P-GLYCOPROTEIN EXPRESSION IN CANINE LYMPHOMAS

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To Mum and Dad

and Dennis

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DECLARATION

The work in this thesis was performed by me, with the following exception. The S-100 staining of lymphomatous samples discussd in chapter 5 was performed by members of the Department of Pathology at the Royal Infirmary under the supervision of Dr. Ann-Marie McNicol.

Jane Stewart
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ABBREVIATIONS

ABC	avidin-biotin immunohistochemical kit
ALL	acute lymphoblastic leukaemia
AP	alkaline phosphatase
APC	antigen presenting cell
ARG	antigen receptor gene
ATL	adult T-cell leukaemia/lymphoma
bp	base pair
cDNA	complementary deoxyribonucleic acid
CLL	chronic lymphocytic leukaemia
CNS	central nervous system
cpm	counts per minute
CSU	Colorado State University
CUVC	Cambridge University Veterinary College
D	dalton
DNA	deoxyribonuceic acid
DNase	deoxyribonuclease
EDTA	ethylenediamine tetra-acetic acid
FDC	follicular dendritic cell
FeLV	feline Leukaemia virus
g.	gramme / gravitational units
GI	gastro-intestinal
GUVC	Glasgow University Veterinary College
HIV	human immunodeficiency virus
hr	hour
HTLV-1	human T- cell lymphotropic virus type 1
IHC	immunohistochemistry/immunohistochemical
IDC	interdigitating cell
kb	kilobase
kD	kilo-Dalton
λ	bacteriophage lambda
MDR	multidrug resistance
MHC	major histocompatibility complex
mins	minutes
ml	millilitre
MLSA	multicentric lymphosarcoma
mRNA	messenger ribonucleic acid
NCSU	North Carolina State University
NHL	non-Hodgkins lymphoma
OD	Optical density
pcr	polymerase chain reaction

P-gp	P-glycoprotein
RNA	ribonucleic acids
RNase	ribonuclease
RFLP	restriction fragment length polymorphism
RPA	RNAse protection assay
S.E.	standard error
SDS	sodium dodecyl sulphate
TCR	T cell receptor
TTF	time to treatment failure
uCi	micro-curie
uM	micromolar
UV	ultraviolet
v/v	volume/volume
w/v	weight/volume

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ABSTRACT

P-glycoprotein (P-gp) is a membrane glycoprotein which can act as an efflux pump for certain chemotherapeutic drugs and thereby confers resistance to these drugs on the host cell. The P-gp isoform encoded by the human *mdr1* gene has been implicated as a factor in clinical resistance in human tumours. To investigate the role of P-gp in acquired clinical resistance, dogs with malignant lymphoma which were referred to Glasgow University Veterinary College for treatment, were given an anthracycline based protocol until first relapse. Tumour samples were obtained from these dogs at diagnosis and at time of disease progression. P-gp expression in these samples was assessed by immunohistochemistry using a monoclonal antibody which detects all known P-gp isoforms and by dot-blot analysis using a human *mdr1* specific probe.

Multiple members of the *mdr* gene family exist in every species studied and only the *mdr1* isoform(s) is implicated in drug resistance. Investigations of the canine *mdr* gene family revealed that the dog has four potential members of this family. The homologue of the human *mdr1* gene was expressed in normal canine liver, kidney and adrenal and at a lower level in the caudal gastro-intestinal tract. Skeletal and cardiac muscle tissue has strong P-gp expression but this did not appear to be of the *mdr1* homologue isoform.

Immunohistochemistry revealed that normal, reactive and lymphomatous nodes contain a P-gp positive dendritic cell population. These P-gp positive cells are morphologically identical to S-100 positive cells and so may represent an antigen presenting cell population. The presence of these cells could confound accurate interpretation of the dot-blot analysis and so imunohistochemistry was used as the main criterion to determine P-gp positivity in the malignant cell population.

Within tumour cells, P-gp expression at time of diagnosis was a rare occurrence (less than 5%) but was significantly more common in drug resistant tumours ($p=0.0113$). However, even within the drug refractory tumours, P-gp expression was not ubiquitous; only 25% of tumours were P-gp positive and therefore P-gp does not appear to be a major cause of treatment failure in canine lymphoma. Statistical analysis of the results revealed that P-gp was associated with advanced stage disease. Advanced stage at presentation seemed a more important predictor of subsequent P-gp expression than the amount or type of drugs received prior to relapse. Treatment with corticosteroids prior to chemotherapy had a profound effect on the ability to achieve remission but again this was not related to the presence of P-gp.

Canine T cell lymphomas are reported to have a poorer clinical performance than B cell tumours. T cell tumours were positively identified in this group of dogs by genotyping using a feline constant region probe of the T cell receptor β chain gene. 10/45 tumours were identified as having β chain rearrangements by this technique. The T cell genotype was a poor prognostic indicator by univariate analysis but none of the T cell tumours expressed P-gp, either before treatment or at relapse, indicating that P-gp is not a major cause of treatment failure in T cell lymphomas.

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1.1 NON-HODGKINS LYMPHOMAS

1.1.1 CLINICAL DRUG RESISTANCE IN NON-HODGKINS LYMPHOMA

Chemotherapy can be an extremely effective cancer treatment. Cures, resulting from intensive chemotherapy, are now obtainable in Hodgkin's disease, certain forms of non-Hodgkin's lymphoma, acute lymphoblastic leukaemia and testicular cancer (Chabner *et al*, 1984). This list of neoplastic diseases represent a uniquely chemosensitive group of cancers. Unfortunately though, when a relapse does occur in these cancers, it is usually associated with the development of clinical drug resistance and further response to chemotherapy is limited.

Clinical resistance to chemotherapeutic agents is complex; the drugs which are rendered apparently ineffective at relapse can be of divergent structure and function. Tumours can acquire resistance to drugs, which are usually effective in first line treatment, even without prior exposure (Armitage *et al*, 1990). The breadth and diversity of acquired resistance in these once chemosensitive tumours then resembles the inherent resistance of tumour types such as colonic and hepatic carcinomas and melanomas.

In the first part of this introductory chapter the heterogeneous group of tumours collectively known as non-Hodgkins lymphoma (NHL) will be described. This group contains tumours which are chemocurable yet at the other extreme contains indolent tumours whose natural course is undeterred by chemotherapy. The majority of NHL show a pattern of initial response to chemotherapy followed by relapses which are increasingly unresponsive to chemotherapy. This epitomises the problem of acquired drug resistance in clinical oncology. Socioeconomically, NHL is an important tumour group; it is the tenth most common cancer-related death in males in the U.K. (CRC Factsheet, 1988). The biological factors which contribute to the heterogeneity of NHL will be examined including the histologic and cytogenetic events which occur during the progression of certain forms of this disease.

In the second section, canine lymphomas are introduced; the clinical and biological similarities to NHL are highlighted. Paramount among these similarities is the chemosensitivity of canine lymphomas which then gives way to profoundly drug-resistant disease, usually within one year. Like NHL, canine lymphoma is a numerically important disease with an annual incidence of between 24 and 30.5 cases per 100,000 dogs (Dorn *et al* 1967; Schneider, 1983). This is higher than the human incidence of NHL which is about 9 per 100,000 in the U.K. (Cancer Research Campaign, Factsheet 1988).

In the final section, the chemotherapeutic drug-resistance mechanism mediated by P-glycoprotein (P-gp) is discussed. Due to widespread research effort in the past five years, this represents one of the best defined resistance mechanisms

applicable to clinical oncology. The activity of P-gp as a resistance mechanism can be successfully ameliorated *in vitro* using non-cytotoxic drugs (reviewed in Bellamy *et al,* 1990) and inevitably this has generated great excitement that it may be possible to overcome clinical multiple drug resistance using P-gp modulating drugs in conjunction with normal chemotherapy (Dalton *et al,* 1989; Salmon *et al,* 1991). This has fuelled research to identify the tumour groups which are likely to be amenable to modulation. Simultaneously, efforts have been directed towards identifying the events which lead to P-gp expression and whether this expression is avoidable by alterations in the existing chemotherapeutic protocols.

1.1.2 HISTOPATHOLOGICAL CLASSIFICATION OF NHL

NHL is a heterogeneous group of tumours derived from cells of lymphocytic origin. The natural course of NHL varies from a slow-growing indolent form, to a fulminating leukaemic type illness. The majority of tumours fall in between these two extremes and show an unremitting progression if left untreated. Histopathology is the traditional method of defining these different groups.

Table 1.1 Working Formulation Histopathological Grades

Low Grade	small lymphocytic follicular small cleaved follicular mixed
Intermediate Grade	follicular large cell diffuse small cleaved cell diffuse mixed diffuse large cell
High Grade (Burkitts)	diffuse small non-cleaved cell
	diffuse immunoblastic[1] lymphoblastic adult T cell leukaemia/lymphoma[2]

1. The National Cancer Institute (NCI) has adapted the WF slightly and considers immunoblastic tumours to be intermediate grade based on their clinical behaviour.
2. Adult T cell leukaemia/lymphoma associated with HTLV-1 infection was not included in the original classification scheme but is included in the NCI clinical schema for malignant lymphomas (DeVita *et al.* 1989)

The original classification system devised by Rappaport (Rappaport *et al.* 1956) differentiated primarily according to the pattern of growth (follicular versus diffuse) rather than on cellular characteristics. It became apparent that with the Rappaport scheme, there was still considerable heterogeneity among some of the

higher grades of tumour (DeVita *et al. 1982*; Levine *et al, 1985a*). Other classification schemes were devised, including the Lukes-Collins and Kiel systems which placed greater emphasis on the cellular morphology (Lukes *et al.* 1974; Lennert *et al.* 1975). Eventually in 1982 these schemes were amalgamated into the "Working Formulation" (The non-Hodgkins lymphoma pathologic classification system, 1982), which is illustrated in table 1.1.

1.1.3 IMMUNOPHENOTYPING NHL

(i) Basic principles

Traditional methods of immunophenotyping lymphoid malignancies were based on the detection of surface and /or cytoplasmic Ig on B cells and the ability of T cells to rosette sheep erythrocytes. Detection of heavy and light chain Ig remains one of the most specific determinants of B cell lineage but T cell rosetting has been superseded by the availability of monoclonals against lineage specific antigens.

There has been a rapid increase in the number of monoclonals against lymphoid markers in recent years. Knapp *et al* (1989), when commenting on the remarkable progress in this area, also noted that as surface membrane investigations proceed, the borders between different cell types (including myeloid cells as well as lymphoid cells) are being fudged rather than strengthened; many of the cluster determinant (CD) groups are no longer considered to be absolutely lineage specific. With this proviso in mind, clinical immunophenotyping depends on the use of panels of monoclonal antibodies rather than one or two single reagents.

It must be appreciated that because NHL is, overall, composed of relatively mature cell types, the role of CD10 (CALLA) and many of the other surface antigens found in very early lymphoid cells (e.g. CD7) in distinguishing prognostic groups is not so critical as it is in ALL (Foon & Todd, 1986). These complexities plus the advantages and disadvantages of individual monoclonals have been discussed in detail by Norton & Isaacson, (1989b) and Foon & Todd, (1986). The range of CD groups which at present would appear critical and are most commonly used for lineage determination in NHL are shown in table 1.2

Table 1.2 Immunophenotyping NHL

B cell lineage	T cell lineage
Ig heavy and light chain (*surface or cytoplasmic*) CD19, CD20, CD40, CD72	CD2, CD3, CD7, CD4, CD8

(ii) Limitations to immunophenotypic and histopathologic classification of NHL

Immunohistochemistry and detailed histopathology are labour intensive techniques which require a great deal of expertise. It is difficult to provide quality control and reproducibility between institutes and even individuals. When the expert pathologists who developed the Working Formulation were tested for the consistencies of their diagnosis, the rate of reproducibility varied from 53-93% (NCI non-Hodgkins classification project writing committee).

An editorial, commenting on phenotyping of haematologic malignancies, (Hanson, 1991) maintains that the problem of "background" in immunohistochemistry is frequently ignored or minimized in the literature. Low level expression of antigens, at the limit of the methodology, is another important limiting factor (Warnke *et al.*, 1985). The loss of antigen expression, either through tissue handling and fixation artefacts, or through genuine downregulation of antigen expression can seriously hamper phenotyping. Most of the lymphocyte surface antigen monoclonals work best on frozen sections rather than paraffin embedded blocks (Norton & Isaacson, 1989a). This is much less convenient for routine work and also results in much poorer cellular morphology (Schlaifer *et al.* 1990a and 1990b).

It would therefore appear that the problems of reproducibility and subjectivity inherent in histopathology and immunohistochemistry combine to limit their uses in adequately defining prognostic groups in NHL.

1.1.4 IMMUNOGENOTYPING NHL

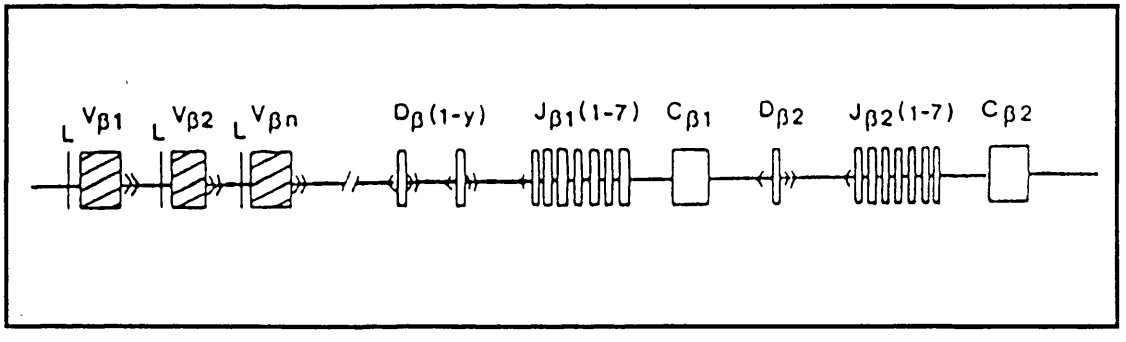
(i) Use of antigen receptor gene rearrangements as a marker for clonal proliferations

Although an individual neoplasm may be polymorphous as regards cell size & phenotype, it is increasingly evident that most neoplasias are composed of almost genetically identical progeny derived from a common clonal stem cell (Farber and Cameron, 1980; Nowell, 1990). Abnormalities within the genome of a neoplastic clonal population can be manifest as gross morphological changes in the chromosome, such as translocations (Croce *et al* 1984), or subtle changes such as point mutations (Balmain and Brown, 1988). These genetic lesions can be used as unique tumour markers (Stetler-Stevenson *et al.* 1988). Lymphoid tumours carry an additional set of unique tumour markers which are the rearranged antigen receptor genes (ARG) of B and T lymphocytes.

Antigen receptors are expressed on B cells as immunoglobulin (Ig) and on T cells as T cell receptors (TCR). The genes encoding these structures are arranged as discontinuous DNA segments organized into variable, diversity and joining regions.

Within each region, multiple similar but non-identical segments are found. As a mandatory step towards mature lymphocyte differentiation, these ARG undergo somatic recombination whereby the rearrangement and joining of one of each V-D-J segments leads to the assembly of an intact antigen receptor. The organisation of the human TCR β chain locus is shown in figure 1.1. The β chain contains two constant regions which are approximately 10kb apart; the 5' region is designated C_{B1} and the 3' region C_{B2} . The coding sequences of the two constant regions are highly homologous having only a four amino acid difference between them. However the introns and the 3' non-coding region of each constant region are not conserved (Toyonaga *et al.* 1985). The detailed arrangement of these genes and the manner in which recombination occur is discussed elsewhere (Tonegawa, 1983; Hedrick *et al,* 1984; Honjo, 1985; Toyonaga and Mak, 1987).

Figure 1.1 T cell receptor β locus



V, variable; D, diversity; J, joining; C, constant region

During lymphocyte ontogeny an orderly pattern of gene rearrangement occurs. Within each gene, and where diversity segments exist, D-J joining precedes V-D-J joining. Incomplete V-D-J joining can lead to the production of truncated mRNA. Aberrant joining can also occur which produces a non-functional gene. In this situation it is common to find functional rearrangement of the other allele. Within pre-B cells, Ig heavy chain gene rearrangement precedes κ light chain. If both κ genes are aberrant rearranged or deleted, then λ light chain rearrangement commences (Altenburger *et al.*, 1980; Korsmeyer *et al.* 1981; Heiter *et al.* 1981). A similar hierarchy of gene rearrangement occurs in T cells. Analysis of transcripts of murine foetal thymic cells revealed that TCR γ gene rearranges first followed by the β gene and lastly by the α gene activation (Raulet *et al.* 1985 and Samelson *et al. 1985*). The δ gene has an unusual location between the variable and joining segments of the α locus, and it appears to rearrange before the β locus. It is deleted if the α locus undergoes rearrangement (Chien *et al* 1987).

It was Korsmeyer & co-workers (1981) who first proposed that the analysis of patterns of ARG rearrangements could be a powerful and useful tool to assess clonality. A monoclonal lymphoid population can be distinguished from a polyclonal population because unique restriction enzyme fragment patterns are generated by the recombinatorial process which are detectable on Southern blots. Polyclonal benign proliferations are not seen using conventional Southern blot hybridisation because the many rearrangements result in a wide range of fragment sizes, none of which is represented in sufficient quantity to be detectable.

The exception to this rule are γ chain rearrangements. Rearrangements are detectable even among polyclonal T cells (Uppenkamp *et al.* 1987) because there is a limited number of V segments in the γ gene and these give rise to a limited number of combinatorial joinings (Kranz *et al.* 1985). Therefore caution has to be exercised when interpreting the presence of γ chain rearrangements in lymphoproliferative disease.

Heritable restriction fragment length polymorphisms (RFLP) exist in the human population which can produce patterns that can be mistaken for rearrangements (Lefranc, 1985; Robinson *et al.* 1985). This pitfall can be avoided by analysing tumour samples in parallel with non-lymphoid tissue from the patient (e.g. granulocytes from the buffy coat of a blood sample). Alternatively, multiple restriction enzymes can be used which will verify the rearrangement encompasses a region of DNA and is not simply a point mutation giving rise to a RFLP.

All mature B cells will have rearranged Ig genes and in humans the majority of monoclonal B cells have rearranged the κ light chain. Only about 30% of normal and neoplastic human B cells and 10% of mouse B cells express the λ chain (Yancopoulos *et al.* 1986). Lambda expressing cells have generally rearranged (or deleted) their κ alleles before productively rearranging their λ gene. For practical purposes this makes a probe of the J κ region the most specific probe for detecting rearrangements in Ig expressing B cell neoplasms.

The vast majority of mature T cells in man express α/β receptors (Kronenburg *et al.* 1986) and less than 5% of peripheral blood T cells are γ/δ (Triebel *et al.* 1989). Malignant T cells seem to reflect the normal situation in that most mature T cell neoplasms have α/β receptors. The TCR α gene is an extremely large gene; the V segments are spread over 750,000bp and the numerous J segments (greater than 50) extend over another 85kb (Griesser *et al*, 1988). This enormous size makes it a difficult gene to analyze using conventional restriction fragment separation techniques. The difficulties of working with α probes plus the hierarchical rearrangement of β before α means the β chain gene probes are more widely used for routine immunogenotyping.

(ii) ARG Rearrangements in lymphoid neoplasia

Virtually all B cell neoplasms have rearrangements of the Ig_H regardless of the stage of differentiation or Ig gene expression (table 1.3). In this way common acute lymphoblastic leukaemia has been shown to be predominantly a precursor B cell neoplasm because most show Ig_H rearrangement and about 50% also have κ gene rearrangement. Most of these ALL's have yet to express fully assembled protein (Korsmeyer *et al,* 1981; Korsmeyer *et al,* 1983). More mature B cell neoplasms such as CLL, follicular lymphoma, large cell lymphoma, and Burkitts lymphoma display rearrangements of both heavy and light chain genes and are therefore easy to assign to a B cell lineage based on genotyping.

Within phenotyped T cell tumours, immunogenotyping has been equally confirmatory of cell lineage in both immature malignancies such as ALL and in mature malignancies like NHL and CLL (table 1.3). Within T-NHL, β chain rearrangement is almost a ubiquitous event. The small percentage which have retained the germline configuration of the β chain tend to be those NHL which are classed as "lymphoblastic" i.e. thymocytic phenotypes with a propensity for intrathymic growth (deVillartey *et al.* 1989; Williams *et al. 1987*).

(iii) Simultaneous Ig and TCR gene rearrangements

The use of Ig and TCR gene probes has revealed complex gene rearrangements in some lymphoid malignancies involving both TCR and Ig genes. The possibility of two different clonal populations of cells co-existing in these tumours has been excluded since immunocytochemically these tumours are of a single phenotype. In addition the equal intensity of rearranged Ig and TCR bands on Southern hybridisations would support the hypothesis that the rearrangement has occurred in cell populations of equal size.

Analysis of the literature, (table 1.3) reveals that the frequency of bigenotype is greater in B cells than in T cells. In up to 11% of mature B cell neoplasias, and 24% of immature B cell tumours (ALL) the TCR genes may be simultaneously rearranged. Hara *et al,* (1987) examined 29 samples of precursor B cell ALL for rearrangements of all four TCR genes and found TCR δ gene rearrangements in 20 cases (69%), TCR α rearrangements in 59% and TCR γ rearrangements in 52% of the cases. TCR β was the least likely to be simultaneously rearranged with the Ig genes at 31%. The high frequency of γ/δ rearrangements in pre-B cell tumours is not unexpected given that γ/δ rearrangement occurs early in ontogeny. In mature T cell malignancies, the incidence of bigenotype is much lower at only 1.5% for T-NHL.

Table 1.3 T cell receptor β chain and Immunoglobulin heavy chain rearrangements in lymphoid malignancies.

Tumour	No of cases	TCR B	Ig_H	dual genotype	References
B ALL	88	20	83	24%	3,8,10, 14,16,17
B NHL	101	7	101	6.9%	10,14,18, 20,21,22
B CLL	45	5	45	11.1%	1,10,13
T ALL	126	125	18/113	16%	1 - 14
T NHL	70	65	1/63	1.5%	2,4,9,10,14 15,18,19,20
T CLL	33	33	0/25	0%	4,8,9,10 11,15.

References for table 1.3

1. Aisenberg et al. (1987)
2. Bertness et al. (1985)
3. Davey et al. (1986)
4. Flug et al. (1985)
5. Hara et al. (1987)
6. Kitchingham et al. (1985)
7. Minden & Mak et al. (1986)
8. Minden et al. (1985)
9. O'Conner et al. (1985)
10. Pelicci et al. (1985)
11. Rabbits et al. (1985)
12. Tawa et al. (1985)
13. Waldmann et al. (1985)
14. Williams et al. (1987)
15. Isaacson et al. (1985)
16. Asuo et al. (1987)
17. Felix et al. (1987)
18. Griesser et al. (1986a)
19. Matsuoka et al. (1987)
20. Liang et al. (1990)
21. Tkachuk et al. (1988)
22. Griesser et al. (1986b)

Given that bigenotypic tumours are not uncommon, especially among immature and/or B cell tumours, is there any way of differentiating among these bigenotypic tumours? Where it has been examined there are quantitative differences in the patterns of ARG gene rearrangement such that only one Ig_H allele is found rearranged in phenotypic T cell neoplasia and the light chain genes are still in the germline configuration. Conversely, there is a tendency for only one TCR allele to rearrange in B cell disorders as opposed to both alleles as seen in T cell neoplasias. It is very rare to find a complete V-D-J recombination of Ig_H in T cell neoplasias or of TCR β chain in B cell disorders (Tawa *et al* 1987: Kitchingham *et al.* 1985; Williams *et al.* 1987).

It is generally accepted that the enzymatic processes underlying ARG rearrangements is very similar between T and B cells. In 1986, Yancopoulos and co-workers introduced T cell variable region segments into pre-B cells and showed that

the T cell receptor gene fragment rearrangements could proceed in the B cell environment. It has been proposed that the accessibility of the Ig and TCR gene loci to the common recombinase enzyme complex (encoded by the RAG genes) could allow dual rearrangement (Yancopoulos *et al.* 1986). Davey *et al.* (1986) speculate that the high incidence of dual genotype in precursor B cell leukaemias may be due to the lack of signals, which are present in more mature cells, that normally terminate gene rearrangements. The signal to switch off recombinase activity seems to require the functional activation through the ARG with subsequent release of second messengers rather than merely the presence of mature ARG. Recombinase activity associated with the RAG1 and RAG2 gene products is decreased by agents which mimic the second messenger effects of AGR cross-linking. Phorbol esters (e.g.. phorbol myristate acetate) plus calcium ionophores can switch off RAG gene expression in both T cells and B cells. (Menetski *et al,* 1990; Turka *et al,* 1991). Because a functional ARG appears to be a prerequisite for stopping further gene rearrangement, the corollary of this is that gene rearrangement, perhaps of both Ig and TCR genes, may continue even after a translocation event or defective rearrangement has occurred on one allele.

The underlying mechanism which leads to bigenotype may take many years to elucidate because it is probably one of the key areas where transformation events are affecting normal cellular differentiation. Several practical points can be suggested to minimize the misinterpretation of immunogenotyping in immature phenotypic tumours.

Wherever possible digests and probes should be used which permit the assessment of the number of alleles rearranged and the extent of that rearrangement (i.e. D-J rearrangements versus complete V-D-J). This will reveal the quantitative differences discussed previously. Light chain probes should be used in conjunction with IgH probes to determine the likelihood of complete Ig assembly taking place. The presence of complete or truncated mRNA for the TCR genes will also help to assess the completeness of gene rearrangement. In this way it can be determined whether the TCR genes or the Ig genes are closer to producing a mature receptor molecule.

1.1.5 KARYOTYPIC ABNORMALITIES AND TRANSLOCATIONS IN NHL

Recurring cytogenetic abnormalities have been identified in NHL and significant correlations have been made between these abnormalities and NHL morphology, immunophenotype and in some cases, prognosis. Despite the large number of NHL cases which have been karyotyped, the conclusions are not always consistent. This is probably due to a combination of the complexity of these karyotypes plus the non-uniformity of morphologic classifications and treatment schedules between institutes.

A comprehensive account of the translocations found in NHL is not attempted; reviews of these translocations and the mechanisms leading to them are available elsewhere (Levine and Bloomfield, 1990; Tycko and Sklar, 1990). Instead attention is focussed on a few abnormalities which have established association with certain morphologic and phenotypic NHL groups. Some of the recurring cytogenetic abnormalities within histologic subtypes are listed in table 1.4 which is adapted from Levine and Bloomfield (1990).

Correlations of chromosomal abnormalities can be made with immunophenotype. The t(14;18) and t(8;14), which involve Ig gene translocations, occur in B-cell NHL. Analogous to this, T cell NHL chromosomal abnormalities can affect the T cell receptor genes. The α locus at 14q11-13 is disrupted in tumours with morphologies usually associated with T cell phenotype i.e. diffuse mixed, immunoblastic and diffuse intermediate sized with nuclear polymorphism (Fifth International Workshop, 1987; Maseki *et al* 1987). However, the frequency of translocations involving TCR genes does not seem to be as frequent as Ig translocations are in B cell lymphomas (Levine *et al.* 1986; Levine *et al,* 1990; Maseki *et al,* 1987). In fact, both arms of chromosome 1, 2p and 6q are equally or in some studies more commonly involved with T cell lymphomas than the TCR gene sites (Levine *et al,* 1986; Berger *et al* , 1988; Fifth International Workshop, 1987; Maseki *et al,* 1987).

Molecular dissection of some of the translocations has already shed light on the oncogenic mechanisms contributing to lymphomagenesis. The activation of *c-myc* by ARG (TCR and Ig) gene translocations in some of the high grade lymphomas has highlighted the role of the *c-myc* family in malignant transformation. This subject has been reviewed recently by DePinho *et al,* (1991) and Klein (1991).

Table 1.4 Recurrent cytogenetic abnormalities within NHL histologic subgroups.

Lymphoma type*	Associated abnormalities
Follicular small cleaved cell	t(14;18), del(6q)
Follicular, mixed small and large cell	+8, t(14;18), del(2q), +3/3q, 10q23-25
Follicular, large cell	+7, t(14;18), 17q21-25
Diffuse, small cleaved cell	del(8p), del(20q)
Diffuse, mixed small and large cell	+3, +5, 11p
Diffuse large cell	+4, +7, -8, -13, +21, +X, 1q, 2q, 3q21, del(6q), 4q, 7q, 9q, 14q, 18q
Immunoblastic	+5, +18, +X, del(3p), del(5q), del(6q), 5p, 5q, 13q, 16, 19p
Small noncleaved cell	t(14;18)

* *Lymphoma type is classed according to the Working Formulation.*

Significant insights into the functional role of the t(14;18)(q32;q21) translocation has emerged in recent years. In this translocation, the putative oncogene *bcl-2* is fused to the joining region segment of the IgH gene. There is accumulating evidence that the constitutive expression of *bcl-2* protects B-cells from apoptotic death (Nunez *et al,* 1990; Hockenberry, *et al,* 1990). The ability of the *bcl-2* gene to confer a survival advantage to B cells is thought to be an initiating event (Rabbitts, 1991) which allows time for other mutations or physiological events to contribute to the neoplastic process, as shown in a transgenic mouse model (McDonell and Korsmeyer, 1991).

One of the problems in karyotypic analysis of NHL and the interpretation of the results is the separation of abnormalities into those present at diagnosis versus those acquired at relapse. The evolutionary cytogenetic changes are, like the primary abnormalities, non-randomly distributed within histological classes. Among these sequential changes are deletions of all or part of 17p and acquired changes in chromosomes 1 and 2 (Levine *et al,* 1990; Sanger *et al,* 1987; Fukuhara *et al,* 1983). Duplication of part of 1q occurs significantly more often in tumours with t(8;14) than with t(14;18) (Fifth International Workshop, 1987; Fukuhara *et al,* 1983). The opposite frequency is found with acquired trisomy 7 or duplication of 7q. This is more commonly in tumours with preexistant t(14;18) than t(4;8) tumours. Trisomy 7 is also more commonly associated with diffuse rather than follicular morphology

(Bloomfield *et al,* 1983). Armitage *et al,* (1988) examined a total of 69 tumours with t(14;18) and found trisomy 7 in 52% of the tumours with a diffuse histology but only 15% of the tumours with follicular histology.

Some acquired abnormalities are not confined to a particular histologic type of tumour. The del(6q) abnormality is significantly associated with progression of follicular forms, small cleaved cell, diffuse large cell and immunoblastic lymphomas (Bloomfield *et al,* 1983; Levine and Bloomfield, 1990). Del(6q) has also been described in other tumour types and may be a prevalent abnormality associated with general tumour progression (Vogelstein *et al,* 1989; Fountain *et al,* 1990).

From this brief overview, it is apparent that the morphologic heterogeneity of NHL is reflected in the heterogeneity of karyotypic abnormalities. The underlying difference in oncogenic events between, for instance t(14,18) follicular tumours versus t(8;14) small non-cleaved tumours helps to explain the difference in the natural history of these two diseases (Klein *et al,* 1991). It is also evident that NHL not only have different cytogenetic origins, they progressively acquire different genetic alterations.

1.1.6 PROGNOSTIC FACTORS IN NHL

The Working Formulation histopathologic classification scheme identified three major prognostic groups based on survival; the terms "low-grade", "intermediate grade" and "high grade" were used to define patients with favourable, intermediate and unfavourable prognosis. This classification scheme is based on the natural history of the tumour groups. However, with the advent of effective combination chemotherapy, the survival of complete responders in the intermediate and high grade NHL groups in the long term (over 15 years) can be greater than for low-grade tumours (Rosenberg, 1985).

Once NHL is classified into low-, intermediate- or high-grade, the influence of histopathologic sub-type on prognosis is often minimal compared to other prognostic factors. Hayward *et al* (1991) analysed the survival data on over nine-hundred patients with intermediate or high-grade lymphomas and found that in this unselected group, the histologic sub-type did not influence survival. Leonard *et al,* (1983) using the Kiel classification system to divide 199 cases of NHL into low or high grade also found that the sub-type within the two grades was not a prognostic indicator.

The influence of immunophenotype on prognosis is still an area of debate. Some analyses did not find cell lineage to influence outcome (Horning *et al,* 1984 and 1986; Shimoyama *et al,* 1988) but others have found that T cell phenotype does have a poorer prognosis (Greer *et al,* 1984; Coiffer *et al,*1988; Armitage *et al,* 1989).

In the study reported by Armitage *et al.* (1989), the influence of phenotype was profound. In a total of 110 uniformly treated patients, stage IV B-cell patients had a higher complete response rate than stage IV T-cell tumours (67% versus 0%) as well as a four year survival of 44% compared to 0%. So although it is not universally detected, T cell phenotype may adversely affect prognosis.

Hayward *et al* (1991) defined a poor prognostic group using multivariate analysis which showed the following factors to be important (in declining order of power): advanced age (over 70 years), worsening performance status, central nervous system/liver involvement, abnormal white count, systemic symptoms ("B" symptoms) and advanced clinical stage. The importance of age as a prognostic indicators over some of the other factors is in agreement with Leonard *et al* (1983), Danieu *et al,* (1986), Horning *et al,* (1984) and Al-Katib *et al,* (1984).

Poor physiological reserve, as indicated by poor performance status and "B" symptoms, have repeatedly been associated with poor prognosis in NHL (Leonard *et al,* 1983: Horning *et al,* 1984; Al-Katib *et al,* 1983). The tendency for randomized trials of new chemotherapy regimes to use younger, fitter patients, either through deliberate exclusion or through the inevitable bias of the referral population at a tertiary centre, can lead to serious difficulty in the interpretation of the results of these trials (Armitage and Cheson, 1988).

The importance of clinical stage, often based on the Ann Arbor classification, (Carbone *et al,* 1971) as a prognostic tool is equivocal. Hayward *et al,* (1991) did identify stage III and IV as having a poorer survival than stage I and II but Leonard *et al,* (1983) found that apart from stage I having a better prognosis than more advanced stage, the difference in survival between the more advanced stages were minimal. Danieu *et al,* (1986), Al-katib *et al,*(1983), Shimoyama *et al,* (1988) and Armitage *et al* (1982) did not find stage a significant prognostic factor in the intermediate and high grade tumours in their studies. This inability for clinical stage to accurately predict clinical outcome is not unexpected. In the lucid article "Staging of lymphomas:Practical thoughts on impractical practices" written by Raubitscheck *et al, (*1990), it was emphasized that the anatomic definition of each stage is fundamentally arbitrary and that the main purpose for staging patients is to allow meaningful comparisons of treatment results from multiple institutions. It was emphasized that within any one staging category, there will be heterogeneity of prognosis depending on patient and tumour characteristics. This is in contrast to many solid tumours, for example, cervical cancer, where patients of a certain stage represent a fairly homogeneous population.

