/B6 antigen was co-expressed with LRP/MVP The question then arose whether these two proteins shared homology The glycosylation pattern of the 3/B6 antigen and LRP/MVP were similar however, studies also showed that these MAbs raised to these antigens recognised different proteins, 3/B6, expressed primarily on the plasma membrane and LRP/MVP which was expressed exclusively in the cytoplasm

Immunohistochemical analysis of normal and malignant tissue of different histological origin revealed that the 3/B6 antigen was widely distributed in foetal tissues and was also

expressed in the distal collecting tubules of adult kidney, suggesting a role in transport of biomolecules. The strongest evidence that 3/B6 may be associated with the MDR phenotype came for studies on pre- and post-chemotherapy tumour tissue where 3/B6 was expressed in the post-chemotherapy tissue blocks only. No expression of the 3/B6 antigen was found in normal and pre-chemotherapy ovarian tissue sections. These results were in agreement with immunocytochemical studies on cultured cells where low-level expression of the 3/B6 antigen was observed in drug-sensitive cell lines. Is there then, a direct role for the 3/B6 antigen in drug resistance? Evidence from drug accumulation studies would suggest that this is not the case. The 3/B6 antigen may simply be an indicator of the MDR phenotype.

The question remains as to the identity of the 3/B6 antigen. The observation that the 3/B6 protein stained the cytoplasm of some MDR cell lines and tissue sections suggested an association with subcellular organelles or vesicles which interacted with the plasma membrane. The apparent co-expression of the 3/B6 protein with LRP/MVP suggested that the protein may be associated with clathrin coated pits and vesicles. Vault proteins are also known to be associated with clathrin coated pits (Kedersha and Rome, 1986 a, b). The observation that the 3/B6 protein stained the cytoplasm of some MDR cell lines and tissue sections suggested an association with subcellular organelles or vesicles which interacted with the plasma membrane

Clathrin coated pits and coated vesicles are associated, at plasma membrane, with the early stages of receptor-mediated endocytosis (Goldstein *et al.,* 1985, Robinson *et al.*, 1987) and in the Golgi region with organisation of the non-constitutive pathway of exocytosis (Ahel *et al.*, 1988). The major structural components of the coat are the light and heavy chains of clathrin assembly proteins (Ungewickell 1993). AP2 is a complex made up of several kinds of polypeptide chains large (100-115KDa), medium (45-50KDa) and small (17-20Kda) (Kirchhausen *et al.*, 1989), medium and small chains are designated AP50 and AP20 respectively.

However, data base searches with the 14 a.a. peptide derived from 3/B6 did not reveal any sequence homology with the subunits of this complex. The 3 α_{C2} species (115 kDa)

yet to be sequenced may contain this 14 a a sequence The data so far suggests that 3/B6 and LRP/MVP are closely expressed in cell lines and tissues Coupled with the reduced expression of the 3/B6 antigen in the LRP ribozyme and antisense transfected OAW42-SR cells and the increase in the expression in the LRP/MVP cDNA transfected A2780 cells it appears that the 3/B6 antigen may constitute part of the clathrin adapter protein complex Similarly, the major subunits of the AP2 complex are also un-glycosylated If clathrin associated vault particles are up-regulated in multidrug resistant cell lines than it would be expected that the expression of other clathrin associated proteins may also be up regulated

Further sequence and possibly cDNA sequence data will be necessary to determine whether the 3/B6 antigen forms part of the clathrin transport pathway For the moment however, evidence suggests that this protein is a novel MDR associated antigen

4.14. Immunofluorescence studies with MAb 5/C4

Analysis of OAW42-SR cells by immunofluorescence with the anti-OAW42-SR MAb 5/C4 revealed that this antibody recognised a cytoplasmic antigen The absence of a fluorescent ring around OAW42-SR cells confirmed this observation This antigen was revealed following fixation of OAW42-SR cells with a glutaraldehyde fixative (Becton Dickinson facslyse solution, (Figure 3 3 1 1)

4 14 1 Immunocytochemical studies with MAb 5/C4

Immunocytochemical analysis indicated that this antibody stained the cytoplasm of all the cell lines assayed (section 3 3 2) Generally, a large majority of cell lines displayed low level cytoplasmic staining The highest level of staining was observed in the OAW42 variants, OAW42-SR and OAW42-S with approximately 90% of cells exhibiting granular cytoplasmic staining with MAb 5/C4 Staining was also observed in the LRP cDNA transfected variants A2780/AC12 and AC16 However these cell lines were stained with less intensity than the OAW42 variants There was some variation in the staining in the LRP ribozyme and antisense transfected variants There did not appear to be any significant down regulation in 5/C4 antigen expression in these variants compared to that observed with MAb 3/B6 in preceding sections Low level staining was observed in the GLC4 and COR-L23 series of cell lines and the mouse fibroblast line MOP-8 with negligible staining observed in the DLKP variants

Analysis of these results indicates that this antibody appears to have a slightly higher specificity for the OAW42 variants than for the majority of other cell lines tested Generally, this antibody appeared to have a low specificity for lung tumour cell lines with low level staining observed in the COR-L23 and DLKP series The differences in staining between these two groups of variants may suggest that the antibody is more specific for large-cell lung tumour cell lines (COR-L23) rather that non-small cell (DLKP) Expression of the 5/C4 protein did not appear to be associated with resistance to any particular cytotoxic drug In fact these results suggest that this antigen is probably a constituent protein in most cell types

4 14 2 Determination of molecular weight by western blotting with MAb 5/C4

The molecular weight of the antigen/s recognised by MAb 5/C4 were determined by western blotting The doublet with Mw of approximately 110 kDa and 85 kDa were consistently detected in a number of OAW42-SR and OAW42-S preparations, (figure 3 3 3 1) A new MAb which was raised to the human MVP was included for comparison purposes (Abbandonza *et al*, unpublished) This antibody also appears to detect a doublet by western blotting in OAW42-SR and SW1573/2R120 The lower 85 kDa band appeared to be intense in the OAW42-SR while the 110 kDa band was stronger in the SW1573/2R120 line with MAb 1032 There did not appeared to be difference in the expression of the bands detected by MAb 5/C4

4 14 3 Investigation of a panel of MDR cell lines with MAb 5/C4

A panel of drug resistance/sensitive cell line pairs was also assayed for the expression of the 5/C4 antigen These results appeared to correlate with those obtained for by immunocytochemistry except for the A2780 line Immunocytochemical analysis revealed that approximately 10% of cells exhibited immunoreactivity with MAb 5/C4 whereas investigation by Western Blotting shows an intense doublet The different results may be due in part to fixation of the cells for immunocytochemistry i e fixation may have reduced the number of sights available to the MAb Western Blotting may have allowed more epitopes to be recognised by this MAb

4 14 4 Cross reactivity studies with purified rat vaults

Purified rat vaults were probed by Western Blotting MAb 5/C4 failed to detect a band at 104 kDa This strongly suggests that MAb 5/C4 is not an anti-MVP antibody It also suggests that this antibody does not detect an LRP/MVP-like protein or cross-react with the LRP/MVP

4 14 5 General discussion for MAb 5/C4

The possibility that MAbs 5/C4 and 1032 (a MAb raised to the MVP by Abbadonza *et al,* personal communication) detected the same antigen then this information would conflict with the previous staining pattern observed for anti-MVP antibodies, LRP-56 and LMR-5 (Flens *et al* 1997 (a) and Schroeyers *et al* 1998) Granular outer cytoplasmic staining was observed with 1032 in SW1573/2R120 and A2780-AC16 cytospins while the A2780 were only slightly positive and the MOP-8 cells were negative (Rome *et al*, personal communication) This is consistent with staining observed for LRP-56 MAb 5/C4 stained A2780 and MOP-8 cell lines but in a similar cytoplasmic fashion Rome *et al* have also, on occasion, observed a second band at approximately 85 kDa when probing preparations of multidrug resistant cells by Western Blotting with the anti-rat vault polyclonal antibody, N2, which may be due to a previously unidentified breakdown product of the MVP or alternatively this band could simply be due to non-specific staining (Rome *et al,* personal communication) This second band was observed when OAW42-SR cells were probed with the N2 polyclonal antibody in experiments with MAb 3/B6 (Figure 3 2 6 2)

MAb 5/C4 may recognise a vault associated protein It has been shown that vault proteins associate with coated vesicles (and ferritin) in rat liver (Kedersha and Rome 1986a, b) It is possible that MAb 5/C4 could recognise a constituent protein of coated vesicles Fractions collected from agarose gel electrophoresis purified rat microsomes and separated by SDS-PAGE revealed a series of protein species between 38-180 kDa including the 104 kDa rat MVP If MAb 5/C4 recognises a protein from this range then this may account for the presence of peripheral granular cytoplasmic staining in the MOP-8 and A2780 lines

4 15 Characterisation of the anti-OAW42-S MAb 3/E3

Immunofluorescence studies indicated that this MAb detected a cell surface epitope on OAW42-S cells Investigation of cytospins of OAW42-S and OAW42-SR cells revealed that the staining was predominantly localised to the plasma membrane in OAW42-S cells while it appeared that this MAb detected discrete cytoplasmic structures in OAW42-SR cells (figure 3 4 2 1) however immunocytochemical analysis revealed no difference in the staining pattern or intensity of staining among drug resistant and drug sensitive cell lines (figure3 4 3 1) It was not possible to determine the molecular weight of this antigen by western blotting or immunoprecipitation From the evidence obtained so far with this MAb it is probable that the antigen detected by MAb 3/E3 is a constituent protein found in all cells and therefore is unconnected with drug resistance The protein is probably not useful as a marker in ovarian cancer

It is evident from the work outlined here that there is a complex series of interactions which lead to the development of the MDR phenotype Investigation of the antigenic expression in *in vitro* cell culture models and *in vivo* will allow a greater understanding of the processes involved in chemotherapeutic drug resistance

5 0 Conclusions and Future Work

Conclusions and future work

Monoclonal antibodies were raised to drug resistant and sensitive variants of the ovarian carcinoma cell line OAW42 From a series of positive clones, 3 were chosen on the basis strong immuno-reactivity to these cell lines by ELISA for further characterisation The anti-OAW42-SR monoclonal antibodies 3/B6 and 5/C4 and the anti-OAW42-S MAb 3/E3 were characterised by a range of immunological and biochemical methods The results of these experiments are summarised below

Immunofluorescence studies, initially carried out on the OAW42-SR cell line with MAb 3/B6, revealed that this antibody recognised a cell surface antigen This was confirmed by further immunofluorescent studies on acetone fixed OAW42-SR cells which also showed that the 3/B6 antigen was expressed mtracellularly Expression of this antigen was also apparent on a number of drug multidrug resistant cell lines

Immunocytochemical analysis of an expanded series of MDR cell lines which included a number LRP/MVP over-expressing cells lines indicated that the 3/B6 antigen was predominantly associated with LRP/MVP over-expressing cell lines Staining with this antibody was both plasma membrane and cytoplasmic Close co-expression was observed in the LRP/MVP cDNA transfected A2780 and LRP/MVP ribozyme and antisense transfected OAW42-SR cell lines

A series of studies were initiated to determine the expression of the 3/B6 antigen in a range of normal adult and foetal tissues Expression was compared with that of the LRP/MVP since our previous results from studies in MDR cell lines suggested some degree of co-expression of these two antigens These studies concluded that the 3/B6 antigen and the LRP/MVP were probably differentially regulated in normal tissue Expression of the 3/B6 antigen was confined to the distal collecting tubules of adult kidney while LRP/MVP expression was found mainly in the proximal tubules Neither antigen was expressed in the adult thyroid Foetal expression of the 3/B6 antigen was observed in the distal tubules of the kidney, placenta and small intestine while LRP-56 staining was observed in the proximal and distal tubules of the kidney LRP-56 staining was also observed in the liver, placenta and small intestine Staining was absent from the foetal adrenal gland with this antibody

In further tissue surveys, co-expression of these two antigens was observed in a limited study of 2 paired pre-and post-chemotherapy breast samples This indicated that the 3/B6 antigen might be a indicator of drug resistance Staining was absent from the pre-chemotherapy tissue blocks while over expression was observed in the post-chemotherapy samples Staining was also absent from 2 normal ovarian and 10 pre-chemotherapy samples indicating that the 3/B6 antigen may not play a major role in the normal ovary or in untreated tumours Analysis of 1 post-chemotherapy tumour block gave inconclusive results In order to fully understand the role of the 3/B6 antigen as a possible indicator of drug resistance it would be necessary to complete a more detailed tissue survey using a greater panel of archival tissue samples Izquierdo *et al* ,(1995) have shown that the LRP/MVP is a negative indicator of survival in ovarian tumours treated with platinum based and alkylating drugs which are more commonly used in the treatment of ovarian cancer It would be necessary to evaluate 3/B6 expression in relation to tumours treated with these drugs Similarly, it would be useful to determine whether 3/B6 is associated with more recently developed drug such as taxol The preliminary results from the pre-and post-chemotherapy breast tumour samples indicated a role for 3/B6 an indicator of drug resistance in breast cancer Given that these two malignancies are sometimes linked (through the expression of the BRCA1 gene in familial ovarian cancer), it would also be useful to determine the importance of 3/B6 expression in this tumour type

The inability to detect the 3/B6 by Western Blotting suggested that this antibody recognised a conformational epitope The development of an immunoprecipitation protocol for MAb 3/B6 and LRP-56 based on biotin labelling of protein allowed the characterisation of the 3/B6 antigen as an un-glycosylated protein with a molecular weight of 115 kDa which was similar but not identical in molecular weight the that of the LRP/MVP Further immunoprecipitation confirmed pervious observations that this antigen was closely co-expressed with the LRP/MVP in MDR cell lines

Cross-reactivity studies with anti-LRP/MVP monoclonal antibodies, and-rat vault polyclonal antibody and purified rat vault indicate that MAb 3 B6 did not recognise the LRP/MVP It was also possible to conclude from these studies that the 3/B6 antigen was probably not an alternative form of the LRP/MVP

It was found that incubation of OAW42-SR cells with MAb 3/B6 did not have any effect on drug accumulation. Therefore the antigen recognised by MAb 3/B6 does not appear to play a direct role in drug resistance in this cell line.

This MAb does not appear to play any significant role in cellular proliferation in the OAW42-SR. It has been observed that some MAb can affect cellular proliferation of MDR cells (Yang and Page 1995).

Protein sequencing results showed that the 3/B6 antigen shared low percentage homology with the blood coagulation factor XII a inhibitor and the MHC class 1 alpha 2 protein.. These proteins however, have significant glycosylation. Their molecular weights are also lower than the 3/B6 antigen. In view of this information and the relatively low homology, it is possible to conclude that the 3/B6 antigen is probably not a member of the coagulation cascade family of proteins or an element of the MHC. The 3/B6 protein does however, share some similarities (particularly molecular weight i.e. major subunits are 115 kDa) with the alpha and beta subunit of the clathrin adapter protein 2 complex which is a multi-subunit protein expressed in association with clathrin coated pits. In view of the apparent frequent co-expression between the 3/B6 protein and the coated pit associated vault proteins, it is possible that the 3/B6 protein might be a subunit of this complex. In order to confirm this hypothesis it would be necessary to conduct studies with available anti-AP-2 antibodies to determine the expression of this protein complex in MDR cell lines. Isolation of purified coated pits (Kedersha and Rome 1986) and analysis by immunoprecipitation or dot blots with MAb 3/B6 would indicate whether the 3/B6 antigen is associated with coated pits.

Micro-sequencing of the 3/B6 protein did not reveal homology with any of the large subunits, however, the $\alpha_C 1$ subunit (115 kDa) has yet to be sequenced and is therefore a possible candidate. Further work on cDNA libraries may give some insight into the identity of the 3/B6 antigen. Preliminary investigation of a cDNA library derived from a normal human kidney cell line has already been carried out.. Lifts taken from phage infected *E. coli* .Y1090 colonies have been probed with MAb 3/B6. Initial results

indicate some possible positive colonies Sequence data from isolated clones would allow comparisons with sequence data already obtained for the β heavy chain (115 kDa) and may suggest the identity of the protein rather than comparisons of short peptide sequences

Coated pits are known to be quickly recycled Labelling experiments, where F(ab) fragments or whole IgG molecular of 3/B6 could be labelled with gold particles (1-5 nm) might allow the subcellular location of the 3/B6 antigen identified Similarly this process could also be carried out with fluorescent labelled dyes and the process assayed by confocal laser microscopy

Immunofluorescence studies with the anti-OAW42-SR MAb 5/C4 indicated that this antibody recognised a cytoplasmic antigen which was not expressed on the cell surface

immunocytochemistry results revealed that the antigen recognised by this antibody was expressed in all of the cell lines tested with little degree in variation of staining It is possible to conclude from these studies that the expression of this antigen is not associated with any particular drug resistance mechanism (by comparing it to the predominant mechanisms which are expressed in these cell lines)

Western Blotting results revealed that this antibody detected 2 bands at 110 kDa and 85 kDa which was similar to the anti-MVP antibody 1032 This suggested that the MAb 5/C4 might recognise the MVP However further studies with purified rat vault particles showed no cross-reactivity The 2 protein bands at 110 kDa and 85 kDa suggest that the MAb 5/C4 might also detect elements of clathrin coated pits Further work with isolated coated pits (Kedersha and Rome 1996) would confirm this hypothesis

The MAb 3/E3 was raised to the OAW42-S cell line which also over-expressed the LRP/MVP In characterising this antibody it was found that the 3/E3 antigen appears to be expressed on the cell surface of all the cell lines tested save for the OAW42-SR where it appeared to be associated with particular cytoplasmic bodies It was not

possible to obtain a molecular weight for this antigen It is probable that this antigen is a common cell surface antigen which is ubiquitously expressed on all cell types

6 0 References

References

Ahle S, Mann A, Eichelsbacher U and Ungewickell (1988) Structural relationship between clathrin assembly protein from the Golgi and the plasma membrane *EMBO J* 7 919-929

Akey CW (1990) Visualisation of transport-related configurations of the nuclear pore transporter *Biophys J* **52**(2) 341-345

Akey CW, and Goldfarb DS (1989) Protein import through the nuclear pore complex is a multistep process *J Cell Biol* **109**(3) 971-982

Alberts B, Bray D Lewis J, Martin R, Roberts K and Watson J (1994) *Molecular Biology of the Cell* Garland Pub London (3rd Edition)

Anzano MA, Roberts AB, Smith JM, Sorn MB and De Larco JE, (1983) Sarcoma growth factor from conditioned medium of a virally transformed cells is composed of both type alpha and type beta transforming growth factors *Proc Natl Acad Sci USA* **80** 6264-6268

Arno S, Suwa H, Mandai M, Tashiro H, Miyazaki K, Okumura H, Nomura H, Hiai H and Fokumoto M (1994) Expression of multidrug resistance gene and localisation of P-glycoprotein in human primary ovarian cancer *Cancer Res* **54** 1355-1359

Artega CL, Hanauske AR, Clark GM et al (1988) Immunoreactive transforming growth factor activity in effusions of cancer patients as a marker of tumour burden and patient prognosis *Cancer Res* **48** 5023-5028