Attempts at direct measurement of tumour cell proliferation in NHL has also produced prognostic information. Overall a high proliferative index is associated with

the poor prognostic categories (as reviewed by Child, 1991). Unfortunately, because of the technical variations and the small number of tumours assessed, it is impossible to incorporate this information into a useable prognostic index (Coiffer *et al*, 1991).

The effect of specific chromosomal abnormalities is equally difficult to include in a prognostic index. The common abnormalities t(14;18) and t(8:14) correlated with histologic phenotype and are not independent factors over and above morphology. Levine and Bloomfield (1990) summarized the evidence for correlating genetic abnormalities with clinical outcome. Abnormalities in 17p or q have been identified as adversely affecting prognosis in three studies (Levine *et al*, 1990; Yunis *et al*, 1989; Rodriguez *et al*, 1991). Yunis *et al*, (1989) also identified trisomy of 2p or dup2p as having a negative impact on survival whereas dup3p or +3 gave an improved prognosis. Levine *et al*. (1988) found that breaks in 2p was associated with better survival; chromosome 2 would therefore appear to affect prognosis differently according to whether loss or gain of information occurs. Cabanillas *et al* (1989) related abnormalities in chromosome 7 to poor response and survival in lymphoma. The basis for these prognostic factors in terms of genes affected by the karyotypic abnormalities are unknown although sparse data may suggest that losses and mutations in p53 may account for the poor prognosis of the tumours with chromosome 17 abnormalities (Rodriguez *et al*, 1991).

Because of the inadequacy of the existent staging based prognostic schemes, editorial comments by Bunn (1988) and Child (1991) both called for a replacement of the currently used system. The first workshop on prognostic factors in large cell lymphomas was reported by Coiffer *et al*, (1991). In this workshop the prognostic indices developed by independent cancer groups were described. These schemes were broadly similar; all involved certain aspects of tumour mass and distribution plus some reflection of tumour growth characteristics such as LDH or B_2 microglobulin (Jaganneth *et al*. 1986). The conclusions of the international workshop was that a new Prognostic Index was needed for NHL and that this index should take account of the major tumour factors described above plus patient factors such as performance, symptoms and age. It is therefore hoped that in the near future a common prognostic scheme will be used for reporting NHL trials and hence facilitate the identification of risk groups and treatment benefits.

1.2 CANINE LYMPHOMA

1.2.1 INTRODUCTION

Canine lymphosarcoma (alternatively known as malignant lymphoma) is the most common haematological malignancy in the dog. All breeds can be affected by lymphoma but there are certain breeds which appear to be at higher risk of developing the disease. Carter *et al* (1987) identified a greater preponderance of medium and large breed dogs within their series of 41 lymphomas. German Shepherd dogs and boxers are overrepresented in surveys (Priester, 1967; MacEwan *et al*, 1987 and Squire, 1973) and a variety of other "at risk" breeds have also been identified including Poodles and Scottish terriers (MacEwan *et al*, 1987). A prospective study of three households of bull-mastiffs revealed that 9 out of 59 bull mastiffs died of lymphoma within a three year period (Onions, 1984). This corresponds to an annual incidence of 5000 cases per 100,000 dogs. The aetiologic significance of this familial predisposition is still unknown. This time-space clustering could suggest an infectious origin (such as a retrovirus) but attempts to identify a causative retrovirus have failed (reviewed by Squires, 1990).

The dog shows an increasing incidence of lymphoma with age (Schneider, 1983) and although very young dogs can occasionally be affected, lymphoma is mainly a disease of middle to old age. Male dogs are more commonly affected than females and the difference is comparable to male/female incidence in humans for NHL. Schneider (1983) estimates that entire male dogs have a 20% increased incidence of the disease compared to female dogs whereas human males have 10-20% greater incidence than women (Scottish Cancer Registration Scheme, 1984), but in certain small studies the disparity between men and woman can be considerably greater (Slymen *et al*, 1990).

Malignant lymphomas can be classified according to their anatomical location. The most common forms are multicentric, thymic, cutaneous and gastrointestinal. *The work in this thesis primarily pertains to the multicentric form of lymphoma (MLSA). In the remaining sections of this introductory chapter, the MLSA (and occasionally thymic forms) are discussed rather than the other types of lymphoma.*

Like NHL, MLSA is a chemosensitive malignancy which relapses with chemoresistant disease in virtually all cases. The time-scale is inevitably shortened compared to humans; the majority of dogs will relapse within one year (Squire *et al*, 1973; Weller *et al*, 1980; Postorino *et al*, 1989; Greenlee *et al*, 1990). Tumour progression is rapid; if left untreated, the majority of cases will die or be presented for euthanasia within one month of diagnosis although from owner history lymphadenopathy can have been present for several weeks prior to presentation

(Bloom and Meyer, 1945; Squire *et al,* 1973) Relapse can be equally swift and devastating. Within a two or three week period between treatments, dogs can progress from clinical remission to large bulk disease. The clinical history of this group of tumours is therefore similar to intermediate and high grade NHL. It has been used as a model for human NHL in the past (Appelbaum *et al,* 1985; MacEwan, 1990; Greenlee *et al,* 1990).

The remainder of this section will describe some of the similarities of canine lymphoma to NHL with particular emphasis on the efforts to understand the heterogeneous response of these tumours to treatment.

1.2.2 HISTOLOGIC CLASSIFICATION

The Rappaport classification scheme has been applied to canine MLSA but has been disappointing in its ability to identify prognostically useful groups (Squire *et al,* 1973; Holmberg *et al,* 1976; Weller *et al,* 1980 and Greenlee *et al,* 1990). This may be due to the emphasis which the Rappaport classification places on diffuse versus follicular patterns of tumour growth. In the dog, the percentage of tumours which have a follicular pattern is low (see table 1.5)

Table 1.5 Follicular histology in MLSA

Reference	*Follicular Number*	*lymphomas %*
Squires *et al,* 1973	1/100	1%
Weller *et al,*1980	7/72	9.7%
Appelbaum *et al,* 1984	1/40	2.5%
Greenlee *et al,* 1990	10/176	5.6%
Holmberg et al, 1976	8/24	33%

The paucity of follicular tumours in the dog may be due to the bias created by only being able to obtain lymph nodes at a biologically advanced stage of disease. Compared to humans most dogs present with more widespread disease. Careful examination of longitudinal sections from multiple lymph nodes has suggested that about one third (8/24 cases) may be follicular (Holmberg *et al,* 1976). Holmberg *et al* (1976) admitted that the canine nodular pattern was less obvious than in humans and that it was not always recognizable in all parts of the node nor was it found in all nodes at necropsy. Thus it is not unreasonable to propose that some of the diffuse tumours may have initially been nodular in pattern and had progressed to a more diffuse form by the time the biopsy (or necropsy) was performed. Progression from follicular to diffuse forms is reported in humans (Hubbard *et al,* 1982).

Taking into consideration that histological grade is not a static situation and that it is not possible to take multiple lymph node biopsies merely to ensure that an underlying follicular pattern is not overlooked it is obvious that alternatives to the Rappaport scheme are needed. Greenlee *et al* (1990) undertook a comprehensive comparison (involving 176 lymphomas) of the merits of four major classification systems; Rappaport, Lukes-Collins, Kiel and the Working Formulation. The Kiel system appeared to be best able to accommodate canine cellular morphologic types and at the same time provide some correlations between morphology and clinical performance.

1.2.3 IMMUNOPHENOTYPIC CLASSIFICATION

The panels of monoclonals used to type human leukaemias/lymphomas do not commonly cross-react with canine lymphocytes. Immunophenotyping MLSA out of necessity is still based on the presence or absence of surface/cytoplasmic immunoglobulin (sIg or cIg) and the use of a few putative T cell markers. The incidence of B cell tumours based on sIg has been in broad agreement for over 10 years: using an indirect method of detecting sIg based on rosetting techniques Onions (1977) found only 51% (17/33) of tumours but when sIg is detected using anti-immunoglobulins reagents, 70-80% of the tumours appear to be sIg positive (table 1.6).

Table 1.6 Immunoglobulin positive MLSA

Reference	*sIg positive tumours*	
	number	*%*
Holmberg *et al*, 1976	8/11	73%
Appelbaum *et al*, 1984	31/40	77.5%
Ladiges *et al*, 1988	42/54	78%
Greenlee *et al*,1990	48/64	75%
Teske *et al*, 1992	52/66	79%

Positive identification of phenotypic T cells has not been so straightforward. The presently available T cell monoclonals such as DT2 and LQ_1, commonly react with sIg positive B cell tumours. Appelbaum *et al* (1984), Ladiges *et al* (1988) and Greenlee *et al* (1990) all report a sizeable proportion of sIg positive tumours to also stain with either DT2 or LQ1. These dual staining tumours represented 67%, 68% and 44% respectively of the total tumour populations. The frequency of this dual staining is beyond that which could be believably explained by "lineage promiscuity" and would more likely represent (excluding technical problems) the fact that the antigens recognized by DT2 and LQ1 are not confined to T cells. Using these pan-T

cell monoclonals (but excluding those tumours which are both pan-T/sIg positive), the percentage of tumours which are phenotypically T cells falls in the range of 9-20% (Ladiges *et al*,1988; Greenlee *et al*, 1990).

Greenlee identified a correlation between immunophenotype and morphologic features using the Kiel classification. All of the tumours with immunoblastic morphology were sIg positive and conversely most of the tumours with a significant small cleaved cell population (the centrocytic and centrocytic/centroblastic forms) were identified as T cells. Teske *et al* (1992) has also identified a correlation between small cleaved cells and T cell phenotype.

1.2.4 IMMUNOGENOTYPING CANINE LYMPHOMAS

There are no published results of genotyping canine lymphomas. However Greenlee *et al* (1990) allude to unpublished genotyping which yielded results consistent with their phenotyping in the few cases in which it was performed. This lack of genotyping information would seem a fairly serious deficit in the classification of canine lymphomas given the problems which have been encountered with the pan-T cell markers. None of the canine specific T cell monoclonals currently available recognize components of the TCR/T_3 complex and hence will not be inherently limited to cells containing TCR gene rearrangements. The proportion of tumours which stain for both sIg and pan-T cell markers has already been described as suspicious. Given that these markers may not be truly T cell specific, there is the possibility that some of the sIg positive, pan-T cell positive tumours may not be phenotypic T cells but could indeed be B/pre-B cells which do not express sIg. Thus it would seem prudent to assume that the current proportion of tumours identified as T cell lineage represents the uppermost limit. Genotyping would help clarify this situation.

1.2.5 TREATMENT OF CANINE LYMPHOMA

(i) First Line Treatment

Combination chemotherapy protocols are generally considered more effective than single agent therapy. Treatment with prednisolone with or without cyclophosphamide produces a temporary response (usually less than 3 months) in less than half of the treated dogs (Brick *et al*, 1968; Squire *et al*, 1973). The introduction of more intensive regimes has not only increased the length of remissions but has also improved the percentage of dogs which attain a complete response. Using a relatively simple protocol of cyclophospamide vincristine, and prednisolone (COP) it is possible to achieve remissions in more than 80% of cases (Cotter *et al*, 1983). Reviews of the treatment of canine lymphomas are available (Madewell, 1985;

Cotter, 1986) and indicate that of the plethora of currently available protocols, no single protocol has gained uniform acceptance as the most efficacious. Median remissions remain in the 5-8 month range with overall survival times of 10 -14 month. Little improvement has been made in remission rate or length or in survival times since the early reports of COP protocols (Squire *et al,* 1973). However it is notoriously difficult to compare protocols between institutions since selection and staging of patients plus methods of calculating results varies between establishments. With these caveats in mind, there is some evidence that the addition of doxorubicin into the basic COP protocol does improve remission lengths (Carter *et al,* 1987; Cotter and Goldstein, 1986). Indeed single agent cytotoxic therapy with doxorubicin alone achieved response rates, remission durations and survival times which compare favourably with the COP protocol (Postorino *et al,* 1989).

Chemotherapy for MLSA is still largely palliative. Given that cure is impossible for the majority of patients, then quality of life for both the patient and the owner are an important goal. In this regard, single agent treatment with an anthracycline does offer the advantage of infrequent out-patient attendance (once every three weeks) and freedom from complicated oral dosing of drugs.

(ii) Rescue therapy following relapse

Chemotherapy in canine lymphoma is usually given throughout the remission period. Depending on the exact protocol, there may be a more intensive induction course of drugs followed by chronic maintenance treatment until clinical signs of relapse are apparent. In other protocols (Postorino *et al,* 1989) the same protocol is given for induction and maintenance. For historical reasons (Squire *et al,* 1973), canine lymphoma is usually treated with maintenance chemotherapy throughout the lifespan of the animal. A recent retrospective survey suggests that chronic treatment gives no improvement in remission or survival over short term administration of anthracycline single-agent chemotherapy (Hahn *et al,* 1992). This has yet to be tested in a randomised prospective trial.

Most protocols give first remissions of about 6 months. The chance of obtaining a second remission is less than for the first, and if achieved, is usually shorter. The length of the first remission often correlates with the length of the second remission; achieving a long first remission gives a greater chance of obtaining a durable second remission (Cotter, 1986). This clinical impression emphasizes the profound inherent biological variability within canine lymphomas such that some tumours fail multiple drug treatment rapidly and then fail subsequent alternate drug protocols (Hohenhaus and Matus, 1990).

The importance of achieving a solid first remission means most drugs are used during induction and consequently, at relapse, there is a limited number of new drugs available. Dogs which have received variations on the COP protocol can be rescued with doxorubicin (Calvert and Leifer, 1981) but despite doxorubicin having a different mechanism of action from cyclophosphamide, vincristine or prednisolone, Calvert and Leifer (1981) could only obtain responses in 4 out of 12 dogs. Dacarbazine can also induce second remissions in dogs resistant to doxorubicin (Gray *et al*, 1984) but again in only a limited number of cases. Hohenhaus and Matus (1990) reported disappointing results using etoposide to rescue dogs relapsing after a vincristine containing multi-drug protocol. However Carter *et al* (1987) remarked that dogs which relapsed following doxorubicin treatment were more likely to achieve a second remission than dogs which had received the COP protocol. Therefore the extent of clinical multiple drug resistance may be greater in the COP treated dogs. Asparaginase, which has a unique mechanism of action compared to the other commonly used cytotoxic drugs, is a useful drug in relapse and can induce remissions in dogs resistant to both COP and anthracycline protocols (personal clinical experience; N.T.Gorman, personal communication). The limited success of "rescue" therapy highlights that multiple drug resistance in relapsed canine lymphomas is just as prevalent as in human NHL.

1.2.6 PROGNOSTIC FACTORS IN CANINE LYMPHOMA

The use of histologic classification as a prognostic indicator in human NHL is not easily emulated in canine NHL. It has been difficult to identify sub-groups with a consistent clinical outcome. This may not only be because canine lymphomas do not exactly "fit" the human cellular descriptions (as discussed by Greenlee *et al.* 1990). It was already mentioned that in NHL, there is considerable heterogeneity of performance within any of the three grades (low, intermediate and high) of the Working Formulation . In the most comprehensive study of canine MLSA histology yet undertaken, Greenlee *et al,* (1990) established that 60% of canine lymphomas are intermediate grade, 29% are high grade and only 11% are low grade. In this study, the high grade (immunoblastic) tumours had longer remissions and survivals than other histologic types, but this difference was not statistically significant. Carter *et al* (1987) also found a trend towards a longer remission in dogs with high grade tumours, but this trend was not statistically significant. Both Greenlee and Carter remarked that the high grade tumours were a more homogeneous group in terms of response than the intermediate or low-grade tumours. Histiocytic type tumours have been associated with good responses (Squire *et al*, 1973: Weller *et al*, 1980) but have also been reported to respond poorly to single agent doxorubicin (Gray *et al*, 1984). If

the human NHL experience is also relevant for MLSA, it is probably unlikely that histology alone will be sufficient to reliably detect prognostically useful subgroups within these intermediate grade tumours. Given that this group makes up the majority of MLSA, this is a disincentive for undertaking detailed histological examinations of canine lymphomas.

Clinical staging is commonly performed in canine MLSA, predominantly by non-invasive means. Depending on the exact scheme used, patients are divided into four or five stages with stage V representing disseminated disease. An example of a staging scheme is given in chapter 7. The dogs can then be sub-classified according to the severity of the clinical signs and history of illness at time of presentation. Assignment according to clinical signs does inevitably involve a degree of subjectivity but has actually been of use in providing prognostic information. Cotter *et al* (1983) found that dogs with severe clinical signs were less likely to achieve a complete response; for stage IIIA and IIIB dogs the CR was 91% and 60% respectively. These workers did not identify an effect of clinical signs on the remission length but other groups have suggested dogs with severe clinical signs do have shorter remissions (Greenlee *et al*, 1990).

Staging according to disease extent has had mixed success in predicting response. Greenlee *et al* (1990) and MacEwan *et al* (1987) did not find stage to be predictive of response whereas Squire *et al* (1973), Cotter *et al* (1983) and Carter *et al* (1987) showed that stage III dogs did perform better than stage IV/V dogs. The consensus takes the view that there is a loose correlation of stage with prognosis but it is by no means clear whether the poorer prognosis of advanced stage tumours is due to inherent drug resistance or due to the susceptibilty of these dogs to the toxic effects of the treatment. Cotter's results (1983) would imply that stage IV tumours are less drug sensitive than stage III tumours (CR rates of 47% and 83% respectively). Squire *et al* (1973) and Carter *et al* (1987) also found that stage IV tumours were less likely to obtain a complete response but the difference was not so marked.

Does phenotyping contribute to the ability to identify different prognostic groups? Unfortunately remarkably little is published on this point; Ladiges *et al* (1988) could not correlate immunophenotype with subsequent response but Greenlee *et al* (1990) did reveal significant correlation of shorter remissions and hypercalcaemia with the T cell phenotype. Hypercalcaemia has been recognized as a poor prognostic factor by other groups (Weller *et al.* 1982). The numbers of treated dogs in the B cell/T cell categories established by Appelbaum *et al* (1984) were too small to make meaningful comparisons but rather curiously they did identify a tendency for T cell tumours to have smaller peripheral lymph nodes than B cell tumours.

A myriad of other clinical factors have also been proposed to influence response including age, sex and weight. The clinical impression that small dogs survive longer (probably due to relative overdosing in comparison to large dogs) is not statistically significant nor is age a significant factor (MacEwan *et al*, 1987). However females can have a significantly prolonged remission compared to males in some studies (MacEwan *et al*, 1987). Cytogenetic studies in canine MLSA have not been carried out, presumably due to technical difficulties, and so no prognostic information is available from this source.

In summary, advanced clinical stage, severe clinical signs, hypercalcaemia and T cell phenotype are all (to a variable extent) associated with poor prognosis. Other factors, including age, sex and body size may have minor effects.

1.3 MULTIDRUG RESISTANCE

1.3.1 MUTATIONAL BASIS FOR DRUG RESISTANCE

Failure of a chemotherapeutic protocol to cause tumour remission may be due to many factors. Physiological and pharmacological factors undoubtedly play a large role in the absorption, distribution and metabolism of chemotherapeutic drugs. In relapsing haematological malignancies, a substantial portion of the tumour burden is in the bone marrow and peripheral circulation. In these malignancies, it is difficult to envisage pharmacologic factors which can alter drug serum concentrations sufficient to result in treatment failure. Research on acquired and inherent clinical resistance in these malignancies has therefore focussed on cellular factors which could account for resistance to chemotherapeutic drugs.

Malignant cell populations are characterised by a genetic instability which leads to the spontaneous generation of variant forms with diverse phenotype and genotype; the genetic origin of cellular resistance to a variety of drugs has been shown (reviewed by Goldie and Coldman, 1984). The dependence of resistance on mutations at drug resistance genes suggest that the higher the mutation rate in a given tumour population, the earlier in its growth curve resistant variants may appear (Goldie and Coldman, 1985).

Albertini *et al* (1990) compiled the results from numerous studies on somatic cells mutation rates and illustrated that the normal mutation rate was between 10^{-5}-10^{-6} at a given gene locus per generation. The implications of this somatic rate of mutation on models of multi-stage carcinogenesis are discussed by Stein *et al*, (1991) and Loeb (1991). The latter emphasizes that most human malignancies contain a multiplicity of genetic abnormalities which would seem to indirectly suggest that the mutation rate within tumours may be greater than the frequency calculated for normal

cells *in vivo*. Loeb (1991) also highlights the deficiencies in the current data directly assessing mutation rates in tumours. Most of the work has been performed in rodent models which because of the differences in DNA repair capacity may not be directly relevant to human neoplasms. If Loeb's prediction that part of carcinogenesis involves the induction of a "mutator phenotype" is correct, then this would lend greater credence to the original Goldie-Coldman hypothesis on the genetic origin of drug resistance.

The likelihood of a resistant cell arising in a population is not merely dependant on the mutation rate but also on the number of cell doublings. When the stem cell population in a tumour is low, the tumour must undergo many more cell doublings to reach a clinically detectable size. Added to this is the selection pressures occurring during this prolonged slow-growth phase; some cell lineages will have died due to environmental pressure. The surviving fraction in these slow-growing tumours will have a higher likelihood of having undergone mutations towards resistance than a tumour of similar mass with a higher growth fraction because of the extended environmental selection pressure. This mutational hypothesis could help explain the intrinsic chemoresistance of the slow-growing indolent NHL in comparison to the potentially curable higher grade tumours. It would appear that the mutational events underlying inherent versus acquired resistance need not be different. In the former case, the resistant variants represent the majority of the cell types in the tumour mass whereas in the chemosensitive group they only represent a tiny fraction of the total cell population at the start of chemotherapy. Ultimately, the ability to completely cure patients will depend on the ability to kill the tumour stem cells. The pessimistic clinical implications of this fact are discussed by Butturini and Gale (1991) and Jasmin (1988).

1.3.2 DISCOVERY OF P-GLYCOPROTEIN

The characterisation of *in vitro* derived drug resistant cell lines has been fundamental in discovering resistance mechanisms with clinical relevance. Foremost among these discoveries were the observations of Kessel *et al*, (1968), Biedler and Rheim (1970), Bech-Hansen *et al*, (1976) and other groups, that chronic *in vitro* selection with a single agent (for example actinomycin D or colchicine) could result in resistance to a wide range of lipophilic drugs which do not share a common structure or target. This *in vitro* phenomenon, which became known as pleotropic or multi-drug resistance (MDR) was intensively investigated because it seemed to reflect the complexity observed in clinical resistance.

In 1974, Ling and Thompson observed that MDR cells which had been selected in colchicine did not accumulate the drug to the extent of the parent cell line.

Two years later, these colchicine resistant cells were shown to overexpress an integral membrane protein of approximately 170,000 daltons (Juliano and Ling, 1976). Roninson *et al*, (1984) and others (Riordan et al, 1985; Scotto et al;1986; Gros et al, 1986c) noted that MDR cells contained amplified DNA. These amplified sequences were found to contain a small family of genes (Van der Bliek et al. 1986); one of these genes, termed *mdr*1 encoded a 4.5 -5.0kb mRNA which in turn coded for the P-glycoprotein (Riordan et al, 1985; Ueda et al, 1987b).

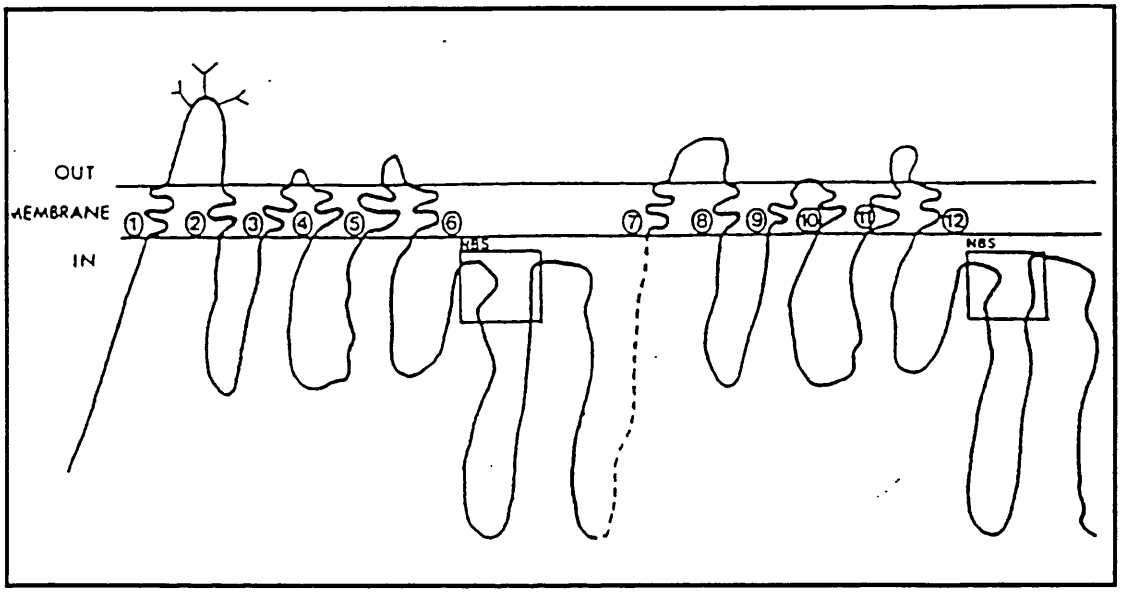
The nucleotide sequences of human *mdr1* (Chen *et al,* 1986), and what were designated mouse *mdr1* (Gros *et al,* 1986b) and hamster *Pgp1 (*Gerlach *et al,* 1986*a*) became available in rapid succession. The complete sequences of all these genes showed that the genes encode proteins of between 1276-1280 amino acids. The sequence of the *mdr* genes placed *mdr* encoded P-gp in the ABC (ATP Binding Cassette) superfamily of transport proteins. Other members of this family include a number of bacterial transport proteins and the "shock" sensitive nutrient uptake systems found in gram-negative bacteria (Gros *et al,* 1986b). The closest homology is to Haemolysin B, a self contained membrane protein which mediates the energy dependent extrusion of the protein haemolysin A. Proteins of the haemolysin group include a hydrophobic region with six transmembrane segments and a nucleotide binding domain, thus resembling one half of P-gp. This general homology to bacterial permeases, provided a potential mechanistic basis for the MDR phenotype. From sequence homology, it would appear likely that P-gp could act as an energy dependant drug efflux pump, lowering the intracellular drug concentration and thereby permitting cell survival.

The functional significance of the *mdr-1* encoded P-glycoprotein (P-gp) in the multi-drug resistance phenomenon was later confirmed by transfection experiments. Full length P-gp cDNA transfected into sensitive cells could confer the MDR phenotype following drug selection of recipient clones (Gros *et al,* 1986a; Croop *et al,* 1987; Ueda *et al,* 1987a) and even without drug selection (Guild *et al,* 1988). The postulated structure of P-glycoproteins has been deduced from the nucleotide sequence and is shown in figure 1.2.

The coding sequence of P-gp comprise two halves (which are almost a direct repeat of each other) connected by a linker of about sixty amino acids. Each half can be divided into two domains of functional significance: an N-terminal domain which has little similarity between the halves, and a C-terminal domain which contains nucleotide binding sites and is largely identical between the two halves. Upstream of the nucleotide binding sites in both halves of the molecule are six hydrophobic segments which are predicted to span the membrane as shown in figure 1.2. It has been postulated that these membrane loops could form a membrane channel through

which drugs are extruded. In conjunction with the results of Hamada and Tsuruo (1988) demonstrating that isolated P-gp containing membranes have ATPase activity, all of the evidence is compatible with the postulated role of P-gp as an ATP-driven pump which removes drug from the cell and thereby prevents cytotoxicity.

Figure 1.2 Proposed P-glycoprotein structure.



NBS, nucleotide-binding sequence consensus region.
The potential N-linked glycosylation sites are indicated by the branches sprouting from the polypeptide. Dashed lines indicate the linker region. (Adapted from Kirschner et al. 1992)

Over five years later, the supra-molecular structure of P-gp has still not been resolved. Freeze fracture electron microscopic studies suggest that *mdr1* probably exists as a higher oligomer, most likely a dimer or a trimer (Arsenault *et al.*, 1988; Sehested *et al.*, 1989; Weinstein *et al.*, 1989). These findings would be consistent with other integral membrane transporters such as Band 3, the anion transporter of red blood cells (Jay and Cantley, 1986). This raises the possibility that *mdr1* monomers may need to organize into higher oligomers for the formation of transport pores that are large enough to accommodate lipophilic drugs moving out of the cell.

1.3.3 MDR GENE FAMILY

The manner by which the coding sequences for two human, three mouse *mdr* genes, and two complete hamster P-gp genes were obtained is summarised by Van der Bliek and Borst, (1989). A single gene has been cloned and sequenced in the rat (Silverman *et al,* 1991). All of these genes have strong homology to each other.

The nomenclature of the *mdr* gene family in different species is confusing. The mouse genes were originally named according to the chronological order in which they were isolated (Gros *et al,* 1986b; Gros *et al,* 1988; Devault *et al,* 1990). A second group led by Horwitz adopted a functional based nomenclature (table 1.8) in which the two murine genes which were most closely related to each other were called *mdr1a* and *mdr1b* (Hsu *et al,* 1989). Both of these genes are capable of conferring the MDR phenotype by gene transfection. The remaining gene, known as *mdr2* by both Horwitz and Gros, does not confer drug resistance in transfection experiments. The second human *mdr* gene was called *mdr3* by Van der Bliek's group (1987) because of its homology to the third hamster gene. The human *mdr3* gene also fails to confer resistance in transfection experiments and is more homologous to the *mdr2* gene of the mouse than the murine *mdr1* genes. The sequence homology between the different *mdr* genes is given in table 1.7 which is adapted from Silverman *et al,* 1991 and the different nomenclatures in table 1.8.

Table 1.7 *mdr* gene homologies (% nucleotide homology)

Gene	Hamster mdr1a	mdr1b	Mouse mdr1a	mdr1b	mdr2	Human MDR2
Human						
MDR1	83.5	75.7	82.2	78.7	71.1	74.9
MDR2	64.2	47.7	71.6	70.6	86.1	100
Mouse						
mdr2	65.0	77.9	71.1	69.4	100	
mdr1b	77.9	83.4	84.2	100		
mdr1a	89.3	76.8	100			
Hamster						
mdr1b	82.0	100				
mdr1a	100					

The sequence comparison in table 1.7 shows that the human *mdr1* gene is analogous to the *mdr1a* gene in rodents. The *mdr1a* and *mdr1b* of rodents are closely related and have been suggested to have arisen by a gene duplication event which is presumed to have arisen after the divergence of rodents and primates. It is also noteworthy that the *mdr2* genes are more closely related to each other than they are to the *mdr1* genes of the same species. The conserved nature of the *mdr2* gene indicates that the protein probably has an important cell function, which is as yet unknown.

Table 1.8 *mdr* gene nomenclature

Suggested Designation	Current Designation Human	mouse	hamster
mdr1a	MDR1	*mdr3*	*pgp1*
mdr1b		*mdr1*	*pgp2*
mdr2	MDR2/*mdr3*	*mdr2*	*pgp3*

Along the length of the gene, the degree of cross-species and cross-isoform homology varies. The areas surrounding the nucleotide binding sites are the best conserved; generally the homology increases towards the 3' end of each half of the gene. The linker region, which joins the two duplicate halves of the gene, is poorly conserved (Hsu *et al,* 1989). This has implications for the ability to generate gene specific probes.

1.3.4 DRUGS AFFECTED BY MDR

Some of the drugs which are generally considered to be affected by *mdr* encoded P-gps are listed in table 1.9. These drugs have diverse cellular targets and structure (Bowman and Rand, 1980) . The common feature of these drugs is there hydrophobicity and, in the majority of cases, their flexible planar ring structure (Beck and Qiang, 1992). The sensitivity to hydrophilic drugs such as bleomycin and cisplatin is generally unaltered or even slightly decreased in MDR cells (Schurr *et al.,* 1989; Keizer *et al.,* 1989). In this regard, the MDR phenotype falls short of being a complete mimic of clinical multiple drug resistance in that it is not uncommon for clinical drug resistance to be accompanied by resistance to drugs such as methotrexate, cisplatin and bleomycin (Armitage *et al,* 1990).

The MDR phenotype has been most studied in drug selected cell lines. These *mdr1* expressing cell lines often show a quantitative variation in the degree of cross-resistance to the MDR spectrum drugs (Akiyama *et al,* 1985; Gekelar *et al.,*1990). Generally resistance is highest to the selecting drug (Akiyama *et al,* 1985) but this is not universal (Guild *et al,* 1988). A notable exception is the colchicine selected chinese hamster ovary cell line CH_RC5, which is 180 times resistant to colchicine but is 5000 fold resistant to gramicidin D (Gerlach *et al.* 1986b). In the chronically selected cell lines it is difficult to exclude other resistance mechanisms contributing to this variation. However after transfection of functional P-gp cDNA among a series of recipient clones (Shen *et al.,* 1986b; Ueda *et al.,* 1987b) variation still exists in the quantitative resistance, even in the absence of drug selection (Guild *et al,* 1988).

Table 1.9 MDR phenotype drugs

actinomycin D	etoposide	mitoxantrone
doxorubicin	epirubicin	azidopine
vincristine	vinblastine	taxol
daunomycin	puromycin	gramicidin D
colchicine	colcemid	valinomycin

Because the primary interest in P-gp stems from the field of cancer chemotherapy, most of the drugs commonly used to screen MDR cell lines are chemotherapeutic drugs rather than some of the membrane perturbing agents used originally in the characterisation of these lines (Bech-Hansen *et al.,* 1976). This concentration of effort is understandable from a clinical viewpoint. Of the drugs included in the MDR spectrum, the anthracyclines, podophyllotoxins and the vinca alkaloids are of major importance in many treatment protocols. Doxorubicin has formed the crux of many lymphoma treatment regimens since the efficacy of the CHOP protocol (cyclophosphamide, doxorubicin, vincristine and prednisolone) was demonstrated in the seventies (McKelvey *et al,* 1976).

In those studies which have not been confined to a cancer perspective, interesting results regarding potential P-gp substrates have been found. Early work suggested that some MDR cell lines have altered sensitivity to certain steroids (Bech-Hansen *et al.,* 1976) and in addition, P-gp is present at high levels in the steroid producing adrenal cortex (illustrated by Bradley *et al,* 1990). This possible connection with normal P-gp function spawned an interest in steroids as substrates for the P-gp pump. Naito *et al.,* (1989) and Yang *et al* (1989) examined the ability of different steroids to inhibit vincristine and azidopine binding to MDR cell membranes. Progesterone, of the steroids tested, inhibited drug binding most efficiently. Curiously, subsequent work has suggested that the two *mdr1* genes in the rodent can be differentiated by their differential binding of progesterone. *Mdr1b,* the isoform found in adrenal and gravid uterus, binds progesterone more effectively than the *mdr1a* isoform. However, effluxing of progesterone by the *mdr1b* expressing cell line could not be shown (Yang *et al,* 1990). Thus although there is a link between P-gp and endogenously occurring steroids, there is still no definite proof of efflux of endogenous substrates via P-glycoprotein.

1.3.5 HOW DOES P-GP RENDER CELLS DRUG RESISTANT?

There is ample evidence that MDR cells avoid cytotoxicity by keeping the intracellular drug concentration low (Ling and Thompson, 1974; Fojo *et al,* 1985a; Willingham *et al.,* 1986). Numerous studies have demonstrated that MDR drugs can bind to P-gp containing membranes (Cornwell *et al,* 1986; Naito *et al,*1988; Safa *et*

al. 1989) and in the inverted vesicle model of Horio *et al* (1988) some of these drugs have been shown to be transported across the membrane. Agents, such as verapamil, which are known to reverse the MDR phenotype, also bind to P-gp and can competitively block MDR drug binding (Safa *et al,* 1987; Foxwell *et al,* 1989). These findings all support the general model of P-gp active transport, which was based on the nucleotide similarities between *mdr1* and the ABC superfamily. However, despite the acceptance of the active drug transport model in its' broadest sense, there are still many uncertainties about the system. Firstly, what is the biochemical basis of the diverse substrate specificity of P-gp? Secondly, how can P-gp confer resistance to a large fold increase in external drug concentration yet reduce the intracellular concentration by a much smaller fold difference (Fojo *et al,* 1985a)? Until these two points are adequately answered, it is unlikely that the full benefits or limitations of *mdr* modulator drugs will be understood.

West (1990) speculated about the mechanistic basis of P-gp substrate specificity and discussed whether P-gp could be a transport system for diverse xenobiotics which are recognised via a common chemical tag placed on them by enzymatic drug-conjugating systems such as glutathione transferase. However, the inverted vesicles of Horio *et al.* (1988) could accumulate vinblastine without any exogenously added glutathione. Therefore, even if P-gp does transport glutathione-conjugated xenobiotics, the glutathione moiety is not an absolute requirement and would seem unlikely to be the major recognition feature.

West (1990) also drew attention to some of the inexplicable aspects of the data on drug binding and transport in MDR cells. Horio *et al,* (1988) noted that daunomycin effectively blocked vinblastine binding. However neither colchicine nor puromycin were particularly effective inhibitors. Earlier work by Cornwell *et al,* (1986) had illustrated the same phenomenon; colchicine was a poor inhibitor of vinblastine binding to MDR cell membranes. This led West to propose that there were possibly separate and non-interacting drug binding sites on the P-gp for different drugs.

The idea of binding site specificity affecting drug interactions was strengthened by the work of Choi *et al,* (1988) who demonstrated that an alteration in a single amino acid can have marked effects on the cross resistance profile of MDR cells (Choi *et al,* 1988). A cluster of point mutations in the human *mdr1* gene which resulted in a glycine to valine substitution was associated with increased resistance to colchicine relative to the vincaalkaloids. The altered amino acid (residue 185) was located immediately adjacent to the cytoplasmic side of the third transmembrane segment (TM3) and was therefore proposed to be involved either in initial drug binding or at some point in the translocation of the drug (figure 1.3). To resolve these

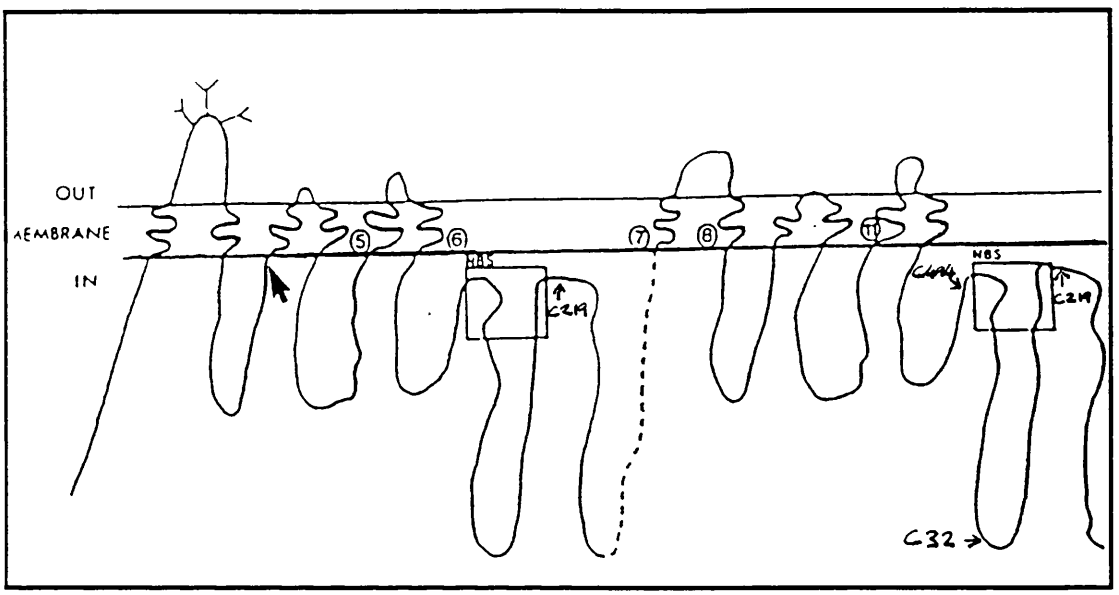
alternatives, Safa *et al,* (1990) compared the binding of photoactive drug analogues to the wild-type versus mutant P-glycoprotein. This work suggested that this mutation did not affect initial drug binding but did affect the ability to translocate vincristine across the membrane.

P-gp is a protein of two virtually identical halves and hence one would expect the homologous cytoplasmic loop in the other half of the molecule to also contribute to drug binding and efflux. Georges *et al,*(1991) proteolytically cleaved P-gp into its two halves and showed that both halves labelled with azidopine. They then used monoclonal antibodies against specific regions of P-gp to perturb drug and ATP binding. The positions of the binding sites of these monoclonals on the intact protein are shown in figure 1.3. C219 binds in both halves of the molecule whereas the binding sites of C494 and C32 are limited to the C-terminal half of the protein. Despite this large separation in the linear drawing of P-gp, C494 and C32 effectively inhibited ATP binding to both halves of P-gp suggesting cooperativity between the two domains, perhaps as a result of close proximity in the native protein. This work has given intriguing insights into P-gp tertiary structure but still does not define the biochemical basis of substrate specificity.

The group led by Philippe Gros approached the question of P-gp substrate specificity by exploiting their knowledge of the murine *mdr* genes. Transfection experiments had shown that the *mdr1a/1b* genes could confer the MDR phenotype (but that this resistance was not identical) whereas m*dr2* could not transfer drug resistance (Gros, *et al,* 1988; Devault and Gros, 1990). Initially they created chimeric proteins where regions of the *mdr1b* protein was replaced by regions from the inactive *mdr2* protein. When *mdr1b* transmembrane domain regions were replaced by the equivalent regions of *mdr2,* biological activity was abolished. Replacement of as few as two TM regions (e.g.. TM5-6 or TM7-8) was sufficient to destroy activity (Buschman and Gros, 1991). The group then went on to investigate whether differences in the TM segments between *mdr1a* and *mdr1b* could account for the difference in drug-resistance spectrum of transfectants. A comparison of the aminoacid sequence of the two genes revealed a difference in TM11. Site directed mutagenesis of a single amino acid at position 941 (Ser to Phe) in TM11 altered the degree of drug resistance. For *mdr1b,* the substitution produced a mutant protein which had retained the ability to confer resistance to vinblastine but had lost the ability to confer doxorubicin or colchicine resistance. This segregation of vinblastine resistance from colchicine resistance in the mutant validates the biochemical observations of competitive binding and drug efflux carried out by Horio *et al,*(1988), Cornwell *et al,* (1986), Safa *et al,* (1989) and others, which suggested that

the binding sites for VBL may be non-overlapping with the colchicine and doxorubicin binding sites.

Figure 1.3 P-gp illustrating some functionally critical sites.



Arrow marks position of the glycine/valine substitution

As a way of unifying some of the apparently divergent information on the binding and efflux capabilities of P-gp and its structural implications, Higgins and Gottesman (1992) have proposed an alternative view of the P-gp pump. Rather than basing P-gp substrate specificity on some "enzyme-like" recognition site somewhere on the cytoplasmic side of the protein, Gottesman proposes that drugs initially interact with the plasma membrane and then interact with the P-gp transporter from the lipid phase. After interaction has occurred, the substrates are "flipped" either into the outer leaflet of the membrane or directly to the exterior. This model assumes that substrates partition themselves into the lipid phase: it is well recognised that despite the size variation, MDR drugs are all cationic, lipid soluble planar molecules which would be expected to intercalate into membranes (Beck and Qian, 1992). In this way, the primary determinant of substrate specificity would be the ability to insert into the lipid membrane in a recognisable fashion, and only secondarily would come the ability to bind to a substrate site on the molecule. Substrate specificity based on lipid membrane intercalation would help explain the P-gp mediated resistance to the hydrophobic peptides gramicidin D and valinomycin which would not seem likely to interact with a binding site positioned in an aqueous part of the molecule.

The "flippase" model could also explain some of the complexities of kinetic data in that the amount of drug available for transport will depend on the amount

partitioned into the membrane at any one time and not on the external or intracytoplasmic concentration. If P-gp acts in a sentinal role, intercepting and expelling drugs prior to entry into the cytoplasm, P-gp would be a particularly effective resistance factor. However, although this "flippase" model is very attractive, it still does not explain the second question posed at the start of this section. This question relates to how the presence of P-gp can give cells a large fold resistance relative to the parental cell line and yet still allow considerable intracellular drug accumulation.

Confocal microscopy of daunorubicin distribution in sensitive and resistant cell lines show that both drug sensitive lines and their resistant counterparts have a similar rapid distribution of drug from the plasma membrane to the perinuclear region within the first two minutes of drug exposure (Gervasoni *et al,* 1991). It is only in the subsequent redistribution that resistant and sensitive cells differ. In the sensitive cells, drug redistributed to the nucleus and cytoplasm in a *diffuse* pattern. In contrast, in the resistant cell lines, daunorubicin redistributed from the perinuclear region into vesicles distinct from the nucleus, which gave the cell a *punctate* pattern. This altered sub-cellular distribution of MDR drugs into a punctate pattern begs the question of whether P-gp is present in internal membrane structures as well as the external membrane.

Remarkably little is known about the sub-cellular distribution of P-gp. In the tumour cell line KB-C4 (which is a high expressor of P-gp), Willingham *et al,* (1987) localised P-gp to the external plasma membrane but also to the lumenal side of the Golgi stack membranes. A small amount was also located in the endoplasmic reticulum. However, the distribution in normal cells, and in tumour cells *in situ,* may be considerably different from cultured cells. Numerous studies (discussed in chapter 5) have described P-gp in what would appear to be the Golgi region of cells or in a punctate distribution. In the hamster and human adrenal, the pattern of staining with anti-P-gp monoclonals suggests that P-gp is distributed throughout cytomembranes and is not localised to the cell surface (Bradley *et al,* 1990).

In summary, P-gp appears to act as a hydrophobic vacuum cleaner expelling hydrophobic drugs which interact with transmembrane portions of the protein. Certain regions within the intracytoplasmic loops are also essential for effective efflux. MDR cells can have an altered intracellular distribution of MDR drugs but the role of P-gp in this redistribution is unknown.

1.3.6 NORMAL TISSUE DISTRIBUTION OF THE MDR GENE FAMILY

Fojo *et al* (1987b), reported the first large survey of normal human tissues for the expression of *mdr1*. They measured total *mdr1* mRNA in blocks of normal tissue

and found high expression in liver, kidney, adrenal and colon. Expression in most other organs was low. Using the *mdr1* specific monoclonal antibody MRK16, Theibaut *et al,*(1987) found P-gp localised to the apical surface of colonic epithelium, brush border of proximal tubular epithelium in the kidney and the canalicular surface of hepatocytes. Suguwara *et al,* (1988) also used MRK16 and confirmed expression of P-gp in the adult (but not neonate) adrenal glands. *Mdr1* encoded P-gp has also been localised to the surface of endothelial cells of capillaries in the brain and testes (Cordon-Cardo *et al* 1989, Thiebaut *et al.* 1989). Van der Valk *et al,* (1990) surveyed normal human tissue using three monoclonal antibodies (C219, JSB-1 and MRK16) and found endothelial staining not only in the brain and testes but also in the lung, prostate, stomach, intestine and glomeruli.

Because of the existence of the human *mdr3* gene, whose tissue distribution was unknown, some confusion arose over the use of the monoclonal antibody C219. This antibody detects a conserved epitope in P-gp which is conserved in all known isoforms (Georges *et al,* 1990). When immunohistochemical analysis of human muscle tissue revealed a strong reactivity which was not detected with *mdr1* specific monoclonals nor with *mdr1* gene probes, this muscle tissue reactivity was considered to be a spurious cross-reactivity. Immunoblotting of electrophoresed protein gels with C219 suggested that the protein detected in muscle tissue was of a higher molecular weight than the other tissue P-gp and so this cross-reactivity was reputed to possibly be to a myosin protein. Reassessment of this work in the light of subsequent information would suggest that C219 does not cross react with myosin but that the reactivity in muscle is detection of the *mdr3* gene product. Detailed examination of the distribution of P-gp isoforms in the hamster using a panel of isoform specific monoclonals (Bradley *et al,* 1990) showed that hamster muscle tissue expresses the equivalent of the human *mdr3* gene. N-linked glycosylation of P-gp in normal human tissue can alter the apparent size of the protein by up to 15kD (Ichikawa *et al,* 1991), this coupled to the poor resolution of large proteins on the gel system used by Thiebaut *et al* (1989) could account for the size discrepancy between liver and heart muscle P-gp.

There are reports of *mdr1* encoded P-gp in human ureter (Weinstein *et al,* 1990a) and in certain epithelium of the female reproductive tract (Finstad *et al,* 1990). This work, and other work using C219 and JSB-1, is now open to reinterpretation following the revelation that certain batches of these monoclonal antibodies may be contaminated with antibodies which react with blood group antigens, in particular blood group A antigens (Finstad *et al,* 1991). Blood group antigens can be expressed by a variety of epithelia and endothelia so it is not

inconceivable that some of the reported P-gp expression in epithelia (and epithelial tumours) and endothelia, may not be genuine.

The detailed immunohistochemical analysis of P-gp isoform tissue distribution in the adult hamster by Bradley *et al,* (1990) showed that rodents have a very similar tissue distribution to humans, with the major exception that P-gp could not be unequivocally demonstrated in the kidney. This point is discussed further in chapter 5.

Rodents have two *mdr1* isoforms, and judging from the results of Croop *et al,* (1989) and Bradley *et al,* (1990), the normal function of the *mdr1b* protein is likely to be quite distinct from the *mdr1a* function. *Mdr1b* is localised to the adrenal cortex and to the epithelium of the uterus in mid to late stage of pregnancy. In the adrenal, reactivity was localised to the *zona fasciculata* and *z. reticularis*; there was no expression in the medulla or *z.glomerulosa*. This strong adrenal cortex staining was limited to males; females showed only weak staining of the *z.glomerulosa*. This would suggest that the expression of *mdr1b* is under tight and probably complex control.

Inevitably, the tissue distribution of P-gp has led to speculation about the normal physiological role of the different isoforms. The association of the *mdr1a* isoform with secretory and excretory epithelial surfaces such as liver, gastro-intestinal tract and kidney, has invoked the idea that *mdr1a* is an efflux pump for noxious xenobiotics or potentially harmful lipophilic endogenous substances. There is remarkably little direct evidence to further substantiate this hypothesis. There are a number of *in vivo* and *in vitro* models that show that *mdr1* may increase in response to various noxious stimuli (Thorgeirsson *et al,* 1987; Burt and Thorgeirsson, 1988; Hill *et al,* 1990, Marino, *et al,* 1990; Chin *et al,* 1990a and 1990b; Gant *et al,* 1991), but these studies have not succeeded in identifying the normal substrates for *mdr1*. The putative role of the *mdr1b* isoform in rodents has centred around vague ideas about steroid transport and metabolism but again although binding of progesterone to P-gp has been demonstrated *in vitro* and *in vivo*, it is by no means clear if these substances are actually transported (Yang *et al,* 1989 and 1990).

The function of the *mdr2* isoform is even less clear. Immunohistochemistry shows that it is present in a complex striated pattern in both cardiac and skeletal muscle fibres. In skeletal muscle, only certain fibres are P-gp positive; these appear to be the Type 1 fibres (slow twitch fibres) identified by their alkaline labile, acid-resistant ATPase activity (Thiebaut *et al,* 1989). The potential substrates of the *mdr2* isoform are completely unknown. Recent evidence suggests that the *mdr1* isoform may be a volume-regulated, chloride-selective channel (Valverde *et al.* 1992); this makes it similar to the cyclic AMP regulated chloride channel encoded by the cystic

fibrosis transmembrane regulator (CFTR) gene (Tabcharani *et al,* 1991). It is interesting to speculate that the *mdr2* isoform may also have ion channel properties that participate in the complex ion fluxes which occur during muscle contraction and relaxation.

1.3.7 P-GP/*MDR* EXPRESSION IN HUMAN TUMOURS

(i) Methodological considerations

P-gp represents a resistance mechanism which can be detected in tumour material using standard techniques. Naturally numerous studies have examined P-gp in tumours and attempted to relate this to inherent or acquired resistance. Before proceeding to the results of some of these studies, some methodological considerations have to be discussed. In a recent editorial Baer and Bloomfield (1991) commented "studies of MDR1 expression are of great potential significance in cancer therapeutics, and it is imperative that methodologic problems be conquered". It is a reflection of the quality of work in this field that this comment was still necessary six years after the first measurement of *mdr*1 in clinical samples (Bell *et al,* 1985). Some of the technical problems and misunderstandings which have arisen (plus ways of avoiding them) are outlined in the first half of this section.

In broad terms it is possible to divide the clinical studies into *mdr* mRNA versus protein measurement studies. The methodologies can be further subdivided into those which use homogenates of tumour tissue versus techniques which allow assessment of P-gp distribution within a tumour sample such as immunohistochemistry (IHC) and *in situ* hybridisations. Historically, *mdr* gene probes became available more rapidly than monoclonal antibodies directed against P-glycoprotein. Consequently, the majority of the earliest studies used dot-blot and Northern hybridisations to measure *mdr* mRNA to categorise tumours into high/intermediate/low expressors of P-gp. Now that IHC information is available on cellular distribution of P-gp some of the generalities which arose from the dotblot work must be reassessed (see below).

A second level of misinterpretation arose because initial studies were carried out before the implications of the *mdr* gene family were appreciated. One of the first antibodies widely available was C219 (Kartner *et al,* 1985) which is not isoform specific (Georges *et al,* 1990). Many studies (predominantly of haematological malignancies) have used C219 alone, even since this potential source of inaccuracy has become apparent (Carulli *et al,* 1990a, 1990b, 1990c; Kuwazuru *et al,* 1990a, 1990b; 1990c; Ma *et al,* 1987; Weide *et al,* 1990; Kato *et al,* 1990; Musto *et al,* 1990; Musto *et al,* 1991; Epstein *et al,* (1989). These authors, if they make reference at all to this source of error, have justified their experimental design with the comment that

this particular normal tissue does not express *mdr2*. In many tissues this may be true but it has certainly not been unequivocally shown. One of the few studies of *mdr2* expression in normal human tissues (using pcr analysis) did not examine peripheral blood but did detect *mdr2* in spleen homogenate (Chin *et al.* 1989). Given that spleen is part of the lympho-reticular system and a site of blood storage, it would seem unsafe to conclude that peripheral blood leucocytes and leukaemias will be *mdr2* negative.

Misinterpretation of *mdr1/mdr2* can be avoided using *mdr1* specific probes. Fortunately, most *mdr* mRNA studies (with a few exceptions such as Holmes *et al,* 1990) have used probes which were *mdr1* specific under the conditions employed. In IHC, the use of a panel of antibodies (as recommended by Grogan *et al* 1990) including *mdr1* specific monoclonals such as MRK16 and JSB-1 (Schinkel *et al,* 1991) minimizes the risk of misinterpretation.

Characterisation of *mdr* cell lines has indicated that only a little surface P-gp in an otherwise P-gp negative cell type, can alter drug resistance (Noonan *et al,* 1990; Chan *et al,* 1988; Bradley *et al.* 1989). The realisation that a low level of P-gp is relevant *in vitro*, has focussed attention on the threshold sensitivity of the techniques employed to analyse clinical samples. Generally it is accepted that Northerns and mRNA slotblots are more sensitive than Western immunoblots but these techniques do have the advantage of being quantifiable, whereas IHC is more subjective. Many of the papers analysing *mdr1* mRNA have adopted the use of the KB drug resistant cell line series as their positive and negative controls (Goldstein *et al,* 1989: Noonan *et al.* 1990; Fojo *et al,* 1987a and 1987b; Kakehi *et al,* 1988; Lai *et al,* 1989; Rothenburg *et al,* 1989; Kanamuru *et al,* 1989; Herweijer *et al,* 1990). KB8-5, the most widely used P-gp positive cell line, has a low level of resistance to MDR drugs (approximately four fold to doxorubicin) and has a reproducible amount of *mdr1* mRNA on dotblot or northern blot. *Mdr1* mRNA from both normal and tumour samples can yield values greater than KB8-5 (Goldstein *et al,* 1989; Fojo *et al,* 1987a) thus it is a realistic control. The *mdr* mRNA in KB8-5 is then used as a threshold between "intermediate" and "high" expressing tumours.

Chan *et al* (1988) and Grogan *et al,* (1990) have established that certain immunocytochemical techniques can be equally sensitive as mRNA detection techniques. Both groups have used cell lines other than the KB8-5 to determine the detection threshold of their IHC but both have established that their technique can detect P-gp on cells with a low fold resistance to MDR drugs. Unfortunately not all studies have determined a meaningful threshold for their methodology and this makes the comparison of results difficult. Some authors do not stipulate what they use as a positive control (Rubin *et al* 1990) and others use highly drug resistant cell lines with

gross overproduction of P-gp well above that in the tumour samples (Keith *et al.* 1990a; Schneider *et al.* 1989; Wishart *et al.* 1990; Schlaifer *et al,* 1990), making it impossible to judge the limit of their sensitivity.

The quest for improved sensitivity has led to *mdr*1 mRNA analysis by pcr technology (Noonan *et al* 1990) and this has shown that low-level *mdr1* expression, undetectable by conventional assays, is common in most tumour types. Given that normal endothelia and tissue macrophages can have P-gp (Van der Valk *et al* 1990), it remains to be proved whether pcr based studies can produce clinically meaningful results.

The problem of comparing between studies is not a trivial one. Often a very small number of samples are analysed and hence it is difficult to determine if variation in results is because of differences in technique sensitivity, biological variation between small sample groups or even due to artefact. Artefacts may be prevalent in this work because very few papers validate their findings by repetition (Chan *et al,* 1990; Marie *et al,* 1991) and hence conclusions are often formed from a single dot-blot reading or on a single IHC section (Goldstein *et al,* 1989; Pirker *et al,* 1991; Solary *et al,* 1991; Ito *et al,* 1988; Musto *et al,* 1991; Weide *et al,* 1990; and many others).

(ii) *Mdr1* in solid tumours

The possible sources of error in clinical P-gp work are many and in reviewing the publications, it is not possible to exclude all papers which do not meet high technical standards; in many tumour types this would reduce the number of properly analysed samples to a mere handful. Table 1.10 divides solid tumours into three groups according to the frequency and amount of P-gp found in pre-treatment samples. This table is adapted from Nooter and Herwiejer (1991) but includes only malignancies which have had at least twenty independent samples analysed by at least two different groups of researchers.

From this table it is apparent that tumours which arise from P-gp expressing normal tissue like the liver, kidney and gastrointestinal tract, have a tendency to retain P-gp following neoplastic transformation. It is also accepted that the tumours in Group 1 tend to fall into the clinical category of intrinsically resistant; i.e. chemotherapy rarely produces complete remission. It is important to note that Group 3, the tumour group which is invariably P-gp negative at time of diagnosis, does not symbolise a group of chemosensitive tumours. Non-small cell lung cancer and head-and-neck tumours have a fairly poor response to chemotherapy; both ovarian tumours and small cell lung cancer often relapse with chemoresistant disease.

Table 1.10 Expression of *mdr1* in solid tumours
References in bold type used immunohistochemical techniques

Group I High *mdr1* expression levels at a high frequency	
Renal cell carcinoma	Fojo *et al*, 1987b Goldstein *et al, 1989* Kakehi *et al*, 1988 Kanamura *et al*, 1989 **Schlaifer *et al*, 1990**
Colon cancer	Fojo *et al*, 1987a Goldstein *et al*, 1989 **Weinstein *et al*, 1991** **Schlaifer *et al*, 1990**
Hepatocellular carcinoma	Goldstein *et al*, 1989 **Schlaifer *et al*, 1990**
Adrenocortical tumours	Fojo *et al*, 1987a **Schlaifer *et al*, 1990**
Group II Intermediate *mdr1* expression levels at a lower frequency	
Neuroblastoma	Fojo *et al*, 1987a Goldstein *et al*, (1989) Bourhis *et al*, (1989a) **Chan *et al*, 1991**
Soft tissue sarcomas	Gerlach *et al*, 1987 **Chan *et al*, 1990** Noonan *et al*, 1990 **Schlaifer *et al*, 1990**
Phaeochromocytoma	Fojo *et al*, 1987a Goldstein *et al*, 1989 **Schlaifer *et al*, 1990**
Breast cancer	Goldstein *et al*, 1989 Moscow *et al*, 1989 Salmon *et al*, 1989 Keith *et al*, 1990a **Schneider *et al*, 1989** **Schlaifer *et al*, 1990** **Wishart *et al*, 1990** **Verelle *et el*, 1990**

Table 1.10 (continued)

Group III Almost always low/undetectable *mdr1* expression	
Ovarian cancer	Gerlach *et al, 1987* Goldstein *et al,* 1989 Bourhis *et al,* 1989b Moscow *et al.* 1989 **Rubin *et al,* 1990**
Lung tumours (small cell and non-small cell)	Goldstein *et al,* 1989 Moscow *et al,* 1989 Lai *et al,* 1989

It must be appreciated that within the three groups, it is possible to select a study which gives results divergent to the rest. Some studies of Group 1 tumours have failed to detect P-gp in tumour cells. Goldstein *et al* (1989) remarked on the variation in dotblot values for this tumour type whereas Moscow *et al,* (1989) failed to find *mdr1* in 10/12 colon samples by slot blot analysis. IHC studies are revealing that this variation between individuals (and presumably between studies) may be a genuine biological phenomenon. Schlaifer *et al,* (1990a) examined two colonic carcinoma samples using immunohistochemistry; neither had P-gp positive tumour cells but did have P-gp positive macrophages. Other IHC studies have also revealed that colonic tumours are not uniformly positive: Weinstein *et al* (1991) found 30 out of 95 tumours were P-gp negative.

IHC analysis in addition to showing inter-patient variability, is also showing that intra-tumour P-gp distribution is heterogeneous (Rubin *et al,* 1990; Finstad *et al,* 1990; Wishart *et al* 1990; Weinstein *et al,* 1991). Eventually this may necessitate regrouping the tumours in table 1.10. The heterogeneous distribution of P-gp in tumour cells is usually described in terms of the potential for selection of a pre-existant drug refractory sub-population by subsequent chemotherapy. However it is still unclear if P-gp positivity in relapsing tumours is due to a drug selection mechanism, as suggested by the Goldie and Coldman hypothesis. For ethical reasons, patients receive drug combinations which are not limited to MDR drugs. Consequently, even if the expression of P-gp does increase at time of relapse in an individual tumour it is not possible to state that this is due to drug selection of mutant phenotypes by MDR drugs.

Determining the frequency of P-gp in relapse tumours versus pre-chemotherapy tumours is still not adequately established for most tumour types. The inter-patient variability of P-gp expression at time of diagnosis necessitates the use

either of large cohorts of pre-treatment and post-treatment samples or preferably the use of paired samples from the same patient before treatment and at relapse. This has not been achieved in most tumour types because of the difficulties of sample procurement.

In those tumour types that are not uniformly P-gp positive at diagnosis, there is accumulating evidence that P-gp may be a poor prognostic indicator, even when non-MDR drugs are included in the treatment protocols. In a collection of solid tumour papers, small numbers of tumours which are P-gp positive at diagnosis are associated with poor clinical response in a variety of tumour types including breast cancer (Salmon *et al,* 1989; Verelle *et al,* 1991), neuroblastoma (Bourhis *et al,* 1989a) and ovarian cancer (Rubin *et al,* 1990). No multivariate analyses was attempted in any of these studies to take account of other prognostic factors.

Chan *et al,* (1990) performed a longitudinal study of 30 cases of childhood soft tissue sarcoma, including twelve patients sampled after treatment. This IHC study is important for various reasons. Firstly, it meets all the technical criteria mentioned previously. Secondly, it provides patient details and actually uses this information to assess whether the presence of P-gp is of prognostic importance independent of other factors. It would appear that in this group of childhood sarcomas the presence of P-gp at diagnosis, or subsequent to treatment, was associated with poor survival. The difference in survival between the P-gp positive group (9/30) and the 21 negative tumours was profound. No statistical analysis of clinical response was attempted (which bypasses the difficulties inherent in objectively assessing solid tumours) but there was a definite trend for the P-gp positive group to be clinically chemoresistant.

(iii) *Mdr1* in haematological malignancies

Many of the early studies of leukaemias and lymphomas analyse only a few patients (often with sub-standard methodology) and inevitably, there is a large range of positive tumours before treatment and at relapse between studies even within each individual leukaemic type (table 1.11). However, the availability of clinical material has brought more rapid results in this area of oncology than in solid tumours.

It is possible to correlate P-gp positivity with poor response (or short duration of response) in most haematologic malignancies (Weide *et al,* 1990; Ito *et al,* 1989; Musto *et al,*1990; Carulli *et al,* 1990b; Ubezio *et al,* 1989). Unfortunately it is difficult to determine if this is due to drug resistance imparted by P-gp or due to the co-segregation of P-gp with other poor prognostic variables, as discussed by the editorial comments of Dalton (1991) and Baer and Bloomfield, (1991). Table 1.11 shows that post-chemotherapy myeloma samples are more likely to be P-gp positive

at relapse but that P-gp expression in AML and ALL is very variable even in relapse samples.

Compiling the results of the NHL studies, P-gp has been found in about a tenth of the tumours at diagnosis whereas about one half have acquired P-gp at relapse. The vast majority of the drug resistant samples were from patients who had received complex drug protocols so it has not been possible to correlate P-gp expression at relapse to one particular drug or drug regime (see references in table 1.12).

Table 1.11 *mdr1* expression in haematologic malignancies.

Acute Myeloid Leukaemia	Untreated	Treated
List *et al*, 1991	2/7	
Goldstein *et al*, 1989	3/24	
Ito *et al*, 1989	0/14	0/11
Pirker *et al*, 1991	45/63	
Noonan *et al*, 1990	27/51	
Marie *et al*, 1991	12/19	5/7
Suguwara *et al*, 1989		1/6
Herweijer *et al*, 1990	13/17*	
Kuwazuru *et al*. 1990c	9/17	
Acute Lymphoblastic Leukaemia	Untreated	Treated
Musto *et al*. 1990	5/11	8/9
Fojo *et al*. 1987a	0/9	
Rothenburg *et al*. 1989	1/9	3/15
Goldstein *et al*. 1989	2/15	
Ito *et al*. 1989	1/3	3/5
Kuwazuru *et al*. 1990c	4/11	
Marie *et al*. 1991	1/2	1/3
Herweijer *et al*. 1990	8/11*	
Ubezio *et al*. 1989	0/5	0/5
Myeloma	Untreated	Treated
Salmon *et al*. 1991		10/15
Carulli *et al*. 1990c		5/10
Epstein *et al*. 1989	6/10	10/12
Salmon *et al*. 1989	4/7*	
Dalton *et al*. 1989		4/6
Musto *et al*. 1990	0/5	2/5
Solary *et al*. 1991	3/12	2/4

* *Treatment status not stated*

Five of the NHL papers used techniques which allowed direct visualisation of the lymphoma samples. However, Umeda *et al,* (1990) used flow cytometry of peripheral mononuclear cells, without making reference to the leukaemic state of the NHL patients. Their criteria for positivity was to have a higher percentage of positive cells in the peripheral blood from NHL patients than in samples from normal healthy volunteers. The normal range was 0-1.5% positive; all of the NHL positive samples were in the range 1.5% to 5.4%. The authors stated that ICC was used to show P-gp in tumour (blast) cells but this data from NHL patients was not presented. It is very unclear from Umeda's flow cytometry work how prevalent P-gp is in the actual tumour cell population.

Several of the studies of leukaemic samples use flow cytometry to document P-gp in blood samples and the criteria for defining positive varies (Epstein *et al.* 1989; Cumber *et al.* 1990; Suguwara *et al.* 1989). Many of these "positive" samples, even from drug-resistant tumours, do not have more than 50% positive cells (Epstein *et al.* 1989; Cumber *et al.* 1990; Suguwara *et al.* 1989;). This is in contrast to the IHC work of Miller *et al,* (1991) and Dalton *et al,* (1989) who categorically state that P-gp is present in essentially all of the tumour cells in a "positive" tumour. Whether this difference in prevalence of P-gp positive cells is a genuine phenomenon or created by the difference in technique is not clear. However, the reservations forwarded by Hanson (1991) regarding flow cytometry and the inability to match staining results with cell morphology is a valid criticism of the P-gp work.

Table 1.12 *mdr1* in non-Hodgkins Lymphomas

Non-Hodgkins Lymphoma	Untreated	Treated	Method
Goldstein *et al,* 1989	4/18	3/5	dotblot
Dalton *et al,* 1989		1/1	IHC
Salmon *et al,* 1989	1/2	2/4	IHC
Suguwara *et al,* 1989	0/2		Flow
Musto *et al,* 1990	0/2	1/2	ICC
Noonan *et al,* 1990	0/1		pcr
Umeda *et al,* 1990	1/2	5/12	Flow
Schlaifer *et al,* 1990a	0/6	0/9	IHC
Miller *et al,* 1991	1/39	5/9	IHC
Total	7/72	19/42	

Methods: dotblot=mRNA quantitation with MDR5A.
IHC = immunohistochemistry with multiple P-gp monoclonal antibodies (except Musto et al,who used C219 alone).
Flow = flow cytometry using MRK16

Attempts to link acquisition of P-gp with particular NHL subgroups have not been successful. Potentially relevant tumour and patient information such as histologic type, stage etc are not automatically given in the papers, perhaps because the small numbers in each study do not appear to merit such detail. Schlaifer *et al,* (1990a) provide the working formulation classification of their NHL samples but since all of the tumour cells in their study were negative, this information is of limited use. The P-gp positive relapse sample in Dalton *et al,* (1989) was a diffuse large cell lymphoma but in the larger study from the same group (Miller *et al,* 1991), the tumours were divided into low, intermediate and high grade without giving further detail. All of their positive samples were in the intermediate-grade subgroup, which is likely to include other diffuse, large cell lymphomas.