Aure JC, Hoeg K and Kalstad P (1971) Clinical and histological studies of ovarian carcinoma Long term follow-up of 990 case studies *Obstet Gynecol* **31**(1) 331-345

Baker TG (1963) Quantitative and cytological study of germ cells in human ovaries *Proc R Soc Sec B* **158** 417-420

Baker VV, Borst MP, Dixon D *et al*, C-myc amplification in ovarian cancer *Gynecol Oncol* **38** 340-342

Bakos V, Hegedeus T, Hollo Z, Welker E, Tusanady GE, Zaman GJR, Flens M, Varadi A and Sakardi B Membrane topology and glycosylation of the human multidrug resistance-associated protein *J Biol Chem* **271** 12322-12326

Bar JK, Hartozinska A, Sobanska E and Mieczyslaw C (1994) Relation between ovarian carcinoma-associated antigens in tumour tissue and detached cyst fluid cells of patients with ovarian carcinoma *Tumori* **80** 50-55

Bast R Jacobs I and Berchuck A (1992) Malignant transformation of ovarian epithelium *J Nat Cancer Inst* **84**(8) 556-558

Bast RC, Feeney M, Lazarus H, Nadler LM, Colvin RB and Kapp RC (1981) Reactivity of a monoclonal antibody with human ovarian carcinoma, *J Clin Invest* **68** 1331-1337

Bates S, Sheih C and Tsokos M (1991) Expression of the mdr-1/P-glycoprotein in human neuroblastoma *Am J Pathol* **139** 305-315

Beck J, Bohnet B, Brugger D, Bader P, Dietl J, Scheper RJ, Kandolf R, Liu C, Niethammer D, and Gekeler V (1998) Multiple gene expression analysis revals distinct differences between G2 and G3 stages breast cancers, and correlations of PKCη with MDR1, MRP and LRP gene expression *Br J Cancer* 77(1) 87-91

Beck W and Danks M (1991) Characteristics of multidrug resistance in human tumour cells In *Molecular and cellulsr biology of multidrug resistance in tumour cells* Roninson IB (ed) Plum Press, NY

Beck W, Grogan TM *et al*, (1996) Methods to detect P-glycoprotein-associated multidrug resistance in patient tumours Consensus recommendations *Cancer Res* **56** 3010-3020

Bellamy (1996) WT P-glycoprotein and multidrug resistance Ann Rev *Pharmacol Toxocol* 26 161-183

Bellamy WT, Dalton WS and Dorr RT (1990) The clinical relevance of drug resistance (Review) *Cancer Invest* **8** 547

Belllamy WT (1990) Multidrug resistance in human tumours (Review) *Curr Opin Oncol* **2** 1126

Berchuck A and Carney M (1997) Human ovarian cancer of the surface Epithelium *Biochem Pharmacol* **54** 541-544

Berchuck A, Olt GJ, Everitt L, Soisson AP, Bast RC, and Boyer CM (1990) The role of peptide growth factors in epithelial ovarian cancer *Obstet Gynecol* **75** 255-262

Berchuck A, Rodriguez GC, Olt GJ, Boenet MP, Whitaker RS, Arrick B, Clarke-Pearson DL and Bast RC (1992) Regulation of growth of normal ovarian epithelial cells and ovarian cancer cell lines by transforming growth factor beta *Am J Obstet Gynecol* **166** 676-684

Bernstein A and Bernstein LB (1991) Comparison of CA125 lipid-associated sialic acid (LASA-P_, CA19-9, and CEA in monitoring patients with ovarian cancer *J Tumour Marker Oncol* **6** 183-187

Biedler JL (1992) Genetic aspects of multidrug resistance (Review) *Cancer* 70 1799

Blaustein A and Lee H (1979) Surface cells of the ovary and pelvic peritoneum A histological and ultra structural comparison *Gynecol Oncol* **8** 34-50

Blaustein A (1984) Immunoecectron microscopic demonstration of surface tumour antigen CA125 *Ultrastruct Pathol* 7(1) 9-11

Blend MJ, and Ostrowski GJ (1994) Recent advances in detection of ovarian cancer A review *JAOA* **94**(4) 305-317

Borg, HG , burgess, R , Scheper, RJ and Lin Yin, JA High expression of LRP in acute myeloid leukaemia is associated with poor response to chemotherapy B J *Haemo* 1996

Boring CC, Squires TS and Tong T (1993) Cancer statistics *Ca Cancer J Clin* **43**(1) 7-26

Borst P (1991) Genetic mechanisms of drug resistance (Review) *Acta Oncol* 30 87
Borst P, and Schinkel AH 91996) What we have learned thus far from mice with disrupted P-glycoprotein genes? *Euro J Cancer* **32A** 985-990

Broxterman HT and Schuurhus (1997) Transport proteins in drug resistance detection and prognostic significance in acute myeloid leukaemia *J Intern Med Suppl* **740** 147-151

Brandenberger AW, Tee MK and Jaffe RB (1998) Estrogen receptor alpha and beta mRNAs in normal ovary, ovarian serous cystadenocarcinoma and ovarian cancer cell lines down regulation of ER-beta in neoplastic tissues *J Clin Endocrinol Metab* **83**(3) 1025-1028

Bruggerman EP, Curnier SJ, Gottesman MM, and Pastan I 919920 Characterisation of azidopine and vinblastine binding site of P-glycoprotein *J Biol Chem* **267** 21020-21026

Bruggerman EP, Germann UA, Gottesman MM, and Pastan I (1989) Two different regions of the P-glycoprotein are photo-affinity labelled by azidopine *J Biol Chem* 264 15483-15488

Buller RE, Niemann T, Connor JP, Squatrito RC, Skilling JS and Anderson B (1995) Isolation and preliminary characterisation of an ovarian carcinoma cell line for ma patient with familial ovarian cancer *Gynecol Oncol* **56** 34-44

Buller RE, Vaseliv S, DiSaia PJ, (1996) CA125 kinetics, a cost-effective clinical tool to evaluate clinical trial outcomes in the 1990's *Am J Obestet Gynecol* **174** 1241-1254

Busche R, Tummler B, Riordian JR and Cano-Gauci DF(1989) Preparation and utility of a radioidiniated analogue of daunomycin in the study of multidrug resistance *Mol Pharmacol* **35** 414-421

Byrne D PhD thesis (1998) Investigation on the role of LRP in multidrug resistance

Chambers TC, Zheng B and Kuo JF (1992) Regulation by phorbol esters and protein kinase C inhibitors and by protein phosphatase inhibitor (okadaic acid) of P-glycoprotein phosphorylation and relationship to drug accumulation in multidrug resistant human KB cells *Mo l Pharmo* **41** 1008-1015

Chan H, Haddah G, Thorner P, de Boer G Lin Y, Ondrusek N, Yeger H and Ling V (1991) P-glycoprotein expression as a predictor of the outcome of therapy for neuroblastoma *New Eng J Med* **325** 1608-1614

Chen C, Chin J, Ueda K, Clark D, Pastan I, Gottesman M, Roninson I, (1986) Internnal duplication and homology with bacterial transport proteins in the mdr-1 (p-glycoprotein) gene from multidrug resistant cells *Cell* **47** 381-389

Chow S-N, Chien C-H and Chen C-T (1996) Molecular Biology of Human Ovarian Cancer *Int Surg* **81** 152-157

Chu NM, Janckila AJ Wallace JH and Yam LT (1989) Assessment of a method for immunochemical detection of antigen on nitrocellulose membranes *J Histochem Cytochem* **37**(2) 257-263

Chu TM, Tsung H and Kawinski E (1994) Detection of soluble P-glycoprotein in culture media and extracellular fluids Biochem Biophys Res Comm 203(1) 506-512

Chugani DC, Kedersha N and Rome LH (1993) Evidence that vault ribonucleoprotein particles localise to the nuclear pore complex *J Cell Sci* **106**(1) 23-29

Cleary, I Ph D thesis (1995) Drug accumulation studies in multidrug resistant human cell lines

Cleary I, Doherty G, Moran E and Clynes M (1997) The multidrug resistant human lung cell line, DLKP-A10, expresses novel drug accumulation and sequestration systems *Biochem Pharmocol* **53** 1493-1520

Clynes M, Redmond A, Moran E and Gilvarry U (1992) Multiple drug resistance in variant from human non-small cell lung carcinoma cell line DLKP-A *Cytotechnology* **10** 75-89

Clynes M (1993) Cellular models for multiple drug resistance in cancer *In vitro Cell Dev Biol* **29**A 171-179

Codegoni AM, Nicoletti MI, Bruaggi G et al , (1998) Molecular characterisation of a panel of human ovarian carcinoma xenografts *Euro J Cancer* **34**(9) 1432-1438
Cole MD (1986) The myc-oncogenes its role in transformation and differentiation *Ann Rev Genet* **20** 361-384

Cole MD (1986) The myc-oncogenes its role in transformation and differentiation *Ann Rev Genet* **20** 361-384

Cole SPC, Bhardwaj G et al , (1992) Overexpression of a transporter gene in multidrug-resistant human lung cancer cell lines *Science* **258** 1650-1654

Coley HM (1997) Drug resistance studies using fresh human ovarian carcinoma and soft tissue sarcoma samples *Keio J Med* **46** (3) 142-147

Coley HM, Twentyman P and Workman P (1989) Improved cellular accumulation is characteristic of anthracyclines which retain high activity in multidrug resistant cell lines, alone or in combination with verapamil or cyclosporin *Biochem Pharmo* **38** 4467-4475

Coop JM (1993) P-glycoprotein structure and evolutionary homologues In *Multidrug resistance in cancer cellular molecular and clinical approaches* Clynes M (Ed) Kluwer Academic Press London 1-32

Cordon-Cardo C and O'Brien JP (1991) The multidrug resistance phenotype in human cancer *Important Adv Oncol* **10** 19-58

Czernobilsky B (1987) Primary epithelial tumours In *Blausteins Pathology of the female genital tract* Kurman RJ (ed) Springer-Verlay 511-560

Dalton W, Grogan T, Rybski J, Scheper R, Richer W, Kailey J, Broxterman H, Pinedo H, and Salmon S (1989) Immunohistochemical detection and quantification of P-glycoprotein in multidrug reisitant human myeloma cells association with levels of drug resistance and accumulation *Blood* **73** 747-752

Dano K (1973) Active outward transport of daunomycin in resistant Ehrlich ascites tumour cells *Biochem Biophys Acta* **323** 466-483

Davis HM, Zurawski VR, Bast RC and Klug TL (1986) Characterisation of CA125 antigen associated with human epithelial ovarian carcinomas *Cancer Res* 46 6143-6148

de Bruijn HWA, ten Hoor KA, Boonstra H, Marrik J, Krans M and Aalders JG (1993) Cancer associated antigen CA195 in patients with mucinous ovarian tumours, a comparative analysis with CEA, TATI and CA125 in serum specimens and cyst fluids *Tumour Biol* **14** 105-115

de Bruijn HWA, van der Zee AGJ and Aalders JG (1997) The value of cancer antigen 125 (CA125) during treatment and follow-up of patients with ovarian cancer *Curr Opin Obstet Gynecol* **9** 8-13

den Boer ML, Pieters R, Kazemier KM, Rottier MMA, Zwaan CM, Kaspers GJL, Janka-Schaub G, Henz G, Creutzing U, Scheper RJ, and Veerman AJP (1998) Relationship between major vault protein/lung resistance-associated protein, p-glycoprotein expression, and drug resistance in childhood leukaemia *Blood* **91**(60 2092-2098

den Boer ML, Zwaan CM, Pieters R, Kazemier KM, Rottier MMA, Flens MJ, Scheper RJ and Veerman AJP (1997) Optimal immunocytochemical and flow cytometric detection of P-gp, MRP and LRP in childhood acute lymphoblastic leukemia *Leukemia* **11** 1078-1085

den Boer, ML *et al* (1996) Multidrug resistance in childhood leukemia is related to accumulation and LRP expression but not to non-pgp or MRP expression. *Amer Soc Cancer Res* Abstract 1996

Derynck R (1986) Transforming growth factor alpha Structure and biological activities *J Cell Biochem* **32** 203-204

de Vita VT (1989) Principles of chemotherapy In *Principles and practice of Oncology* de Vita, Helman S and Rosenberg SA (Eds) JP Lippincott Company, Philadelphia, 276-300

Diebold, J *et al* Bcl-2 expression, p53 accumulation and apoptosis in ovarian carcinomas *Am J Pathol* **105** 3 341-349 (1996)

Dietel M, Arps H, Lage H and Hiendorf A (1990) Membrane vesicle formation due to acquired mitoxantrone resistance in human gastric carcinoma cell line EPG85-257 *Cancer Res* **50** 6100-6106

Dingemans AMC, van Ark-Otte J, van der Valk P, Apolinario RM, Scheper RJ, Postmus PE and Giaccome G (1996) Expression of the human major vault protein LRP in human lung cancer samples and normal lung tissue *Ann Oncol* 7 625-630

Dixon A, Bell J, Elston C, and Blarney R (1992) P-glycoprotein expression in locally advanced breast cancer treated by neoadjuvant chemotherapy Br J Cancer **66** 537-541

Donaldson E Nagell J, Pursell S et al , (1980) Multiple biochemical markers in patients with gynecological maliganacies *Cancer* **45** 948-953

Duffy CP, Elliott RA, O'Connor R *et al*, (1998) Enhancement of chemotherapeutic drug toxicity to human tumour cells in vitro by a subset of non-steroidal anti-inflammatory drugs (NSAIDs) *Euro J Cancer* **34(8)** 1250-1259

Edman P (1956) *Acta Chem Scand* **10** 761-768

El Ferink RPJO, Tytgat GNJ and Groen AK (1997) The role of mdr-2 P-glycoprotein in hepatobiliary lipid transport *FASEB J* **11** 19-28

Endicott JA and Ling V (1989) The biochemistry of P-glycoprotein mediated multidrug resistance Ann Rev Biochem **58** 137-171

Epenatos A, Shepard J, Britton K, Hawkins L, Nimmon C, Taylor-Papadimitriou et al, (1984) Radioimmunodiagnosis of ovarian cnacer using $^{[123]}$I labelled tumour-assoociated monoclonal antibodies *Cancer Detect Prev* **7**(1) 45-49

Favarot M, Combaretaret V, Goillot E, Wagner J, Bouffet E, Mazmgue F, Thyss A, Bordigoni P, Delsol G, Bailly C, Fontainiere B, and Philip T (1990) Expression of P-glycoprotein restricted to normal cells in neuroblastoma biopsies *Br J Cancer* **64** 233-238

Favrot MC, Combaret V, Gilliot E, Tabone E, Bouffet E, Dolbeau D, Bouvier R, Coze C, Michon J and Philip T (1991) Expression of P-glycoprotein restricted to normal cells in neuroblastoma biopsies *Br J Cancer* **64**(2) 233-238

Feldherr CM and Akin D (1990) EM visualisation of nucleoplasmic transport processes *Electron Microsc Rev* **3** 73-86

Filipitis M, Suchomel RW, Zochbauer S, Brunner R, Lechner K and Pirker R (1997) Multidrug resistance-associated protein (MRP) in acute myeloid leukaemia *Clin Cancer Res* 3 1419-1425

Flens M (1997) Novel drug resistance-associated proteins PhD thesis Free University of Amsterdam

Flens M *et al* The endothelial protein C receptor is a novel drug resistance associated glycoprotein *Int J Cancer* (1998 in press)

Flens MJ, Scheffer GL, van der Valk P, Broxterman HJ, Eijdems EWHM, Huysamns ACLM, Izquierdo MA, Scheper RJ (1997) Identification of novel drug resistance-associated proteins by a panel of rat monoclonal antibodies *Int J Cancer* **73**(2) 249-257

Fojo A, Akiyama M, Gottesman M, and Pastan I (1985) Reduced drug accumulation in multidrug resistant human KB carcinoma cell lines *Can Res* **54** 4557-4563

Friedel P *et al* (1998a) Migration of coordinated cell clusters in mesenchymal and epithelial cancer explants *in vitro Cancer Res* **55** 4557-4560

Futscher BW, Abbaszadegan MR, Domann F, and Dalton WS (1994) Analysis of MRP mRNA in mitoxantrone-selected, multidrug-resistant human tumour cells *Biochem Pharmacol* **47** 1601-1606

Gadducci A, Capriello P, Bartolini T, Barale E, Cappelli N, Facchini V and Fioretti P (1988) The association of ultrasonography and CA125 test in preoperative evaluation of ovarian carcinoma *E J Gynecol Oncol* **9** 373-376

Gadducci A, Ferdeghini M, Ceccarini T, Prontera C, Facchini V Biachi R and Fioretti P (1990) A comparative evaluation of the ability of serum CA125, CA19 9, CA15 3, CA50, CA72 4 and TATI assays in reflecting the course of disease in patients with ovarian carcinoma *Eur J Gynaec Oncol* **11** 127-133

Gallion HH, Hunter JE, van Nangel JR, Averette HE, Cain JM et al , (1992) The porgnostic implications of low serum CA125 levels prior to the second-look operation for stage II and IV epithelial ovarian cancer *Gynecol Oncol* **46** 29-183

Gamboa G Carpenter PM, Podnos Y et al , (1995) Characterisation and development of UIC 107, a primary human ovarian cancer cell line *Gynecol Oncol* **58** 336-343

Ganapathi R and Grabowski D (1988) Differential effect of calmodulin inhibitor, trifluorpreazine in modulating cellular accumulation, retention and cytotoxicity of doxorubicin-resistant L1210 mouse leukaemia cells *Biochem Pharmacol* **37**(2) 185-193

Georges E, Bradley G, Gariepy J, and Ling V (1990)Detection of P-glycoprotein isoforms by gene-specific monoclonal antibodies. *Proc. Natl. Acad. Sci. USA* **87** 152-156.

Gerace L, and Burke B 91988). Functional organisation of the nuclear envelope *Ann Rev. Cell. Biol.* **4** 335-374.

German U (1993). Molecular analysis of the multidrug transporter. In Multiple Drug Resistance in Cancer: Cellular, Molecular and Clinical Approaches Clynes M (Ed). Kluwer Academic Publishers London.

Germann U (1996). P-glycoprotein-a mediator of multidrug resistance in tumour cells. *Euro. J. Cancer* **32A** 927-944.

Gervasoni J, Fields S, Kirshna S, Baker M, Rosado M, Thuraisamy K, Hindenberg A and Taub R (1991). Subcellular distribution of daunorubicin in P-glycoprotein positive and negative cell lines using laser-assisted confocal microscopy. Cancer Res. **51** 4955-4963.

Goasguen JE, Dossot JM, Fardel O, Lemee F, Legall E, et al., (1993). Expression of the multidrug resistance-associated P-glycoprotein (P-170) in 59 cases of *de novo* acute lymphoblastic leukemia-prognostic implications. *Blood* **81** 2394-2398.

Godwin AK, Tesa JR, Handel LM, Liu Z, Vanderveer LA, Tracy PA and Hamilton TC (1992). Spontaneous transformation of rat ovarian surface epithelial cells: association with cytogenetic changes and implications of repeated ovulation in etiology of ovarian cancer. *J. Natl. Cancer Inst.* **84**(8) 592-601.