The immunophenotype of the relapse samples is unknown. It is therefore unknown if the incidence of P-gp expression in the relapsing T cell tumours and B cell tumours is equal. Adult T cell leukaemia/lymphoma, associated with HTLV-1 infection, is a subgroup of lymphoid malignancies which is categorised as a high grade lymphoma in the NCI modified version of the Working Formulation. These lymphomas are exclusively T cell neoplasms. Four studies (table 1.13) have shown that the incidence of *mdr1*/P-gp in ATL is higher both in untreated and treated cases compared to other forms of NHL. The numbers involved, especially in the relapse samples is still too small to make firm conclusions.

Table 1.13 *mdr1* expression in ATL

Adult T-cell leukaemia/lymphoma	Untreated	Treated
Su *et al.* 1991	4/7	
Umeda *et al.* 1990		0/3
Kuwazuru *et al.* 1990a	8/20	6/6
Kato *et al.* 1990	4/6	4/5
Total	16/33	10/14

So at this time it is only possible to state that more NHL express P-gp at relapse than before treatment. Several lines of indirect evidence suggest that drug selection may not necessarily be the underlying mechanism for P-gp expression in these relapse samples.

Nooter and Herweijer (1991) remarked that in most types of leukaemias, elevated *mdr1* levels can be found and that this is in discordance with normal haemopoietic cells which do not express *mdr1*. Unfortunately, the latter half of this statement, often repeated, is based on old data, from the slot-blot and Northern

analysis data of a handful of unpurified blood samples (Fojo *et al.* 1987a; Holmes *et al*, 1990). Once more sophisticated techniques were applied to this complex group of cell types, Chaudury and Roninson (1991) demonstrated that haemopoietic stem cells in bone marrow express a functional P-gp. That an extremely primitive cell type within the bone marrow expresses P-gp is significant because it helps rationalise the results of the leukaemic samples. P-gp expression in untreated samples could reflect the retention of part of the primitive stem cell (as indicated by concomitant CD34 expression) phenotype due to neoplastic transformation (List *et al,* 1991).

CLL and multiple myelomas have a more mature phenotype than the ALL group and so it is less easy to attribute their P-gp to a stem cell phenotype. However it is important to remember that P-gp in mature white blood cells has not been addressed in any detail in pathologic states. Indirect evidence suggests that the presence of P-gp in lymphocytes should not be discounted. McGown *et al,* (1991) measured daunorubicin uptake in the (normal) lymphocytes of ovarian cancer patients who were randomly sampled during the course of their MDR/non-MDR drug regime. The accumulation of drug in the lymphocytes from the chemotherapy patients could be increased by up to 123% by the addition of verapamil whereas accumulation in samples from healthy volunteers remains unchanged. These lymphocyte populations were not analysed for P-gp expression but it does raise the possibility that the cellular stress of chemotherapy could (temporarily) elevate P-gp in normal lymphocytes.

In one of the few published examples of a (mouse) B-lymphocyte population (presumed to be a cell line) examined by Northern analysis, Teeter *et al* (1990) detected strong *mdr1b* expression. However, Weide *et al.*(1990) and Suguwara *et al.* (1989) failed to detect P-gp in the peripheral blood of 30 healthy individuals using immunocytochemistry and flow cytometry respectively. It would therefore appear that P-gp in normal healthy lymphocytes is rare. However, the lymphocytes from people receiving lymphotoxic /immunosuppressive drugs have not been studied in detail. If mature lymphocytes can express P-gp (perhaps only under certain circumstances), this would not only provide a normal tissue precedence for P-gp in fairly mature lymphocytes, it would also emphasize the importance of sorting leukaemic from normal cells prior to analysis of bulk samples. In the work by Herwiejer's group (Herwiejer *et al.* 1990 and 1991; Nooter *et al.* 1990), unsorted peripheral blood samples were analysed by RNAse protection assay. The authors state that all samples contain at least 50% leukaemic cells, but the opposite view would say they could contain 50% normal cells! It is curious that their technique has detected the highest incidence of *mdr1* in both AML and ALL.

Returning to the question of MDR drug selection of P-gp positive mutants, there is interest in the work with CML and CLL. These malignancies can undergo a

blast crises which is characterised by an enormous production of a cell type which is morphologically dissimilar and more primitive than the original neoplastic cell. Some of the oncogenic events that accompany this transformation have been determined (Gale and Butturini , 1989; Daley and Ben-Neriah, 1991; Klinken, 1991). The blast crises of these diseases are not infrequently P-gp positive. Blast crises can occur even in untreated patients and although sufficient numbers of these drug naive patients have not yet been analysed, it would appear that prior drug treatment is not a pre-requisite for P-gp expression (Marie *et al,* 1991; Tsuruo *et al,* 1987; Goldstein *et al,* 1989; Carulli *et al,* 1990b, Weide *et al,* 1990).

Further work with leukaemic samples will help unravel the crucial question of whether P-gp in relapse samples is drug selected or an unfortunate event arising from progressive genetic events which are an inherent part of the disease process.

1.4 SUMMARY

Both NHL and canine MLSA represent a heterogeneous group of tumours which overall are chemosensitive but are often associated with a high incidence of drug-resistant relapse. In NHL, certain morphologic types are associated with specific oncogene-activating translocations and these affect the natural history and treatment response of the disease. Canine histologic types parallel human NHL except there is a preponderance of intermediate and high grade tumours. The histologic progression from follicular to diffuse forms is less evident in the dog compared to humans, perhaps because dogs present with biologically more advanced disease. Cytogenetic studies have revealed that NHL progressively acquire non-random genetic lesions, some of which are associated with a poor prognosis. Other prognostic factors influencing remission and survival in both humans and dogs are broadly similar. Advanced stage, poor performance status, "B" symptoms and T cell phenotype are all associated with poor prognosis in some studies.

Mdr1 encodes a membrane spanning glycoprotein (P-gp) which acts as an ATP-dependant efflux pump lowering the intracellular concentration of a variety of chemotherapeutic drugs and allowing cells to survive drug exposure. The drugs affected include clinically important agents such as some anthracyclines and the vinca-alkaloids. P-gp is a normal tissue protein and tumours which arise from tissues which express the *mdr1* isoform are generally chemoresistant. The currently available evidence suggests that normal lymphocytes do not express P-gp and many haematological malignancies which are drug-sensitive are also P-gp negative.

The role of P-gp in contributing to the heterogeneous response of NHL tumours is unclear. P-gp is rare in untreated tumours but is acquired in about 50% of relapse samples. The influence of drug treatment, in particular MDR drug treatment,

in selecting P-gp expressing subclones is not known; all of the relapse patients have received multiple agent protocols. High grade T cell lymphomas may have a higher incidence of P-gp expression than other NHL groups. Indirect evidence from other haematological malignancies suggest that events unconnected with drug selection may influence P-gp expression in lymphoid malignancies.

1.5 STUDY DESIGN AND THESIS AIMS

Determining the role of MDR drugs in the selection of P-gp positive tumour cell populations cannot be properly studied in humans because it is not ethically possible to give humans single agent protocols of MDR (or non-MDR) drugs. The inability to obtain adequate clinical samples from patients has further hindered the study of P-gp in clinical oncology. Within human NHL studies, basic biological factors (such as immunophenotype) have not been incorporated into study design.

MLSA in dogs is a naturally occurring tumour which displays acquired resistance to MDR and non-MDR drugs. In dogs, it is already known that single agent treatment of MLSA with an anthracycline such as doxorubicin (which is an MDR drug) can give acceptable disease free survival times (Postorino *et al,* 1989). This study was designed to take advantage of the efficacy of the anthracyclines in MLSA to study the role of P-gp in contributing to acquired anthracycline resistance in intermediate and high grade lymphomas.

Dogs with MLSA were actively recruited into the study from referral cases and treated with a simple chemotherapy protocol which is detailed below. Epirubicin is a derivative of doxorubicin which is still part of the MDR drug spectrum (Mirski *et al,* 1987). Dogs were scheduled to receive this anthracycline until relapse was evident. Epirubicin is less cardiotoxic than doxorubicin (Young *et al.* 1989), hence decreasing the likelihood that the MLSA dogs would show dose-limiting cardiac side effects prior to relapse.

Table 1.14 Epirubicin chemotherapy protocol

MLSA Chemotherapy Protocol	
Epirubicin	25mg/m^2 intravenously, every three weeks until relapse.
Prednisolone	2mg/kg *per os*, daily for seven days 1mg/kg, daily for seven days 1mg/kg, every other day until relapse

Lymph node samples were collected from these dogs at time of diagnosis and then at relapse for analysis of P-gp/*mdr1* expression. Samples from a smaller number of dogs which had received a standard multi-drug protocol prior to relapse were also obtained. P-gp expression in lymph node sections was assessed by immunohistochemistry using the non-isoform specific monoclonal antibody C219. A human *mdr1* probe was used to confirm the presence *mdr1* isoform in P-gp positive nodes using slot-blot analysis.

The lymphomas were genotyped; rearrangements in the T cell receptor β chain were used to positively identify potential T cell tumours. Genotyping of the canine tumours was carried out using a feline TCR β chain constant region probe. The most appropriate restriction enzyme digests, with a low incidence of restriction fragment length polymorphisms, was determined from the digestion of a phenotypically immature canine T cell line and normal germline samples.

Clinical performance, tumour characteristics (ie P-gp status and TCR gene rearrangement) plus relevant host factors were statistically analysed by multivariate analysis to determine the important prognostic factors in the anthracycline treated dogs.

The main aim of this thesis was to use canine lymphomas as a means of determining the relevance of P-gp as a potential resistance mechanism to anthracyclines in a clinical setting. The most cited hypothesis on acquisition of drug resistance suggests that drug selection is a major factor influencing the nature of the resistance mechanisms in tumours. The chronic treatment with epirubicin would be expected to favour the acquisition of P-gp more than treatment with a multi-drug regime (i.e. COP) which contains a smaller MDR drug component. The frequency of P-gp expression in the epirubicin treated dogs would therefore be expected to approach the maximum likely in a lymphoma population. From the percentage of treated lymphomas which express P-gp following epirubicin administration it will be apparent whether P-gp modulation or MDR drug avoidance is worth pursuing as part of salvage treatment of resistant lymphomas.

The basis for the poor clinical performance of T cell tumours relative to B cell tumours is unknown. The combination of the genotyping and the P-gp expression information will help establish if this difference is due to a propensity for T cells to express P-gp.

The relative importance of P-gp as a prognostic factor in NHL is unclear; other factors may identify poor risk groups more effectively and easily. The statistical analysis of clinical performance taking into account both patient and tumour details

should identify if P-gp is a critical prognostic indicator. Through statistical analysis, it may also be possible to identify groups which are "at risk" of expressing P-gp.

In summary, it is hoped to establish the role of P-gp in contributing to acquired drug resistance by building up a reasonably complete picture of P-gp in canine lymphomas, without excluding other biological factors, and without excluding the possibility that mechanisms other than simple drug selection may result in P-gp expression.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

The following section lists routinely used materials. Less frequently used materials are described in the appropriate Methods' section.

2.1.1 CHEMICALS

All chemicals were of the highest available quality and were obtained from BDH Chemicals Ltd, Gibco BRL, Pharmacia LKB or Sigma Chemicals except the following;

Acrylamide	Severn Biotech, Kidderminster
Bacto Agar	Difco, Detroit, USA
Doxorubicin	Farmitalia Carlo Erba
Epirubicin	Farmitalia Carlo Erba
Formamide	Fluka, Buchs, Switzerland
Methocell MC4000	Fluka, Buchs, Switzerland
Phenol	Rathburn, Walkerburn, UK
RNAzol B	Biogenesis Ltd
Vinblastine	Eli Lilly, Basingstoke

2.1.2 RADIOCHEMICALS

(α^{32}P)dCTP, (γ^{32}P)ATP plus (α^{32}P)UTP for labelling probes were obtained from Amersham International plc.

2.1.3 EQUIPMENT

Routine equipment which would be an integral part of any laboratory is not listed.

1. Dotblot manifold: BRL
2. Electroblotting System: Milliblot SDE, Millipore, Watford.
3. Gel Tanks: tanks for agarose & acrylamide gels were from IBI Ltd, Cambridge & Biorad Labs Ltd, Watford, Hertfordshire.
5. Hybridisation membranes: Genescreen, NEN, Boston. Hybond-N, Amersham International. Nitrocellulose 0.45um, Schleicher & Schuell via Anderman Lab Supplies, Kingston-upon-Thames.
6. Hybridisation Oven & Bottles: Hybaid Ltd, Middlesex.
7. Laser densitometer: autoradiographs were analysed at a SUN workstation using a Molecular Dynamic Densitometer and PDI Quantitation 1 software.
8. Microdismembranator: Braun, Germany
9. Plate reader: Biorad Labs Ltd, Watford.

2.1.4 RESTRICTION ENDONUCLEASES & OTHER ENZYMES

Restriction Enzymes

The majority of restriction endonucleases (RE) were from Pharmacia Ltd, Boeringer Mannheim Corporation or Northumbria Biologicals. Bulk ordering of RE's by the Department meant that the same "brand" of RE could not be used throughout this work.

Other Enzymes

DNAse 1 (RNAse free)	Pharmacia LKB
RNAse A	Pharmacia LKB
RNAse T_1	Boerhinger Mannheim
RNAse Inhibitor	Pharmacia LKB
T_4 Polynucleotide kinase	Northumbria Biologicals
Proteinase K	Boerhinger Mannheim

2.1.5 SIZE MARKERS

DNA size markers;

(i) *Hind III* digested phage λ, BRL

(ii) *Eco RI/BamHI* digested Adenovirus, IBI Ltd.

RNA size markers; 0.24 - 9.5kb RNA ladder, BRL

Protein size markers; Prestained standards, 14,300-200,000, BRL.

2.1.6 BUFFERS, SOLUTIONS & MEDIA

Where mentioned, autoclaving was carried out at 121^{o}C for 20 minutes.

General Buffers & Solutions

Denhardts (x100)
2% Ficoll
2% polvinylpyrrolidone
2% bovine serum albumin.
Aliquoted & frozen at -20^{0}C.

MOPS(x10)
200mM MOPS (3-[N-morpholino}propanesulphonic acid)
10mM EDTA
50mM Na Acetate
Made to pH 7.O with glacial acetic acid then autoclaved.

RBC Lysis Buffer
8.3g Ammonium chloride
O.037g EDTA
1g Potassium carbonate. Make up to 1 litre with distilled water.

RNA Lysis buffer
0.3M Na acetate
0.5% SDS
5mM EDTA. Made to ph8 with 10M NaOH then autoclaved.

TAE(1x) pH8
40mM Tris base
2mM EDTA
20mM NaCl
20mM NaAc

TBE(1x) pH8
89mM Tris borate
89mM Boric acid
2.5mM EDTA

TBS(10x) pH7.6
116.9 NaCl
24.2g Tris base
6.7g EDTA. Make up to 2 litres at pH7.6.

Loading Buffers

Dye loading buffer
125mM Tris HCL (autoclaved)
20% w/v Glycerol
5mM EDTA (autoclaved
0.25% w/v Bromophenol blue
0.25% w/v Xylene cyanol

Northern Loading Buffer
1x MOPS buffer
50% v/v formamide
2.2M formaldehyde
0.5ug/ml ethidium bromide

Acrylamide Gel Loading Buffer (RNAse Protection Assays)
80% Formamide
1 x TBE
1mM EDTA
0.1% Xylenecyanol
0.1% Bromophenol blue

Western Loading Buffer(x2)
60g Urea
20mls "Solubilisation " buffer. *See below*
33.7mls distilled water.
This urea buffer is then aliquoted & stored at -70^{0}C.

Solubilisation buffer
25g Sucrose
0.3g Tris base
1.54g Dithiothreitol
0.005M EDTA
5g SDS
2.5mg Pyronin Y

Make to Ph8 with HCl and final volume of 50mls with distilled water.

Transfer solutions

Phosphate buffer "Genescreen"(20x) *used to transfer both RNA & DNA.*
0.5M Na_2HPO_4
0.5M NaH_2PO_4

Electroblotting solution *used to transfer proteins.*
48mM Tris base
39mM Glycine
0.037% (vol/vol) SDS
20% methanol

Hybridisation solutions & Wash solutions

Formamide hybridisation mix (makes 100mls)
50mls formamide
25mls SSC(x20)
10mls "Genescreen"(x20)
5mls Denhardts(x100)
10% SDS
8mls distilled water
1ml salmon sperm DNA (*boiled 5 mins then cooled on ice)*

Non-Formamide hybridisation mix
50mM Pipes
100mM Nacl
50mM Na_2PO4
50mM Nah_2PO4
1mM EDTA
5% SDS

Hybridisation Mix for Polyd(T)
2.5mls Denhardts (x100)
1ml "Genescreen" (X20)
12.5mls SSC(x20)
34mls distilled water

Standard Saline Citrate(SSC) (20x)
700g NaCl
353g tri-Sodium citrate. Make to 4 litres with distilled water.

Tissue culture media

RPMI
88 mls RPMI-1640 *from Gibco BRL*
800mls sterile distilled water
26.6mls Na carbonate
10mls Na pyruvate
10mls L-glutamine
1ml 1M NaOh

Dulbecco's Modified Eagles media (DMEM)
50mls Dulbecco's MEM (10X) *from Gibco BRL*
400mls sterile distilled water
5mls Na pyruvate
5mls Glutamine
25mls Na bicarbonate

2.1.7 CELL LINES

3132

This cell line was derived from the ascitic fluid of an adult male Belgian Shepherd dog with multicentric lymphoma (Strandstrom & Rimaila-Parnanen, 1979). This cell line has been reported to produce a retrovirus (Strandstrom, personal communication reported in Squires, 1990) but subsequent attempts to induce retroviral expression have been unsuccessful (Squires, 1990). 3132 expresses surface immunoglobulin (sIg) and Fc receptors (Holmes, 1989). This cell line was maintained in RPMI 1640 plus 20% foetal calf serum and grows in suspension forming small spheroids of about 10 cells. Every 3-5 days, 80% of the media was removed and replaced with fresh media.

CL-1

This cell line was obtained from Dr.Hajime Tsujimoto, Department of Veterinary Internal Medicine, University of Tokyo. It was established from a spontaneous case of thymic lymphosarcoma in a dog. FACS analysis of CL-1 carried out by Dr. Ryo Goitsuka, also of Tokyo University, suggested that Cl-1 is immunophenotypically an immature T cell reacting with the monoclonals DT-2 (Wulff *et al,* 1982) and E11 (Szer *et al* 1985). This information was made available from Dr.Tsujimoto by personal communication. Cl-1 grows as a single cell suspension in RPMI 1640 plus 20% foetal calf serum. Every 3-5 days 80% of the media was removed and replaced by fresh media.

KB3-1, KB-Ch^{R}-8-5, KBV-1

This series of cell lines was derived from human KB epidermoid carcinoma cells. The lines were obtained from the European Collection of Animal Cell cultures, Porton Down, Salisbury. KB3-1, the parent cell line for the drug resistant mutants, was derived from a single clone of human epidermoid carcinoma cells after two subclonings. The drug resistant mutants, KB-CH^{R}-8-5 (also known as KB8-5) and KBV-1 were obtained after chronic selection in colchicine and vinblastine respectively (Akiyama *et al,* 1985; Shen *et al,* 1986c). All three cell lines grow as monolayer cultures in DMEM and are split using 0.125% Trypsin. KB8-5 is maintained in 10ng/ml of colchicine and is 3-6 fold more resistant to colchicine than the parent. KBV-1 is grown in DMEM plus 1ug/ml of vinblastine.

AuxB1 & CH$_{R}$C5

AuxB1 is a drug sensitive chinese hamster ovary cell line from which the drug resistant line CH$_{R}$C5 was derived (Ling & Thompson, 1974; Bech-Hansen *et al,* 1976). CH$_{R}$C5 was obtained following chronic selection in colchicine and is approximately 150 fold resistant to colchicine: it is grown in DMEM plus 10ug/ml

colchicine. CH_RC5 overexpresses Pgp1 in comparison to the parental line and is used as a positive control in the western blots.

3201

This cell line is derived from a specific pathogen free kitten which developed an intrathoracic tumour following inoculation with the FeLV negative thymic tumour cell line. 3201 has a paucity of lynmphocyte surface markers but does rosette guinea-pig erythrocytes (Rojko *et al* 1989). It has β chain rearrangements (J Neil, personal communication) and is used as a positive control for β chain expression on northern blots. RNA from this cell line was kindly donated by J Neil, Glasgow University.

2.1.8 MONOCLONAL ANTIBODIES & IMMUNOLOGICAL REAGENTS

Monoclonal Antibodies

C219 This IgG2a monoclonal antibody was produced following immunization of mice with drug-resistant hamster & human cell lines (Kartner *et al*, 1985) and detects all known isoforms of P-gp in all the species studied so far. It has been epitope mapped to a hexapeptide sequence (VVQEAL) in a conserved, cytoplasmic portion of P-gp (Georges *et al*, 1990). Synthetic peptides containing this hexapeptide sequence have been used to block specific C219 binding (Georges *et al* ,1990). It is obtained from CIS UK Ltd, High Wycombe.

MAC 387 This IgG1 monoclonal was raised against purified human blood monocyte components. It reacts with a cytoplasmic antigen in many cells of the monocyte/macrophage series including infiltrating & reactive histiocytes, tumour infiltrating macrophages and sinus histiocytes (Flavell *et al* 1987). However other macrophage populations are not labelled including germinal centre macrophages. It can also react with human granulocytes. MAC 387 is obtained from Dako Ltd, High Wycombe.

MRK 16 is an IgG2a monoclonal that was raised against the human leukaemia cell line K562/ADM. It reacts with an external epitope on the human *mdr1* encoded P-gp. This monoclonal does not react with the human *mdr3* gene product nor with rodent isoforms. MRK-16 was obtained from Dr. Tsuruo, Cancer Chemotherapy Center, Toshima-ku, Tokyo.

S-100 This is a rabbit polyclonal sera raised against ox brain. It was obtained from Dako and they claim it recognizes both α and β chains of S-100.

Immunological Reagents

10 amino acid peptide (spanning C219 epitope)

V-V-Q-E-A-L-D-K-A-R was obtained from Peptide & Protein Research, University of Reading. The peptide was purified to "immunological grade purity" of over 80%.

15 amino acid peptide (spanning C219 epitope)

V-V-Q-E-A-L-D-K-A-R-E-G-R-T-C was obtained from Biomac, Department of Biochemistry, Glasgow University. This was screening grade (70%-95% pure) quality.

Clonab LNC was described by the manufacturers as "a murine antibody against an unrelated antigen and is provided as a control to assess the degree of non-specific binding of the primary antibodies of the Clonab product range. Clonab LNC is a mouse immunoglobulin of class IgG" (Biotest Product Information sheets, 1991). It was used as a negative control primary antibody in the early work with C219. Curious results were obtained with this product and subsequent requests for further information on this product revealed that this was actually a 5ug/ml IgA solution, and not an IgG product as initially published (Anita Baker, Biotest UK, personal communication). Clonab LNC was obtained from Biotest UK , Solihull, West Midlands.

Rabbit anti-mouse Ig Alkaline Phosphatase conjugate

This was obtained from Dako Ltd and was used at a 1 in 50 dilution in routine immunohistochemistry.

Sheep anti-mouse Ig Horseradish peroxidase conjugate

This was obtained from Amersham International and was used at a 1 in 5000 dilution in Western immunoblots.

Vectastain ABC *Elite* Kit

This kit contains all the reagents necessary for a peroxidase, avidin/biotin based detection system for murine primary monoclonals. Details of its use are given in later sections. It was purchased from Vector Laboratories, Peterborough, UK.

Vector Blocking Kit

This kit contains reagents which are used to block nonspecific binding of the Biotin/Avidin components of the Vector ABC kit. It was purchased from Vector Laboratories, Peterborough, UK.

Normal sera

Normal dog, rabbit & mouse serum was obtained from Sigma chemicals.

2.1.9 MOLECULAR PROBES

MDR5A

The MDR5A clone represents a portion from the middle third of human *mdr1* (Ueda *et al*, 1987a). The 1383bp produced by *EcoR1* digestion of the pHDR5A cDNA clone (corresponding to positions 1178 -2561 of the full length cDNA) was subcloned into pGEM4 (Promega, Biotech). This plasmid was kindly supplied by Dr. M Gottesman, National Cancer Institute, Bethesda. The pGEM4 plasmid permits production of riboprobes from SP6 or T7 RNA polymerase transcription start sites. Riboprobes for use in RNAase protection assays were produced following linearisation of the MDR5A construct with *Xmn 1* (figure 4.1). Detailed sequence maps of this probe are provided in chapter 4. Riboprobes for Southern blots were linearised with *BamH1*. All dot-blots and Northern blots were hybridised with full length MDR5A which had been released from pGEM4 with *EcoR1* digestion and then gel purified prior to random prime labelling of the DNA.

pEX1/172

This 172 base pair probe encodes the last exon of the hamster Pgp2 gene (Ng *et al*, 1989). This exon was described as exon 1 in this original paper but it is the equivalent of exon 27 or 28 (depending on species) in later publications. This short sequence does not contain any recognition sites for any of the common 6bp restriction enzymes. This exon has a high homology to other *mdr* genes; 98% to Pgp1 and Pgp2, 94% and 95% to murine *mdr*1a and *mdr*1b, 92% and 91% to human *mdr*1 and *mdr*3. Ng *et al* (1989) have used this conserved sequence to identify *mdr* gene family members in the hamster.

7S

This probe is a clone of the murine abundant cytoplasmic 7S ribosomal RNA (Balmain *et al*, 1982) which is highly conserved across species. It hybridizes to a 0.3kb band in the mouse, human and dog. The 7S probe is used to normalize for loading on Northern transfers.

POLYd(T)

Polyd(T) hybridizes with polyadenylated RNA and therefore provides an estimate of total mRNA loading. It is used on dot-blot hybridisations to assess RNA loading. It was obtained from Pharmacia LKB.

ACTIN (γ)

A *Bam -Hind* III fragment of a clone of human γ actin (Gunning *et al*, 1983) was previously subcloned into psP64. This clone is linearised with *Hinf 1* to generate a template for transcription with SP6 Polymerase. Human γ actin protects an 145 base pair fragment of this probe (Enoch *et al*, 1986).

FELINE TCR β CHAIN REGION PROBE

This clone represents V, D, J & C sequences of the feline TCR β chain which had become transduced by Feline Leukaemia virus (FeLV). This viral-*tcr* was isolated from field case of thymic lymphosarcoma in a young adult cat, code name T17 (Fulton *et al,* 1987). The clone was obtained by screening a T17 genomic library using the human constant region probe pB400 (Collins *et al,* 1985). The intronless FeLV transduced β chain has been partially sequenced within the constant region and is identical to the normal feline constant region within the sequenced portion (Neil *et al,* 1988). The transduced β chain was subcloned into pUC18; digestion of this construct with *Bgl II* releases a 390bp fragment which encompasses part of the constant region (from nucleotide 584 to 974 in the map provided in Fulton *et al.* 1987). The position of this fragment relative to the four constant region exons is illustrated in figure 6.1. The 5' *Bgl II* site is 58bp downstream of the start of exon 1. The 390bp fragment therefore spans the majority of the large first exon, all of the 2nd exon and part of the third exon. The 3' part of the 3rd exon, which encodes the transmembrane segment of the polypeptide, is not included in the *Bgl II* fragment.

The purified *Bgl II* fragment of the feline β chain was used in the majority of the Southerns in this study. Occasionally, the unpurified construct containing V-D-J sequences was used. Where this is the case it is mentioned in the text.

2.1.10 CANINE TISSUE SAMPLES

Samples of normal canine tissue were obtained from freshly euthanased dogs at the local Dog Home. Samples were snap frozen in liquid nitrogen and stored at -70^{o}C until use. Lymphomatous nodes were primarily collected from canine patients presented to the Department of Veterinary Surgery at Glasgow University. Excisional lymph node biopsies were carried out for diagnostic purposes; part of these biopsies were snap-frozen for future use. Dogs which were treated for lymphoma but subsequently relapsed were euthanased & the appropriate samples collected immediately after death. Samples were collected from dogs which died either at home or at the referring veterinary surgery as soon as was logistically possible but sometimes a delay of up to 12 hours had occurred. Some samples came direct from referring veterinarians or from cadavers brought in for necropsy. Limited clinical information is available on these cases.

2.2 EXTRACTION OF NUCLEIC ACIDS & PROTEINS

All work with RNA & DNA was carried out using autoclaved solutions & where appropriate DEPC treated solutions & equipment. Disposable gloves & plasticware were used throughout. DNA & RNA quantitation was performed using a combination of visual assessment on an agarose gel when run against known standards and spectrophotometrically using O.D.$_{260}$.

2.2.1 MICRODISMEMBRANATING TISSUE SAMPLES

Tissue samples used for nucleic acid or protein extraction were first powdered using the microdismembranator. The samples and the dismembranator chamber were kept frozen using liquid nitrogen during the procedure.

2.2.2 RNA EXTRACTION

RNA was extracted from powdered tissues & cells using RNAzol B. Monolayer cultures were lysed in the flasks with RNAzol. Suspension cultures were spun down & pelleted before extraction. The extraction procedure followed the manufacturers instructions. RNA for use in RNAse protection assays was reprecipitated following ethanol precipitation using 0.2M NaCl plus isopropanol. All of the normal tissues & cell line RNA was prepared using RNAzol B. However some of the earliest lymphoma samples underwent simultaneous DNA & RNA extraction detailed in 2.2.3, the section below.

2.2.3 DNA EXTRACTION

Powdered tissues & cell lines were prepared as above then lysed with 0.3M Na Acetate equilibrated phenol pH7.6. Blood samples (usually about 8mls) were prepared for DNA extraction by first lysing red blood cells using a five fold excess of RBC Lysis Buffer. After mixing for 10 minutes, the intact WBC were pelleted by centrifugation (1500g, 10mins). Approximately 0.5-1g of tissue was extracted with 15 mls of phenol; about 10mls of phenol was used per 175cm^2 tissue culture flask or for blood samples. An equal volume of lysis buffer and chloroform isoamyl alcohol was added & the samples mixed for 20 minutes. The samples were then spun at 3000g for 20 minutes at room temperature. The upper aqueous phase was collected & precipitated with an equal volume of isopropanol at 4^0C for at least one hour. The DNA was spun down at 3000g 20 minutes at 4^0C, airdried then resuspended in sterile water. This procedure simultaneously extracts DNA & RNA.

When RNA was the prime requirement, the procedure was essentially as above except the phenol was equilibrated with 0.3M Na Acetate at pH7.0 rather than pH7.6, the initial spin was carried out at 4^0C and the first ethanol precipitation was at -20^0C. These samples were then resuspended in TNM (0.14M NaCl, 0.01M Tris pH7.4, 1.5mM $MgCl_2$) to facilitate the use of RNAse free DNAse 1 at a future date.

2.2.4 PROTEIN EXTRACTION FROM TISSUES & CELL LINES

Tissues were powdered as described in 2.2.1 & the powder transferred into a 15ml Falcon tube. 2mls of protein lysis buffer was added, mixed thoroughly and put on ice for 15 minutes. The samples were then spun at 3,500g for 10 minutes to pellet debris. The supernatant was collected & protein estimation was carried out using the Biorad Kit method.

Protein lysis buffer:
0.1M Tris pH8
10% glycerol
0.5% Nonidet P40
1ug/ml pepstatin
1ug/ml aprotinin
1ug/ml chymostatin

2.3 PREPARATION OF ^{32}P RADIOLABELLED PROBES

With the exception of end labelled polyd(T), all other probes were separated from unincorporated ^{32}P-labelled nucleotides using disposable Sephadex containing "NICK" columns from Pharmacia. An aliquot was removed to measure incorporation in a scintillation counter.

2.3.1 EXTRACTION OF DNA FROM LOW MELTING POINT GELS

Plasmid DNA fragments for use as probes were prepared by digestion with the appropriate restriction enzyme followed by separation in a 1% Low Melting Point agarose gel made with 1xTBE containing 0.5ug/ml ethidium bromide. The DNA fragments were visualized on a UV transilluminator & the bands of interest were excised from the gel and placed in an clean tube. The gel slice was melted at 65^0C and approximately 3 volumes of TBE added. The DNA solution was maintained at 37^0C where an equal volume of equilibrated phenol was added & mixed thoroughly. The upper aqueous phase was removed following centrifugation at 13,000g for 5 minutes. The samples were then reextracted with phenol and chloroform before precipitation with 2 volumes of ethanol plus 0.1 volume of 3M Na acetate at -20^0C for at least one hour. The DNA was recovered by centrifugation at 13,000g, 15 minutes, washed, dried and finally resuspended in TE.

2.3.2 RIBOPROBE PRODUCTION

Radiolabelled single-stranded RNA probes (riboprobes) were prepared using the SP6/T7 Transcription Kit from Boehringer Mannheim. Riboprobes of the mdr5a plasmid for filter hybridisation used 1ug of template DNA which was linearised with *BamH1* and labelled according to the kit instructions for 90 minutes. Riboprobes for the RNAse Protection Assays used 1ug of *Xmn* I linearised mdr5a template DNA which was labelled for 30 minutes. In both cases, the DNA template was removed by DNAse 1. Riboprobes for the RNAse Protection Assays were phenol & chloroform extracted prior to use.

2.3.3. RANDOM PRIMING OF dsDNA

^{32}P labelled dsDNA probes were produced with the aid of the "Prime-it" random primer kit from Stratagene. Between 25ng and 100ng of template DNA was used & the protocol followed Stratagene's recommendations.

2.3.4 POLYMERASE CHAIN REACTION LABELLED PROBE

The probe pEX/172 (Ng *et al,* 1989) was obtained from Dr. Ling of the Ontario Cancer Institute, Toronto. The pEX/172 DNA template was provided, at an unknown concentration, in conjunction with pcr templates of an unidentified length. Labelling was performed exactly as detailed by Dr. Ling.

Labelling instructions;

5ul Template
2.5ul primer "A" (100ng/ml)
2.5ul primer "B" (100ng/ml)
5ul dATP, dGTP, dTTP mix. (2.5nM each)
5ul cold dCTP, 5uM
5ul ^{32}P-dCTP
20ul distilled water
5ul 10 x PCR buffer*
0.5ul Taq 1 Polymerase*
(* supplied from Northumbria Biologicals)

The mixture was covered with 2 drops of sterilised mineral oil. 25 pcr cycles were as follows;

Denature 94^0C, 30secs
Anneal 48^0C, 30secs
Extend 72^0C, 1 min

2.3.5 LABELLING POLYD(T)

Polyd$(T)_{20}$ (Pharmacia) was labelled using T_4 Polynucleotide Kinase (PNK) at 37^0C for 45 minutes as follows;

15ul polyd$(T)_{20}$ 1ug/ul
2.5ul "One-Phor-All" buffer (Pharmacia)
2ul $\gamma\,^{32}P$-ATP
4.5ul water
1ul T_4 PNK

The reaction was terminated by 20ul of 0.5M EDTA; the resultant labelled oligonucleotide was used without further purification.

2.4 SEPARATION & HYBRIDISATION OF NUCLEIC ACIDS

2.4.1 DIGESTION, SEPARATION & SOUTHERN TRANSFER OF DNA

Separation and transfer of DNA was essentially as described by Southern (1975). 20ug of genomic DNA was digested for at least 16 hours in a total volume of 150ul using 15ul of the required restriction enzyme. The digested DNA was then precipitated with 0.1 volume of 3M Na Acetate and an equal volume of isopropanol at -20^{o}C for 1 hour. The DNA was pelleted by centrifugation at 13,000g for 15 mins followed by air-drying and resuspension in 25ul of sterile water. Resuspension continued overnight at 37^{o}C and then transferred to 4^{o}C until use (usually within one week). 5ul of Dye Loading Buffer was added to the DNA and then run on a 0.6%-1.0% TAE gel. Gels were run overnight at about 40mA using a buffer recirculation system. The next morning, gels were stained with Ethidium bromide for 20 minutes to allow polaroid photography on a transilluminator. DNA was denatured for 20 minutes in 1.5M NaCl/0.5M NaOH followed by neutralization in 3M NaCl/0.5M Tris-HCl pH7.0 for 30 minutes. The gel was then rinsed in Genescreen buffer & transferred onto either Genescreen or Hybond N membranes overnight using Genescreen buffer. The membranes were rinsed in Genescreen prior to baking at 80^{o}C for 2 hours to fix the DNA on the membrane. Occasionally filters were UV fixed onto Hybond membranes.

2.4.2 SEPARATION & NORTHERN TRANSFER OF RNA

Methodology for separation and transfer of RNA followed instructions in Maniatis *et al,* (1982). 1% (w/v) agarose gels were prepared by dissolving 3g of agarose in 216mls of water then cooling to 60^{0}C. 54mls of 37% formaldehyde plus 30mls of MOPS(10x) was then added, mixed and immediately poured. 20ug of total RNA was freeze-dried to give a volume less than 5ul then redissolved in 15ul of RNA loading buffer plus 2ul of Dye Loading buffer. The samples were heated to 65^{o}C for 10 minutes then chilled on ice before loading on the gel. Gels were electrophoresed for 6-16 hours at 30-60mA. The gels were photographed then soaked in 50mM NaOH/10mM NaCl for 30 minutes prior to transfer onto Hybond N using Genescreen as the transfer buffer. Membranes were rinsed & baked as previously described.

2.4.3 RNASE PROTECTION ASSAY

Riboprobe production from *Xmn I* linearised MDR5A is detailed in section 2.3.2. The method is adapted from Melton *et al,* (1984).

Hybridisation: 20ug of total RNA plus 500,000cpm of riboprobe were dried in a speedi-vac & then dissolved in 20ul of hybridisation solution;

80% Formamide	
400mM NaCl	*Hybridisation*
40mM Pipes pH6.4	*buffer*
1mM EDTA	

The samples were incubated at 85°C, then the water bath thermostat was turned down to 45°C and held at 45°C for 3 hours. The water bath was then allowed to cool to room temperature overnight.

Digestion: Digestion was carried out at 30°C for 30 minutes in 300ul digestion buffer;

10mM Tris pH7.6
5mM EDTA
0.3M Na acetate
2ug/ml RNAse A
30U/ml RNAse T_1

Digestion buffer

Following digestion, the samples were incubated with 2.5ul Proteinase K (10mg/ml) plus 3.2ul SDS (20%) at 37°C for 15 minutes. The samples were phenol/chloroform extracted, precipitated with ethanol in the presence of 1ul of glycogen (Pharmacia) and dried in the speedi-vac.

Acrylamide gel electrophoresis:

The products of the above hybridisation/digestion were run on an 8M urea denaturing acrylamide gel prepared as follows;

37g Urea
15.98mls Acrylamide (30% acrylamide/0.8% bis acrylamide)
8mls TBE(10x)
Make up to 80mls once urea is dissolved.
Degas, add 30ul TEMED (Biorad) & 300ul Ammonium persulphate

Dried samples were resuspended in 4ul of RNAse loading buffer, heated to 95°C for 5 minutes, chilled on ice and then electrophoresed at 30mA until the gel had run the desired distance. The gel was peeled off the plates onto blotting paper, wrapped in clingfilm and exposed to radiographic film. Occasionally, gels were dried under vacuum prior to autoradiography.

2.4.4 DOTBLOTTING OF RNA

20ug of RNA in TNM buffer (total volume of 150ul) was pipetted along the 1st and 5th rows of a 96 well plate. The RNA was DNAsed for 30 minutes to remove any trace DNA in the samples. One third titrations of the samples was then performed in the microtitre plates; the final volume of each well was 100ul. The contents of each well was then transferred onto Hybond N using a dotblot manifold.

2.4.4 HYBRIDISATION IN FORMAMIDE SOLUTIONS

Hybridisations in formamide were performed in a shaking water bath for a minimum of 16 hours.

Northern blots were initially hybridised in formamide hybridisation mix (see section 2.1.5) at 56°C but this method was found to be less sensitive than non-

formamide hybridisation at 65°C and hence was abandoned in favour of the latter methodology for the Northerns shown in this thesis and all dot-blot hybridisations.

Southern blots were routinely hybridised in non-formamide solutions. However one Southern blot using the MDR5A probe was hybridised at 42°C in formamide hybridisation solution. This Southern is indicated in the text.

2.4.6 HYBRIDISATION IN NON-FORMAMIDE SOLUTIONS

Hybridisations in non-formamide solution (recipe in section 2.1.6) were carried out at 65°C (for a minimum of 16hours) using a Hybaid oven and Hybaid roller bottle system.

2.4.7 DOT-BLOT HYBRIDISATIONS

Dot-blot hybridisations with the MDR5A probe were carried out in non-formamide hybridization solutions as detailed in 2.4.6. Hybridisation with polyd(T) was carried out in polyd(T) hybridisation solution for 1 hour at room temperature.

2.4.8 WASHING FILTERS

Following formamide hybridisations, filters were initially washed in 1 x SSC plus 1% SDS (60 minutes) followed by a 65°C wash with 0.1 x SSC, 1% SDS for 30 minutes.

Following non-formamide hybridisation, membranes were washed at 65°C using 1 x SSC plus 5% SDS for at least 90 minutes with a minimum of 5 changes of wash buffer.

Following room temperature polyd(T) hybridisation, dot-blots were washed at room temperature in 1 x SSC/5%SDS for 1 hour with 4 changes of wash buffer.

2.4.9 STRIPPING FILTERS

Stripping filters, especially northern blots, was avoided where possible. When necessary, blots were stripped by agitating briefly in 1% boiling SDS and allowing to cool in the SDS solution.

2.4.10 AUTORADIOGRAPHY

Following washing, filters were blotted dry, wrapped in clingfilm and exposed to Kodak AR film in a film cassette with fast tungstate intensifying screens. Loaded film cassettes were held at -70°C until developed. Southern blot hybridisations required up to 3 weeks exposure time whereas RNA hybridisations with mdr5a probes required 2-7 days. 7S and polyd(T) probes gave adequate exposures within hours.

2.5 SEPARATION & IMMUNODETECTION OF PROTEINS

2.5.1 SAMPLE PREPARATION

Protein extracts were prepared & quantified as detailed elsewhere. 150ug of total protein in 50ul was mixed with an equal volume of 2 x Protein loading buffer and loaded without boiling.

2.5.2 GEL & RUNNING CONDITIONS

Denaturing protein gels were cast & run in the Protean gel tank system (Biorad). Gels were prepared as follows;

54g Urea

18.6mls Acrylamide(30% Acrylamide with 0.8% bis acrylamide)

10mls Electrophoresis buffer

5mls SDS(20%)

23.7mls water.

The mixture was degassed & then set with 1ml of 15% ammonium persulphate plus 25ul Temed (Biorad).

Gels were run overnight at 25mA in electrophoresis buffer containing 1% SDS.

2.5.3 ELECTROBLOTTING

Electroblotting was performed using a millipore semi-dry electroblotter. The membrane was wetted in Electroblot Transfer Buffer; 6 sheets of 3M Whatmans filter paper was sandwiched next to the anode & cathode with the membrane & gel layered in between. Transfer took place over 1 hour at 200mA. The membrane & gel were then stained in Poinceau's stain for 5 minutes and destained in 5% acetic acid. This allowed a visual assessment to be made of the evenness of the transfer and the integrity of the proteins.

2.5.4 IMMUNODETECTION OF PROTEINS ON WESTERN BLOTS

Biotin/iodinated streptavidin method

Blocking 3% BSA in TBS with 0.05% Tween 20 was used as a blocking solution. Membranes were blocked for at least 1 hour at room temperature with 3 changes of block solution in the first 30 minutes to remove any acetic acid residues.

Primary antibody (C219) was used at 1ug/ml in a 3%BSA/TBS/0.05% Tween solution. The membrane was sealed in a plastic bag and incubated overnight at 4^0C on a shaker. The membrane was washed in TBS for 15 minutes with 3 changes of wash. Anti-mouse IgG Biotin conjugate (Sigma) was applied at a 1 in 20,000 dilution in block solution for 1 hour. A second TBS wash of 30 minutes with 3 changes of wash preceded the final readout system. The membrane was incubated for 30 minutes at room temperature with 50uCi of ^{125}I Streptavidin (Amersham International) in

50mls of block. The filter was then washed extensively for three hours with numerous changes of buffer before autoradiography.

Chemiluminescence method

Blocking, and all antibody dilutions, were in 10% Marvel, TBS/0.05% tween 20. Washes were in TBS/0.05% tween. All procedures were carried out at room temperature. Block was applied for 1 hour prior to C219 (10ug/ml) for 2 hours. The first wash in TBS was for 15 minutes with 3 changes of buffer. The second antibody, anti-mouse IgG Horseradish peroxidase conjugate (Sigma), was diluted 1 in 5000 and applied for 15 minutes. The second wash for 30 minutes had a further 3 buffer changes. The membrane was then incubated with a chemiluminescense substrate (ECL kit from Amersham) as per manufacturers instructions & exposed to radiographic film.

2.6 IMMUNOHISTOCHEMISTRY

2.6.1 PREPARATION OF SLIDES & SECTIONS

Slides were pre-coated with Vectabond (Vector Labs). Frozen tissue sections were cut at 5uM by Mr. Ian McMillan of the Veterinary Pathology Department & Mr. Angelo Kyriakides of the Veterinary Surgery department. Cytospins of 3132 & Cl-1 cells were prepared using approximately 200,000 cells per slide. The KB cells were grown on coverslips and then the coverslips were stuck onto slides using DPX mountant. Fixation was in acetone at room temperature for 10 minutes.

2.6.2 ALKALINE PHOSPHATASE CONJUGATE STAINING

All lymph nodes, cell lines and the majority of canine normal tissues were stained using this methodology, which was adapted from Wishart *et al,* 1990. The evolution of this particular protocol is discussed in the relevant results section. The procedure was carried out at room temperature as follows;

Wash: TBS/0.05% tween
Block: 1.5% rabbit serum, 1.5% dog serum in TBS/0.05% tween, 20 mins.
Primary Antibody: C219 5ug/ml in block solution.
Wash: 2 x 2mins in TBS/tween
Second Antibody: Rabbit anti-mouse Ig Alkaline Phosphatase conjugate (Dako), 1 in 20 dilution in block solution
Wash: 2 x 2mins in TBS/tween
Fast Red substrate: (see below) 20 mins
Wash: 2 x 2mins in water
Counterstain: Haematoxylin.
Sections were then mounted in Glycergel (Dako)

Substrate Solution

A batch of substrate (without Fast red salt) was prepared in advance and then aliquoted & frozen. First Veronal Acetate buffer was made;

0.97g Na acetate(trihydrate)

1.47g Na barbitone

2.5mls 0.1M HCl

247.5mls water. Final pH is 9.2

To make 20 x 5ml aliquots;

50mg Napthal AS B_1 was dissolved in 20 drops of dimethyl formamide. Add 100mls of Veronal Acetate buffer plus 100ul of 1M levamisole.

The 5ml aliquots were defrosted as needed and 2.5mg of Fast Red TR salt added within 10 minutes of use. The substrate solution is filtered through a 45um disposable filter (Gelman) before use.

2.6.3 ABC PEROXIDASE STAINING

This methodolgy was used for tissues such as the gastrointestinal tract plus central nervous system which gave unacceptable background with alkaline phosphatase staining. This was carried out at room temperature using the Vectastain *elite* ABC kit. The procedure was as outlined in the manufacturers instructions with some modifications. Prior to application of primary antibody, non-specific avidin/biotin binding was blocked using the Blocking Kit provided by Vector Labs. After the biotinylated second antibody was washed off, endogenous peroxidase activity was quenched using 0.3% peroxide in methanol for 30 mins. This was washed off before proceeding with the rest of the protocol. The final substrate was diamminbenzidine tetrahydrochloride (DAB).

2.6.4 AUROPROBE STAINING

This system is based on the reaction between a biotinylated secondary antibody and streptavidin coated gold spheres. The protocol was identical to the Alkaline phospatase method until the secondary antibody:-

Second antibody: Goat anti-mouse Ig biotinylated antibody (Sigma) at 1 in 10,000 dilution in block solution.

Wash: 2 x 5 mins TBS

Streptavidin-Gold: 1 in 50 dilution in TBS, 2 hours.

Wash: 2 x 5 mins TBS then 2 mins in water.

Silver Enhance: 15 mins

Rinse in water then counterstain with saffrenin.

2.7 DRUG SENSITIVITY ASSAYS

2.7.1 TETRAZOLIUM BASED ASSAY (MTT)

This assay is based on the principle that live cells (but not dead cells) can reduce the tetrazolium dye, MTT, to a coloured product that can be read in an ELISA plate reader and follows the protocol suggested by Plumb *et al,* (1989).

Cells were plated at 500 per well in RPMI onto triplicate 96 well plates (leaving the first row blank as a control) and left to acclimatize overnight in the incubator. Cytotoxic drug was then added at different concentrations per row and left for 24 hours. Drug was then removed and replaced by fresh media. (3132 and CL-1 grow in suspension so before any media changes, the 96 well plates were spun at 1000g for 10 minutes to pellet the cells.) The cells were left for 3 days (which is approximately 2 doubling times for these cell lines) before replacing the media with fresh RPMI containing 10mM Hepes buffer . MTT was then added at a concentration that was predetermined to give maximal absorbence without cellular toxicity in this system. Plates were wrapped in foil & incubated for a further 4 hours. The media was then carefully removed & the insoluble formazan crystals dissolved in DMSO plus Sorenson's buffer (0.1M glycine, 0.1M NaCl pH10.5). Plates were then read in an ELISA plate reader at 570nm.

2.7.2 SOFT AGAR CLONOGENIC ASSAY

Cells were plated at 500 cells per 6cm plate in 3% Bacto agar/RPMI 20% foetal calf serum. Prior to plating, 3132 cells were gently pipetted up & down to disaggregate cell clumps. Cytotoxic drugs at different concentrations were incorporated into the soft agar mixture, each concentration was in triplicate. The plates were then incubated for 3 weeks before colonies were counted. A colony was defined as a group of 50 or more cells.

2.7.3 METHOCELL CLONOGENIC ASSAY

Clonogenic assays in methocell were essentially as detailed for Bacto agar except the cells were plated in 0.9% methocell.

2.7.4 CONDITIONED MEDIA PRODUCTION

Cells were scaled up to roller bottle quantities. 150mls of fresh media was added and left for three days. The media was then harvested, cleared of cells by centrifugation and filter sterilized. It was frozen at -20^{o}C in aliquots.

CHAPTER 3
DEVELOPMENT OF AN IMMUNOHISTOCHEMICAL TECHNIQUE FOR THE DETECTION OF P-GLYCOPROTEIN

3.1 C219 DETECTS P-GP IN CANINE TISSUE

Monoclonals against P-gp have been easily available for several years. MRK16 (Hamada and Tsuruo, 1986) JSB1 (Scheper *et al,* 1988) and C219 (Kartner *et al,* 1985) represent three of the most widely used reagents.

MRK16 is commonly used in human cancer research and has the advantage of being *mdr1* specific. The external epitope recognised by MRK16 is thought to be human specific because it does not react with rodent cells (Tsuruo *et al,* 1989). There is no published work mentioning the reactivity of MRK16 in species other than human and rodents. Despite its' lack of reactivity with rodent tissue, it was felt that it should be established whether or not this reagent could be useful in the dog. The MRK16 epitope is known to be best preserved in unfixed or formalin fixed tissue (Grogan *et al,* 1990) so MRK16 was tried on both unfixed and formalin fixed canine liver tissue using an alkaline phosphatase/fast red methodology (detailed in section 2.6.2) and 10ug/ml of MRK16. Liver was selected as the test tissue because it has expression of an *mdr1* isoform in every species studied (Fojo *et al,* 1987a; Bradley *et al,* 1990; Gant *et al,* 1992) including canine (chapter 4). No positive staining was found in the canine sections (data not shown) which supports the view that MRK16 is not suitable for analysing canine lymphomas.

Of the two other monoclonal antibodies JSB-1 is also *mdr1* specific and is not species specific (Scheper *et al,* 1988). Unfortunately JSB-1 was reported to be less dependable than C219 in immunohistochemistry (Krishan *et al,* 1991; Van der Valk *et al,* 1990; G. Wishart, personal communication) and was particularly sensitive to most tissue fixation techniques (Pavelic *et al,* 1991). It was not used in canine tissue.

C219, which detects all known P-gp isoforms, has already been shown to detect P-gp in canine tissue (Lieberman *et al,* 1989) and was considered dependable (Grogan *et al,* 1990; Krishan *et al,* 1991). Using the alkaline-phosphatase immunohistochemical technique described by Wishart *et al* (1990) (and adapted in section 2.6.2), C219 produced staining localised to the canalicular surface of the canine hepatocyte (figure 3.1a). A concentration of 10ug/ml of C219 was used in these first slides. In the negative control (figure 3.1b), a 1 in 20 dilution of Clonab LNC (see section 2.1.8) showed a complete absence of reactivity.

A western immunoblot (methodology given in section 2.5) using C219 and an iodinated biotin-streptavidin system, confirmed that canine liver and kidney contain a protein recognized by C219 (fig 3.2). This protein is slightly smaller than the human and hamster P-gp (figure 3.2). Lieberman *et al* (1989) also reported the canine renal protein to be smaller than the human counterpart; 160kD compared to 170kD. Accurate sizing on the upper portion of the gel shown in figure 3.2 was not possible,

but the sizes would appear to be similar to Lieberman's. The canine lymphoma cell lines CL-1 and 3132 were negative for P-gp on Western immunoblot.

Figure 3.1 Canine liver C219 immunohistochemistry (AP)
(AP alkaline phosphatase)
a Canine liver plus C219, x40 magnification *(Top picture)*
b Canine liver plus Clonab LNC, x20 magnification *(Bottom picture)*

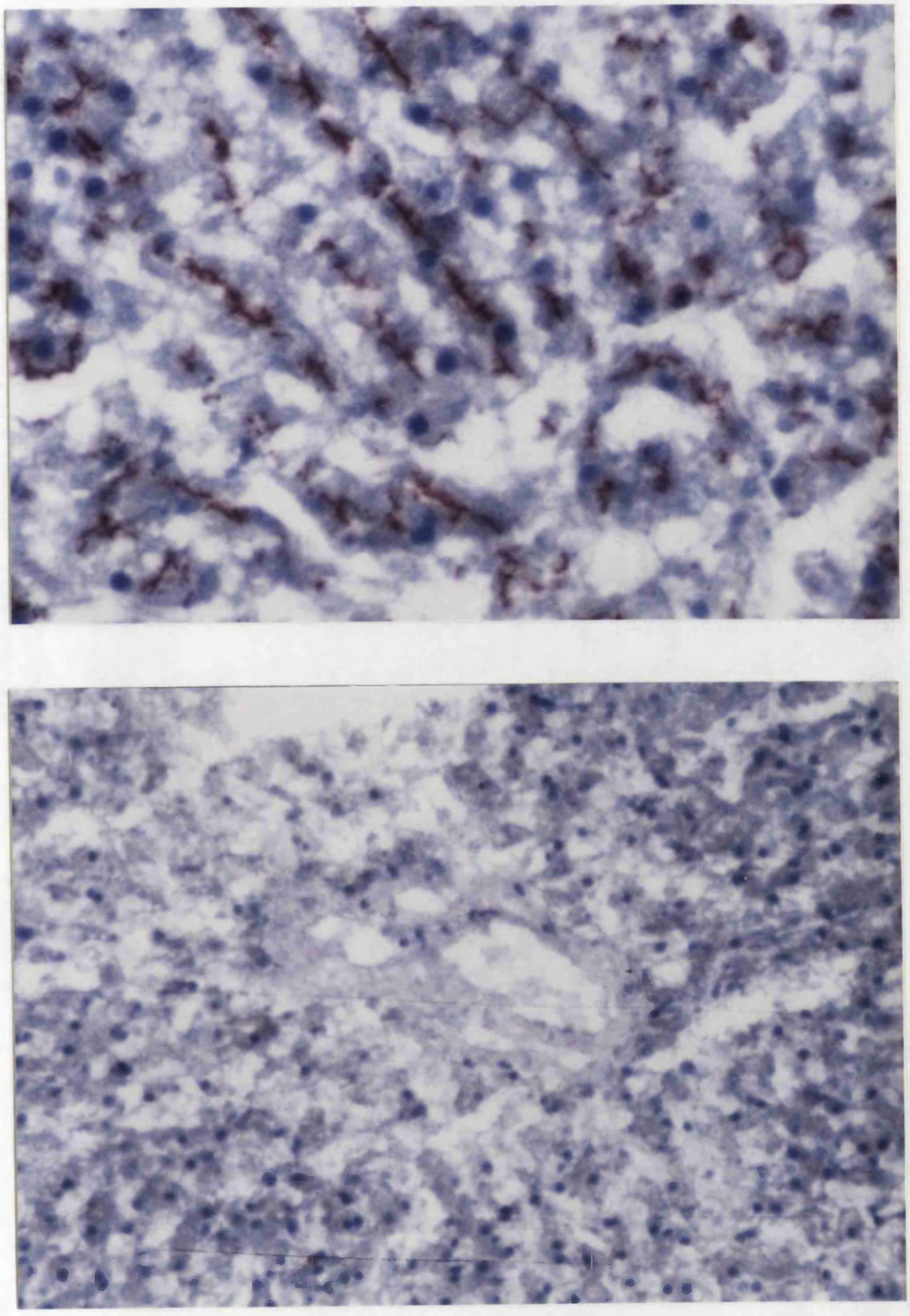
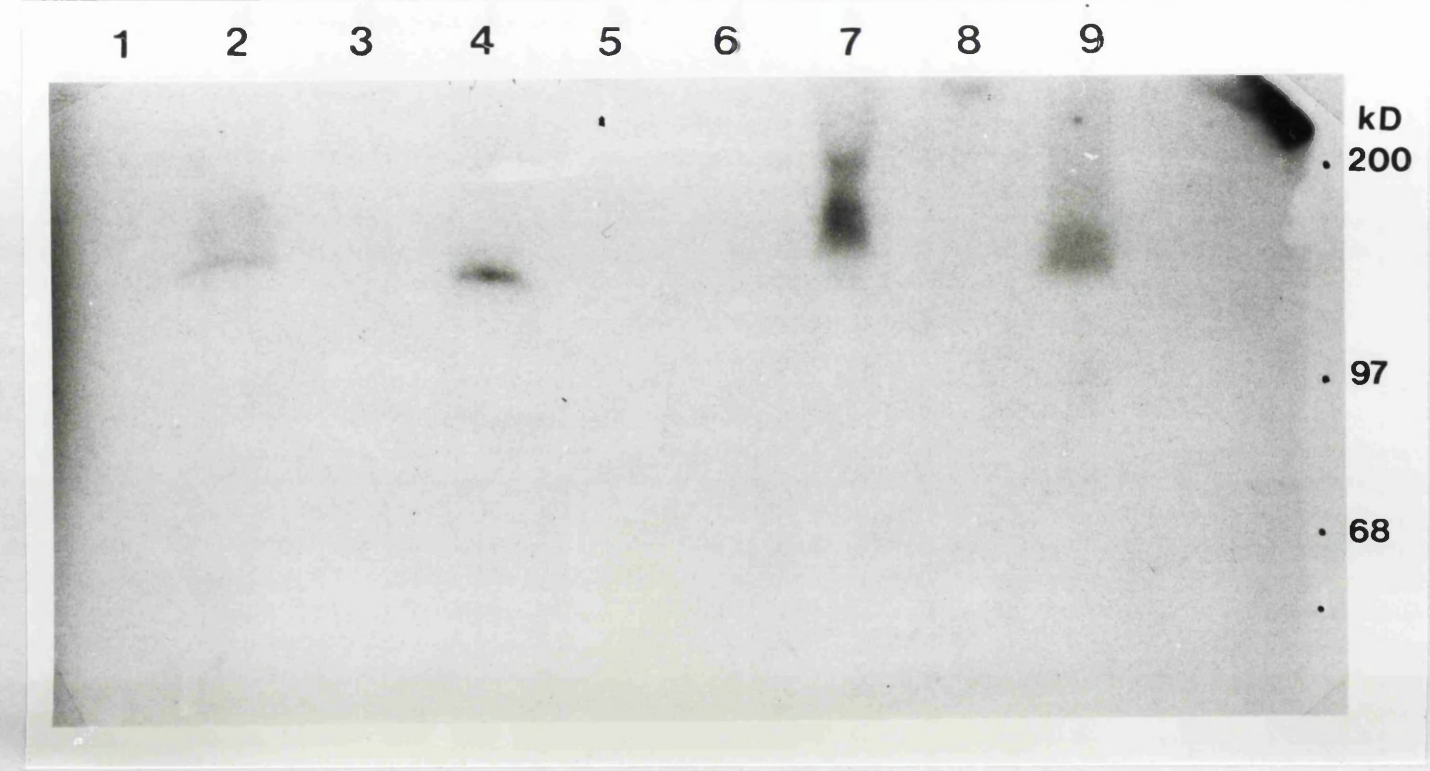


Figure 3.2 C219 Western Imunoblot of canine tissues and canine lymphoma cell lines

Lanes 1. 3132
2. dog kidney
3. dog pancreas
4. dog liver
5. CL-1
6. AuxB-1
7. CH_RC5
*8. A2780**
*9. A2780 AD**

***Methodology:** Samples were prepared and run according to section 2.5 using the biotin/iodinated streptavidin detection method.*

**A2780 AD is an adriamycin selected MDR cell line derived from the human ovarian carcinoma cell line A2780 (Rogan et al,* 1984)



3.2 TECHNICAL PROBLEMS WITH C219 IMMUNOHISTOCHEMISTRY (IHC)

The initial success with the first IHC protocol (based on Wishart *et al*, 1990) led to its use in subsequent IHC. This original protocol exposed sections to C219 before exposure to any other protein products i.e. there was no "blocking" step using normal serum prior to the application of the primary antibody. This protocol relied on the use of a Clonab product "LNC" as a negative control. Clonab supply this reagent specifically to act as a control for their monoclonal products. The LNC product was described as a mixture of IgG's suitable for use as a negative control in IHC.

During the investigation of the P-gp content of a canine sarcoma, it became apparent that there were problems with the use of the Clonab product. The sarcoma, shown in figure 3.3a, gave very intense cytoplasmic staining with C219 in a spindle shaped cell population. The Clonab negative control was completely negative (figure 3.3b). However, serial sections which had been exposed to 5% normal human serum instead of Clonab, gave the same positive result as the C219 section (figure 3.3c). The positive reactivity with normal human serum suggested that both the C219 and the serum staining was spurious, possibly through binding to Fc receptors. If this was the case, it seemed curious that the Clonab reagent did not show up this effect.

C219 is an IgG2a monoclonal and the normal serum would contain a mixture of isotypes. Given that within the Clonab range of monoclonals, there are IgM, IgG1, IgG2a and IgG2b products, one would expect the LNC reagent to have a mixture of all of these isotypes. The manufacturers were contacted to confirm the Ig content of their LNC product. After testing this product, they found it was a 9ug/ml solution of IgA. Thus, once Clonab LNC had been diluted 1 in 20 prior to use (following the protocol of Wishart *et al*, 1990), an IgA solution of 0.45ug/ml was being matched as a negative control for a 10ug/ml solution of an IgG2a monoclonal. Negative controls should ideally use isotype and concentration matched "irrelevant" monoclonals (Hanson, 1991). Clonab LNC obviously fails to meet these criteria. Isotype matching is important because Fc receptors can vary in their ability to bind monomeric IgG of different subclasses (Fanger et al, 1989) hence use of unmatched isotypes in controls can lead to spurious results.

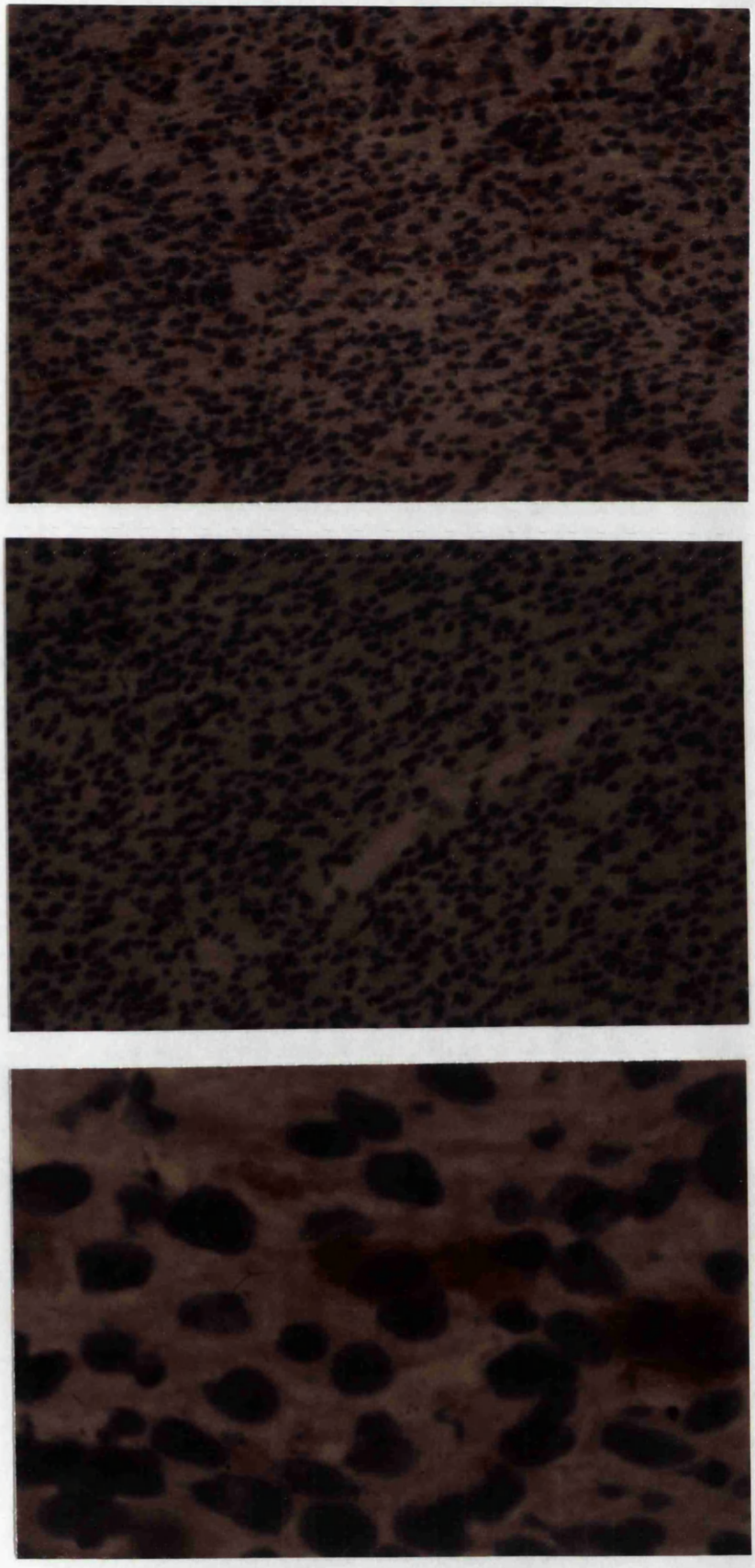
Clonab LNC was abandoned and alternate negative control reagents were investigated (see below).

Figure 3.3 Sarcoma immunohistochemistry with C219 and controls

3.3a Sarcoma plus C219, x10 *(Top picture)*

3.3b Sarcoma plus Clonab LNC x10 (*Middle picture*)

3.3c Sarcoma plus 5% normal human serum, x 50 (*Bottom picture)*



3.3 USE OF C219 EPITOPE SPECIFIC PEPTIDES IN COMPETITIVE IMMUNOHISTOCHEMISTRY AND WESTERN BLOTTING

Georges *et al*, (1990) determined the C219 recognition sequence using a series of overlapping hexapeptides covering the entire 211 amino acid fragment of the C-terminal of hamster *Pgp1*. C219 reacted most strongly with the sequence VQEALD and had very little activity with the adjacent overlapping peptides.

The different *mdr* isoforms in human and rodents were examined for the presence of this hexapeptide sequence in the C- and N- terminal halves of the P-gp. There was some variation in the exact hexapeptide sequence at the different sites between species (see table 3.1); new peptides representing these subtle variations were then examined for reactivity in the C219 ELISA based binding assay (Georges *et al*, 1990). The results, are illustrated in table 3.1.

Table 3.1 C219 binding to peptide analogs of recognition epitope

C-terminal domain	Peptide	Signal
Classes I, II, III (Rodent Classes I and III (Human)	VQEALD VQEALD	++ ++
N-terminal domain		
Classes I, II, III (Rodent) Class III (Human) Class I (Human: *mdr1*)	VQAALD VQAALD VQVALD	++++ ++++ +

Non-conserved amino acid residues are in bold script

From the results of Georges *et al*, (1990), several predictions can be made about the use of C219. Firstly, the combined signal from the N- and C-terminal binding sequences of the rodent class I and II isoforms would appear to be stronger than the human class I isoform i.e. C219 as an IHC reagent may perform better in rodent than human tissues. Indeed Thiebaut *et al*, (1989) reported that, under the same conditions, C219 reacted less strongly with human liver compared to rodent liver. The second prediction that can be made based on table 3.1 is that C219 may be more effective at detecting the human class III isoform of P-gp compared to class I. Thiebaut *et al*, (1989) described C219 staining in human muscle tissue (a class III expressing tissue) as "strong localisation" which contrasts with his comments about C219 staining in the liver.