Godwin AK, Testa JR and Hamilton TC (1993). The biology of ovarian cancer development. *Cancer* **71** 530-536.

Goldenberg DM, Larson SM (1992). Radioimmunodetection on cancer identification. *J. Nucl. Med.* **33**(5) 803-814.

Goldstein JL, Brown MS, Anderson RGW, Russell DW and Schnider WJ (1985) Receptor mediated endocytosis concepts emerging from the LDL-receptor system *Ann Rev Cell Biol* **1** 1-9

Goldstein JL, Galski H, Fojo AT *et al*, (1989) Expression of a multidrug resistance gene in human cancers *J Natl Cancer Inst* **81** 116-124

Gorai I, Nakazama T, Miyagi E, Hirahara F, Nagashima Y and Minigaguchi H (1995) Establishment and characterisation of two human ovarian clear cell adenocarcinoma lines form metastic lesions with different properties *Gynecol Oncol* **33** 66

Gottesman MM (1993) How cancer cells evade chemotherapy-Sixteenth Richard and Linda Rosenthal Foundation Award Lecture *Cancer Res* **53** 747

Gottesman MM and Pastarn I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter *Ann Rev Biochem* **62** 385-427

Granai C, Gajewski W and Arena B (1994) Ovarian cancer-issues and management (Review) *Cancer J* 7(1) 7-11

Gros P, Heniah YB, Croop JM and Housman DE (1986) Isolation and expression of cDNA that confers multidrug resistance *Nature* **323** 728-731

Hamada H and Tsuro T (1986) Functional role for the 170-180 kDa glycoprotein specific to drug resistant tumour cells revealed by monoclonal antibodies *Proc Natl Acad Sci USA* **83** 7785-7789

Hamilton TC, YoungRC, McKoy WM, Grotzinger et al, (1983) Characterisation of human ovarian carcinoma cell line HIH OVCAR3 *Cancer Res* **43** 5379-5389

Harlow E and Lane D (1988) Antibodies a laboratory manual Cold Spring Harbour Laboratory (pub)

Hart SM, Ganeshaguru K, Scheper RJ, Pretive HG, Hoffbrand AV and Metha AB (1997) Expression of the human major vault protein LRP in acute myeloid leukemia *Exp Hematol* **25**(12) 1227-1232

Hart SM, Ganeshaguru K, Scheper RJ, Hoffbrand AV and Metha AB (1995) Expression of the human major vault protein in haematological malignancies *Am Soc Haematol* (Abstract 1223)

Heenan M, O'Driscoll L, Cleary I, Connolly L and Clynes M (1997) Isolation from a human MDR lung cell line of multiple clonal subpopulations which exhibit significantly different drug resistance *Int J Cancer* **71** 907-915

Herod, JJ *et al* The prognostic significance of Bcl-2 and p53 expression in ovarian carcinoma *Cancer Res* **56** 9 2178-2184 (1996)

Herrman C, Volknandt W, Wittich B, Kellner R and Zimmerman H The major vault protein (MVP100) is contained in colinergic nerve terminals of electric ray organ J *Biol Chem* **271** 13908-13915

Higgins CF (1992) ABC transporters-from microorganisms to man Ann Rev Cell Biol 8 67-113

Hill BT (1993) Different patterns of cross resistance resulting from exposure to specific anti-tumour drugs or to radiation in vitro *Cytotechnology* **12** 265-288

Hill BT, Whelan RDW, Gribby EM, Sheer D and Hosking LK (1987) Establishment and characterisation of three new human ovarian carcinoma cell lines and initial evaluation of their potential in experimental chemotherapy studies *Int J Cancer* **39** 219-225

Hill, BT *et al* Establishment and characterisation of three new ovarian carcinoma cell lines and initial evaluation of their potential in experimental chemotherapy studies *Int J Cancer* **39** 219-225 (1987)

Hipfner DR, Gauldie SD, Deeley RG, and Cole SP (1994) Detection of the M(r) 190,000 multidrug resistance protein MRP, with monoclonal antibodies *Cancer Res* 54 5788-5792

Hipfner DR, Almquist, K , Stride, B , Deeley, RG , Cole, SP (1996) Location of a protease-hypersensitive region in the multidrug resistant protein (MRP) by mapping of the epitope of MRP-specific monoclonal antibody QCRL-1 *Cancer Res* **56** 3307-3314

Hoffman G and Mattern M (1993) Topoisomerase II in multidrug resistance *Cytotechnology* **12** (1-3) 137-154

Holtzmayer T, Hilsenbeck S, von Hoff DD Rominson IB (1992) Clinical correlates of MDR1 (p-glycoprotein) gene expression in ovarian and small-cell lung carcinoma *J Cancer Inst* **84**(19) 1486-1419

Horibata K and Harris AW (1972) Mouse myelomas and lymphomas in culture *Exp Cell Res* **60** 61-77

Horio M, Chin KV, Currier SJ, Goldenberg S, Williams C, Pastan I, Gottesman MM and Handler J (1989) Transepithelial transport of drugs by the multidrug trnasporter in cultured Madin-Darby canine kidney derived cell line epithelia *J Biol Chem* **264** 14880-14884

Hsu S, Lothstein L, Horwitz S (1989) Differential expression of the 3 mdr gene family members in multidrug resistant J774 2 mouse cells *J Biol Chem* 264 12053-12062

Hsu SM, Raine L and Fanger H (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques *J Histochem Cytochem* **29** 577-580

Huang WM, Gibson SJ, Facer P, Gu J and Polak JM (1983) Improved section adhesion for immunocytochemistry using high molecular weight polymers of L-lysine as a slide coating *Histochemistry* 77(2) 275-279

Hung Y-C and Satyastwroop (1994) The female reproductive system Cell lines from tumours of the ovary and uterus In *Atlas of human Tumour cell lines* Tabibzadeh S (ed) Academic Press NY 359-386

Hurteau J, Rodrigurez G, Whitaker RS et al , (1994) Transforming growth factor-beta inhibits proliferation of human ovarian cancer cells obtained from ascites *Cance*r 74 93-99 '

Ikea K, Oka M, Narasaki F, Nakano R, Kukuda M, Mizuta Y, Yamada Y, Kamihira K, Tomonaga M, and Kohno S (1997) LRP gene expression in human gastric cancers, and adult T-cell leukaemia cells *Proc Am Ass Cancer Res* **390** (abs 2616)

Ilse D and Edman P (1963) *Aust J Chem* 16 411-416

Inoue M, Fujita M, Nakawaza A, Ogawa H, and Tanizawa O (1992) Sialyl-Tn, sialyl-Lewis Xi, Ca 19 9, Ca 125, carcinoembryonic antigen and tissue polypeptide antigen in differentating ovarian cancer from benign tumours *Obstet Gynecol* **79** 434-439

Islam MQ, Szprier J, Szprier C, Islam K and Dasnoy JF (1989) A gene for the suppression of anchorage independence is located in rat chromosome 5 band q22-23 and rat interferon locus maps at the same region *J Cell Sci* **92** 147-162

Izquierdo, MA van der Zee AGJ, Vermorken JB, *et al* (1995) Drug resistance associated marker LRP for prediction of response top chemotherapy and prognosis in advanced ovarian carcinoma. *J Nat Cancer Inst* **87** 16 1230-1237

Izquierdo MA (1996) Non-pgp related multidrug resistance, focus on the major vault protein LRP (PhD Thesis) Free University of Amsterdam

Izquierdo, MA *et al* (1996a) Overlapping phenotypes of multidrug resistance among panels of human cancer cell lines *Int J Cancer* **65** 1-8

Izquierdo, MA *et al* (1996b) Broad distribution of the multidrug resistance related protein LRP in normal tissues and tumors *Am J Pathol* **148** 3 877-887

Izquierdo MA, Neefjes J, El Matahari A, Flens J, Scheffer G, Scheper RJ (1996c) Over-expression of the ABC transporter TAP in multidrug resistant human cancer cell lines *Br J Cancer* **72** (12) 1967-7

Izquierdo MA, Scheffer GL, Schroeijers A and Scheper RJ (1998) Vault related resistance to anticancer drugs determined by the expression of the major vault protein LRP *Cytotechnology* in press

Jacobs IJ, Kohler MF, Wiseman EW, Marks JR, Whitaker R, Kerns BA, Humphrey P, Berchuck A, Ponder BA, and Bast RC (1992) Clonal origin of epithelial ovarian carcinoma analysis be loss of heterozygosity, p53 mutation, and X-chromosome inactivation *J Natl Cancer Inst* 84(23) 1793-1798

Jedlitschky G, Leir I, Buchloz U, Barouin K, Center M, and Keppler D (1994) ATP-dependant transport of glutathione S-conjugates by multidrug resistant small cell lung cancer cells *Cancer Res* 544833-4836

Juliano RL and Ling V (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants *Biochem Biophys Acta* 455 152-162

Juranka P, Zastawny R and Ling V (1989) P-glycoprotein multidrug resistance and a superfamily of membrane associated transport proteins *Faseb J* 3 2583-2592

Kabawat S, Bast RC, Bhan A, Welch W, Knapp R and Colvin R (1983) Tissue distribution of coelomic-epithelium-related antigen recognised by the monoclonal antibody OC125 *Int J Gynecol Pathol* 2(3) 275-285

Kavallaris M, Leary LA, Barrett JA and Freidlander ML (1996) MDR1 and multidrug resistance-associated protein (MRP) gene expression in epithelial ovarian cancers *Cancer Lett* 102(1-2) 7-16

Keen JH (1987) Clathrin assembly proteins affinity purification and a model for coat assembly *J Cell Biol* 105(5) 1989-1998

Kedersha N, Heuser JE, Chugani DC, and Rome LH (1991) Vaulti III Vault ribonucleoprotein particles open into flower-like structures with octagonal symmetry *J Cell Biol* 112 225-235

Kedersha N, Miquel M-C, Bittner D, Rome LH (1990), Vaults II Ribonucleoprotein srtructures are highly conserved among higher and lower eukaryotes *J Cell Biol* 110 895-901

Kedersha, NL , Rome, LH Isolation and characterisation of a novel ribonucleoprotein particle large structures contain a single species of small RNA *J Cell Biol* **103** 699-709 (1986b)

Kedersha NL , Rome, LH Preparative gel electrophoresis for the purification of small organelles and particles *Anal Biochem* **156** 161-170 (1986a)

Kenemans P, Yedema CA, Bon GC and Von Mensdorff-Pouilly S (1993) CA 125 in gynaecological pathology-a review *Euro J Obstet Gynecol Reprod Biol* 49 115-124

Kessel D, Bottenrill V, Wodinski I (1968) Uptake and retention of daunorubicin by mouse leukemic cells as factors in drug response *Cancer Res* 28 938-941

Kickhoefer V Kedersha N and Rome LH (1993) Vault ribonucleoprotein particles from rat and bullfrog contain a related small RNA that is transcribed by RNA polymerase III *J Biol Chem* **268**(11) 7868-7873

Kickhoefer VA, and Rome LH (1994) The sequence of the cDNA encoding the major vault protein from Rattus norvegicus *Gene* 151 257-260

Kickhofer VA, Rajavel K, Scheffer GL, Dalton WS, Scheper RJ and Rome LH (1998) Vault proteins are upregulated in multidrug resistant cancer cell lines *J Biol Chem* 273(15) 8971-8974

Kim JW, Lee CG, Lyu MS, Kim HK, Rha JG, Kim DH, Kim SJ and Namkoong SE (1997) A new cell line from human undifferentiated carcinoma of the ovary establishment and characterisation *J Cancer Res Clin Oncol* 123 82-90

Kirby EP and Thornton RD (1987) Isolation and characterisation of an inhibitor of factor XIIa from bovine plasma *I Biol Chem* 262(26) 12714-12721

Klumper E, den Boer ML, Pieters R, Huismans DR, Rottier MMA, Zwaan MC, Scheffer GL, Scheper RJ and Veerman AJP (1995) Non-P-glycoprotein mediated resistance to anthracyclines is associated with the expression of a 110 kDa protein in relapse childhood leukaemia Klumper E PhD thesis, Free University of Amsterdam

Kohler G and Milstein C (1975) Continuous culture of fused cells secreting antibody of pre-defined specificity *Nature* 256 495-497

Kohler MF, Kerns BJ, Humphrey PA, Marks JR, Bast RC and Berchuck A (1993) Mutation and overexpression of P53 in early stage epithelial ovarian cancer *Obstet Gynecol* **81** 643-650

Komarov P, Shtil A, Buckingham L, Robinson I and Coon J (1997) Genes encoding for different drug resistance-associated proteins (MDR-1, LRP and MRP) are differentially regulated by extracellular stimuli *Proc Am Ass Cancer Res* **479** (abs 3207)

Kool M, de Haas M, Scheffer G, Scheper RJ, van Eijk M, Juijn J, Baas F, Borst P (1997) Analysis of expression of cMOAT (MRP-2), MRP-3,MRP-4 and MRP-5, homologues of the multidrug resistance associated protein gene (MRP-1) in human cancer cell lines *Cancer Res* **57** 3537-3547

Krishnamachary N and Center M (1993) The MRP gene associated with a non-P-glycoprotein multidrug resistance encodes a 190 kDa membrane bound glycoprotein *Cancer Res* **53** 3658-3661

Kuiper CM, Broxterman HJ, Bass F, Schuurhuis GJ, Haisma HJ, Scheffer GL, Lankelma J, and Pinedo HM (1990) Drug transport variants without P-glycoprotein overexpression from a human squamous lung cancer cell line after selection with doxorubicin *J Cell Pharmacol* **1** 35-41

Kupryjanczyk J, Thor AD, Beauchamp R, Poremba C, Scully RE and Yandell DW (1996) Ovarian, peritoneal and endometrial serous carcinoma clonal origin of multifocal disease *Mod Pathol* **9**(3) 166-173

Lammeli UK, (1970) Clevage of structural proteins during assembly of the head of bacteriophage T4 *Nature* **277** 680

Langdon SP, Hawkes MM, Lawrie SS Hawkins RA, Tesdale AL, Crew AJ, Miller WR and Smyth JK (1990) Oestrogen receptor expression and the effects of oestrogen and tamoxifen on the growth of human ovarian carcinoma cell lines *Br J Cancer* **62**(2) 213-216

Larbre H, Delatour M, Marechal F, and Delatour G (1990) Comparison of a new tumour marker TATI to other tumour markers in various cancers *J Tumour Marker Oncol* **5** 305-311

Larkin AM, Moran E, Alexander D, Dogherty J, Connolly L, Kennedy SM, Clynes M (1998a) A new monoclonal antibody that specifically recognises the mdr-3 gene product *Int J Cancer* in press

Larkin AM, Moran E, Kennedy SM and Clynes M (1998b) Investigation of multidrug resistance (MDR) protein levels in pre-and post-treatment samples from breast cancer patients *Br J Cancer* **78** S1 (Abstract)

Laurencot CM, Scheffer GL, Scheper RJ and Shoemaker RH (1997) Increased LRP mRNA expression is associated with the MDR phenotype in intrinsically resistant human cancer cell lines *Int J Cancer* **72** 1-6

Lauther D, Canitrot Y, Deeley R, and Cole S (1996) Multidrug resistance mediated by the multidrug resistance protein gene *Biochem Pharmacol* **52** 967-977

Law E, Gilvarry U, Lynch V, Gregory B, Grant G and Clynes M (1992) Cytogenetic comparison of two poorly differentiated human lung squamous carcinoma cell lines *Cancer Genet Cytogenet* **59**(2) 111-118

Lee G and Ng H-T (1995) Clinical evaluations of a new ovarian cancer marker COX-1 *Int J Gynecol Obstet* **49** supp S27-S32

Leier I, Jedlitschky G, Buchholz U, and Keppler D (1994) Characterisation of the ATP dependant leukotriene C4 export carrier in mastocytoma cells *Euro J Biochem* **220** 599-606

Levelle-Webster C, and Arias I (1995) The biology of the P-glycoprotein *J Membrane Biol* **143** 89-102

Levine AJ, Mommahad J, Finlay CA (1991). The p53 tumour suppressor gene. *Nature* **351** 453.

Levy-Toledano R, Caro LH, Hindman N and Taylor SI (1993). Strptavidin blotting: a sensitive technique to study cell surface proteins; application to investigate autophosphorylation and endocytosis of biotin labelled insulin receptors. *Endocrinology* **133**(4) 1803-1808.

Linn SC, Pinedo HM, van Ark-Otte J, van der Valk P, Hoekman K, Honkoop AH, Vermorken JB and Giaccone G (1997). Expression of drug resistance proteins in breast cancer, in relation to chemotherapy. *Int. J. Cancer* **71**(5) 787-795.

List AF (1993). Multidrug resistance: clinical relevance in acute leukaemia. *Oncology (Huntingt)* 7(10) 23-28, 32, discussion 32, 35-38.

List AF (1997). Non-P-glycoprotein drug export mechanisms of multidrug resistance. *Semin. Hematol.* **34**(4) 20-24.

List AF, Spier CS, Grogan TM et al., (1996). Overexpression of the major vault protein lung resistance protein predicts treatment outcome in acute myeloid leukaemia. *Blood* **87** 2464-2469.

Lodish HF (1988). Transport and secretory and membrane glycoproteins from rough endoplasmic reticulum. *J. Biol. Chem.* **263** 2107-2110.

Loe DW, Deeley RG, andCole SP (1996). Biology of the multidrug resistance-associated protein, MRP. *Eur. J. Cancer* **32A** 945-957.

Long HJ, Neilmark RA, Su JQ, Garneau SC, Levitt R, Goldberg RM, Poon MA, Kugler JW (1994). Phase II elevation of 5-fluorouracil and low-dose leucovrin in cisplatin-refractory advanced ovarian carcinoma. *Gynecol. Oncol.* **54** 180-183.

Maker AP, Kristensen GB, Kaern J, Bormer OP et al , (1993) Prognostic significance of pre-and postoperative serum CA125 levels in ovarian cancer new aspects and multivariate analysis *Obstet and Gynecol* **79** 1002-1010

Markowska J, Kojczynski Z, Szenwierski Z and Manys G (1990) CA 125 in monitoring chemotherapy of patients with ovarian cancer *Euro J Gynaecol Oncol* **11** 209-214

Marks JR, Davidoff AM, Kearns BJ et al , (1991) Overexpression and mutation of p53 in epithelial ovarian cancer *Cancer Res* **51** 2979-2984

Mart C, Lang T, Koza A, Mayer I and Daxenbichler G (1990) Transforming growth factor beta and ovarian carcinoma cells Regulation of proliferation and surface antigen expression *Cancer Lett* **51** 221-225

Masterson A, Moran E, Scheper R and Clynes M (1998 in press) Detection of biotin labelled LRP/MVP in multidrug resistant variants of the ovarian cell line OAW42 *Biochemica (*Boehringer Mannheim)

Mattes, JM *et al* Mouse monoclonal antibodies to human epithelial differentiation antigens expressed on the surface of ovarian carcinoma ascites cells *Cancer Res* **47** 6741-6750 (1987)

Meier T, Arin S, Malarkannan S, Poincelet M and Hoessli D (1992) Immunodetection of biotinylated lymphocyte-surface proteins by enhanced chemiluminescence a non-radioactive method for cell-surface protein analysis *Annal Biochem* **204**(1) 220-226

Mc Garry Y, Moran E, Hallahan C, Clynes M and Tipton K (1996) Behavior of a novel monoclonal antibody for investigation of ovarian cancer and with the possible involvement in multiple drug resistance *J Exp Oncol Therapeut* **1** 260-270

McGuckin MA, Ramm LE, Joy GJ, Free KE and Ward BG (1992). Preoperative discrimination between ovarian carcinoma, non-ovarian gynaecological malignancy and benign adexal masses using serum levels of CA125 and the polymorphic epithelial mucin antigens CASA, OSA and MSA. *Int. J. Gynecol. Cancer* **2** 119-128.