Georges *et al* (1990) went on to use a fifteen residue peptide spanning the C219 hexapeptide recognition sequence as a control reagent in IHC. Preabsorption of C219 with a 100 molar excess of the peptide completely obliterated all specific staining in tissues. They remarked that the use of the peptide in a competitive binding

assay clearly resulted in enhanced specificity because it allowed them to identify non-epitope staining in tissues such as the seminal vesicles and colon. This epitope specific peptide therefore seemed an ideal approach to reduce misinterpretation in IHC.

A six aminoacid peptide (VQEALD) was synthesized for use in IHC. Unfortunately, in work carried out by Dr Nicol Keith, this peptide failed to block C219 in IHC or in Western immunoblots. Dr. Georges (personal communication) commented that this inability to block C219 could be due to the small hexapeptide failing to take up the correct conformation in solution (the original epitope mapping had used hexapeptides mounted on polypropylene pins).

Two longer peptides were generated; a ten residue peptide (VVQEALDKAR) and a fifteen residue peptide (VVQEALDKAREGRTC), described in section 2.1.8. The latter was identical to the peptide used in the immunohistochemistry reported in Georges *et al*, (1990). These two peptides were compared for effectiveness in blocking C219 reactivity in both IHC and Western immunoblot. Preabsorption of C219 with a 100 molar excess of the 15 residue peptide for 1 hour at room temperature prior to use in IHC completely obliterated specific activity. The MDR drug resistant cell line KBV-1 (see section 2.1.7) had no residual reactivity with C219 preabsorbed with the fifteen residue peptide (figure 3.4a and b). The ten residue peptide was less successful at blocking C219 reactivity. Even at a 1000 molar excess some staining in KBV-1 cells would remain (figure 3.4c). Figure 3.5 shows two western blot of normal canine liver which were probed with C219 plus different concentrations of each peptide. The ten residue peptide failed to block C219 binding to liver P-gp at a 1000 molar excess whereas the 15 residue peptide was completely effective at the same concentration. The western in the lower panel shows smeared reactivity below 100kD. This is presumed to be due to protein degradation.

The fifteen residue peptide was adopted for use in all further IHC using C219. Despite being completely effective at blocking in IHC at a 100 molar excess, the Western immunoblot suggested that a 1000 molar excess of peptide was needed. The 15 residue peptide was routinely used at a 1000 molar excess in all subsequent IHC. The peptide solution deteriorated with storage at $4^{o}C$ and was discarded and replaced by a fresh solution every two weeks.

Other measures were also adopted to improve the quality of staining. Sections were exposed to 3% rabbit serum for twenty minutes prior to the addition of primary antibody and the C219 concentration was reduced to 5ug/ml. Both of these factors helped to reduce background staining.

Figure 3.4 KBV-1 Immunohistochemistry: comparison of blocking peptides
3.4a KBV-1 plus C219 (*Top*)
3.4b KBV-1 plus C219 preabsorbed with 15 residue peptide (100M excess) (*Middle)*
3.4c KBV-1 plus C219 preabsorbed with 10 residue peptide (1000M excess) (*Bottom*)
All are at x50 magnification.

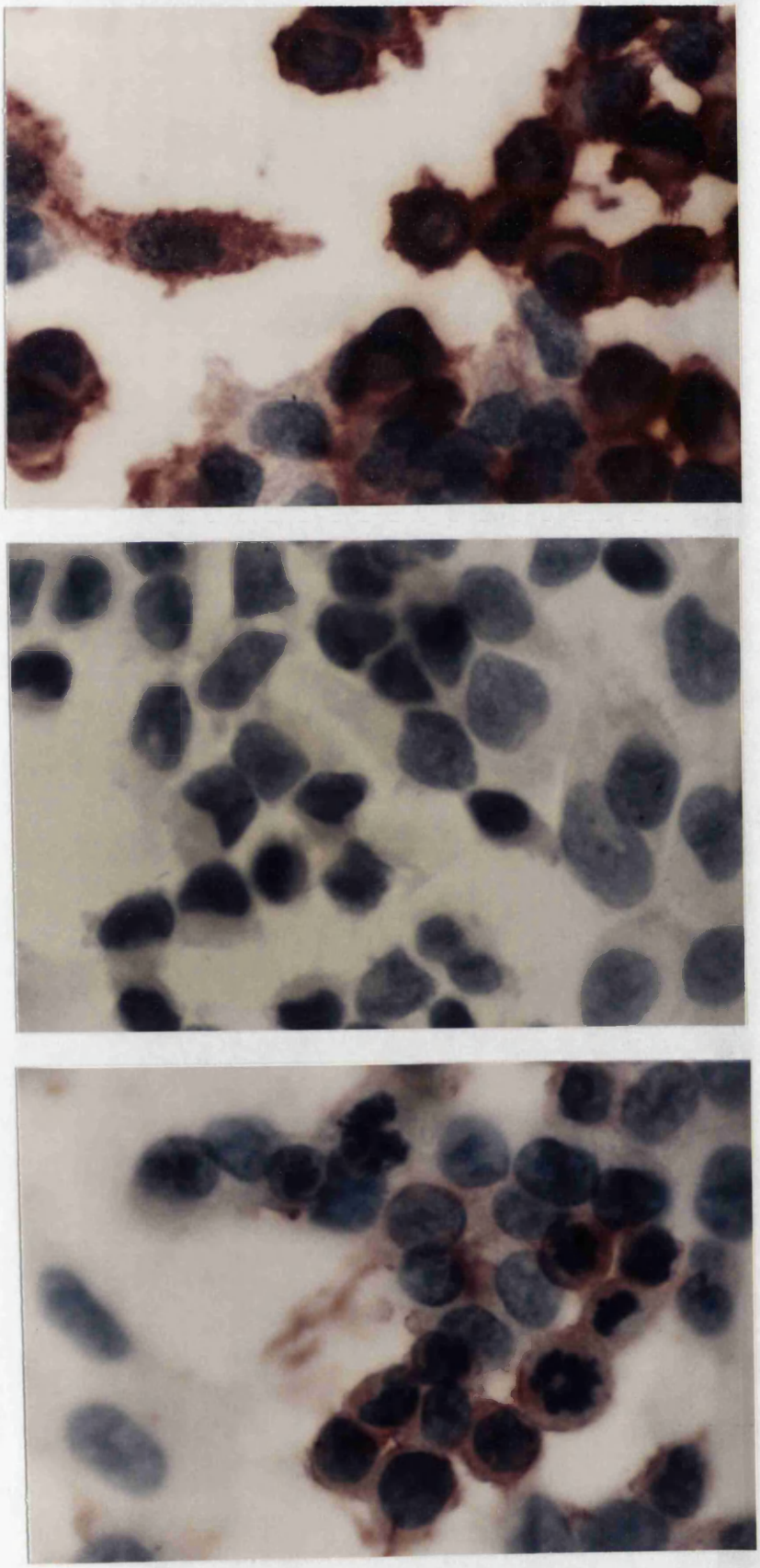
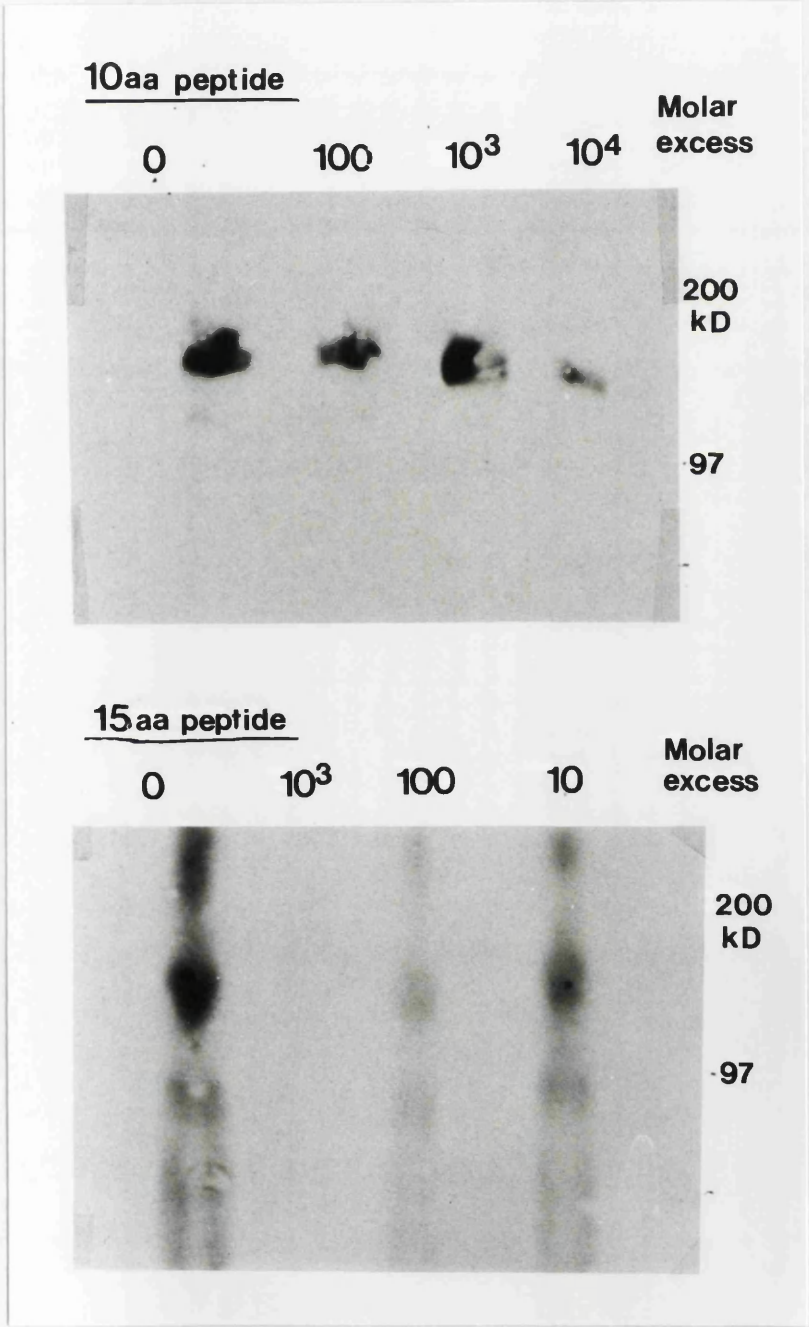


Figure 3.5 Western immunoblot: Canine liver plus C219 with titration of blocking peptide.

Equal quantities of canine liver protein extract were loaded in each well.(Methodology in section 2.5 using a chemiluminescence detection system). Where indicated, C219 was preabsorbed for one hour at room temperature prior to use.

Upper Panel
Titration of 10 residue peptide
1. no peptide preabsorption
2. 100 molar excess
3. 1000 M excess
4. 10^4 M excess

Lower Panel
Titration of 15 residue peptide
1. no peptide preabsorption
2. 1000M excess
3. 100M excess
4. 10M excess



3.4 USE OF COMPETITIVE IMMUNOHISTOCHEMISTRY TECHNIQUE TO DETECT P-GP IN NORMAL CANINE TISSUES

3.4.1 RESULTS

The normal tissue distribution of P-gp isoforms is discussed in the introductory chapter. Both C219 and *mdr* isoform specific antibodies have been useful in the investigation of normal tissue P-gp distribution in other species.

A small study was performed of normal canine tissues using C219 with the twofold aims of determining the similarity of the dog to other species, but also as a means of gaining familiarity with the use of C219 in IHC and hence gain expertise in scoring a range of P-gp expression critical for the assessment of lymphomatous nodes discussed in chapter 5. Tissues from a normal adult male dog were collected shortly after euthanasia.

Most tissues were stained using the alkaline-phosphatase/fast red based system described in section 2.6.2. However, certain tissues, including the gastro-intestinal tract, the CNS, adrenals and pancreas could not be assessed using the alkaline-phosphatase system because of endogenous alkaline phosphatase activity which resulted in a severe background problem. These tissues were stained using an avidin-biotin-peroxidase system provided as an "ABC" kit by Vector labs, detailed in section 2.6.3.

Figures 3.6-3.10 shows cardiac and skeletal muscle, adrenal and colon tissue stained with C219. The adrenal and colon were stained using an ABC-peroxidase system. Positive staining appears as a dark brown colour. Table 3.2 lists the normal tissues examined and indicates which tissues gave background problems such that they could not be adequately assessed by either the alkaline-phosphatase nor the ABC technique.

The most positive tissues were liver, muscle tissue and the adrenal. Lung, testis, bladder and ureter were not adequately assessed due to problems with the quality of the sections. Staining in Leydig cells was seen in some testis sections but was not reproducible. Pancreas and the CNS had background problems which were not overcome by switching to an ABC system nor by the inclusion of steps to block endogenous avidin/biotin binding or by endogenous peroxidase quenching.

Figure 3.6 Cardiac muscle immunohistochemistry with C219

3.6a Cardiac muscle plus C219, x50 (*Top)*

3.6b As above except C219 preabsorbed with blocking peptide (*Bottom*).

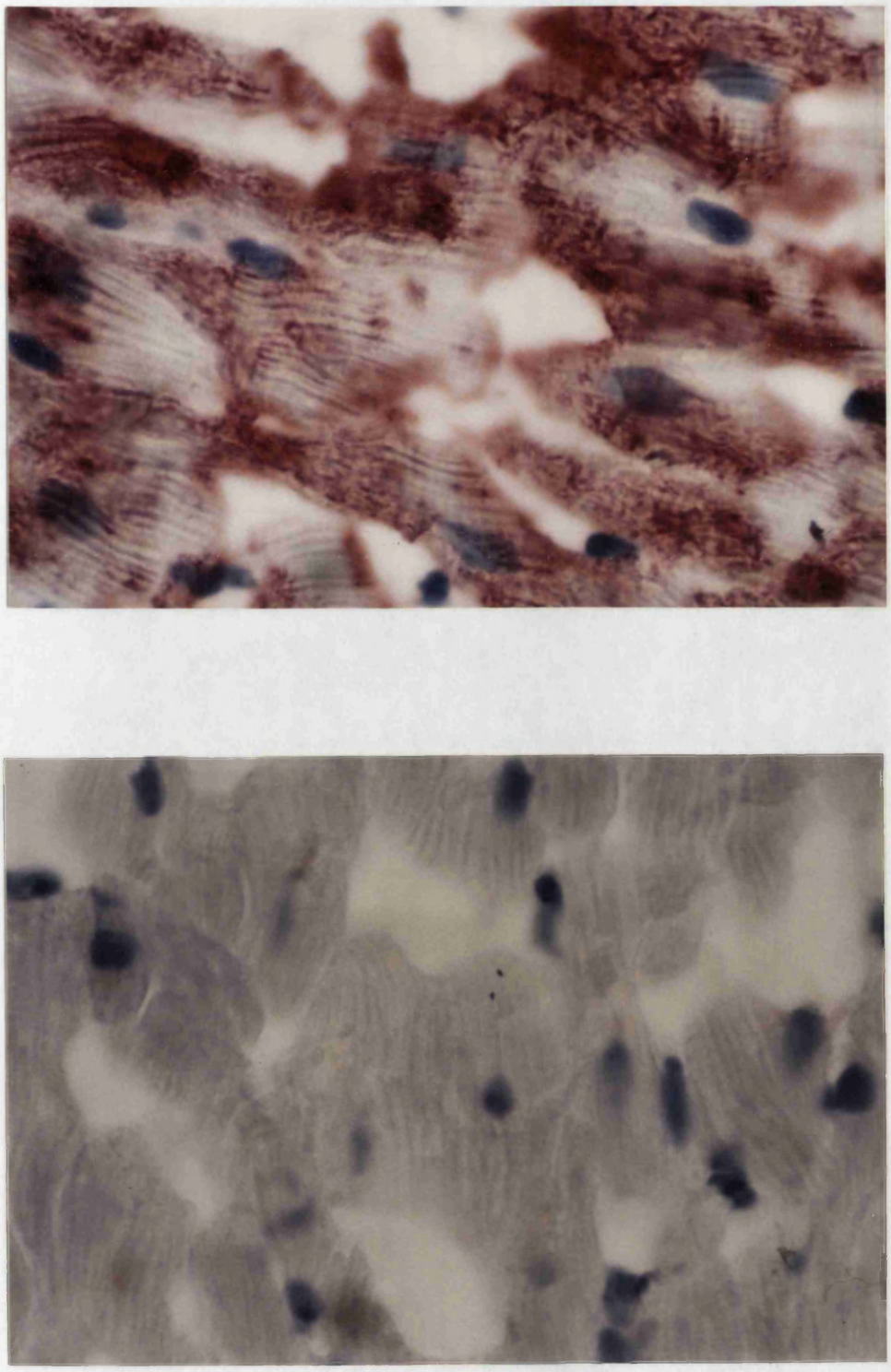


Figure 3.7 Skeletal muscle immunohistochemistry with C219, x20

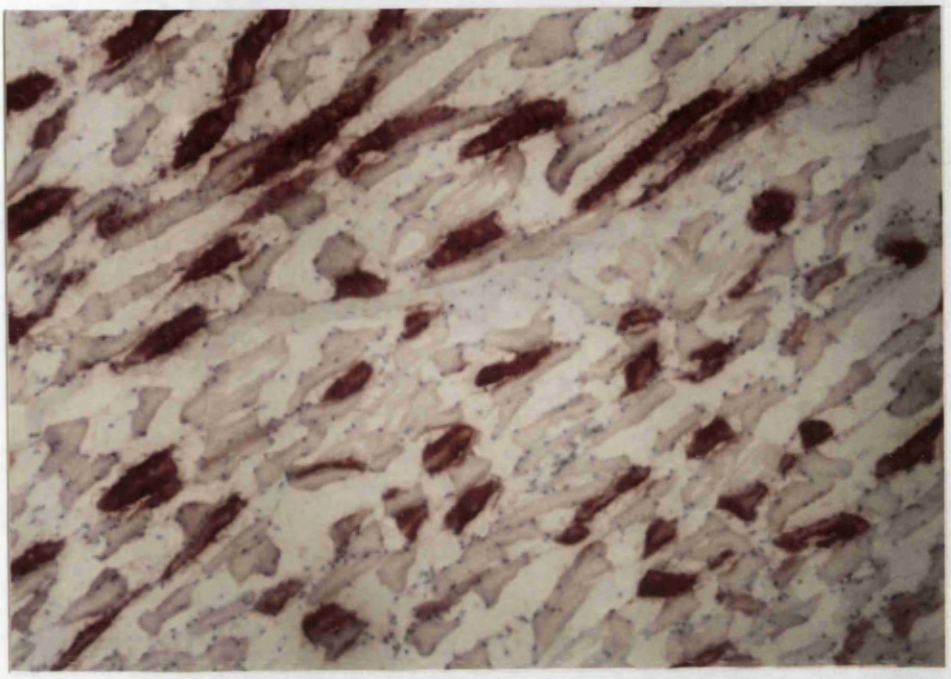


Figure 3.8 Colon immunohistochemistry with C219 (ABC-peroxidase), x40

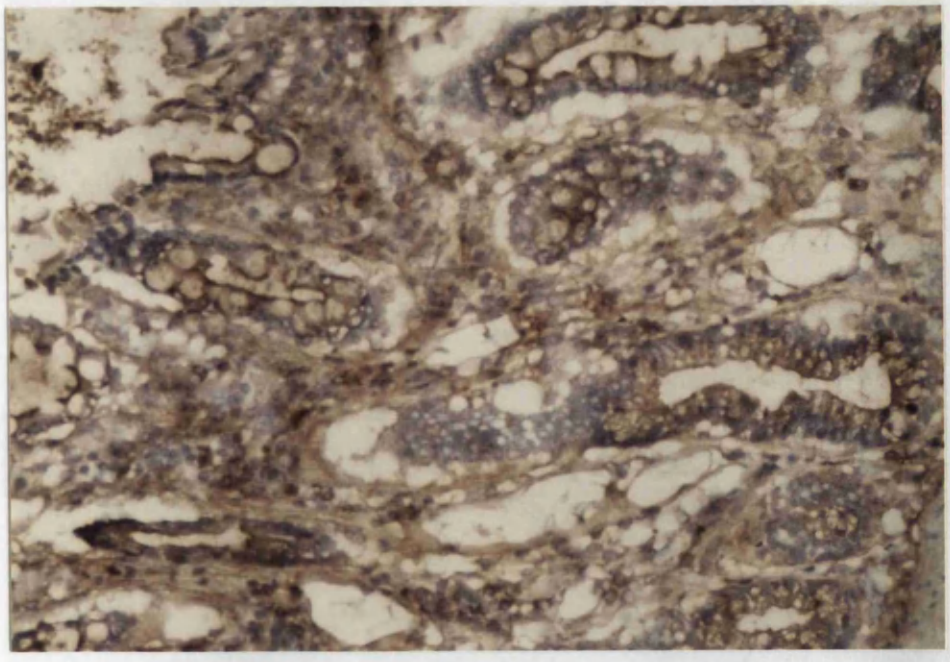


Figure 3.9 Adrenal immunohistochemistry with C219 (ABC-peroxidase technique)

3.9a Adrenal (x 4) *Top picture*

3.9b Adrenal (x 10) *Bottom picture.*

The P-gp negative Z. glomerulosa is the outermost layer (at top of picture) with the P-gp positive Z. fasciculata underneath.

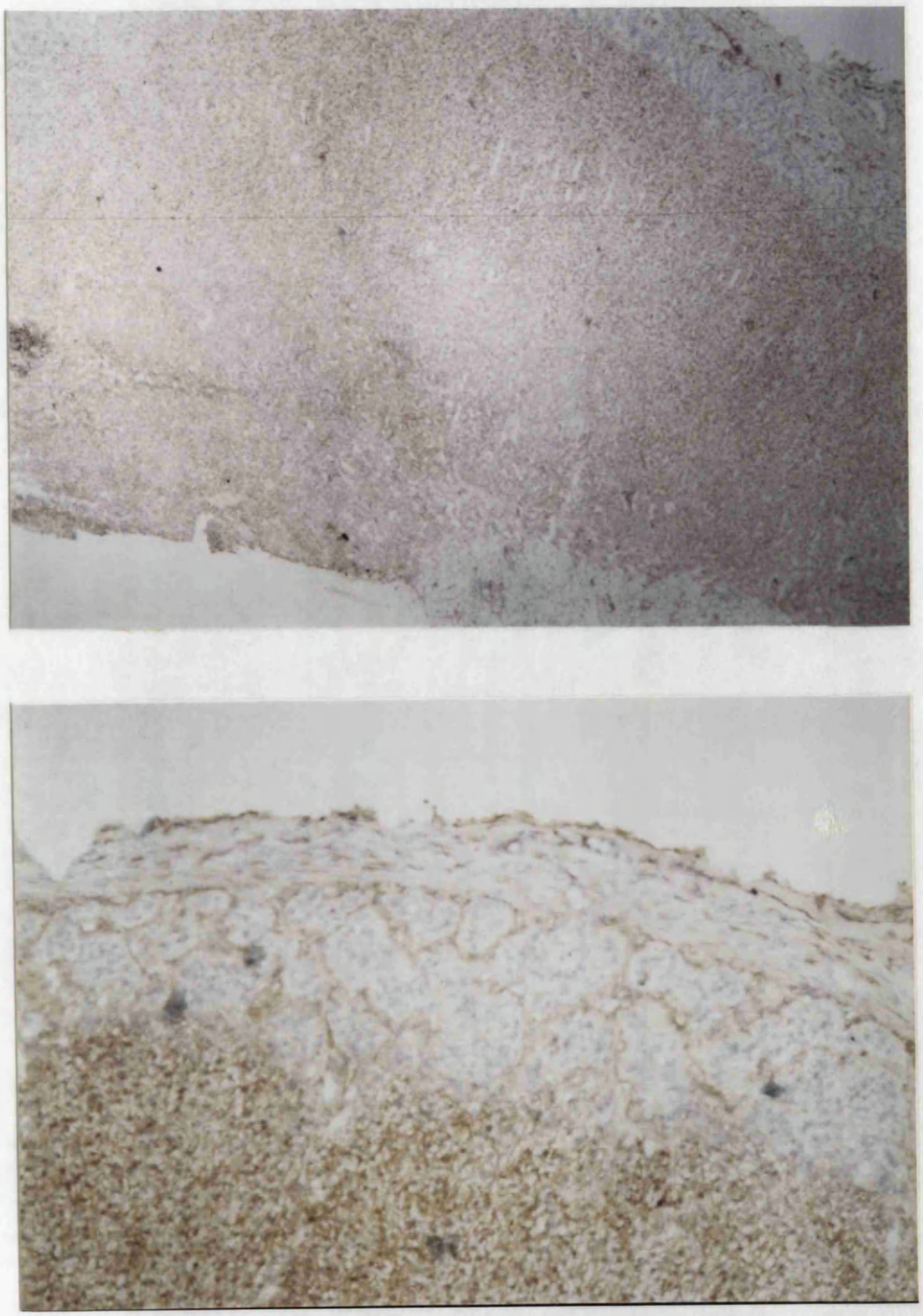


Table 3.2 P-gp in normal canine tissues

Tissue	P-gp	Comment
Liver	+	Hepatocyte canalicular surface
Pancreas	UI	Unacceptable background
Oesophagus	-	
Stomach	-	
Duodenum	-	
Jejunum	-	
Ileum	-	
Colon	+	surface and cytoplasmic staining of epithelial cells
Cardiac muscle	+	Striated staining not limited to cell surface.
Skeletal muscle	+	Striated staining in certain muscle fibres
Kidney	+	Collecting tubules positive Background in cortex
Ureter	UI	Poor tissue morphology
Bladder	UI	Poor tissue morphology
Prostate	-	
Testicle	UI	Staining in Leydig cells? Poor tissue morphology.
Lung	UI	Poor tissue morphology
Spleen	+	Minority dendritic cell population P-gp positive
Cerebrum	UI	Background stain in endothelia
Cerebellum	UI	Background stain in endothelia
Adrenal	+	Innermost zones of the cortex positive in the adult male

+ *positive*, - *negative*, UI, *uninterpretable*

3.4.2 DISCUSSION

In general the canine tissue distribution matched that of other species. Intense P-gp staining was found in skeletal and cardiac muscle, adrenal cortex and liver. Less intense staining was found in tissues like the kidney and colon. The staining of a dendritic-type cell population in the spleen is discussed further in chapter 5.

Liver P-gp was localized to the canalicular surface of the hepatocytes as expected from other species. Bradley *et al*, (1990) and Van der Valk *et al*,(1990) used

isoform specific antibodies to show that the P-gp in hamster and human liver respectively is of the class I isoform.

The pattern of staining in canine skeletal and cardiac muscle was identical to that reported by Bradley *et al* (1990). Cardiac muscle fibres and a subset of skeletal muscle fibres showed intense staining with C219 in a coarse striated fashion which may be due to staining of internal membranes of muscle fibres. This staining of cardiac and skeletal muscle by C219 was originally thought to be a cross-reaction to a muscle protein (Thiebaut *et al* 1989) because it was in discordance to the results of MRK16 staining of muscle tissue. As information became available about the tissue distribution of the different *mdr* isoforms (Croop *et al,* 1989; Bradley *et al,* 1990) it became apparent that this intense staining was due to C219 detecting the class III isoform in muscle tissue. It was predicted from the epitope-mapping of C219 (Georges *et al,* 1990) that C219 would be particularly effective at detecting class III versus Class I or II isoforms. This seems to be borne out by the intensity of staining in canine muscle tissue relative to liver.

Bradley *et al,* (1990) reported sex differences in the P-gp expression in the adrenal cortex of the adult male versus adult female. The male hamster expressed considerable class II P-gp in the adrenal cortex whereas the female did not. The adrenal shown in figure 3.6 is from a male dog and it has the same localisation as the hamster; the inner *zona reticularis* and the *zona fasciculata* stain strongly whereas the outermost *zona glomerulosa* is negative. The adrenals from a neutered bitch and an intact bitch (both of which had received immunosuppressive doses of corticosteroids prior to euthanasia) were examined and both had less intense staining with P-gp than the adult male. However, both nodes had marked adrenal cortical atrophy, presumably as a result of the steroid administration, and hence are not a valid comparison to the adult male. The adrenals from a normal adult female were not examined.

Minor differences between the dog results and published results in other species include the inability to detect P-gp in blood vessels in the CNS compared to rodent and humans (Cordon-Cardo *et al,* 1989, Bradley *et al,* 1990, Van der Valk *et al,* 1990). Unfortunately, in both the alkaline-phosphatase and the ABC stained CNS sections, there was staining of the blood vessels which could not be entirely abolished by the competitive peptide. It was therefore impossible to rule out some spurious staining at this site. Endothelial staining in the capillaries of the testes has been reported by the same groups as reported CNS endothelial staining (Cordon-Cardo *et al,* 1989, Bradley *et al,* 1990, Van der Valk *et al,* 1990). Again it was not possible to confirm this in dog testes primarily because of the poor quality of the sections and diffuse background problems in the interstitial spaces between the seminiferous

tubules. Initial staining suggested that Leydig cells may be P-gp positive, but this was irreproducible. The Leydig cells and blood vessels are packed together into the small areas between the seminiferous tubules so unless morphology is good, it can be difficult to get adequate visualisation of this area.

P-gp in the upper gastro-intestinal tract (stomach and small intestine) of the dog could not be found, but these tissues were particularly problematic regards background. Other workers have failed to unequivocally detect P-gp in the gastro-intestinal tract. Suguwara *et al*, (1988) could not detect P-gp in the GI tract of human samples using MRK16. Ichikawa *et al*, (1991) used a Western immunoblotting technique which could detect P-gp in the positive control cell line KB8-5 (described in section 2.1.7) and detected only minimal P-gp in colon membrane preparations with C219 in comparison to liver and adrenal. Bradley *et al*, (1990) did not detect P-gp in hamster stomach but did locate P-gp in the small and large intestines. However they commented that "the caecum and colon consistently demonstrated the highest levels of P-gp, whereas samples from elsewhere in the intestinal tract had barely detectable levels of P-gp". Van der Valk *et al*, (1990) described the staining in human stomach tissue to be "partly positive". The emerging impression is that P-gp expression in the GI tract may not be as ubiquitous nor as strong as might be implied in some of the first papers studying *mdr* mRNA expression (Fojo *et al*, 1987b; Mukhopadhyay *et al*, 1988; Chin et al, 1989). The ubiquity of P-gp in the GI tract is repeatedly reported in subsequent review articles (Kaye, 1988; Gottesman and Pastan, 1988; Van der Bliek and Borst, 1989), but this may be a simplification of the truth.

P-gp in the canine kidney was localised to the collecting tubules and not to the kidney cortex as might have been expected from previous work (Van der Valk *et al*, 1990). The kidney cortex gave faint staining in the glomeruli and the convoluted tubules but this staining could not be obliterated by the peptide so had to be dismissed as mainly background (data not shown). Other workers have also reported that the kidney cortex contained C219 staining that could not be abolished by peptide competition (Bradley *et al*, 1990) and concluded that the hamster kidney did not contain P-gp. They could offer no explanation for the Western results of Lieberman *et al*, (1989) who found P-gp in the isolated renal brush border membranes of the rat, dog and human also using C219.

This study has detected P-gp in canine kidney both by IHC and western immunoblot. In the western shown in figure 3.2, the signal from the kidney sample (which was from cortical tissue) was less intense than the signal from the liver sample. Yet according to the IHC results, the kidney cortex does not contain staining which can be abolished by competitive peptides and so the distinct and specific band on the Western is unexpected. It cannot be ruled out that the protein preparation

isolated from kidney cortex used in figure 3.2 was inadvertently contaminated with medullary tissue. Nor is it impossible that the canine kidney cortex does contain P-gp but that genuine staining in the cortex is being masked by non-epitope binding of C219 to the glomeruli and convoluted tubules. In support of this are the results of Lieberman *et al,* (1989) who used purified brush border membranes from human, rat and dog in their C219 Western immunoblots and obtained a band of the appropriate size in all their species. They remarked that they had to over-expose the immunoblots relative to the positive controls to get an adequate signal from the kidney tissue. The C219 probed western immunoblots produced by Ichikawa *et al,* (1991), like figure 3.2, showed a less intense signal in (human) kidney samples compared to liver. IHC of kidney cortex using MRK16 has repeatedly detected P-gp in kidney tubules (Theibaut *et al,* 1987 and 1989; Sugawara *et al,* 1988; Van der Valk *et al,* 1990) although Van der Valk *et al* (1990) described the kidney tubule staining as being only "partly positive". From this amalgam of results, it is not unreasonable to speculate that there is P-gp in the normal kidney cortex but due to tissue-specific background problems it is difficult to distinguish "signal" from "noise" using C219. Yet again the emerging impression is that P-gp expression in the kidney, like the GI tract, may be more limited than was first appreciated by initial RNA analysis (Fojo *et al,* 1987b; Kakehi *et al,* 1988).

3.5 SUMMARY

Normal canine tissues were assessed for P-gp presence using the monoclonal antibody C219. Following problems with non-specific reactivity of the monoclonal, a competitive immunohistochemical technique was adopted as a control to show specific P-gp reactivity. This technique used a fifteen residue peptide containing the recognition sequence for C219 to competitively block C219 specific binding. The peptide was shown to block binding at a 100 molar excess on IHC and on Western immunoblots. However, to allow a safe margin of error, the peptide was routinely used at a 1000 molar excess. C219 was preabsorbed with a 1000 molar excess of peptide for 1 hour at room temperature prior to use in IHC.

Strong P-gp expression was found along the canalicular surface of hepatocytes, in the inner zones of the adrenal cortex of the male and in cardiac and skeletal muscle. Less intense staining was found in the kidney and colon. The P-gp distribution in the dog would therefore appear to be similar to the distribution in other species.

CHAPTER 4
DETECTION OF CANINE *MDR1* HOMOLOGUE

4.1 CONSERVED EXON PROBE DETECTS FOUR PUTATIVE *MDR* GENES IN THE DOG

In the introductory chapter, the *mdr* gene family of rodents and man was described. Comparing the sequences available for the three hamster genes, Ng *et al* (1989) created a probe corresponding to the terminal exon (immediately adjacent to the 3' untranslated region) of *pgp2* gene as a means of determining the total number of genes in the hamster *mdr* family. This exon probe, pEX172, has 98% sequence similarity to hamster *pgp1* and *pgp3* genes, 94% and 95% with murine *mdr1b* and *mdr2* genes and 92% and 91% homology with the human *mdr1* and *mdr2* genes respectively. This 172 base pair fragment does not contain the recognition site of any of the common six base-pair cutting restriction enzymes nor does it cross-hybridise with the equivalent exon in the 5' homologous half of the molecule. It can therefore be used to detect individual members of the *mdr* gene family. The position of this probe relative to a prototype *mdr* gene is shown in figure 4.1.