Mckenzie R *et al Photoderm. Photoimmunol. Photomed.* **10** (2) 74-9

Mecheter EB and Roninson IB 919920. Efficient inhibition of P-glycoprotein-mediated multidrug resistance with a monoclonal antibody. *J. Biol. Chem.* **268** 1792-1798.

Meier T, Arni S, Malarkannan S Poincelet M and Hoessli D (1992). Immunodetection of biotinylated lymphocyte-surface proteins by enhanced chemiluminescence: a nonradioactive method for cell-surface protein analysis. *Anal. Biochem.* **204**(1) 220-226

Merino M and Jaffe G (1992). Age contrast in ovarian pathology. *Cancer* (supp) **71**(2) 537-544.

Mettler, L. Radzun HJ, Salmassi A, Kochlingf W and Parwaresch MR (1990). Six new monoclonal antibodies to serous, mucinous and poorly differentiated ovarian adeniocarcinomas. *Cancer* **65** 1525-1532.

Michieli M, Masolini P and Baccarani M (1997). P-glycoprotein and lung resistance-related protein expression and function in leukaemic blast cells. *Br. J. Haematol.* **96** 356-365.

Mobus VJ, Gerharz CD, Press R, Moll R, Beck T, Mellin W, Pollow K and Knapstein PG (1992). Morphological, immunohistochemical and biochemical characterisation of 6 newly established human ovarian carcinoma cell lines. *Int. J. Cancer* **52**-76-84.

Mobus VJ, Moll R, Grteharz CD, Kieback DG, Weikel W, Hoffmann G and Kreienberg R (1994). Establishment of new ovarian and colon carcinoma cell lines: differentiation is only possible by cytokeratin analysis. *Br. J. Cancer* **69** 422-428.

Mok CH, Tsao SW, Knapp RC, Fishbaugh PM, and Lau CC (1992) Unifocal origin of advanced human epithelial ovarian cancers *Cancer Res* **52**(18) 5119-5122

Moran E, Cleary I, Larkin AM, Amhaolaoibh RN, Masterson A, Scheper RJ, Izquierdo MA, Center M, O'Sullivan F, Clynes M (1997a) Co-expression of MDR-associated markers, including P-170, MRP and LRP and cytoskeletal markers, in three resistant variants of the human ovarian cell line OAW42 *Eur J Cancer* **33** 652-660

Moran E, Larkin AM, Doherty G, Kelehan P, Kennedy S and Clynes M (1997b) A new mdr-1 specific monoclonal antibody(6/1C) on paraffin wax embedded tissue without pretreatment of sections *J Clin Pathol* **50**(6) 465-471

Morgan RJ, Speyer J, Doroshow JH, Marglin K, Raschko J Sorich J et al, (1995) Modulation of 5-fluorouracil with high-dose leucovorm calcium activity in ovarian cancer and correlation with CA125 levels *Gynecol Oncol* **58** 79-85

Moscow J and Dixon H (1993) Glutathione related enzymes, glutathione and multidrug resistance *Cytotechnology* **12** 155-170

Muldberjerg M Markussen S, Magnusson S and Halkier T (1993) Bovine factor XIIa inhibitor *Bloob Coagul Fibfinol* **4**(1) 47-54

Muller M, Bakos E, Welker E, Varadi A, Germann UA, Gottesman MM, Morse BS, Rominson IB and Sarkadi B (1996) Altered drug-stimulated ATPase activity in mutants of human multidrug resistance protein *J Biol Chem* **271** 1877-1883

Nagata A, Hirota N, Sakai T, Fujimoto M and Komoda T (1991) Molecular nature and possible presence of a membranous glycan-phosphatidyl-inositol anchor of CA125 antigen. *Tumour Biology* **12** 279-286

Nahaas WA (1997) Ovarian cancer Current outlook on this deadly disease *Postgrad Med* **102**(3) 112-120

Narod, S A (1994) Genetics of breast and ovarian cancer *Brit Med Bull* **50** 3 656-676

Negishi Y, FuruKawa T, Oka T , Sakamoto M, Hirata T, Okabe K, Matoyoshi K, Akiya K and Soma H (1987) Clinical use of CA125 and its combination assay with other tumour markers in patients with ovarian carcinoma *Gynecol Obstet Invest* **23** 200-207

Nesbitt SA and Horton MA (1992) A nonradioactive biochemical characterisation of membrane proteins using enhanced chemiluminescence *Anal Biochem* **206**(2) 267-272

Nicosia SV, Saunders BO, Acevedo-Duncan ME, Setrakin S, DeGregoria R, (1991) Biopathology of ovarian mesothelium In, Familiari G, Makabe S, Motta PM (eds), *Ultrastructure of the ovary* Kluwers Academic Publishers, 287-310

Nustad K, Bast RC, O'Brien TJ, Nilsson O, Segum P, Suresh MR, Sage T, Nozawa S, Bormer OP, de Brujin HWA, et al , (1996) Specificity and affinity of 26 monoclonal antibodies against CA125 antigen First report of the ISOBM TD-1 workshop *Tumour Biol* **17** 196-219

Nustad K, Nillison O, Kierulf B, Varaas T, Thrane-Steen K, Paus E, Okkenberg C, Olsen KH and Bormer O (1994) Immunoassays for the CA125-related antigen using new monoclonal antibodies In *Current Tumour Diagnosis Applications, Clinical Relevance, Research-Trends* Muchen K (ed) Zuckschwerdt Verlag 397-400

O'Brien TJ, Raymond LM, Bannon GA, Ford DH, Harderdottir H, Miller FC and Quirk JG (1991) New monoclonal antibodies identify the glycoprotein carrying the CA125 epitope *Am J Obstet Gynecol* **165** 1857-1864

O'Sullivan F, Meledy P, Mc Bride S and Clynes M (1998) Primary culture In *Animal Cell Culture Techniques* Clynes M (ed) Berlin Springer 115-130

Olopade OI, Jwnkins R, Linnenbach AJ, Cowan and JM (1990) *Proc Am Assoc Cancer Res* **31** (Abs 318)

Panici PB, Scabia G, Baiocchi G, Lacobelli S and Mancuso S (1989). Predictive value of multiple tumour marker assays in second look procedures for ovarian cancer. *Gynecol. Oncol.* **35** 286-289.

Parker R, Mechetner E, Garcia R, Pavich D, and Fruehauf J (1997). Acquired cisplatin resistance in human ovarian A2780/CP70 cells in associated with MRP and LRP over-expression. *Proc. Am. Ass. Cancer. Res.* **481** (abs: 3219).

Parker SL, Tong T, Bolden S et al, (1997). Cancer statistics 1997. *Ca Cancer J. Clin.* **47**(1) 5-27.

Pejovic T, Heim S, Mandahl N et al, (1991). Bilateral ovarian carcinoma: Cytogenetic evidence of unicentric origin. *Int. J. Cancer* **47** 358-360.

Pfeffer SR and Rothman JE (1987). Biosynthetic protein transport and sorting by endoplasmic reticulum and Golgi. *Ann. Rev. Biochem.* **56** 929-852.

Pieters R, Klumper E, Kaspers GJL, and Veerman AJP (1997). Everything you always wanted to know about cellular drug resistance in childhood lymphoblastic leukaemia. *Crit. Rev. Oncol. Hematol.* **25** 11-26.

Potter M (1972). Immunoglobulin-producing tumours and myeloma proteins in mice. *Physiol. Rev.* **52** 613-719.

Pulay T, Csomor S, Meszaros K, Szaraka G, Csabay L and Szinnyai A (1987). Significance of CEA, AFP, SP1 and HGC as tumour markers in ovarian carcinoma. *Neoplasma* **34** 595-600.

Raaijmakers HGP, Izquierdo MA, Lokhorst HM, de Leeuw C, Belien JAM, Bloem AC, Dekker AW, Scheper RJ and Sonnevald P (1998). Lung resistance-relatd protein

expression is a negative predictive factor for response to conventional low but not to intensified does alkylating chemotherapy in multiple myeloma *Blood* **91**(3) 1090-1036

Ramani P, and Drewchand H (1995) Expression of mdr1/P-glycoprotein and p110 in Neuroblastoma *J Pathol* **175** 13-22

Reichelt R, Holzenburg A, Buhle EL, Jarnik M, Engel A and Aebi U (1990) Correlation between structure and mass distribution of the nuclear pore complex and of distinct pore complex components *J Cell Biol* **110**(4) 883-894

Rischin D, and Ling V (1993) Multidrug resistance in leukemia. *Leukemia Adv Res Treatm* **269**-293

Robey-Caffery SS, Rutledge ML, and Bruner JM (1991) Expression of a multidrug resistance gene in esophageal adenocarcinoma *Am J Clin Pathol* **93** 1-7

Robinson MS (1987) Coated vesicles and protein sorting *J Cell Sci* **87**(2) 203-204

Robinson MS (1989) Cloning of cDNAs encoding two related 100-kDa coated vesicle proteins (alpha-adapitns) *J Cell Biol* **108**(3) 833-842

Rome LH, Kedersha NL and Chugani DC (1991) Unlocking vaults organelles in search of a function *Trends in cell biology* **1** 47-50

Rubin, SC *et al* Expression of p-glycoprotein in epithelial ovarian cancer evaluation as a marker of multidrug resistance *Am J Obstet Gynecol* **163** 69-73 (1990)

Runowicz CD (1992) Advances in the screening and treatment of ovarian cancer (Review) *Ca Cancer J Clin* **46**(6) 327-349

Rustin GJS, Nelstrop A, McClean P (1996) Serum CA125 to define response In *Ovarian Cancer 4* Sharp F, Blackett T, Leake R Berek J London Chapman and Hal Medical 129-141

Safa AR, Mehta ND, Agresti M (1989) Photo-affinity labelling of P-glycoprotein in multidrug resistant cells with photoactive analogues of chlochicine *Biochem Biophys Res Commun* **161** 1420-1408

Salmon DJ, Godolphin W, Jones LA et al, (1989) Studies of HER-2/neu proto-oncogene in human breast and ovarian cancer *Science* **244** 707-712

Sawada M, Terada N, Wada A, Mori Y, Yamasaki M, Saga T and Endo K (1990) Oestrogen- and androgen-responsive growth of human ovarian adenocarcinoma heterotransplanted into nude mice *Int J Cancer* **45**(2) 359-363

Schadendorf D, Makki A, Stahr C, Wanner RT, Scheffer GL, Flens MJ, Scheper RJ, and Henz BM (1995) Membrane transport proteins associated with drug resistance expressed in human melanoma *Am J Pathol* **147**(6) 1545-1552

Scheffer, GL Wijngaard PLJ, Flens MJ, Izquierdo MA *et al* (1995) The drug resistance related protein LRP is the human major vault protein *Nature Med* **1** 6 578-582

Scheper, RJ, Broxterman HJ, Scheffer GL *et al* (1993) Overexpression of a M(r) 110,000 vesicular protein in non-P-glycoprotein mediated multidrug resistance *Cancer Res* **53** 1475-1479

Scheper RJ, Scheffer GL, Flens MJ, van der Valk P, Meijer CJLM, Pinedo M, Clevers HC, Slovak ML, Rome LH, Shoemaker RH and Izquierdo MA *et al*, (1996) Role of LRP/major vault protein in multidrug resistance In *Multidrug resistance in cancer cells* Gupta S and Tsuro T (Eds), Wiley and Son, London

Schinkel AH, Myer U, Wagenaar E, Mol CAAM *et al*, (1997) Normal viability and altered pharmo-kinetics in mice lacking mdr-1-type (drug transporting) P-glycoprotein *Proc Natl Acad Sci USA* **94** 4028-4033

Schinkel AH, Smit JJ, van Tellingen O, *et al*, (1994) Disruption of the mouse mdr-1a P-glycoprotein leads to a deficiency in the blood-brain barrier and to an increased sensitivity to drugs *Cell* 77 491-502

Schinkel AH, Wagenaar E, van Deemter L, Mol CAAM and Borst P (1995) Absence of the mdr-1a P-glycoprotein in mice affects tissue distribution and pharmokinitics of dexamethasone, digxin and cyclosporin A *J Clin Invest* **96** 1698-1705

Schnider J (1993) Detection of multidrug resistance marker P-170 protein in cervical carcinomas by using immunohistochemical technique-reply Am J Obstet Gynecol

Schroeijers AB, Scheffer GL, Flens M, Meijer GA, Izquierdo MA, van der Valk P, and Scheper RJ (1998) Immunohistochemical detection of the human major vault protein LRP with two monoclonal antibodies in formalin-fixed paraffin-embedded tissues *Am J Pathol* **152**(2) 373-378

Schroeijers, AB, *et al* Immunohistochemical detection of the human major vault protein (hMVP) in formalin fixed, paraffin embedded human tissues *Proc Am Ass Cancer Res* **38** 34 (abst 3242) 1997

Schuurhuis, GJ *et al* Early multidrug resistance, defined by changes in intracellular doxorubicin distribution, independent of P-glycoprotein *Br J Cancer* **64** 857-861 (1991)

Scully RE (1979) Tumours of the ovary and maldeveloped gonads In *Atlas of tumour pathology*, 2nd series Armed Forces Institute of Pathology

Serova OM (1987) Amplification of c-myc proto-oncogene in primary tumours, metastasis and blood leukocytes of patients with ovarian cancer *Eksop Onkol* **9** 25-27

Shao Y, De Giuli R, Wyler B, and Lehnert M (1995) Overexpression of MDR1/P-glycoprotein and MRP but not LRP is mutually exclusive in multidrug resistant human myeloma cells selected with doxorubicin *Proc Am Ass Cancer Res* **36** 337 (abs 2006)

Shi T, Wrin J, Reeder J, Liu D and Ring D (1995) High affinity antibodies against P-glycoprotein *Clin Immuno Immunopath* **76**(1) 44-51

Shishi J, Ghazizadeh M, Oguro T, Aihara K and Araki T (1986) Immunohistochemical localisation of CA125 antigen in formalin-fixed paraffin sections of ovarian tumours with the use of pronare *Am J Clin Pathol* **85** 595-598

Slovak, ML *et al* The drug resistance related protein LRP maps proximal to MRP on the short arm of chromosome 16 *Cancer Res* **55.** 4214-4219 (1995)

Smit JJ, Schinkel AH, Oude-Elkerink RP, Groen Ak et al , (1993) Homozygous disruption of the murine mdr-2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease *Cell* **75**(3) 451-62

Strathdee C, and Lo R (1989) Cloning, nucleotide sequence and characterisation of genes encoding the secretion function of the P haemolyticaleutoxin determinant *J Bacteriol* **171** 916-928

Sugawara I, Akiyama S, Scheper RJ, and Itoyama S (1997) Lung resistance protein (LRP) expression in human normal tissues in comparison with that of MDR1 and MRP *Cancer Lett* **112** 23-31

Taylor-Papadimitriou J, Peterson J, Arkile J, Burchell J and Ceriani R (1981) Monoclonal antibodies to epithelial specific components of the human milk fat globule membrane production and reaction with cells in culture *Int J Cancer* **28** 17-21

The National Cancer Registry. Cancer in Ireland 1994, incidence and mortality-a summary.

Theibaut F, Tsuro T, Hamada H, Gottesman MM, Pastan I, and Willingham MC (1987). Cellular localisation of the multidrug resistance gene product P-glycoprotein in normal human tissues. *Proc. Natl. Acad. Sci. USA* **84** 7735-7738.

Thornton RD and Kirby EP (1987). Isolation and characterisation of an inhibitor of factor XIIa from bovine plasma. *J. Biol. Chem.* **262**(26) 12714-12721.

Tiffoli G, Frustaci S, Tumitto L, Talamini R, Gherlinzoni F, Picci P and Boicchi N (1992). Expression of MDR-1 and GST pi in human soft tissue sarcomas: reaction to drug resistance and biological aggressiveness *Ann. Oncol.* **3** 63-69.

Tobwin H, Staehelin T and Gordon J (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76** 577-580.

Todaro GJ, Marquardt H, Twardzik DR, Reynolds FH and Stephenson JR (1983). Transforming growth factors produced by viral-transformed human tumour cell lines. In *Genes and Proteins in Oncogenesis*. Vogel H and Weinstein IB (eds). Raven Press 165-182.

Tortora GJ and Reynolds-Gabowski S (1996). *Principles of anatomy and physiology*. HarpersCollins College Publishers New York 848-890.

Tsuandy GE, Bakos E, Varadi A, and Sarkadi B. Membrane topology distinguishes a subfamily of the ATP-binding cassette (ABC) transporters. *FEBS Lett.* **402** 1-3.

Tsuji. Y, *et al.* Identification of two different surface epitopes of human ovarian epithelial carcinomas by monoclonal antibodies. *Cancer Res.* **45** 2358-2362 (1985).

Tuxen M, Soletormos G and Dombernowsky P (1995) Tumour markers in the management of patients with ovarian cancer *Cancer Treat Rev* **21** 215-245

Ueda K, Pastan I and Gottesman MM (1987) Isolation and sequencing of the promotor sequence of human multidrug resistance protein (P-glycoprotein) gene *J Biol Chem* **262** 17432-17436

Van der Bliek A, Kooiman P, Schneider C and de Lange T (1987) The human mdr-3 gene encodes a novel P-glycoprotein homologue and gives rise to alternatively spliced mRNA in liver *EMBO Journal* **6** 3325-3331

van der Pole JP, Blom DJR, Flens MJ, Luyten GPM, de Waard-Siebinga I, Koornneef L, Scheper RJ, and Jager M (1997) Multidrug resistance-related proteins in primary choroidal melanomas and in vitro cell lines *Invest Ophthamol Vis Sci* **38** 2523-2530

van der Valk P, van Kalken CK, Pinedo HM, et al , (1990) Distibution of multidrug resistance associated P-glycoprotein in normal and neoplastic human tissues Analysis with 3 monoclonal antibodies recognising different epitopes of the P-glycoprotein molecule *Ann Oncol* **1** 54-64

van Nieker *et al* (1988) Characterisation of a human ovarian carcinoma cell line, ONT 14, derived from mucinous cystadenocarcinoma *Int J Cancer* **42** 104-111

van Nierkerk C, Poles LG, Jap, PHK, Smeets DFCM, Thomas CMG, Ramaerkers FCS and Vooijs GP (1988) Characterisation of human ovarian cell linr ONT14 derived from a mucinous cystadenocarcinoma *Int J Cancer* **42** 104-111

Vasu Sk, and Rome LH (1995) Dictyostelium vaults Disruption of the major vault proteins reveals growth and morphological defects and uncovers a new associated protein *J Biol Chem* **270** 16588-16594

Vasu SK, Kedersha NL and Rome LH (1993) cDNA cloning and disruption of the major vault protein alpha gene (mvpA) in Dictystelium disctoideum *J Biol Chem* **268**(21) 15356-15360

Veerman AJP, den Boer ML, Pieters R, Kazemier KM, Roitter MMA, Kaspers GJL, Scheper RJ, Janka G, Henze G, and Creutzig U (1997) Intracellular daunorubicin concentrations and LRP are related to drug resistance in childhood leukaemia *Proc Am Assoc Cancer Res* **422** (2958)

Veneroni, N, Zaffaroni MG, Daidone E, Benini R, Villa and Silvestrini R (1994) Expression of P-glycoprotein and in vitro or in vivo resistance to doxorubicin and cisplatin in breast and ovarian cancer *Eur J Cancer* **30A** 7 1002-1007

Verovski VN, van der Berge DL, Devayaeye MM, Scheper RJ, De Veve WJ, Storme GA (1996) Low-level doxorubicin resistance in P-glycoprotein-negative human pancreatic tumour PSN1/ADR cells implicates brefeldin A-sensitive mechanism of drug resistance *Br J Cancer* **73** 596-602

Versantvoort CH, Broxterman HJ, Bagrij T, Scheper RJ and Twentyman P R 91992) Regulation by glutathione of drug transport in multidrug resistant human lung tumour cell lines over-expressing multidrug resistance associated protein *Br J Cancer* **72** 82-89

Versantvoort CHM, Broxterman HJ, Bagrij T, Scheper RJ and Twentyman PR (1995) Regulation of glutathione of drug drug transport in multidrug resistant human lung tumour cell lines overexpressing multidrug rersistance associated protein *Br J Cancer* **72** 82-89

Voet D and Voet J (1990) Biochemistry Wiley and Son (pub) New York

Volm M *et al*, (1989) Detection of drug resistance in human ovarian carcinoma *Arch Gynecol Obstet* **244** 123-128

Volm M, Mattern J and Koomagi R (1997a) Expression of LRP in non-small cell lung carcinomas of smokers and non-smokers and its predictive value for doxorubicin resistance *Anticancer Drugs* **60** (S12) 2469-2477

Volm M, Stammler G, Zintl F, Koomagi R, and Sauerbrey A (1997b) Expression of lung resistance-related protein (LRP) in initial and relapse childhood acute lymphoblastic leukemia *Anti-Cancer Drugs* **8** 662-665

Wang, LF , Du Plessis, DH , White, JR, Hyatt AD, Eaton, BT (1995) Use of gene-targeted phage display random epitope library to map antigenic determinant on blue-tongue virus outer caspid protein VP5 J *Immuno Methods* **178** 1 1-12

Wilson AP (1984) Characterisation of a cell line derived from the ascites of a patient with papillary serous cystadenocarcinoma of the ovary *J Natl Cancer Inst* **72** 513-521

Wilson AP, Dent M, Pejovic T, Hubbold and Radford H (1997) Characterisation of seven human ovarian tumour cell lines *Br J Cancer* **74** 722-727

Wilson, AP, characterisation of a cell line derived from the ascites of a patient with papillary serous cystadenocarcinoma of the ovary *J Nat Cancer Inst* **72** 3 513-521 (1984)

Wolf, CR *et al* cellular heterogeneity and drug resistance in two ovarian adenocarcinoma cell lines derived from a single patient *Int J Cancer* **39** 695-702 (1987)

Wyler B, Shao Y, Schneider E, Cianfriglia M, Scheper RJ, Frey BM, Gieseler F, Schmid L, Twentyman P, and Lehnert M (1997) Intermittent exposure to doxorubicin in vitro selects for multifunctional non-P-glycoprotein-associated multidrug resistance in RPMI 8226 human myeloid cells *Br J Haematol* **97** 65-75

Yang X and Page M 91995) An Mr 7-kDa membrane protein over-expressed in human multidrug resistant ovarian cells *Cancer Lett* **88** 171-178

Yedema CA, Kenemans P, Voorhorst F, Bon G *et al*, (1993) CA125 half-life in ovarian cancer a multivariate analysis **67** 1361-1367

Young RC, Knapp RC, Perez CA et al, Cancer of the ovary In De Vita VT, Hellman S, Rosenberg SA (eds), *Principles and Practice of Oncology*, ed 3, Lippincott Co 884-913

Zaman GJR, Flens M, van Leusden MR, de Hass M, Mulder HS, Lankelma J, Pinedo HM, Scheper RJ, Baas F, Broxterman HJ and Borst P (1994) The human multidrug resistance-associated protein MRP, is a plasma membrane drug efflux pump *Proc Natl Acad Sci USA* **91** 8822-8826

Zaman GJR, Lankelma J, van Tellingen O, Beijen J, Dekker H, Paulusma C, Oude-Elferink RPJ, Bass F, andBorst P, (1995) Role of glutathione in the export of compounds from cells by the multidrug resistance-associated protein *Proc Natl Acad Sci USA* **92** 7690-7694

Zhou DJ Gonzalez-Cadavid N, Ahuja H et al, (1988) A unique pattern of proto-oncogene abnormalities in ovarian adenocarcinoma *Cancer* **62** 1573-1576

Zhou L and Leung BS (1992) Growth regulation of ovarian cancer growth factors alpha and beta 1 *Biochem Biophys Acta* **1180** 130-136

Appendix 1

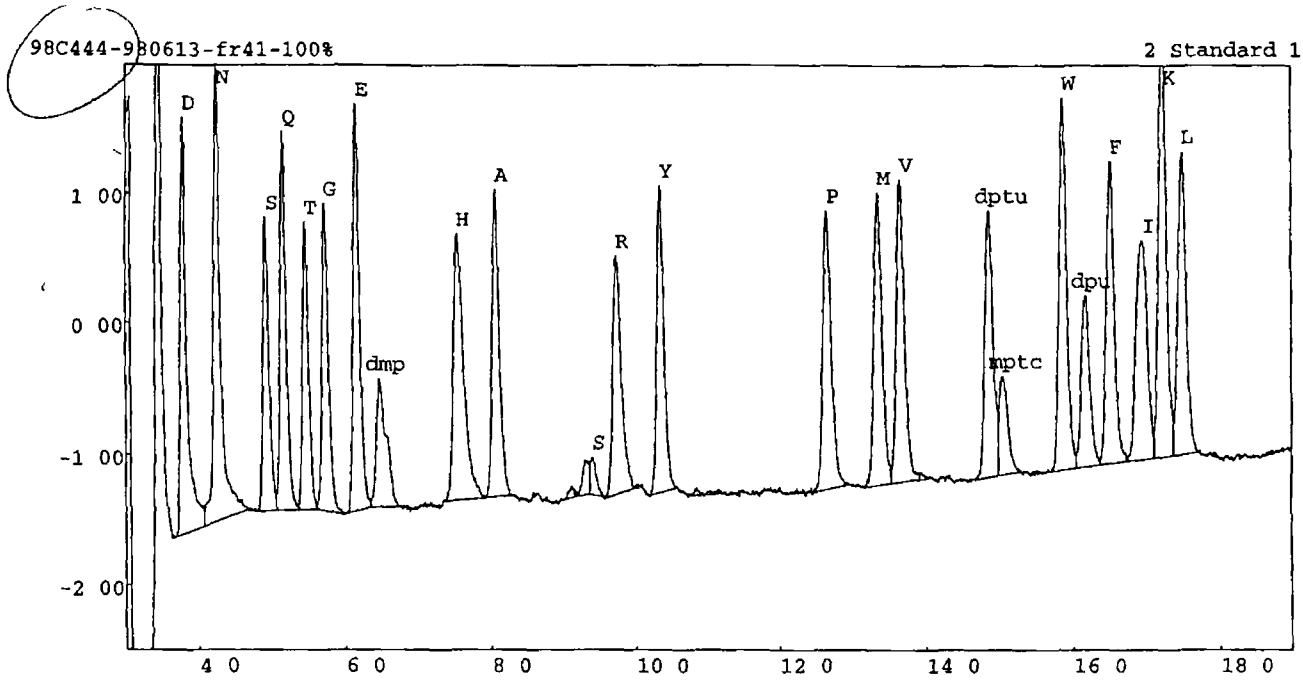
Abbreviations

5-FU	5-Flurouracil
ABC/HRP	Streptavidin/biotin-horseradish peroxidase conjugate
Adr	Adriamycin
ATCC	American Tissue Culture Collection
BCA	Bicinchoninic acid
Biotin-7-NHS	D-biotinyl-ε-aminocaproic acid-N-hydroxysuccinimide
BSA	Bovine serum albumin
CMF	cyclophosphamide, methatrexate, 5-fluoruracil
DAB	Diaminobenzidine
DDSA	Dodecenylsuccinic anhydrate
dH_2O	Deionised water/glass distilled water
DMEM	Dulbeccos Modified Eagles Medium
DMSO	Dimethyl sulfoxinide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin(Adriamycin)
ECL	Enhanced chemiluminescence
ECACC	European Collectionof Animal Cell Culture
EDTA	Ethylene diamino tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
FIGO	Federation of Gynaecology and Obstetrics
FITC	Fluorescein-isocyanate
HAT	Hypoxanthme, aminopterin, thymidine
HCl	Hydrochloric acid
HEPES	4-(2-hydroxethyl-)-piperazine ethane sulphonic acid
RP HPLC	Reverse phase High performance liquid chromatography
HT	Hypoxanthine, thymadine
IC_{50}	Inhibitory concentration 50%
Ig	Immunoglobulin
IMS	Industrial methylated spirits
KDa	KiloDalton
LRP	Lung resistance related protein

MAb/s	Monoclonal antibody/monoclonal antibodies
MDR	Multidrug resistance
MEM	Minimum Essential Medium
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex
mRNA	Messenger RNA
MRP	Multidrug resistance protein
MVP	Major vault protein
Mw	Molecular weight
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NCTCC	National Cell and Tissue Culture Centre
ND	Not done
NEAA	Non-essential amino acids
NSCLC	Non-small cell lung carcinoma
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
P-gp	P-glycoprotein
PMSF	Phenylmethyl sulfonyl fluoride
PNAG	Phenyl-N-acetyl-α-D-galactosaminide
PNPP	P-nitrophenyl phosphate
PVDF	polyvinyldifluoride (blotting membrane)
RID	Radial-immunodiffusion
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
TBS	Tris buffered saline
TEMED	N,N,N,N-tetramethyl-ethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
TV	Trypsin /versene
UV	Ultra violet

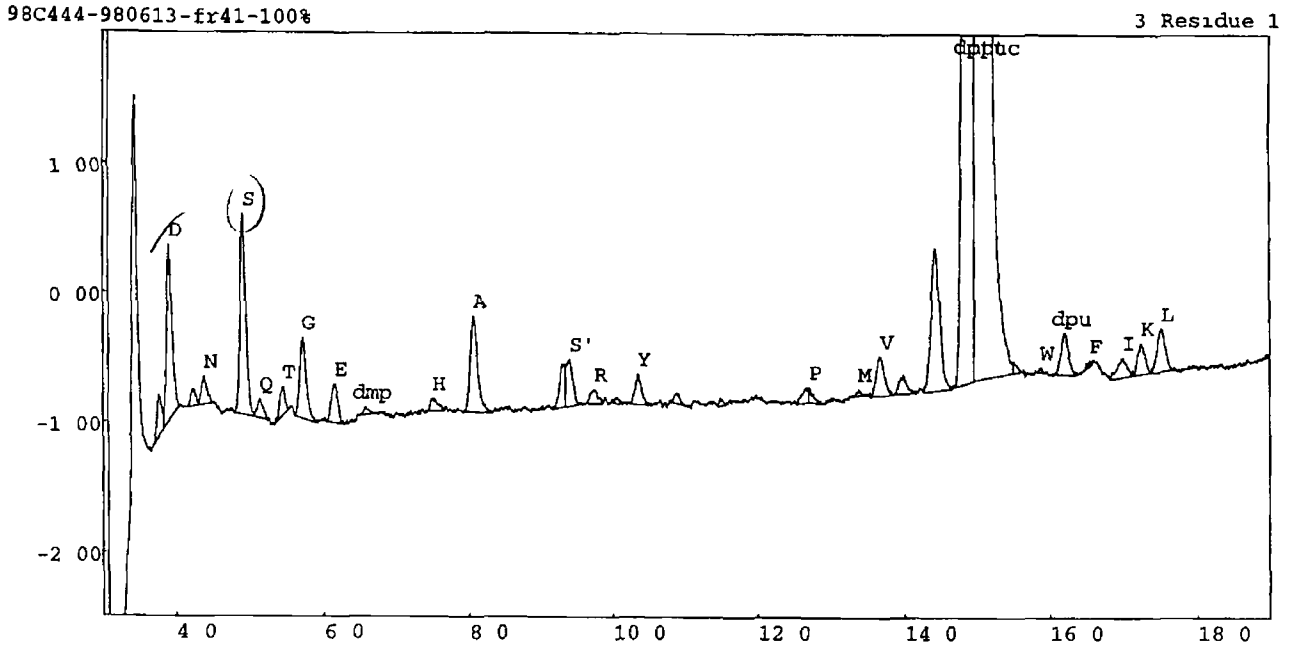
VP-16	Etoposide (Vepesid)
v/v	Volume to volume ratio
w/v	weight to volume ratio

Appendix 2



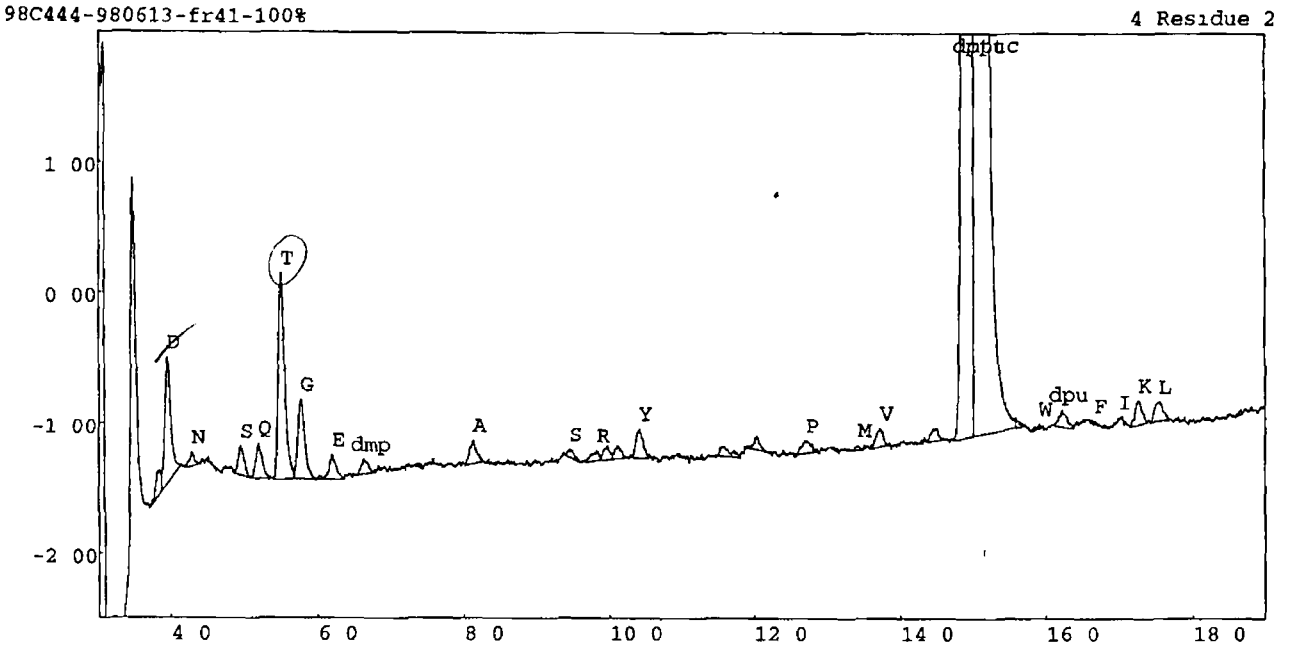
2 Standard 1, Interpolated baseline

Peak No	RT	Peak ID	Type	Height	Pmol Ht
1	3 78	D	c	25966	10 00
2	4 24	N	c	28521	10 00
3	4 91	S	c	18314	10 00
4	5 15	Q	c	23723	10 00
5	5 45	T	c	17942	10 00
6	5 72	G	c	19320	10 00
7	6 15	E	c	25483	10 00
8	6 47	dmp		8005	10 00
9	7 54	H	c	16841	10 00
10	8 07	A	c	19197	10 00
14	9 40	S'		2409	10 00
15	9 74	R	c	14921	10 00
16	10 34	Y	c	19200	10 00
20	12 65	P	c	17503	10 00
21	13 35	M	c	18564	10 00
22	13 66	V	c	19132	10 00
24	14 86	dptu		16871	10 00
25	15 05	mptc	r	6167	10 00
26	15 87	W	c	23497	10 00
27	16 17	dpu		10796	10 00
28	16 51	F	c	19169	10 00
29	16 95	I	c	13982	10 00
30	17 23	K	c	28640	10 00
31	17 49	L	c	19246	10 00