Using this probe, Ng *et al* (1989) detected two bands in genomic digests of human and rhesus monkey DNA and three bands in the mouse and hamster genome. This corresponds to the number of known *mdr* genes in these species. The pEX172 probe was obtained from Dr. Ling and used to investigate the number of potential members of the canine *mdr* gene family.

The results of a Southern blot probed with the pEX172 probe are shown in figure 4.2. The canine samples (in lanes 1-3 and 7-9) were digested with *EcoR1, Hind III* and *Pvu II*. Each digest produced four discrete bands upon hybridisation with pEX172. The *EcoR1* and *Hind III* digests of murine and human DNA samples gave three and two bands respectively. (In lanes 6 and 12, faint bands present on the original autoradiograph have been lost in the photographic reproduction of the gel).

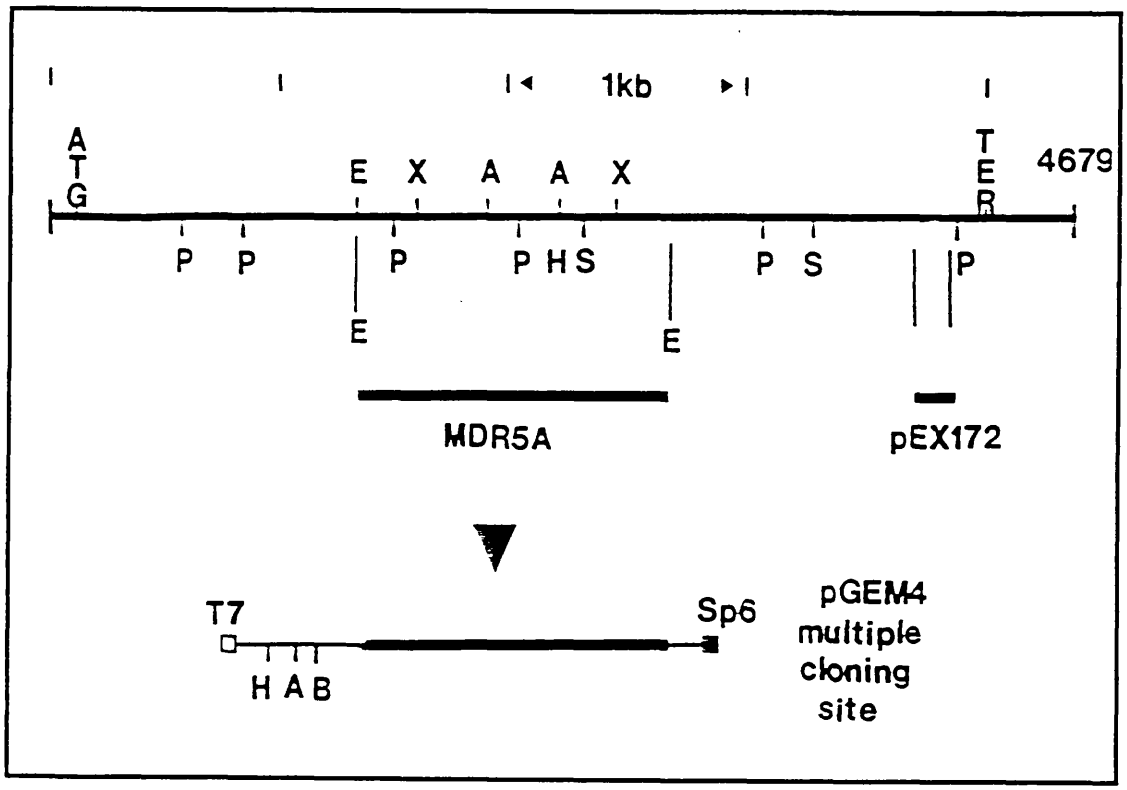
The pattern obtained in the mouse and human samples matches that of Ng *et al*, (1989). The results from the hybridisation to the canine genome suggests that the dog may have four *mdr* genes. The conserved fragment pattern of *mdr* genes between the canine cell lines 3132 and normal canine genome suggest that the cell line has retained all of its *mdr* genes. Curiously, one fragment in each digest of 3132 DNA gives a more intense signal which could represent amplification of that gene. However, in the *Hind III* digest of normal DNA (lane 8), the same band as in 3132 is also more intense. This increase in intensity may be due to sequence differences between the canine genes which results in stronger hybridisation to one particular gene. The difference in band intensity in 3132 could not be satisfactorily repeated so was not investigated further.

Figure 4.1 Position of *mdr* probes relative to human *mdr1* sequence

The base pair positions are calculated from the human mdr1 sequence, EMBL accession code M14758.

The MDR5A was subcloned into the EcoR1 site of pGEM4 (Promega Biotec) as described in materials & methods section 2.1.9. The MDR5A-pGEM4 construct was linearised using BamH1 (which cuts in the plasmid multiple cloning site) for riboprobe production used in Southern blots. Riboprobes for RNAase protection assays were linearised with Xmn I and transcribed using the Sp6 promoter. The Sp6 promoter adds 10 base pairs onto the size of the riboprobe products. The Xmn riboprobe total length is 279 base pairs.

The pEX172 probe is a pcr product using a hamster Pgp2 sequence as a template.



A, Acc 1; B, BamH1; E, EcoR1; H, Hind III; P, Pvu II; S, Stu I; X, Xmn I,

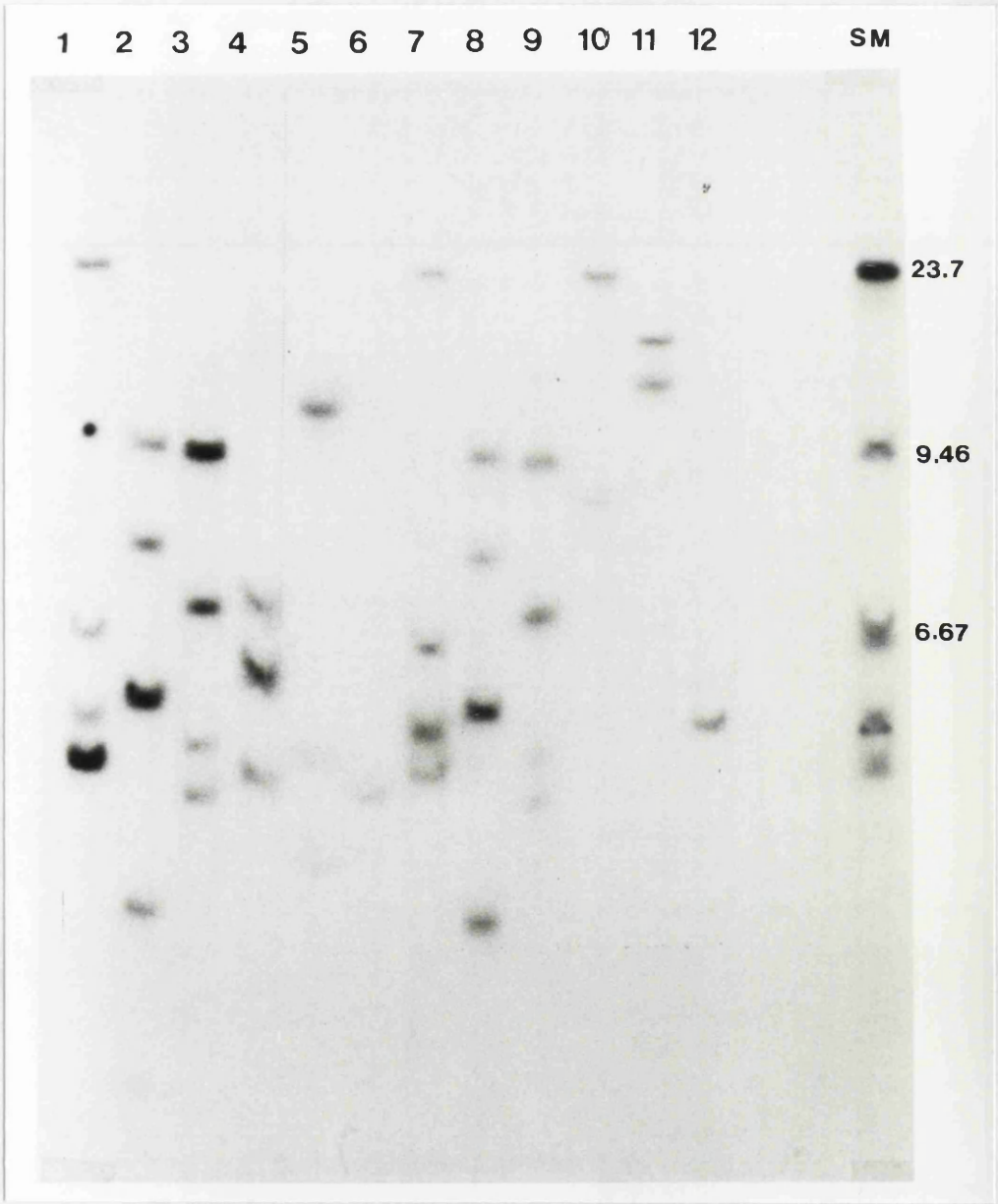
Sp6, Sp6 promoter, T7, T7 promoter.

Figure 4.2 Southern blot of canine genome probed with pEX172

Southern was run according to method in section 2.4.2 and hybridised using the non-formamide conditions (section 2.4.6). DNA sources were: 3132 (canine) cell line, NIH 3T3 mice, dog and human liver samples. SM = size markers

Lanes:

Digest	*3132*	*Mouse*	*Canine*	*Human*
EcoR1	*1*	*4*	*7*	*10*
HindIII	*2*	*5*	*8*	*11*
PvuII	*3*	*6*	*9*	*12*



4.2 HUMAN MDR1 GENE PROBE, MDR5A, HYBRIDISES TO CANINE GENE

The ultimate aim of this study was to investigate the expression of P-gp in lymphoma tissue and to determine if this P-gp expression affected clinical performance. As discussed in the introduction, only the *mdr1a/1b* genes are associated with the multi-drug resistance phenotype; the expression of *mdr2* genes does not have any proven relevance to clinical resistance. It would therefore be important to exclude the expression of *mdr2* when assessing *mdr* mRNA in lymphomatous nodes. This is especially important given that the immunohistochemical analysis of P-gp in canine tissues relies on the C219 monoclonal which does not differentiate between the drug-resistance relevant and irrelevant isoforms.

A human *mdr1* specific probe, MDR5A, was investigated for suitability to detect the canine *mdr1* equivalent gene or genes. The location of the probe within the human *mdr1* gene and its restriction map are given in figure 4.1.

It is not known if the canine resembles the human in having only one *mdr1* gene, or has multiple *mdr1* genes like rodents. The results from section 4.1 suggesting that the dog may possess four *mdr* genes means that multiple *mdr1* genes are a distinct possibility.

From a comparison of rat, hamster, mouse and human *mdr* sequences (Silverman et al, 1991), human *mdr1* more closely resembles the rodent *mdr1a* genes and in this respect one could expect the human MDR5A to cross-hybridise with the canine *mdr1a* equivalent. However the overall homology between *mdr1a* and *mdr1b* genes is high; murine *mdr1a* is 82.2% and *mdr1b* 78.7% homologous to human *mdr1*. Published results using MDR5A in rodent tissues are lacking so there is no information to indicate if this probe does detect the rodent *mr1a* and *mdr1b* genes. It is quite possible that the MDR5A probe could cross-hybridise with both *mdr1* genes if they exist, in canine tissue.

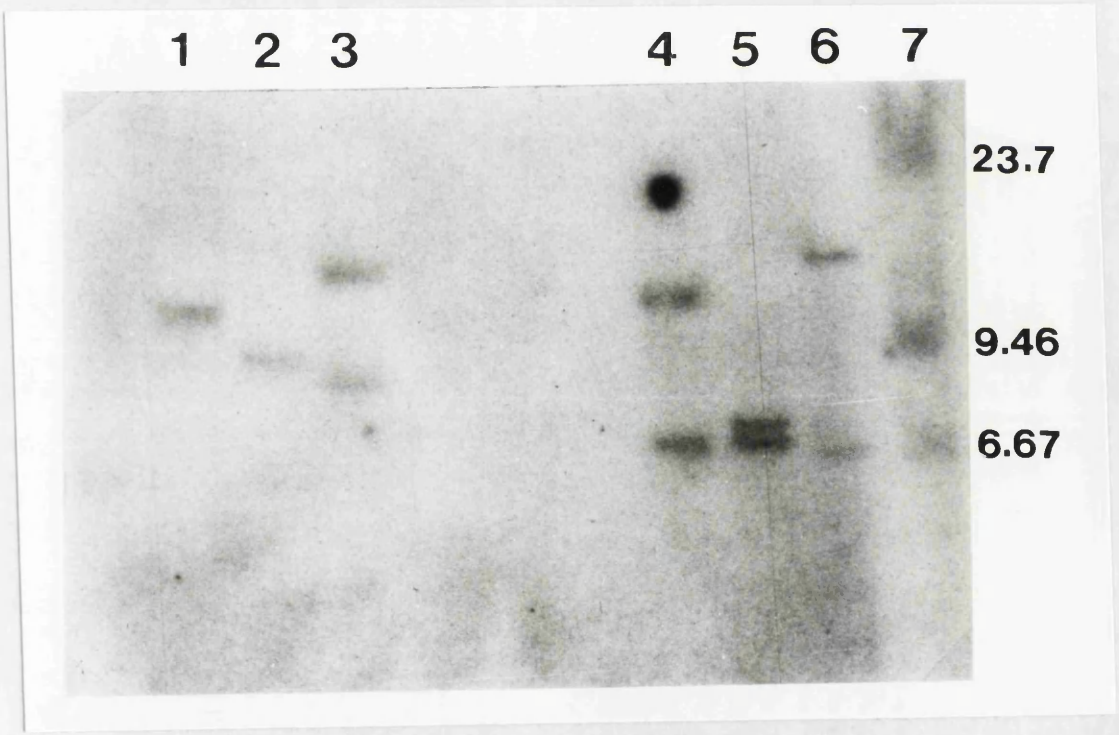
Figure 4.3 shows a Southern blot of canine and murine genomic DNA hybridised with an MDR5A riboprobe produced after plasmid linearisation with *BamHI*. The filter was hybridised in the presence of formamide as described in section 2.4.4. *EcoR1* and *Hind III* digested canine DNA (lanes 1 and 2), give a single band of approximately 12kb and 9.5 kb respectively. *PvuII* digested canine DNA produces two bands about 14kb and 8 kb in size. The murine genomic digests in figure 4.3 gave two bands in all three digests.

The dearth of information on the use of this probe in rodent tissue makes it impossible to determine if the two bands in each of the murine digests represent hybridisation to one or both of the murine *mdr1* genes. However, the canine pattern suggests that under these conditions, only one canine gene is detected, presumed to be an *mdr1* homologue.

Figure 4.3 Southern blot of canine genome probed with MDR5A

Hybridised under formamide conditions. Canine DNA obtained from cell line 3132, mouse DNA from NIH 3T3 liver.

Lanes:
1. *Canine, EcoR1 digest*
2. *Canine, Hind III digest*
3. *Canine, Pvu II digest*
4. *Mouse, EcoR1 digest*
5. *Mouse, Hind III digest*
4. *Mouse, Pvu II digest*
7. Size markers



The MDR5A probe was used to analyse *mdr1* homologue expression in normal canine tissue. RNA was extracted from normal adult male tissue as described in section 2.2.2 and analysed on Northern blots (methodology in section 2.4.2). The filter was probed with random primed full length MDR5A clone (described in sections 2.1.9 and 2.3.3) under non-formamide conditions. The filter was stripped and reprobed with 7S to adjust for RNA loading. The results are shown in figures 4.4. and table 4.1.

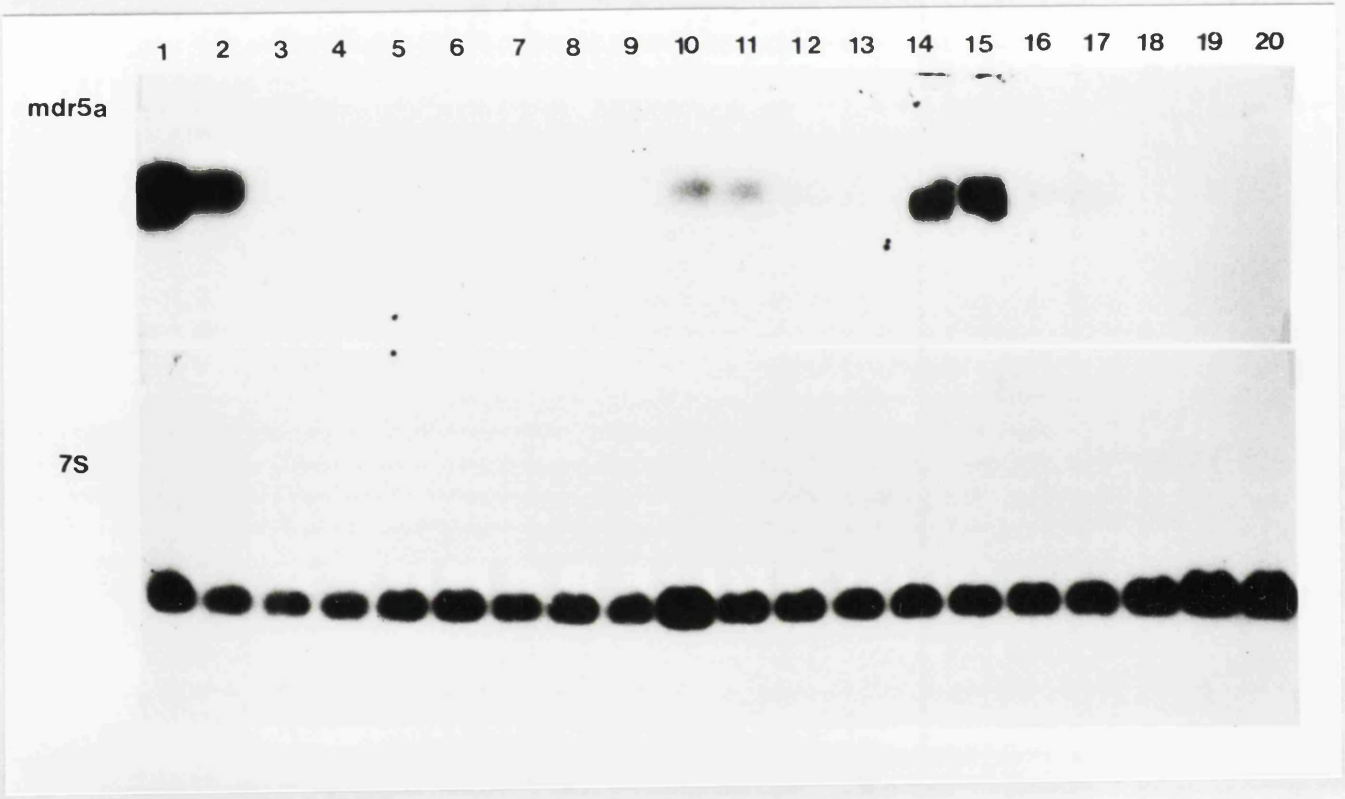
Table 4.1 Tissue expression of mdr1 homologue(s) in the dog

Lane	Tissue	mdr1	Lane	Tissue	mdr1
1	KB8-5	+++	11	colon	+
2	liver	+++	12	prostate	-
3	testicle	-	13	bladder	-
4	skeletal muscle	-	14	kidney	++
5	skin	-	15	adrenal	++
6	cerebrum	-	16	spleen	+/-
7	cerebellum	-	17	lymph node	-
8	stomach	-	18	oesophagus	-
9	duodenum	-	19	lung	-
10	jejunum	+	20	cardiac muscle	-

- negative; + positive; +++ strong positive; +/- marginal expression

Figure 4.4 shows that there is detection of transcripts of the same size as the human *mdr1* message in liver, kidney, and adrenal. Some expression is also seen in the caudal GI tract i.e. jejunum and colon and the spleen may also have a low level of expression. This tissue distribution of expression is in general agreement with RNA analysis of *mdr1* gene expression in humans (Fojo et al, 1987b) and mouse (Croop et al, 1989) .

Figure 4.4 Northern blot of normal canine tissue hybridised with MDR5A, then rehybridised with 7S.
Lane order is given in table 4.1.



4.3 USE OF RNAse PROTECTION ASSAYS TO DETERMINE HOMOLOGY BETWEEN HUMAN *mdr1* AND CANINE *mdr* TRANSCRIPTS FROM DIFFERENT TISSUES

Ultimately to determine the number and class of genes in the canine *mdr* family will require cloning and sequencing of all members of the family. An attempt was made to clone the canine *mdr1* homologue by screening a canine genomic library with the MDR5A probe. Unfortunately, this screening failed to produce any positive clones, possibly because the library was not fully representative and no further attempts were made.

The results of section 4.2 suggest that a canine *mdr1* homologue is detectable by the human *mdr1* probe MDR5A and that the tissue distribution of this gene (or genes) is similar to other species. In chapter 3, C219 monoclonal antibody detected P-gp in cardiac and skeletal muscle yet both of these tissues were negative for *mdr1* homologue expression (figure 4.4); this could represent expression of an *mdr2* homologue in muscle tissue which does not cross-hybridise to MDR5A. Without canine gene-specific probes it is difficult to prove that certain tissues have *mdr1* homologue expression and others have *mdr2* gene expression.

RNAse protection assays provide an indirect method of assessing the homology between RNA species when sequence information is not available. In this section, the aim was to determine the degree of homology between canine *mdr* species and human *mdr1* using RNAse protection assays (RPA). At the same time, the assay was investigated as to its' suitability as a rapid semi-quantitative assay for quantitating *mdr1* from tumour samples, as used by Herweijer et al, (1990).

The RNAse protection assay protocol used in this study is given in section 2.4.3. In summary, it consists of 20ug of total RNA hybridised overnight with *Xmn1*-linearised MDR5A riboprobe, subjected to RNAase A and T1 digestion and the products then run on a denaturing polyacyrylamide gel. The restriction map of the MDR5A plasmid is shown in figure 4.1. MDR5A riboprobes for use in the RNAse protection assays were produced from the *Xmn*-I linearised plasmid for two reasons. Firstly, it was difficult to produce adequate amounts of full length riboprobes from longer MDR5A templates. Secondly, the shorter *Xmn*-I linearised riboprobe does not extend into the "joining" region between the two halves of the *mdr* gene. This joining area is that part of the gene with the lowest homology both between isoforms and between species. It seemed likely that the diversity in this area would compromise detection of homologies on an RPA.

Initially, effort was concentrated on optimizing the conditions of the assay. Figure 4.5 is an RPA of KBV-1 RNA hybridised with (*Xmn*-I linearised) MDR5A and digested with a decreasing amount of RNAse enzymes. Lane 4 represents digestion with the full amount of enzyme detailed in the methods section. Lane 5 and 6 have been digested with a half and a tenth of the total enzyme concentration used in lane 4. The lanes did not run completely straight and so it was not possible to determine if the KBV-1 protected band was in lane 5 or 6 or both. At the lowest concentration of enzyme (0.1), there was no residual probe left in the control lane (lane 8) nor was there a signal from the KB3-1 sample in lane 7. On this basis, a tenth of the RNAse A and RNAse T1 concentration given in the methods was adopted for the next assays.

Considering the degree of gene amplification and *mdr1* over-expression in the KBV-1 cell line, the signal seen on RPA was modest. If RPA were to be of use in a quantitative basis, it was important to ensure that the amount of probe added was not the limiting factor in the intensity of signal achieved. In figure 4.5, 500,000cpm of the MDR5A probe was hybridised to the test RNA. Figure 4.6 shows the results of hybridising KBV-1 with different counts of probe, ranging from 50,000 cpm to 800,000cpm. There was no large increase in the signal intensity achieved in lane 1 compared to lane 6 so it was decided to continue using 500,000cpm of the MDR5A probe per sample.

Herweijer et al, (1990) simultaneously hybridised tumour RNA with *mdr* riboprobes and γ actin probes (as an RNA loading standard) to allow a semiquantitative assessment of *mdr* expression. The γ actin probe used by Herweijer was obtained and figure 4.7 represents human and canine RNA run in conjunction with 50,000cpm of the γ actin probe. The human RNA sample protects a 145bp fragment almost the same length as the actin riboprobe. This is in agreement with the results of Enoch et al, (1986). The canine RNA protected a fragment of approximately 85bp. Thus it appeared that the actin probe could be used as a standard for RNA loading in both human and canine samples.

Figure 4.5 Titration of RNAse concentration with KBV-1 RNA in an RNAse protection assay (RPA)

1. Size markers
2. Xmn 1 MDR5A (undigested)
3. empty
4. KBV-1: 1 x total RNAse
5. KBV-1: 0.5 x total RNAse
6. KBV-1: 0.1 x total RNAse
7. KB3-1: 0.1 x total RNAse
8. MDR5A alone: 0.1 x total RNAse

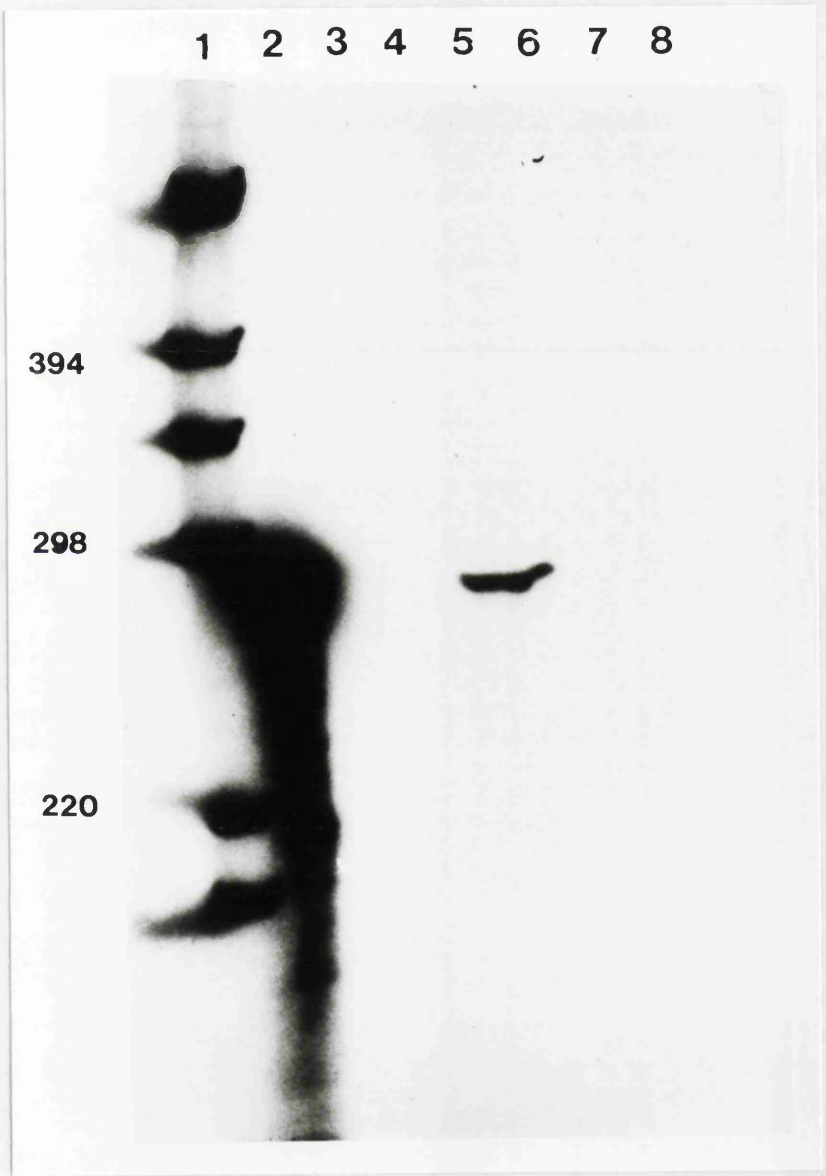


Figure 4.6 Optimisation of MDR5A probe counts in KBV-1 RPA

1. KBV-1 + 800,000cpm
2. KBV-1 + 600,000cpm
3. KBV-1 + 400,000cpm
4. KBV-1 + 200,000cpm
5. KBV-1 + 100,000cpm
6. KBV-1 + 50,000cpm
7. No RNA + 800,000cpm
8. empty
9. Undigested MDR5A

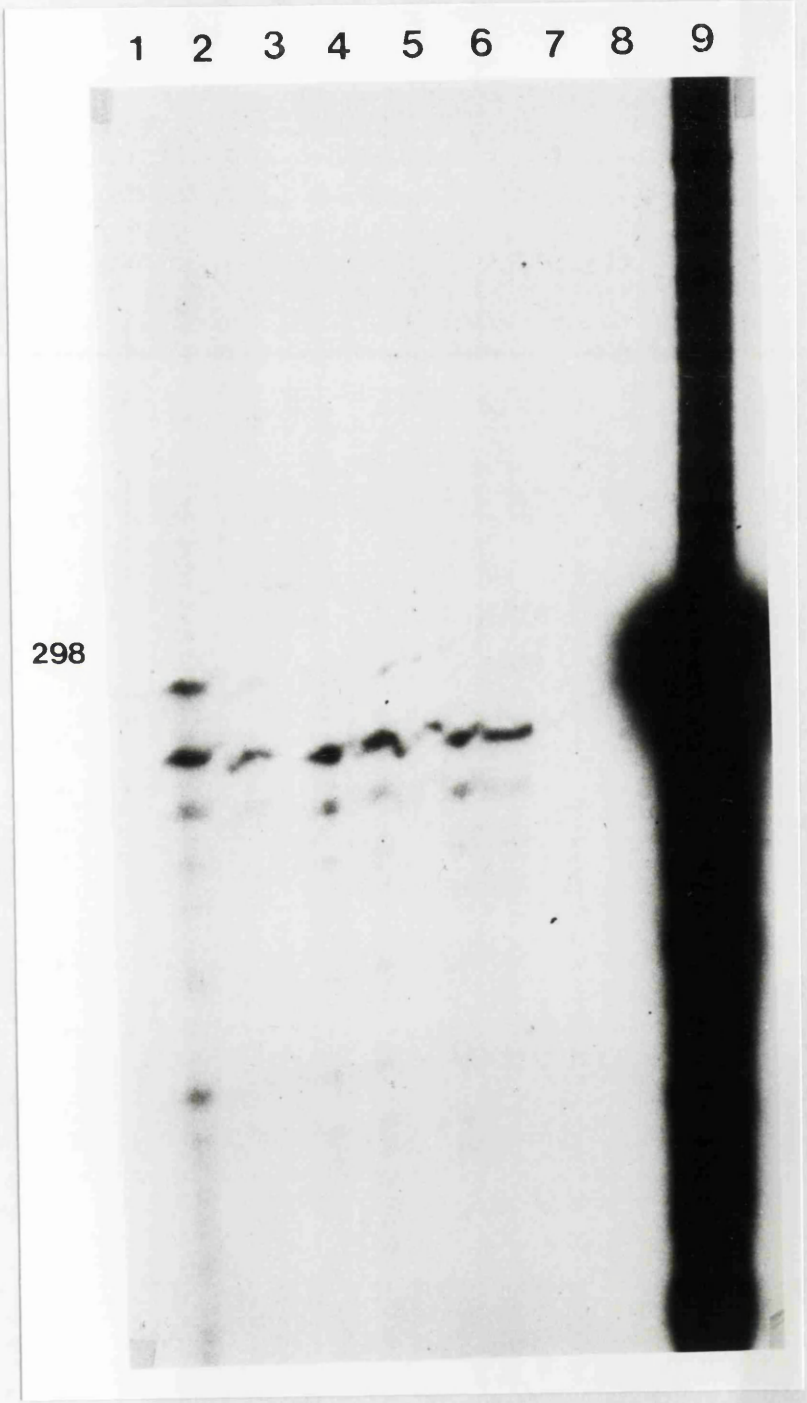
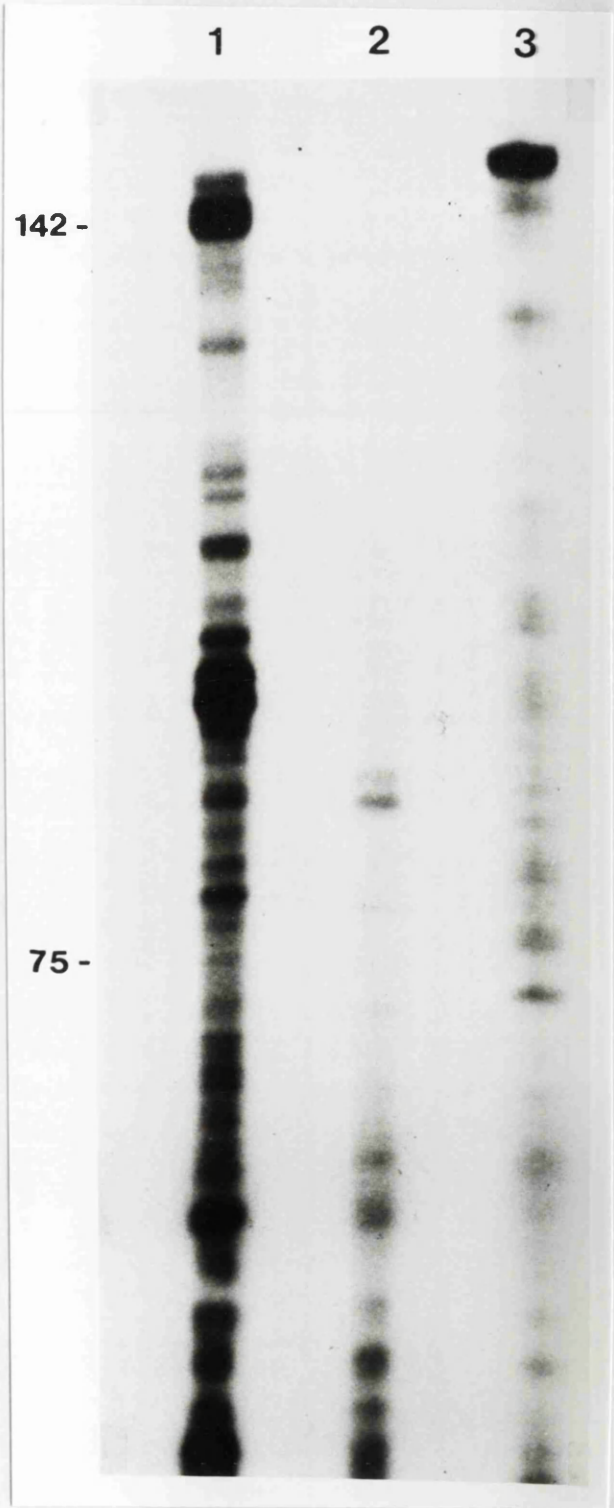


Figure 4.7 RNAse protection assay with γ actin probe

1. Human RNA plus 50,000cpm of γ actin riboprobe
2. Canine (liver) RNA plus 50,000cpm of γ actin riboprobe
3. γ actin riboprobe alone (*undigested).*



The MDR5A and actin probes were used together in the RPA shown in figure 4.8. Lane 6 is KBV-1 RNA hybridised with MDR5A alone whereas in lane 7 and 8, MDR5A and γ actin were hybridised simultaneously to KBV-1 RNA and the drug sensitive parent KB3-1. Lane 9 represents normal dog liver RNA hybridised to MDR5A alone. Comparing lanes 7 and 8, the RNA loading of KBV-1 and KB3-1 looks similar as judged by the intensity of the γ actin band but KBV-1 has a considerably more intense *mdr1* fragment. The detection of low-level *mdr1* expression in the parental cell line contrasts with the negative results of Northern blots (one of which is shown in figure 5.1). The detection of *mdr1* expression on RPA but not on Northern could merely represent the improved sensitivity of the RPA. However, the results in lane 9 (the canine liver RNA sample) were considered suspicious. Canine liver RNA would appear to have given complete protection to the entire length of the *mdr1* riboprobe, implying that there is complete or very strong homology between the RNA species. This degree of protection was unexpected and, in conjunction with the obvious band in the KB3-1 lane, it was thought that this result could be an artefact due to incomplete digestion of the MDR5A labelled riboprobe.