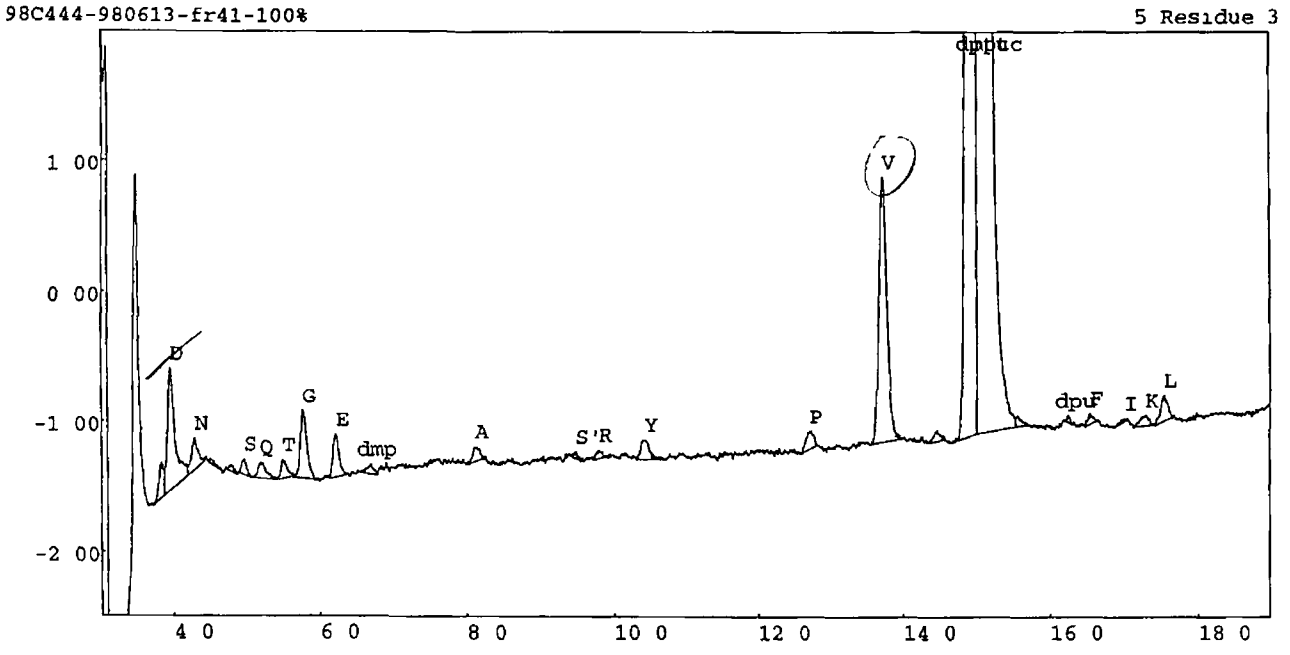
3 Residue 1, Interpolated baseline

Peak No	RT	Peak ID	Type	Height	Pmol Ht
2	3 91	D	c	11026	4 25
4	4 37	N	c	1723	0 60
5	4 90	S	c	12619	6 89
6	5 14	Q	c	1146	0 48
7	5 45	T	c	1849	1 03
8	5 72	G	c	5075	2 63
9	6 15	E	c	2479	0 97
10	6 57	dmp		395	0 49
11	7 50	H	c	731	0 43
12	8 06	A	c	6108	3 18
15	9 40	S'		2949	12 24
16	9 75	R	c	815	0 55
18	10 35	Y	c	1780	0 93
22	12 70	P	c	937	0 54
24	13 38	M	c	279	0 15
25	13 68	V	c	2442	1 28
28	14 88	dptu		143723	85 19
29	15 07	mptc	r	287792	466 66
31	15 88	W	c	264	0 11
32	16 20	dpu		2580	2 39
33	16 54	F	c	460	0 24
34	16 99	I	c	1180	0 84
35	17 24	K	c	1897	0 66
36	17 51	L	c	2702	1 40



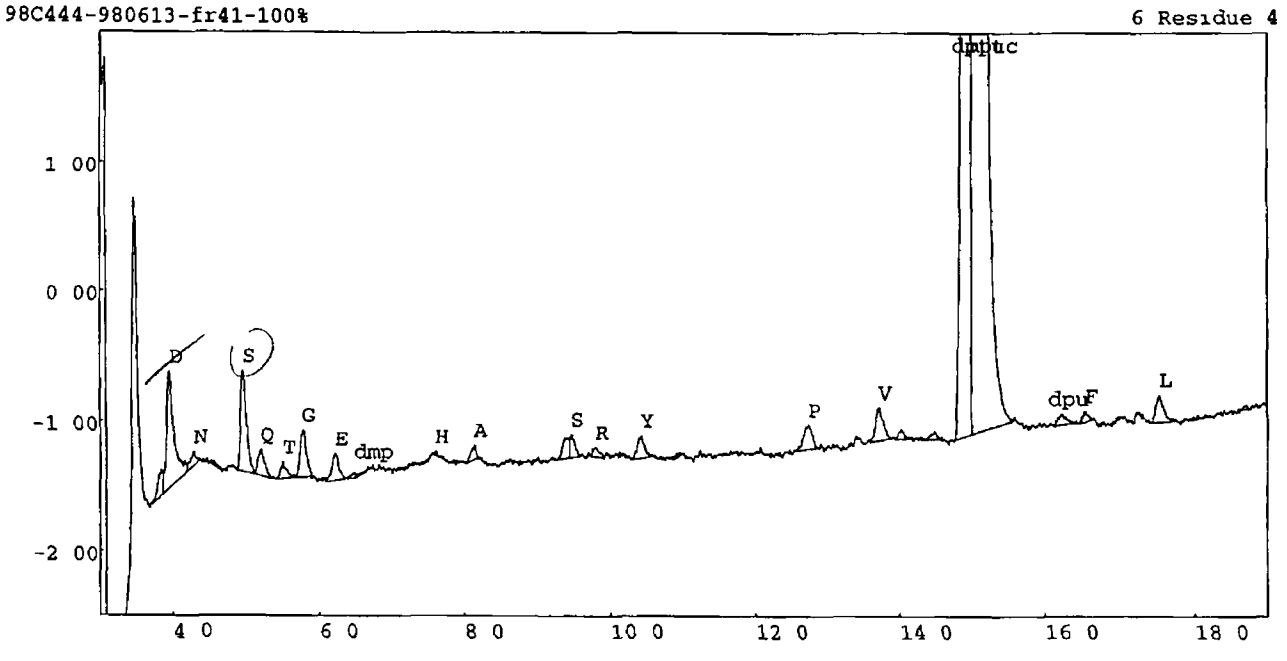
4 Residue 2, Interpolated baseline

Peak No	RT	Peak ID	Type	Height	Pmol	Ht
2	3 95	D	c	7781	3	00
3	4 28	N	c	768	0	27
5	4 94	S	c	1749	0	96
6	5 19	Q	c	2080	0	88
7	5 50	T	c	12855	7	16
8	5 77	G	c	4953	2	56
10	6 20	E	c	1412	0	55
11	6 63	dmp		833	1	04
13	8 13	A	c	1379	0	72
14	9 45	S'		533	2	21
15	9 83	R	c	527	0	35
18	10 41	Y	c	1729	0	90
23	12 70	P	c	725	0	41
25	13 42	M	c	192	0	10
26	13 72	V	c	1118	0	58
28	14 91	dptu		106857	63	34
29	15 10	mptc	r	210768	341	77
31	15 91	W	c	231	0	10
32	16 22	dpu		882	0	82
33	16 67	F	c	154	0	08
34	17 00	I	c	315	0	23
35	17 26	K	c	1396	0	49
36	17 54	L	c	1132	0	59



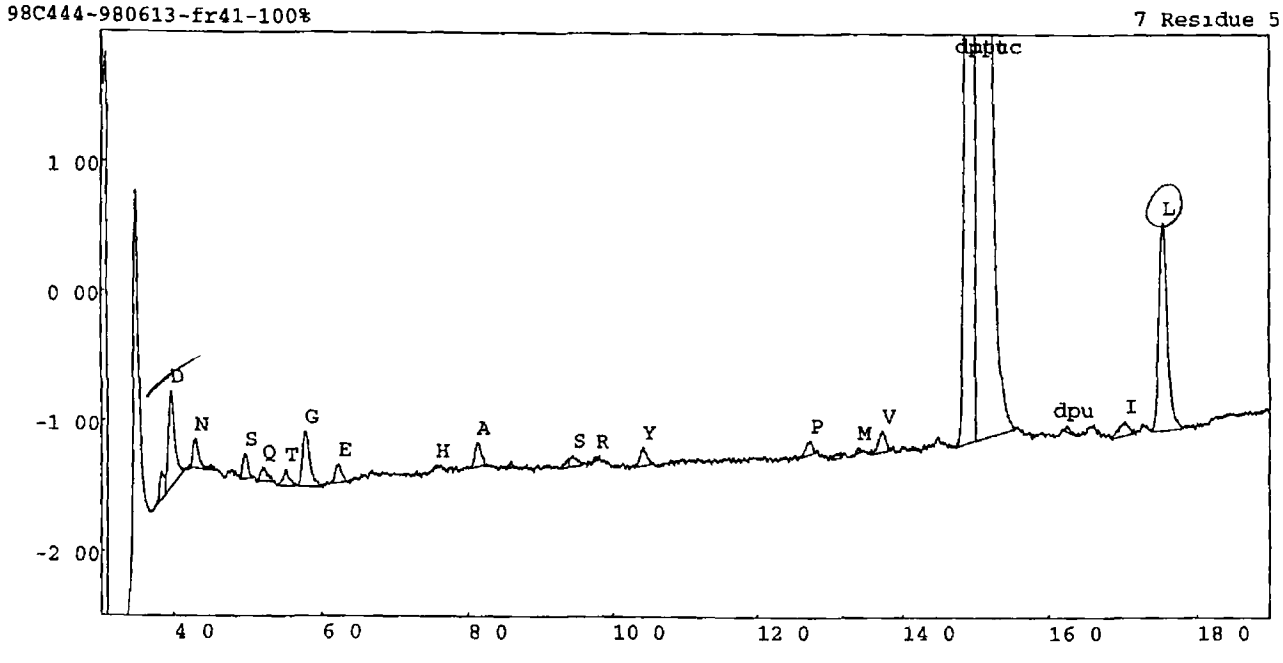
5 Residue 3 Interpolated baseline

Peak No	RT	Peak ID	Type	Height	Pmol Ht
2	3 95	D	c	7602	2 93
3	4 28	N	c	1814	0 64
6	4 96	S	c	949	0 52
7	5 19	Q	c	915	0 39
8	5 49	T	c	1058	0 59
9	5 78	G	c	4174	2 16
10	6 22	E	c	2544	1 00
11	6 69	dmp		448	0 56
12	8 12	A	c	836	0 44
13	9 48	S'		297	1 23
14	9 80	R	c	403	0 27
15	10 42	Y	c	1194	0 62
17	12 71	P	c	1099	0 63
18	13 73	V	c	16796	8 78
21	14 93	dptu		91520	54 25
22	15 12	mptc	r	204241	331 18
24	16 26	dpu		351	0 33
25	16 55	F	c	557	0 29
26	17 01	I	c	280	0 20
27	17 30	K	c	618	0 22
28	17 55	L	c	1526	0 79



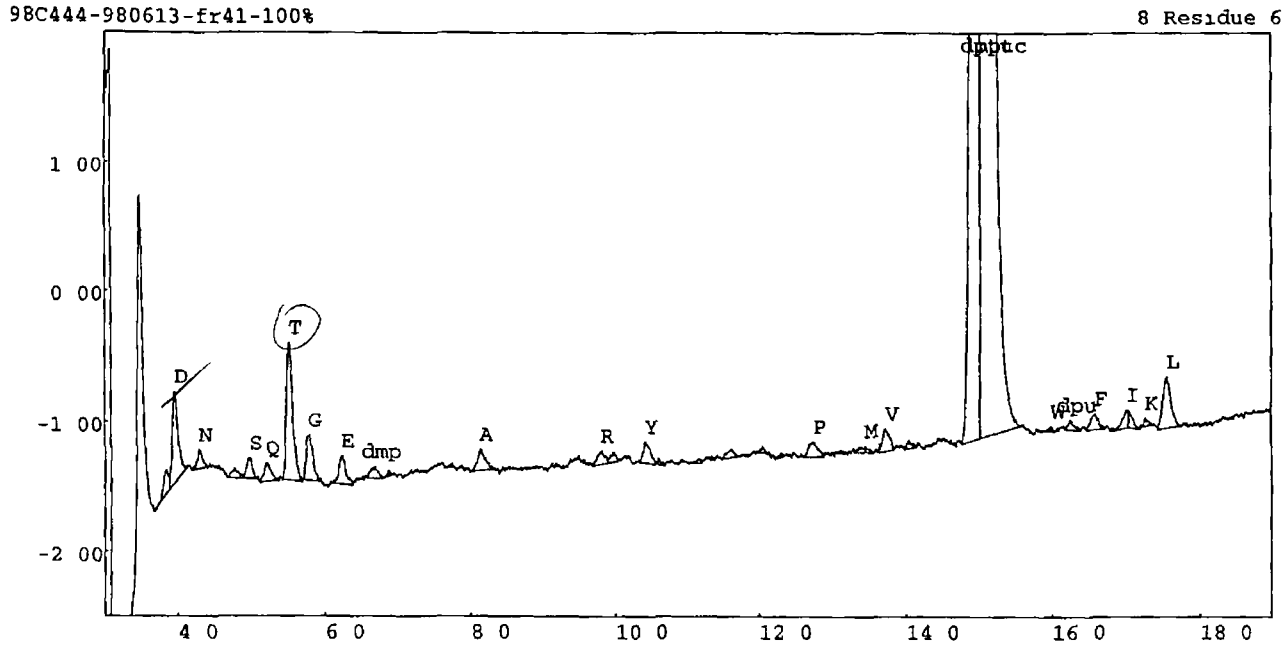
6 Residue 4, Interpolated baseline

Peak No	RT	Peak ID	Type	Height	Pmol Ht
2	3 95	D	c	7152	2 75
3	4 27	N	c	844	0 30
5	4 95	S	c	6337	3 46
6	5 20	Q	c	1631	0 69
7	5 50	T	c	935	0 52
8	5 78	G	c	2931	1 52
9	6 21	E	c	1566	0 61
11	6 66	dmp		320	0 40
13	7 60	H	c	389	0 23
14	8 14	A	c	848	0 44
16	9 46	S'		1413	5 87
17	9 80	R	c	534	0 36
18	10 41	Y	c	1315	0 68
21	12 72	P	c	1483	0 85
22	13 72	V	c	1986	1 04
26	14 92	dptu		79264	46 98
27	15 11	mptc	r	202789	328 83
28	16 23	dpu		604	0 56
29	16 55	F	c	588	0 31
30	17 54	L	c	1630	0 85



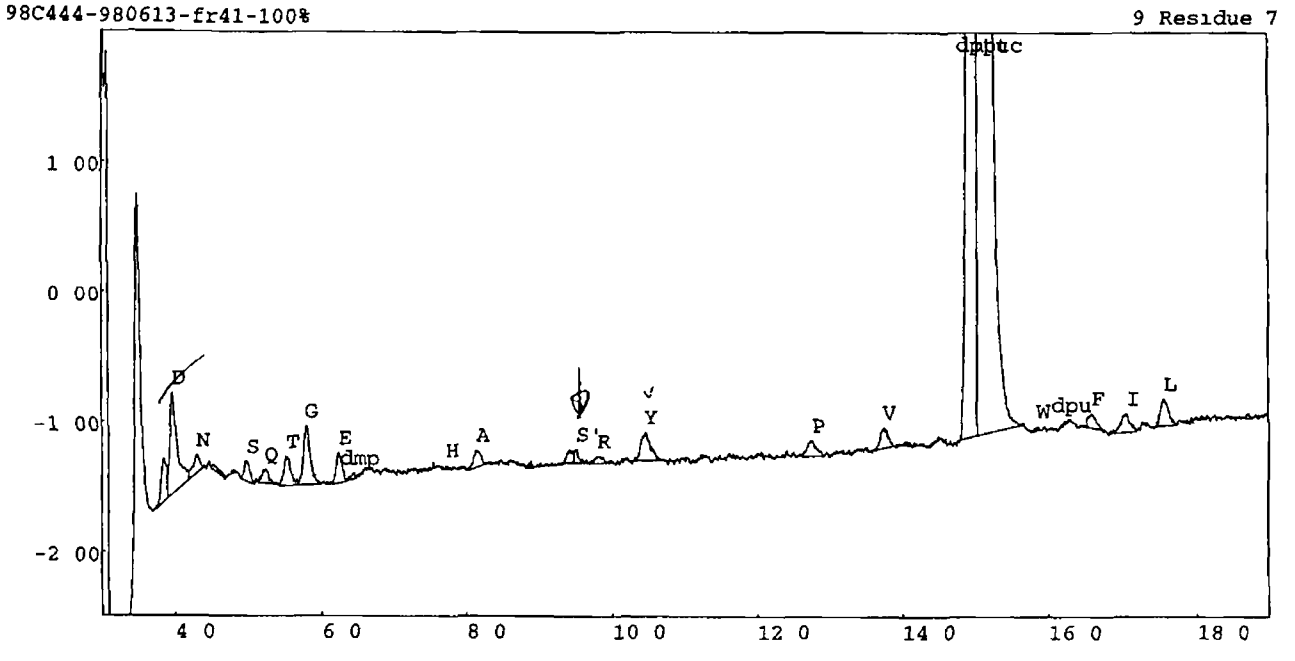
7 Residue 5, Interpolated baseline

Peak No	RT	Peak ID	Type	Height	Pmol Ht
2	3 95	D	c	6008	2 31
3	4 28	N	c	1810	0 63
5	4 95	S	c	1421	0 78
6	5 20	Q	c	786	0 33
7	5 50	T	c	907	0 51
8	5 77	G	c	3402	1 76
9	6 22	E	c	1050	0 41
10	7 56	H	c	262	0 16
11	8 13	A	c	1499	0 78
15	9 45	S'		563	2 34
16	9 77	R	c	228	0 15
17	10 42	Y	c	1022	0 53
18	12 73	P	c	810	0 46
20	13 39	M	c	328	0 18
21	13 72	V	c	1220	0 64
23	14 92	dptu		69712	41 32
24	15 11	mptc	r	196120	318 02
25	16 24	dpu		380	0 35
26	17 02	I	c	801	0 57
27	17 54	L	c	13189	6 85



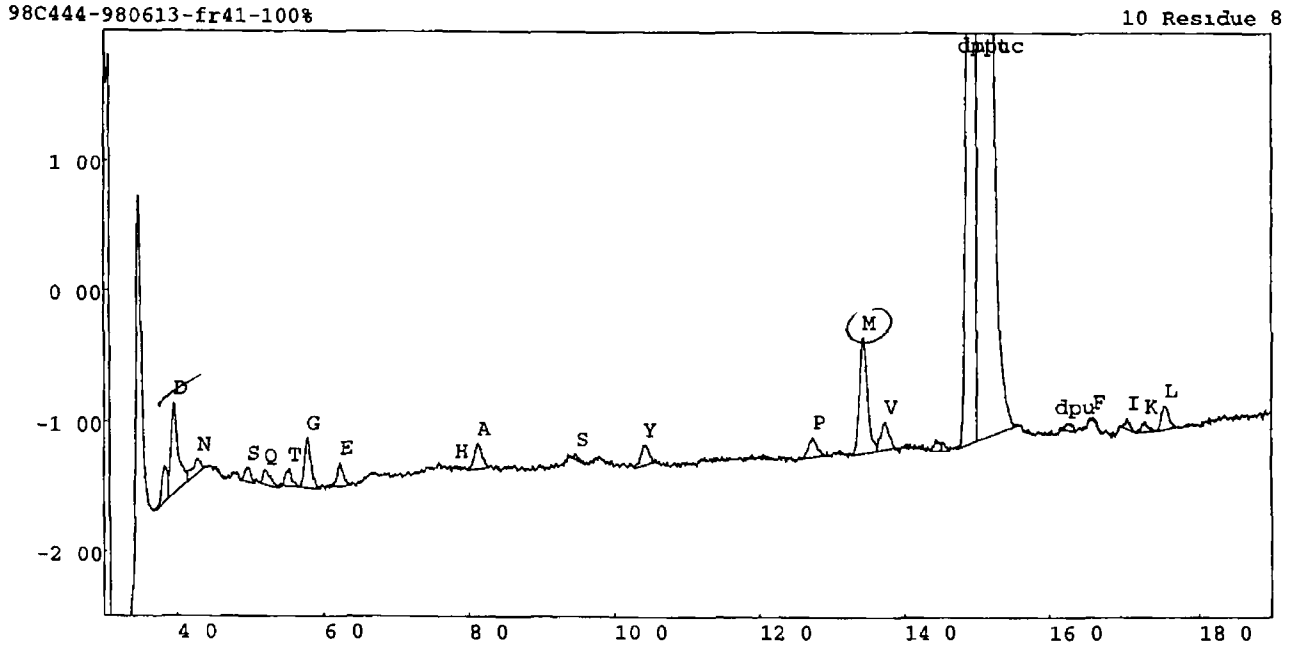
8 Residue 6, Interpolated baseline

Peak No	RT	Peak ID	Type	Height	Pmol Ht
2	3 95	D	c	5775	2 22
3	4 28	N	c	1126	0 39
5	4 96	S	c	1272	0 69
6	5 20	Q	c	1105	0 47
7	5 51	T	c	8556	4 77
8	5 78	G	c	2846	1 47
9	6 22	E	c	1667	0 65
10	6 68	dmp		609	0 76
12	8 13	A	c	1257	0 65
13	9 80	R	c	755	0 51
15	10 42	Y	c	1297	0 68
22	12 73	P	c	913	0 52
25	13 44	M	c	327	0 18
26	13 72	V	c	1291	0 67
28	14 93	dptu		70906	42 03
29	15 12	mptc	r	208055	337 37
30	16 00	W	c	254	0 11
31	16 25	dpu		550	0 51
32	16 58	F	c	880	0 46
33	17 01	I	c	1143	0 82
35	17 27	K	c	393	0 14
36	17 55	L	c	3089	1 61



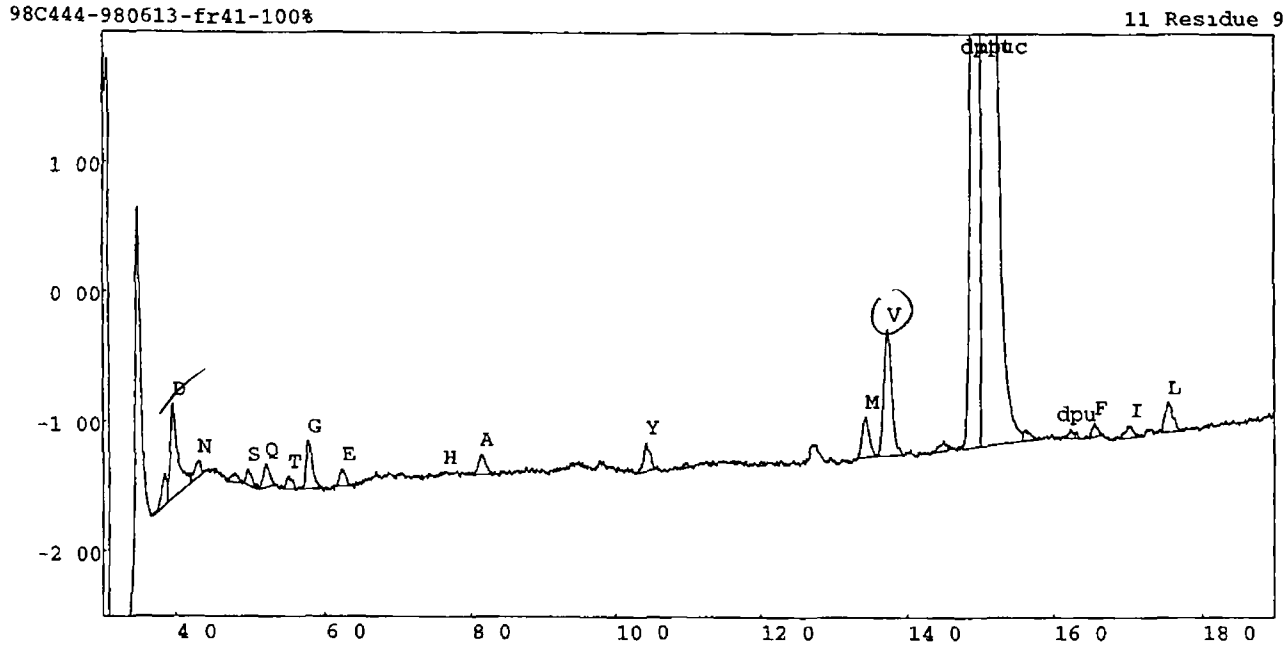
9 Residue 7, Interpolated baseline

Peak No	RT	Peak ID	Type	Height	Pmol	Ht
2	3 96	D	c	6299	2	43
3	4 29	N	c	1110	0	39
6	4 96	S	c	1228	0	67
7	5 22	Q	c	826	0	35
8	5 52	T	c	1782	0	99
9	5 79	G	c	3637	1	88
10	6 23	E	c	1801	0	71
11	6 43	dmp		318	0	40
12	7 73	H	c	170	0	10
13	8 15	A	c	984	0	51
16	9 50	S'		771	3	20
17	9 80	R	c	386	0	26
19	10 45	Y	c	1673	0	87
22	12 75	P	c	908	0	52
24	13 74	V	c	1214	0	63
27	14 95	dptu		67439	39	97
28	15 14	mptc	r	182650	296	17
29	15 86	W	c	217	0	09
30	16 23	dpu		312	0	29
31	16 60	F	c	880	0	46
32	17 06	I	c	1110	0	79
33	17 56	L	c	1645	0	85



10 Residue 8, Interpolated baseline

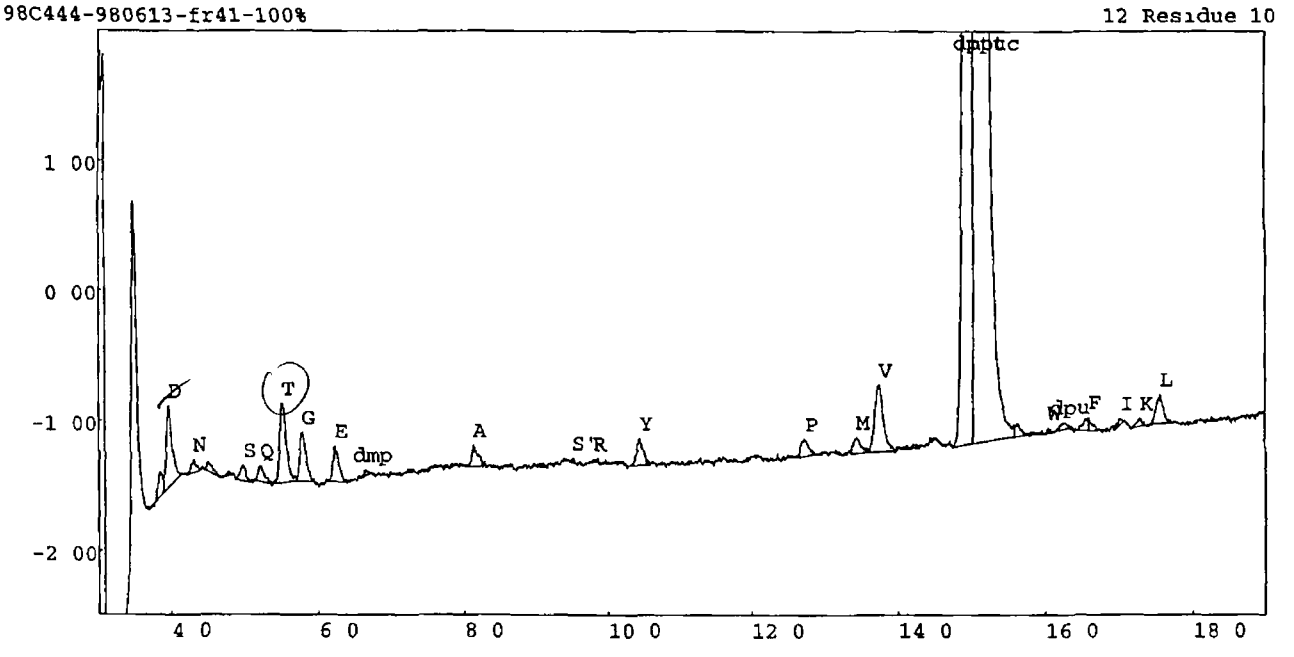
Peak No	RT	Peak ID	Type	Height	Pmol Ht
2	3 95	D	c	5651	2 18
3	4 27	N	c	1010	0 35
5	4 95	S	c	842	0 46
6	5 19	Q	c	912	0 38
7	5 52	T	c	1015	0 57
8	5 78	G	c	3095	1 60
9	6 22	E	c	1387	0 54
10	7 82	H	c	197	0 12
11	8 12	A	c	1560	0 81
12	9 47	S'		347	1 44
13	10 40	Y	c	1228	0 64
17	12 73	P	c	1183	0 68
18	13 42	M	c	7272	3 92
19	13 73	V	c	1757	0 92
24	14 93	dptu		63802	37 82
25	15 12	mptc	r	203769	330 42
27	16 26	dpu		421	0 39
28	16 58	F	c	445	0 23
29	17 04	I	c	612	0 44
30	17 28	K	c	496	0 17
31	17 55	L	c	1433	0 74



11 Residue 9, Interpolated baseline

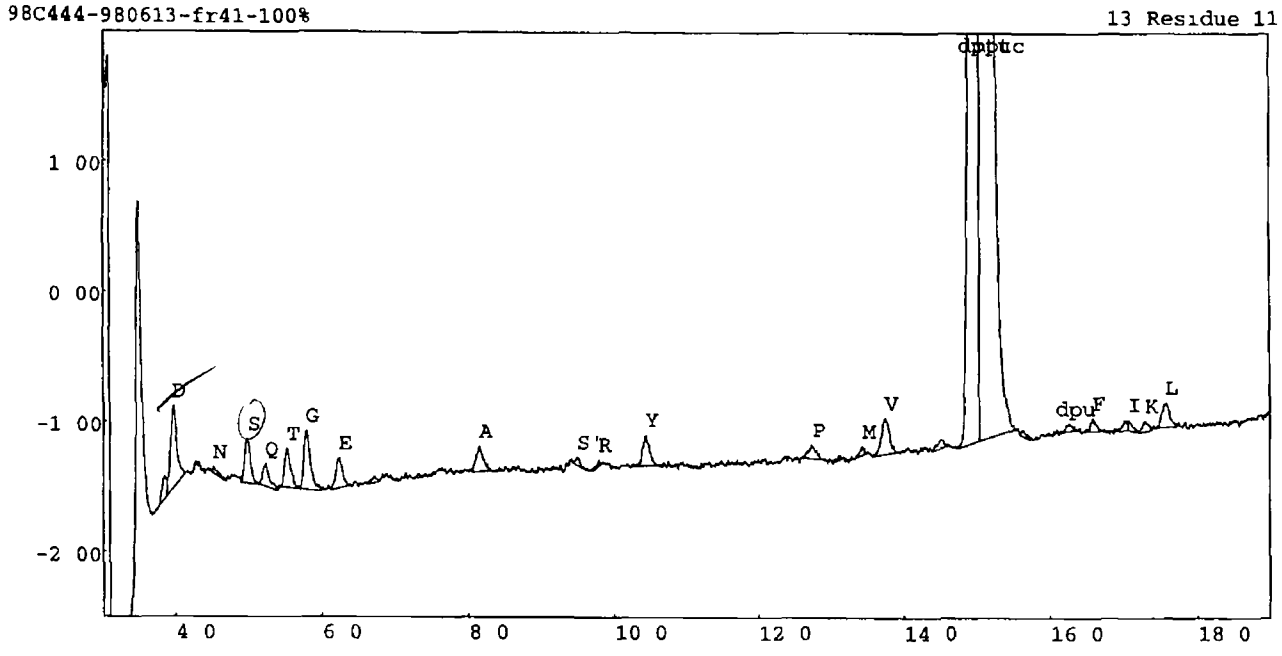
Peak No	RT	Peak ID	Type	Height	Pmol Ht
2	3 95	D	c	5853	2 25
3	4 30	N	c	1057	0 37
5	4 95	S	c	935	0 51
6	5 20	Q	c	1376	0 58
7	5 50	T	c	734	0 41
8	5 78	G	c	2957	1 53
9	6 22	E	c	1057	0 41
10	7 62	H	c	116	0 07
11	8 13	A	c	1250	0 65
12	10 42	Y	c	1675	0 87
15	13 43	M	c	2519	1 36
16	13 73	V	c	7984	4 17
18	14 93	dptu		63790	37.81
19	15 12	mptc	r	196533	318 68
21	16 23	dpu		474	0 44
23	16 56	F	c	759	0 40
24	17 04	I	c	672	0 48
25	17 55	L	c	1746	0 91

28-09-1998 08 48 98C444-980613-fr41-100%



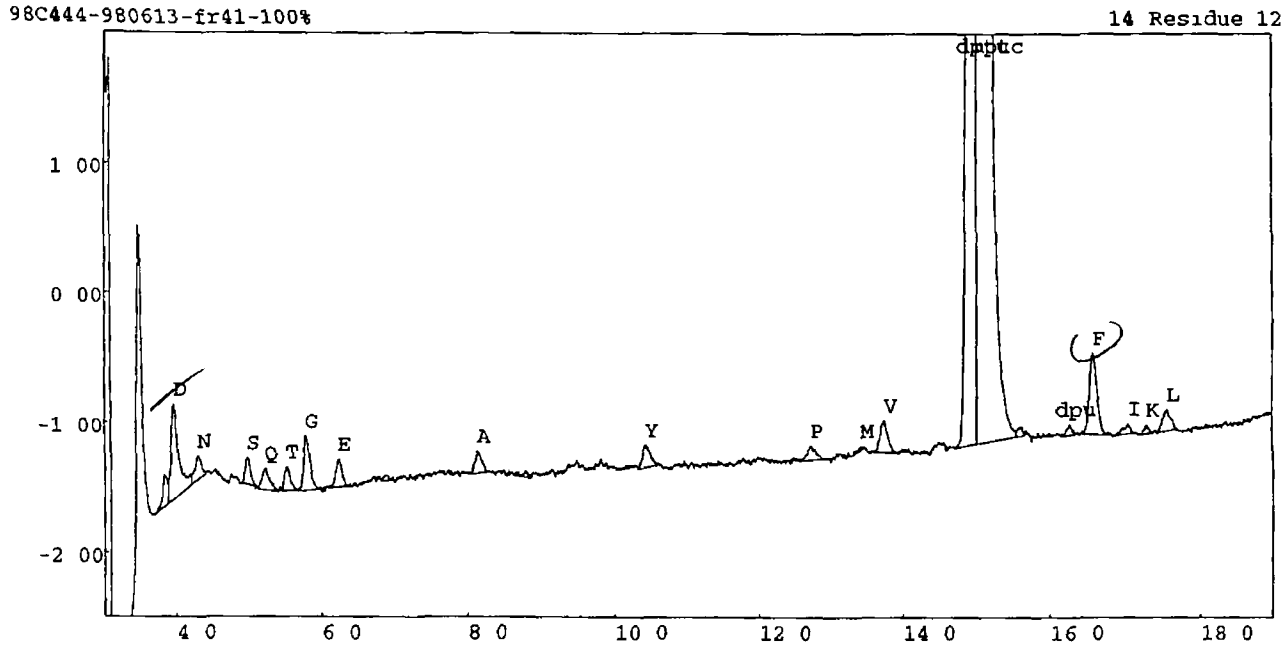
12 Residue 10, Interpolated baseline

Peak No	RT	Peak ID	Type	Height	Pmol Ht
2	3 96	D	c	5045	1 94
3	4 30	N	c	686	0 24
6	4 97	S	c	925	0 51
7	5 21	Q	c	985	0 42
8	5 51	T	c	4996	2 78
9	5 78	G	c	2986	1 55
10	6 22	E	c	2139	0 84
11	6 64	dmp		284	0 35
14	8 13	A	c	1181	0 62
16	9 50	S'		214	0 89
17	9 80	R	c	168	0 11
18	10 44	Y	c	1611	0 84
20	12 73	P	c	1010	0 58
22	13 43	M	c	866	0 47
23	13 74	V	c	4216	2 20
27	14 94	dptu		64854	38 44
28	15 13	mptc	r	181640	294 54
31	16 04	W	c	230	0 10
32	16 26	dpu		456	0 42
34	16 60	F	c	730	0 38
35	17 03	I	c	381	0 27
36	17 30	K	c	494	0 17
37	17 57	L	c	1721	0 89



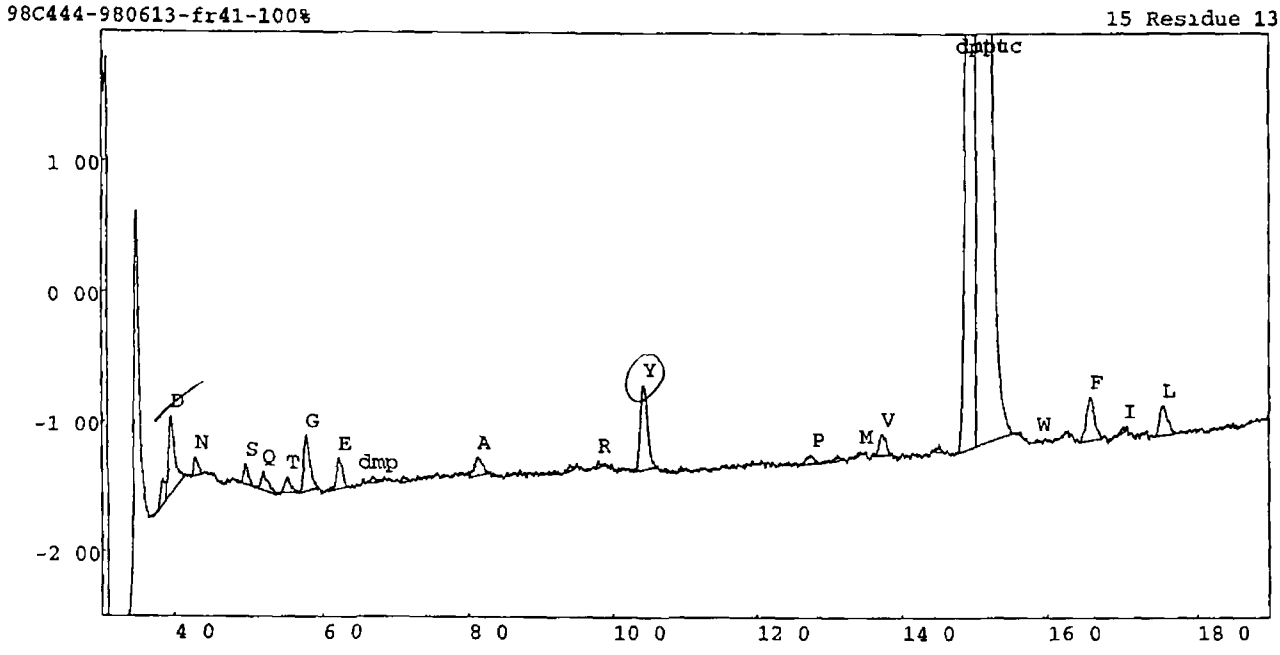
13 Residue 11, Interpolated baseline

Peak No	RT	Peak ID	Type	Height	Pmol Ht
2	3 96	D	c	5167	1 99
3	4 51	N	c	475	0 17
4	4 97	S	c	2738	1 50
5	5 22	Q	c	1303	0 55
6	5 52	T	c	2346	1 31
7	5 79	G	c	3608	1 87
8	6 23	E	c	1790	0 70
11	8 15	A	c	1487	0 77
12	9 48	S'		472	1 96
13	9 79	R	c	333	0 22
14	10 43	Y	c	1792	0 93
15	12 73	P	c	816	0 47
17	13 43	M	c	524	0 28
18	13 74	V	c	2193	1 15
20	14 94	dptu		68315	40 49
21	15 14	mptc	r	163787	265 59
23	16 26	dpu		408	0 38
24	16 58	F	c	721	0 38
26	17 06	I	c	642	0 46
27	17 29	K	c	523	0 18
28	17 57	L	c	1489	0 77



14 Residue 12, Interpolated baseline

Peak No	RT	Peak ID	Type	Height	Pmol Ht
2	3 95	D	c	5986	2 31
3	4 28	N	c	1414	0 50
4	4 95	S	c	1675	0 91
5	5 20	Q	c	1233	0 52
6	5 50	T	c	1409	0 79
7	5 78	G	c	3354	1 74
8	6 22	E	c	1667	0 65
10	8 13	A	c	1274	0 66
15	10 42	Y	c	1313	0 68
16	12 71	P	c	826	0 47
17	13 42	M	c	338	0 18
18	13 74	V	c	2027	1 06
19	14 93	dptu		63194	37 46
20	15 12	mptc	r	176901	286 85
22	16 26	dpu		574	0 53
23	16 57	F	c	5109	2 67
24	17 04	I	c	659	0 47
25	17 28	K	c	498	0 17
26	17 55	L	c	1313	0 68



15 Residue 13, Interpolated baseline

Peak No	RT	Peak ID	Type	Height	Pmol Ht
2	3 95	D	c	4767	1 84
3	4 28	N	c	1089	0 38
4	4 96	S	c	1223	0 67
5	5 20	Q	c	1128	0 48
6	5 52	T	c	937	0 52
7	5 78	G	c	3377	1 75
8	6 22	E	c	1859	0 73
9	6 68	dmp		307	0 38
11	8 14	A	c	1054	0 55
13	9 80	R	c	287	0 19
14	10 43	Y	c	5334	2 78
18	12 75	P	c	508	0 29
20	13 43	M	c	186	0 10
21	13 73	V	c	1285	0 67
23	14 94	dptu		55846	33 10
24	15 13	mptc	r	165800	268 85
26	15 87	W	c	149	0 06
27	16 58	F	c	2696	1 41
28	17 02	I	c	342	0 24
29	17 55	L	c	1873	0 97

EUROSEQUENCE bv

synthesis and analysis of peptides, proteins and oligonucleotides

INTERPRETATION PROTEIN SEQUENCE ANALYSIS RESULTS

See also 'Product Information Protein Sequence Analysis' for explanation of 'Initial Yield' and amino acids in parentheses.

Our order number >> **980613**
Product code >> **98C444** Sample: **980613-(I+III)-T-fr. 41**

Sample amount	. 100%
Date of analysis	: September 25, 1998
Performed	14 cycles
Date of interpretation report	: October 30, 1998
Interpreted by	: Dr. Wicher J Weijer

MAIN SEQUENCE

Position #	1				5					10			14
Amino Acid	Ser-	Thr-	Val-	Ser-	Leu-	Thr-	(Cys?)-	Met-	Val-	Thr-	Ser-	Phe-	Tyr-Pro
Initial Yield	appr 10 pmol												

Remarks

1. Fraction 41 of HPLC 98E178 was analyzed
2. At position 7 there are some indirect indications for the presence of a cysteine

Signature
('investigator')

Signature
('verifier')

Appendix 3

3 Standard protocols

The lysis of cells disintegration of isolated cell membranes and solubilization of proteins is a crucial step for subsequent immunoprecipitation The method should ensure solubilization of the target protein in a form that is immunoreactive undegraded and ideally biologically active Variables influencing the efficiency of solubilization and subsequent immunoprecipitation of proteins are the ionic strength and pH of the lysis buffer type and concentration of detergents used presence of divalent cations and other factors (3 4)
In the following protocols are given for biotinylation of surface proteins on intact cells (3 2) or membrane preparations (3 3) and for total cell homogenates (3 4) These standard protocols serve as general guidelines and first choice protocols but may be varied to meet special needs

Note Biotin 7 NHS reacts with free amino groups Do not use Tris buffers or buffers containing ammonium ions or sodium azide for the biotin labeling reaction In case of contamination with these substances extensive dialyzing or desalting on appropriate columns is recommended

3 1 Buffers required

- **Phosphate buffered saline (PBS)**
  Dissolve 8 g of NaCl 0 2 g of KCl 1 44 g of Na_2HPO_4 and 0 24 g of KH_2PO_4 in 800 ml of redist H_2O Adjust the pH to 7 4 with HCl Add H O to 1 l Dispense the solution into aliquots and autoclave Store at room temperature

- **NaCl 1 M, 100 ml**
  Dissolve 5 84 g of NaCl in 80 ml of redist water Adjust the volume to 100 ml with redist water Dispense into aliquots and sterilize by autoclaving

- **Buffers listed in table 1 (Note** not all buffers are needed for particular applications amounts were calculated for at least 10 labeling reactions) **Prepare the daily requirements only**

Tab 1 Preparation of buffers

Stock solutions	Biotinylation buffer	Final conc	Lysis buffer	Final conc	Homogenizing buffer	Final concentration
Core buffer	4 0 ml	50 mM	4 ml	50 mM	12 ml	50 mM
NaCl	1 8 ml	150 mM	1 8 ml	150 mM	5 4 ml	150 mM
PMSF			0 12 ml	0 1 mg/ml	0 36 ml	0 1 mg/ml
Aprotinin			1 2 µl	1 µg/ml	3 6 µl	1 µg/ml
Leupeptin			1 2 µl	1 µg/ml	3 6 µl	1 µg/ml
Nonidet P 40			1 2 ml	1%		
Sodium deoxycholate			0 6 ml	0 5%		
Redist water	6 2 ml		4 3 ml		18 2 ml	
Final volume	12 0 ml		12 0 ml		36 0 ml	

3 2 Biotin-labeling of cell surface proteins on intact cells, preparation for immuno precipitation

Wash cells at least twice with ice cold PBS to remove any remaining serum proteins from the culture medium Adherent cells should be washed by addition of PBS to the monolayer and the supernatant discarded Cells in suspension should be washed by centrifugation and the pellet resuspended Carefully remove PBS from cells after the last wash

Add biotinylation buffer to the washed cell monolayers (volumes as indicated in table 2) or to the cell sediment at a concentration of 10^6 10^7 cells/ml suspend pelleted cells

Note A cell concentration no higher than 1 x 10^7 cells per ml should be used to ensure optimal vectorial labeling and cell recovery

Tab 2 Correlation between cell number and biotinylation buffer

Volume of biotinylation buffer (ml)	Size of petri dish or well diameter (mm)	Approximate number of cells
1 0	90	10^6 10^7
0 5	60	5 x 10^5 5 x 10^6
0 25	35	10^5 10^6
0 25	30	10^5 10^6

Add 5 µl of biotin 7 NHS stock solution to 1 ml of suspension mix and incubate for 15 min at room temperature

Stop the reaction by adding 50 µl of stop solution per ml (final concentration 50 mM NH_4Cl) and incubate for 15 min at room temperature or on ice

Wash the cells in PBS

Extract cells in lysis buffer (up to 10^6 cells 0 5 ml and 10^6 10^7 cells 1 ml c lysis buffer) for 30 min on ice If suspension cells are used proceed with soni cation of the suspension (three steps ahead)

Scrape the cells to one side of the dish with a suitable device

Transfer to a chilled microfuge tube

Sonicate the suspension (3 pulses 30 s each) on ice and extract for a furth 30 min on ice

Centrifuge the lysate at 12 000 g for 10 min at 4°C in a table top microfuge

Transfer the supernatant to a fresh microfuge tube and store it on ice c at 70°C depending on the sensitivity of the target antigen to freezing an thawing

Continue with the immunoprecipitation procedure (3 5)

3 3 Isolation of membranes, biotin labeling of membrane proteins and their pr paration for immunoprecipitation

Wash cells at least twice with ice cold PBS to remove any remaining seru proteins from the culture medium Adherent cells should be washed by additi of PBS to the monolayer and the supernatant discarded Cells in suspensi should be washed by centrifugation and the pellet resuspended

Add homogenizing buffer (cooled to 4°C) to the chilled washed cell mon layers or to the cell sediment at a concentration of 10^6 10^7 cells/ml (see tat 2) to give a total volume of 1 3 ml If suspension cells are used proceed w ultrasonic treatment of suspension (three steps ahead)

Scrape the cells to one side of the dish with a suitable device

Transfer the suspension to a chilled microfuge tube

Homogenize cells by ultrasonic treatment

Centrifuge the homogenized suspension at 12,000 g 12 min 4°C in a table top microfuge to remove debris
Separate the supernatant and transfer to a tube for ultracentrifugation
Centrifuge the solution at 100 000 g 45 min 4 C to pellet the cell membrane fraction
Resuspend the membrane pellet in 1 ml of lysis buffer Sonicate and incubate 30 min at 4 C
Add 5 µl of biotin 7 NHS stock solution mix and incubate for 15 min on ice
Stop the reaction by adding 50 µl of stop solution per ml (final concentration 50 mM NH_4Cl)
Store sample on ice or at 70 C depending on the sensitivity of target antigen to freezing and thawing
Continue with immunoprecipitation procedure (3 5)

3 4 Biotin labeling of total proteins and recombinant proteins from eucaryotic expression systems and their preparation for immunoprecipitation

The basic protocol provided here may be used with minor modifications for other expression systems like yeast or bacteria

Wash cells at least twice with ice cold PBS to remove any remaining serum proteins from the culture medium Adherent cells should be washed by the addition of PBS to the monolayer and the supernatant discarded Cells in suspension should be washed by centrifugation and the pellet resuspended
Add lysis buffer (cooled to 4 C) to the chilled washed cell monolayers (approx cell number per volume see table 2) or to the cell sediment at a concentration of 10' 10 cells/ml If suspension cells are used proceed with sonication of the suspension (1 2 ml) (three steps ahead)
Scrape the cells to one side of the dish with a suitable device
Transfer 1 2 ml to a chilled microfuge tube
Sonicate (3 pulses 30 s each) on ice and incubate for 30 min at 4 C
Centrifuge the lysate at 12 000 g for 10 min at 4 C in a table top microfuge
Transfer the supernatant to a fresh microfuge tube
Add 25 µl of biotin 7 NHS stock solution to 1 ml of the sample mix and incubate for 15 min at 4°C
Stop the reaction by adding 50 µl of stop solution per ml (final concentration 50 mM NH_4Cl) and incubate for 15 min at 4 C
Continue with the immunoprecipitation procedure (3 5)

3 5 Immunoprecipitation of the target protein

3 5 1 Solutions required

1 M Tris buffer, pH 7 5, 50 ml
Dissolve 12 1 g of Tris base in 80 ml of redist water Adjust to pH 7 5 by adding about 6 5 ml of conc HCl Adjust the volume of the solution to 100 ml with redist water Dispense into aliquots and sterilize by autoclaving Store at room temperature

- **NaCl, 1 M, 100 ml**
  Dissolve 5 84 g of NaCl in 80 ml of redist water Adjust the volume to 100 ml with redist water Dispense into aliquots and sterilize by autoclaving Store at room temperature

- **Buffers listed in table 3** (amounts were calculated for at least 10 labeling reactions) **Prepare the daily requirements only**

Tab 3 Preparation of buffers

Stock solution	Dilution buffer	Final conc	Wash buffer 1	Final conc	Wash buffer 2	Final conc	Wash buffer 3	Final conc
Tris buffer	0 6 ml	50 mM	2 4 ml	50 mM	2 4 ml	50 mM	0 24 ml	10 mM
NaCl	1 8 ml	150 mM	7 2 ml	150 mM	24 0 ml	500 mM		
Nonidet P 40	0 12 ml	0 1	0 48 ml	0 1%	0 48 ml	0 1%		
Aprotinin	1 2 µl	1 µg/ml						
Leupeptin	1 2 µl	1 µg/ml						
PMSF	0 12 ml	100 µg/ml						
Redist water	7 8 ml		38 0 ml		21 1 ml		23 8 ml	
Final volume	12 0 ml		48 0 ml		48 0 ml		24 0 ml	

Not provided with the kit

Control antibody (preimmune serum or irrelevant monoclonal antibody)

3 5 2 Assay procedure

Divide the preparations of antigen (1 ml) into two or more equally sized aliquots and place in microfuge tubes Adjust the volume of each aliquot to 1 0 ml with dilution buffer Add 50 µl of protein A agarose suspension (50 µl beads per 1 ml of sample) to two of these aliquots Gently rock both aliquots for 1 3 h a 4 C

Pellet beads by gravity sedimentation or alternatively by centrifugation a 12 000 g for 20 s in a microfuge Transfer supernatants to fresh tubes

Note In some cases one or two additional absorption steps may be necessar to completely remove proteins that may bind to protein A agarose (e g ant bodies contained in the culture medium and bound to the cell surface)

To one aliquot add antibody directed against the target protein To the othe add the same volume of a control antibody (preimmune serum or an irrelevar monoclonal antibody) *Gently* rock both aliquots for 1 h at 4°C

Note The antibody should be titrated in pilot experiments in which increasin quantities of antibody are used to precipitate a fixed amount of antiger Usually between 0 5 µl and 5 µl of polyclonal antiserum 5 µl and 100 µl c hybridoma tissue culture medium 0 1 and 1 0 µl of ascitic fluid or 1 µg an 5 µg of purified monoclonal or polyclonal antibodies are sufficient for comple immunoprecipitation

Add protein A agarose to the mixture and incubate for at least 3 h (or ove night) at 4°C on a rocking platform 50 µl of the homogeneous protein A-ag rose suspension are usually sufficient the amount of protein A agarose shou at least result in a visible pellet after sedimentation

Note 1 ml of protein A agarose binds about 20 mg of IgG equivalent to abc 2 ml of serum 200 ml of supernatant from cultured hybridoma cells and 1 ascites fluid

Protein A binds the Fc parts of most mammalian IgGs with high affinity by non immune mechanism (4) The binding capacity depends on the anim species and the IgG subclass (see table 4) Protein A does not bind oth immunoglobulins like IgM IgA etc and bovine serum albumin In the case th the primary antibody in use is not compatible with the protein A agarose p vided with this kit switch to protein G agarose or immobilized anti Ig antibc (4) Cut the pipette tip for pipetting the bead suspension

Tab 4 Binding specificities of protein A

Species/subclass		Affinity for protein A	Species/ subclass	Affinity for protein A
Human	IgG_1	++++	Horse	++
	IgG_2	++++	Cow	++
	IgG_3		Pig	+++
	IgG_4	++++	Sheep	+/
Rat	IgG_1		Goat	
	IgG_{2a}		Rabbit	++++
	IgG_{2b}		Chicken	
	IgG_{2c}	+	Hamster	+
Mouse	IgG_1	+	Guinea pig	++++
	IgG_{2a}	++++		
	IgG_{2b}	+++		
	IgG_3	++		

Collect complexes by gravity sedimentation or alternatively by centrifugation at 12 000 g for 20 s in a microfuge
Remove supernatant carefully
Add 1 ml of wash buffer 1 resuspend the beads and incubate for 20 min at 4 C on a rocking platform
Repeat the preceding three steps once
Collect complexes as described add 1 ml of wash buffer 2 to the pellet resuspend incubate for 20 min at 4°C on a rocking platform pellet the beads again and remove the supernatant
Note Different buffers are commonly used to wash protein A antigen antibody complexes The tighter the binding between antibody and antigen the more stringent the washing buffer conditions can be The washing buffers described are used if low stringency conditions are appropriate If higher stringency is needed increase the salt concentration and ionic strength by using 0 5 M NaCl or 0 5 M LiCl for the first wash Additionally SDS (final concentration 0 1% (w/v)) may be applied in the second wash
Repeat previous step
Add 1 ml of wash buffer 3 to the pellet resuspend incubate for 20 min at 4°C on a rocking platform pellet the beads again and remove the supernatant
Remove the last traces of the final wash from the agarose pellet and from the wall and lid of the microfuge tube
Add 25 75 µl of gel loading buffer
Denature proteins by heating to 100°C for 3 min Remove protein A agarose by centrifugation at 12 000 g for 20 s at room temperature in a microfuge Transfer supernatant to a fresh tube
Analyze an aliquot by SDS polyacrylamide gel electrophoresis

3 6 Gel electrophoresis

The biotin labeled and immunoprecipitated proteins can be separated by any type of one and two dimensional electrophoresis system providing sufficient protein resolution (5 6 7)

For a detailed protocol for SDS polyacrylamide gel electrophoresis or two dimensional electrophoresis please refer to one of the standard textbooks or to manuals from manufacturers of electrophoresis equipments

3 7 Western blotting

After electrophoresis blot the gel onto a nitrocellulose or PVDF membrane using a standard Western blot protocol (8 9)

Note To avoid damage or contamination of the membrane always wear gloves when handling

- Hydrophobic membranes such as PVDF must be pre wetted prior to protein transfer moisten the membrane with methanol for a few seconds then soak in transfer buffer for at least 5 min Nitrocellulose should be briefly soaked in water and then for at least 5 min in transfer buffer
- It is essential to thoroughly equilibrate the gel in transfer buffer for 5 10 min prior to transfer
- Blot according to standard protocols
- The blot can be stored dry for several months in a refrigerator if necessary but must be re wetted before starting immunodetection PVDF membranes should be re wetted in methanol or in 5% Tween 20 (v/v)

3 8 Visualization of biotin labeled proteins on membranes

There are a number of reagents and kits available which can be used for the visualization of biotin labeled proteins on membranes (table 5)

Tab 5 Suitable products for the detection of biotin labeled proteins on Western blots

Product	Pack size	Cat No
BM Chemiluminescence Western Blotting Kit (Biotin/Streptavidin)	For 1000 cm^2 of membrane	1559 460
BM Chemiluminescence Western Blotting Reagents	For 1000 cm^2 of membrane For 4000 cm^2 of membrane	1500 708 1500 694
Streptavidin POD conjugate	500 U	1089 153
Streptavidin AP conjugate	1000 U	1089 161
BM Teton POD substrate precipitating	200 mg	1544 845
BM blue POD substrate precipitating	100 ml	1442 066
BM purple AP substrate precipitating	100 ml	1442 074
4 Nitro blue tetrazolium chloride (NBT)	3 ml (300 mg)	1383 213
5 Bromo 4 chloro 3 indolyl phosphate (X phosphate)	3 ml (150 mg)	1383 221
Blocking reagent for nucleic acid hybridization and detection	50 g	1096 176

In principle the membrane is first incubated in a suitable blocking reagent to prevent non specific binding of conjugate or substrate The biotin labeled proteins are then reacted with a streptavidin enzyme conjugate Excess conjugate is removed by washing and bands are visualized by a suitable substrate