The control lane (lane 5) was identical to the other lanes regarding probe counts and hybridisation/digestion conditions but had no test RNA; all of the probe was digested in this control. However, the other lanes contain RNA which has been freeze-dried after a salt precipitation and resuspension step. The presence of these salts may alter the digestion efficiency of the RNAses. A second factor is the RNAse concentration; the RPA in figure 4.8 was digested with one tenth of the enzyme concentration detailed in the original protocol. Because of the concern over the completeness of the digestion, the enzyme concentration was restored to the original concentration for subsequent assays.

When digestions were carried out with the full concentration of enzyme, the 269bp protected fragment seen in figure 4.8 with the canine liver RNA disappeared. Instead, a shorter fragment of 110bp, plus ladder, was produced. This MDR5A ladder overlapped and obscured the γ actin fragment, which made it unsuitable for simultaneous MDR5A and actin hybridisations. Figure 4.9 shows the results of an RPA using the full amount of enzyme with RNA from canine liver, kidney, and skeletal muscle. Kidney and skeletal muscle samples were hybridised with γ actin in adjacent lanes to the MDR5A hybridisation. Lanes 6 and 10 show that canine liver and kidney RNA both protect an MDR5A fragment of 110bp and give identical laddering. Lane 7 and 8 used the same quantity of skeletal muscle RNA in the hybridisations; the γ actin hybridisation protects a band of the expected size whereas in the MDR5A lane, there is no protection of fragments. Comparing intensity of the γ actin fragments of the muscle tissue versus the kidney tissue, it would appear that more RNA was loaded in the muscle lane. So the lack of protection in the muscle tissue is not likely to be due to insufficient starting quantities of muscle RNA.

Figure 4.8 Combined use of MDR5A and actin probes with human RNA samples

1 Size markers
2 undigested actin probe
3 undigested MDR5A probe
4 empty
5 MDR5A + actin (no test RNA)
6 KBV-1 plus MDR5A
7 KBV-1 plus MDR5A + γ actin
8 KB3-1 plus MDR5A + γ actin
9 Canine liver RNA plus MDR5A

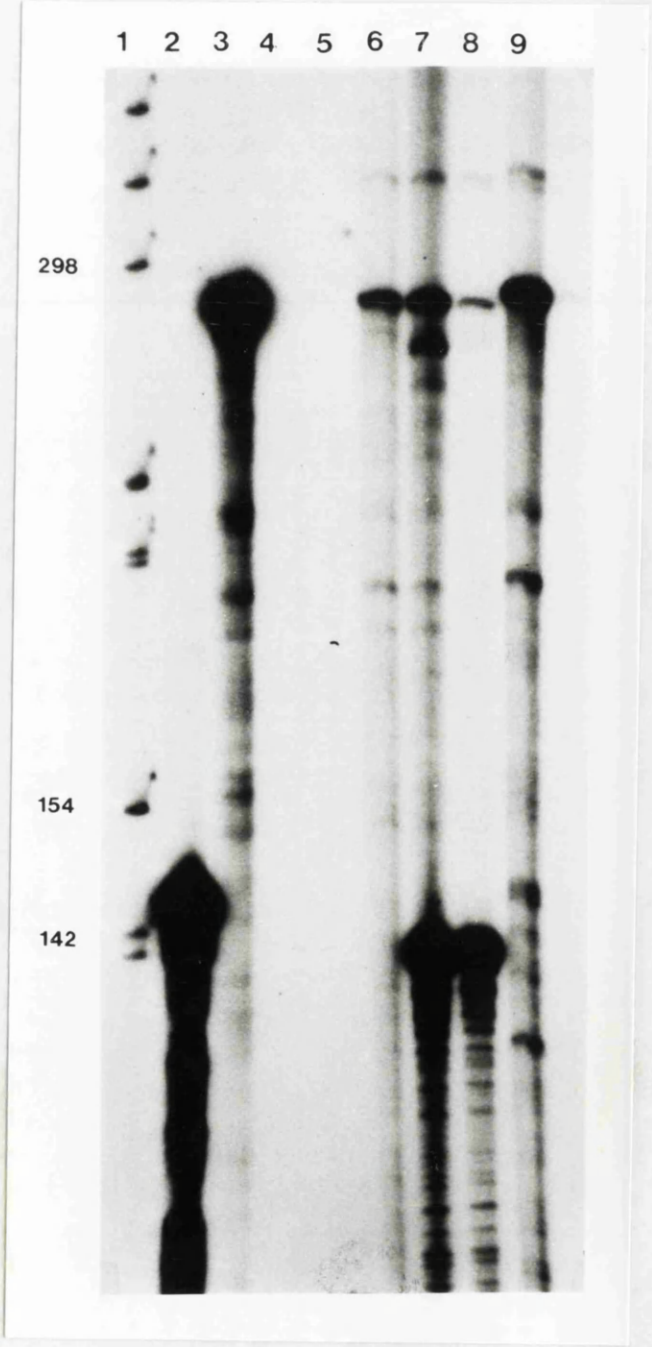
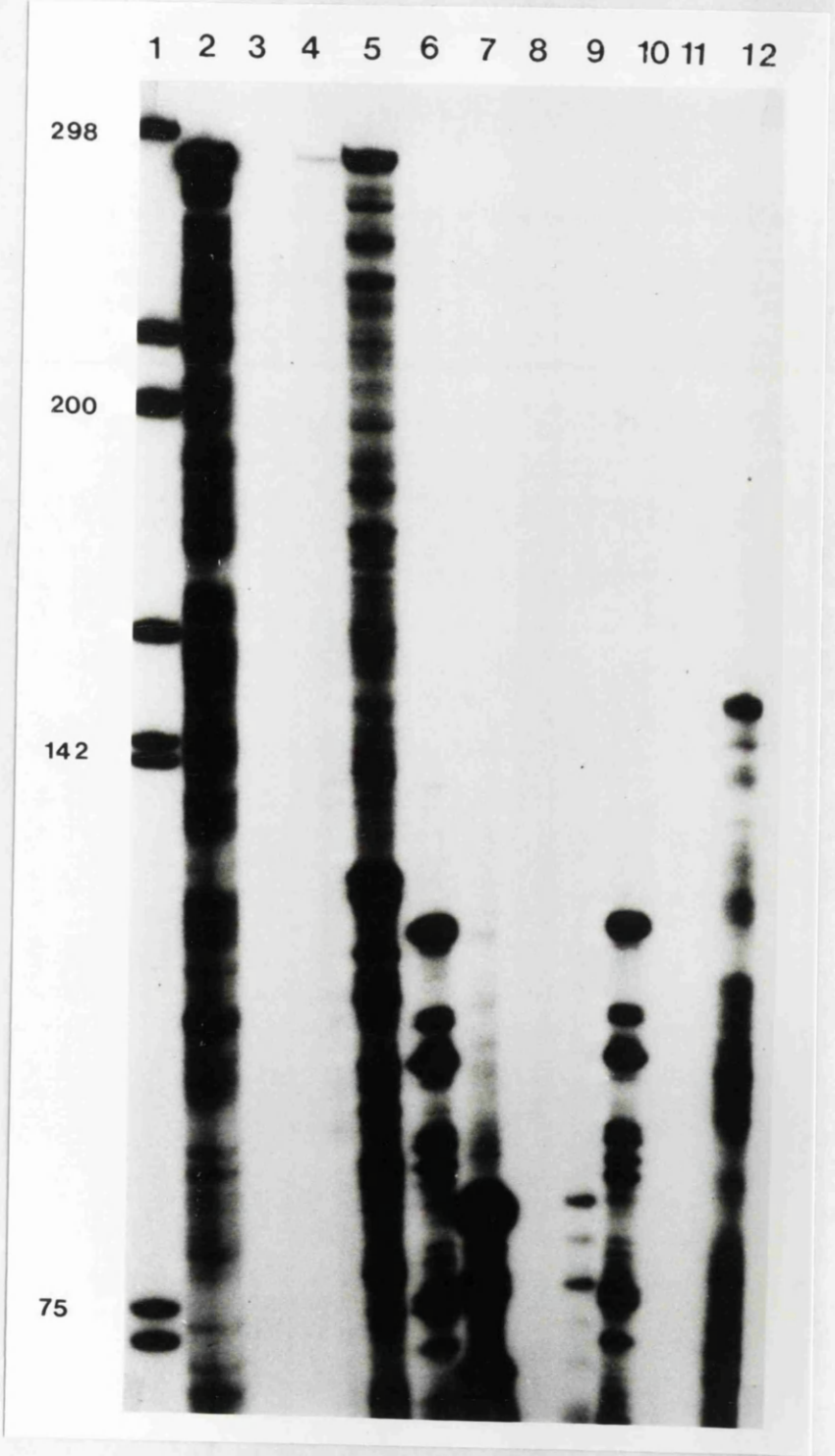


Figure 4.9 Canine RNA hybridised with MDR5A and γ actin probes

1. size markers
2. undigested MDR5A
3. MDR5A (no test RNA)
4. KB3.1 + MDR5A
5. KBV-1 + MDR5A
6. canine liver RNA + MDR5A
7. canine skeletal muscle + γ actin
8. canine skeletal muscle + MDR5A
9. canine kidney RNA + γ actin
10. canine kidney RNA + MDR5A
11. γ actin (No test RNA)
12. undigested γ actin



The conclusion from this piece of work was that liver and kidney contained an *mdr* RNA species which skeletal muscle does not. The identical size and intensity of the protected fragments in the liver and kidney hybridisations suggest that the *mdr* mRNA in these two tissues is identical and given its' partial homology to MDR5A, is likely to be an *mdr1* species.

The *BamHI* linearised MDR5A riboprobe (which is 1383bp compared to the *Xmn-I* riboprobe of 269bp) produced a single band in the *EcoR1* and *Hind III* digested canine DNA shown in figure 4.3. The conclusion from the RPA analysis that liver and kidney express an *mdr1* species is partially dependant on the short *Xmn*-I MDR5A riboprobe retaining the specificity of the longer probe. This was verified at the genomic level by Southern analysis. A southern of dog, human and mouse DNA was divided and one half hybridised with the *BamH1* MDR5A riboprobe, and the duplicate half with the *Xmn-I* digested MDR5A riboprobe. The results are shown in figure 4.10.

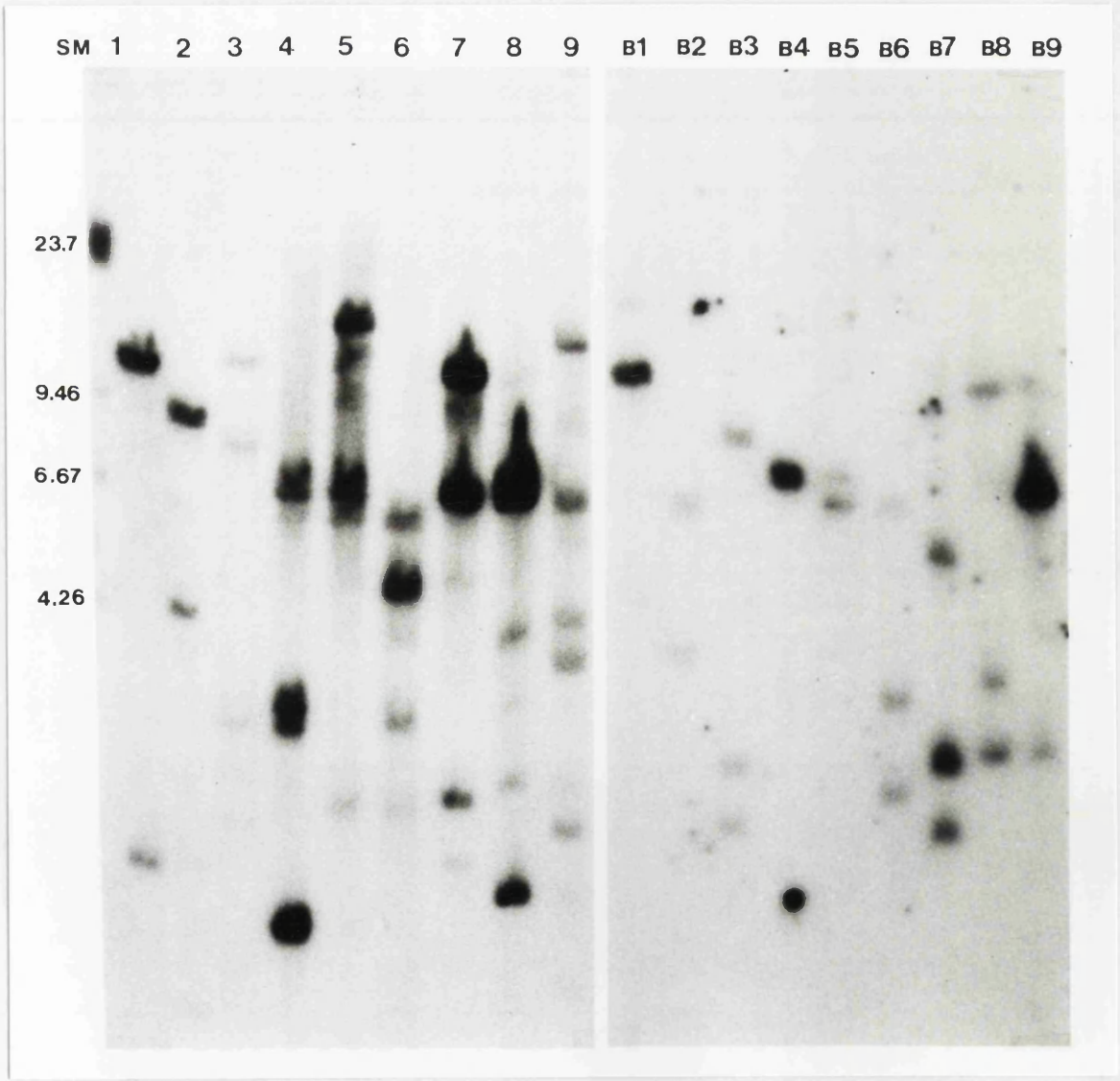
The hybridisations in figure 4.10 were performed in non-formamide buffer at 65^{o}C, whereas the southern in figure 4.3, was hybridised at 42^{o}C in 50% formamide. The high temperature non-formamide system was adopted because it was found to be more sensitive and this is illustrated in figure 4.10. The three digests of canine DNA (lanes 1-3) in figure 4.10, each contain an additional faint band which was not apparent on the southern shown in figure 4.3. The complex and rather fuzzy bands in the human DNA digests (lanes 4-6) give similar results to other workers who use the MDR5A probe. MDR5A hybridisations to human DNA digested with *PvuII*, *HindIII* and *EcoR1* are found in Ueda et al, (1987b), Shen et al,(1988) and Lai et al,(1989) respectively. Therefore, under the hybridisation conditions used in this system, the MDR5A probe retains its specificity for human *mdr1* and does not detect human *mdr2*.

Comparing the equivalent lanes in the two halves, the *Xmn-1* MDR5A hybridisation has lost bands relative to the *BamH1* MDR5A pattern. Given that the *Xmn-I* probe is considerably smaller, this is expected. The quality of this southern hybridisation is not good but there is certainly no compelling evidence that the shorter probe is detecting additional genes. There is a faint band of high molecular weight in lane B1 which is not present in lane 1 but in itself this was not considered to indicate that an *mdr2* gene (or genes) was being detected. The *Xmn-1* MDR5A hybridised murine and human digests do not contain any new bands which corroborates the canine results and gives further credence to the assessment that the shorter *Xmn-1* digested riboprobe retains *mdr1* specificity.

Figure 4.10 Duplicate Southern blots hybridised with MDR5A and *Xmn-1* digested MDR5A.

Left hand panel (1-9) was hybridised with full length MDR5A riboprobe, right hand side (B1-B9) with Xmn-1 linearised MDR5A riboprobe. Lanes 1-9 and B1-B9 are identical. DNA was from outbred dog liver, human clinical sample and murine (NIH 3T3) liver. SM, size markers.

1 Canine: *EcoR1* digest
2 " *Hind III* digest
3 " *Pvu* II digest
4 Human: *EcoR1* digest
5 " *Hind III* digest
6 " *Pvu II* digest
7 Murine: *EcoR1* digest
8 " *Hind III* digest
9 " *Pvu II* digest



As an assay, the RPA did not lend itself to semi-quantitative use in the dog; the size of the *mdr* and γ actin fragments were too similar in size to allow simultaneous hybridisation. Although the technique appeared to be very sensitive ie. could detect *mdr1* in the KB3-1 parental cell line, it did not differentiate between moderate and high expression. The signal from canine kidney was similar to KBV-1 yet from Northern results it is obvious that KBV-1 has considerably more *mdr1* mRNA. It also required RNA of extremely high quality; some KBV-1 RNA which appeared perfect on an ethidium stained gel did not give protection in the assay. The inconsistency of the assay was judged to be too great for routine analysis of canine tumour RNA.

4.4 DISCUSSION

Section 4.1 used a highly conserved probe (pEX172) of exon 28 of the hamster *pgp2* gene to determine the number of *mdr* genes in the dog. This probe detected the appropriate number of bands in human and mouse DNA. The equivalent exon of the rat *mdr1b* gene has also been used for the same purpose in the rat and confirmed that the rat would appear to have three *mdr* genes like other rodents (Silverman *et al*, 1991). The pEX172 probe revealed that the dog may have four *mdr* genes, which would be more than any of the species examined so far. The designation of these four genes into *mdr1 /mdr2* (or more?) categories cannot be carried out without sequencing information.

Unpublished work, which was presented at an American Association of Cancer Research meeting in 1991, suggests that the *mdr* gene family in some mammals may be complex. Using the (pEX172) probe , Childs and Ling discovered that the pig had five potential *mdr* genes. Three genomic clones from the pig were isolated using pEX172 and the 3' untranslated region (UTR) of these clones was sequenced and analysed. The 3' UTR is known to be isoform specific and well conserved across species and may therefore be used to classify genes according to their isoform (Endicott, *et al*, 1987; Ng *et al*, 1989). This analysis revealed that the pig had one gene that was the equivalent of an *mdr1* but the other two, although very homologous to each other, did not correspond to any of the preexisting classes. The fourth and fifth porcine genes have not yet been characterised.

Based on this incomplete information on the pig *mdr* gene family, it would seem unsafe to make predictions about the class organisation of the canine *mdr* family. The pig information also poses questions as to the functional potential of this new class of *mdr*; it is difficult to envisage a class of genes with an essential function which is expressed in the pig but completely absent in humans and rodents. It may be that these new *mdr* genes are actually pseudogenes of no functional significance.

The homology between the different isoforms, and between species, increases towards the 3' end of each half of the P-gp molecule. This corresponds to the conserved intracytoplasmic portions of the P-gp. Because of this, probes from these areas tend to cross-hybridise with other family members; the pEX172 probe in the conserved 3' end of the carboxy-terminal half of the gene is an example of this phenomenon. MDR5A spans the middle third of the human *mdr1* molecule including the conserved nucleotide binding site but avoids problems of cross hybridisation to human *mdr2* presumably by its incorporation of the divergent linker region and some of the external domains. However, other probes in the middle portion of the *mdr* molecule are not always gene specific. The probe, pCHP1, used by Riordan *et al*, (1985) detected approximately ten bands on southern blotting of *EcoR1* digested hamster DNA. Similarly Croop *et al*, (1989) derived the probe pcDR1.3 from mouse *mdr1b* cDNA which includes the highly conserved nucleotide binding site. Croop commented (without actually showing Southern data) that this probe cross-hybridised extensively with murine *mdr1a* and *mdr2* genes.

In contrast to Riordan *et al*, (1985), the fragment pattern seen with MDR5A and *EcoR1* digested canine DNA is very simple: using a formamide hybridisation system, only a single fragment was detected and using a different hybridisation protocol, a second band of less than 5kb appeared. This suggests that MDR5A cross-hybridises with a single gene in the dog.

The results of the Northern analysis of canine tissue also supports the view that MDR5A is cross-hybridising with a single gene and its products. Firstly, the tissue distribution of the MDR5A cross-hybridising species is very similar to *mdr1* expressing tissues in other species. Secondly all of the transcripts from the different tissues were of the same size. In the human and mouse, *mdr1* and *mdr2* transcripts are different sizes and can be differentiated on gel fractionation. In the human, the *mdr2* transcripts are approximately 400bp shorter than the *mdr1* transcripts (Van der Bliek *et al*, 1987). In the mouse the *mdr2* transcripts are again shorter than *mdr1* mRNA. Using the pcDR1.3 probe mentioned above, Croop *et al*, (1989) detected transcripts ranging from 4.5-6kb in normal mouse tissues.

Multiple transcripts have never been seen in canine tissues using MDR5A. This in itself does not rule out that two genes (for example an *mdr1a* and an *mdr1b*) are being detected. In the mouse, the *mdr1a* gene produces transcripts of multiple sizes depending on the transcription initiation site and the polyadenylation signal used (Hsu *et al*, 1990). The size variation means that an *mdr1a* transcript can overlap with an *mdr1b* transcript and appear as a single species. In the mouse, these two transcripts are differentiated using probes from the diverse linker region. (Hsu *et al*, 1989).

Table 4.2 Comparison of mdr gene sequences in the *Xmn 1* MDR5A riboprobe region of the mdr1 gene

Gene	Access Code (EMBL)	Nucleotide Difference/ homology	Longest unbroken run	Longest run: 1bp difference
Hamster mdr1a	M17897	20/ 92.6%	38	57
Mouse mdr1a	M33581	24/ 91.1%	38	57
Hamster mdr1b	M17896	34/ 87.4%	38	57
Mouse mdr1b	M14757	41/ 84.8%	20	38
Rat mdr1b	M62425	42/ 84.4%	38	53
Human mdr2	M23234	59/ 78.1%	17	23
Mouse mdr2	J03398	65/ 75.9%	22	28

Rather than proposing the complex situation of *mdr1a* and *mdr1b* transcripts masking each other, the simplest conclusion which fits the Southern and Northern data is that a single gene cross-hybridises with MDR5A. The RNAse protection assays illustrate the homology between the canine *mdr* mRNA and human *mdr1*. Table 4.2 details the exact number of nucleotide differences between *mdr* genes over the 269 bases covered by the *Xmn-I* MDR5A riboprobe. The percentage homology between the *mdr1a* genes and human *mdr1* in this short region is greater than the homology over the entire gene. A comparison of rat, mouse, hamster and human *mdr* gene sequences is given in table 4.3, adapted from Silverman *et al*, (1991). The overall homology between mouse *mdr1a* and human *mdr1* is 82.2%, but in the riboprobe region rises to 91.1%.

Table 4.3 Overall homology of mdr genes using coding and non-coding sequences *(adapted from Silverman et al, 1991)*

Gene	% nucleotide homology	
	Human mdr1	Human mdr2
Hamster mdr1a	83.5	64.2
Hamster mdr1b	75.7	47.7
Mouse mdr1a	82.2	71.6
Mouse mdr1b	78.7	70.6
Mouse mdr2	71.1	86.1
Rat mdr1b	79.1	71.5
Human mdr2	74.9	100

When the sequence differences are shown in their actual positions (figure 4.11), then it can be seen that most of the base pair changes are not contiguous; there are only a couple of locations in the *mdr1a* which has two adjacent base pair changes. In comparison to *mdr1a*, the murine *mdr2* gene has nine sites with contiguous base pair changes. The RNAse enzymes digest areas of single-stranded RNA. It may be that single nucleotide differences which are well dispersed do not sufficiently disrupt the RNA-RNA duplex to be recognised and destroyed by the enzymes.

The differential in the frequency and dispersion of base pair substitutions probably accounts for the ability to detect protected bands in the canine liver RNA hybridisations but the absence of protection in the muscle RNA samples. From cross-species comparisons, muscle tissue would be expected to predominantly express the *mdr2* gene; if the canine *mdr2* gene resembles the mouse *mdr2* gene then it would not seem likely that protected fragments of over 40 nucleotides would be generated. In contrast, the *mdr1a* gene has long stretches where RNAses may not pick up single base differences; in the middle portion there is a distance of 127 bases with only single nucleotide changes. Both kidney and liver produced the same fragment ladder on RPA so it would seem logical that these two tissues express the same *mdr* gene. If there was a mixture of *mdr1a* and *mdr1b* mRNA in one or both these tissues then one might expect the intensity of the fragments in the two tissues to be different. This is not the case, yet again suggesting MDR5A cross-hybridises to a single species.

Figure 4.11 Nucleotide differences in *Xmn-1* MDR5A riboprobe region of the three murine *mdr* genes relative to human *mdr1*.

Sequence of the human mdr1 gene is given in full with the nucleotide positions numbered according to the human mdr1 cDNA EMBL accession number M14758 (Chen et al, 1986). Mouse mdr1a, mdr1b and mdr2 are aligned (in that order) beneath the human sequence. Identity is indicated by a dash. Adjacent base differences are in bold script.

```
*2716bp
ATTTATTTTA TTACATTTTT CCTTCAGGGT TTCACATTTG GCAAAGCTGG
--------C- ---------- T--------C ---------- ----------
---------G ------A--- T--------C ---------- -------C--
C-C--C--CT ----T--C-- ---------C -----G---- -G--------

AGAGATCCTC ACCAAGCGGC TCCGATACAT GGTTTTCCGA TCCATGCTCA
---------- --------A- ---------- -------AA- --------G-
---------- -------AG- ---------- -------AA- --------G-
---------- ----CAA--- ----G-C--- --CC--TAA- G-G-----A-

GACAGGATGT GAGTTGGTTT GATGACCCTA AAAACACCAC TGGAGCATTG
---------- ---C------ ---------- ---------- C------C--
--------A- A--C-----C -------A-- -G----G--- ---CT--C--
-G-----CA- ---C------ -----T-A-- ------GT-- -------C-T

ACTACCAGGC TCGCCAATGA TGCTGCTGAA GTTAAAGGGG CTATAGGTTC
--C------- -------C-- ---------- --G------- ---C---G--
--C------- -------G-- ----T--AGT ---------- -G-G---GG-
T----A--A- ------CA-- ------G--- --CC----A- -C-CG--AA-

CAGGCTTGCT GTAATTACCC AGAATATAGC AAATCTTGGG ACAGGAATAA
T--------- --G---TT-- ----C----- ---------- --------C-
---------- ---G------ -----G---- ---C--C--- ------G-C-
--A-T-G--- T-----G-A- ----C-C--- A--C-----A --C--T--T-
                  *2985 bp
TTATATCCTT CATCTATGG
-C------C- A--------
-CC-C----- AG-------
-------A-- T--T--C--
```

The overall conclusion remains that MDR5A cross-hybridises to a single gene, almost certainly an *mdr1* homologue. Whether this homologue is an *mdr1a* or *mdr1b* equivalent cannot be determined, indeed it is not clear if the dog has more than one *mdr1* gene. In rodents, the *mdr1a/mdr1b* gene expression in tissues has considerable overlap. However, the major difference between the expression of the two *mdr1* genes is that in both the hamster and the mouse, the *mdr1b* form predominates quite markedly in the adrenal cortex. In the canine, the adrenal *mdr* transcript was an identical size to the liver transcript which suggests that unlike the rodent, the same gene may predominate in both organs. This could suggest the dog is similar to humans and only has one *mdr1* gene.

Even if dogs do possess two *mdr1* genes, human *mdr1* is more homologous to *mdr1a* than *mdr1b* and so the expected cross-hybridisation would be to the *mdr1a* gene. However the difference between *mdr1a* and *mdr1b* is not great and when a rat cDNA library was screened with the human MDR5A probe, it was the rat *mdr1b* gene which was subsequently identified (Silverman *et al.* 1991). Given the complexity of the potential cross-hybridisation and differential tissue expression of the *mdr1* genes, it is not possible to rule out that dogs may have more than one *mdr1* gene but that under the stringent hybridisation conditions used in this study, only one gene is apparently detected at the genomic level.

4.5 SUMMARY

Using a conserved exon probe from the 3' end of the *mdr* gene, four fragments were produced on Southern blot implying that the dog may have four members of the *mdr* gene family. The human *mdr1* specific MDR5A probe appears to only detect one of these genes at the genomic level. Northern analysis of *mdr* gene expression from canine tissue detects transcripts of a single size and the tissue distribution of this transcript expression is consistent with an *mdr1* gene distribution. The strong homology of the canine gene to the human *mdr1* gene is evident in the 110bp fragment which is protected by canine liver and kidney RNA on RNAse protection assay. From comparison with *mdr2* sequences in the human and mouse, it is highly unlikely that a canine *mdr2* homologue could protect a fragment of this length. The gene detected by MDR5A would therefore appear to be a canine *mdr1* homologue.

CHAPTER 5:
P-GP AND *MDR* mRNA DETECTION IN CANINE LYMPH NODES

5.1 DOTBLOT QUANTITATION OF *mdr1* IN CELL LINES

5.2 IMMUNOHISTOCHEMICAL DETECTION OF P-GP: SENSITIVITY AND LIMITATIONS OF THE TECHNIQUE.

5.3 STUDIES WITH CANINE LYMPHOMA CELL LINES

5.3.1 IN VITRO CHEMOSENSITIVITY OF CANINE LYMPHOMA CELL LINES.

5.3.2 LACK OF P-GP EXPRESSION IN CANINE CELL LINES

5.3.3. LACK OF P-GP EXPRESSION IN CANINE CELL LINES FOLLOWING DRUG EXPOSURE.

5.4 DETECTION OF P-GP IN NORMAL, REACTIVE & LYMPHOMATOUS NODES

5.4.1 ACQUISITION OF CLINICAL SAMPLES

5.4.2 CHEMOTHERAPEUTIC TREATMENT OF MLSA

5.3.3 P-GP DETECTION IN A DENDRITIC CELL POPULATION AND ENDOTHELIUM OF LYMPH NODES

(i) P-gp in normal, reactive and lymphomatous nodes.

(ii) Characterisation of the P-gp positive dendritic cells.

(iii) Do the dendritic cells express mdr1?

5.4.4 DETECTION OF P-GP AND *MDR* mRNA IN LYMPHOMA CELLS

5.5 SUMMARY

5.1 BLOT QUANTITATION OF *mdr1* IN CELL LINES.

In the introduction, the problems of quantifying P-gp protein expression or *mdr*1 mRNA were discussed. From that discussion, the importance of using well characterised and well-recognised cell lines as controls was emphasized.

For this work, cell lines from the KB cell series were used in a similar fashion to Goldstein *et al,* 1989. The cell lines are described in section 2.1.7. Table 5.1 documents the relative resistance of these cell lines to doxorubicin and the estimated over-expression of mRNA as calculated in 3 papers.

Table 5.1. KB cell lines

	KB3.1	KB8.5	KBV.1
Doxorubicin resistance[1]	1	3.2	420
mdr1 overexpression			
Shen *et al,* 1986c	n.d.[2]	3[3]	320
Fojo *et al,* 1987b	1	40	>500
Kuwazuru *et al,* 1990a	1	15	n.d

1. *The doxorubicin resistance is relative to KB3.1. 2. not done. 3.Shen et al could not detect any mdr1 signal in KB3.1 so compared KB8.5 to KB8 which has a relative resistance to doxorubicin of only 1.1*

Very highly resistant cell lines, such as KBV-1, are difficult to use in techniques which rely on densitometric reading of autoradiographs. This is because the *mdr1* expression of KBV-1 is so great in comparison to the clinical samples that it is not possible to obtain exposures which have both the sample and KBV-1 in the linear portion of the response curve of the radiographic film. *Mdr1* gene amplification has occurred in KBV-1; the biological relevance of gene amplification in clinical resistance has never been documented. Thus, for both biological and technical reasons it is important to have a control with intermediate *mdr*/Pgp expression.

KB8-5 meets these requirements. In contrast to KBV-1, the *in vitro* resistance of KB8-5 to MDR drugs is modest. The fold increase in mRNA is within what could be described as a realistic range, judging from previous work with human tumour and rodent tissue (Goldstein *et al,* 1989, Fairchild *et al,* 1987). The "fold increase" in *mdr1* mRNA present in KB8-5 is commonly referred to but seldom measured. Most workers who use these cell lines do not obtain detectable *mdr1* hybridisation signal on a Northern with the sensitive parental cell line KB3-1. Shen *et al,* 1986c could not

get any *mdr1* hybridisation with KB3.1 signal so expressed the KB8.5 value relative to another cell line with only very marginal drug resistance. Even the paper which is most quoted as having shown that KB8.5 has a 40 fold increase in *mdr1* mRNA (Fojo *et al,* 1987b) does not have any visible *mdr1* hybridisation to KB3.1 RNA in the figures shown. Thus when the derived cell line KB8.5 is described as having a 40 fold induction of *mdr1* mRNA relative to KB3-1, it has to be assumed that this refers to the background hybridisation that KB3-1 gives in dot-blot analysis. Those groups which use KB8.5 in their quantification system have not independently re-calibrated the KB lines for relative *mdr1* expression in their own particular system. Thus Goldstein *et al (*1989), Bourhis *et al,* (1989a and 1989b) arbitrarily assign a value of "30" to the KB8-5 signal based on Fojo *et al* (1987b) without mentioning what fold difference was actually achieved in their system. Kuwazuru *et al,* (1990a) re-examined KB8-5 and reported *mdr1* expression approximately 15 fold greater than the parental KB3-1 by slot-blot analysis. There is, therefore, some leeway in the accepted relative *mdr1* overexpression of KB8.5 as judged by the current literature.

Figure 5.1 is a northern blot of the KB cell lines plus two canine lymphoma cell lines probed with MDR5A. These RNA sources are the panel of controls run on every dot-blot. Figure 5.1 illustrates the extremely strong signal obtained from KBV-1 in comparison to KB8-5; it is not possible to accurately quantitate this signal. No signal is seen with the canine cell lines.

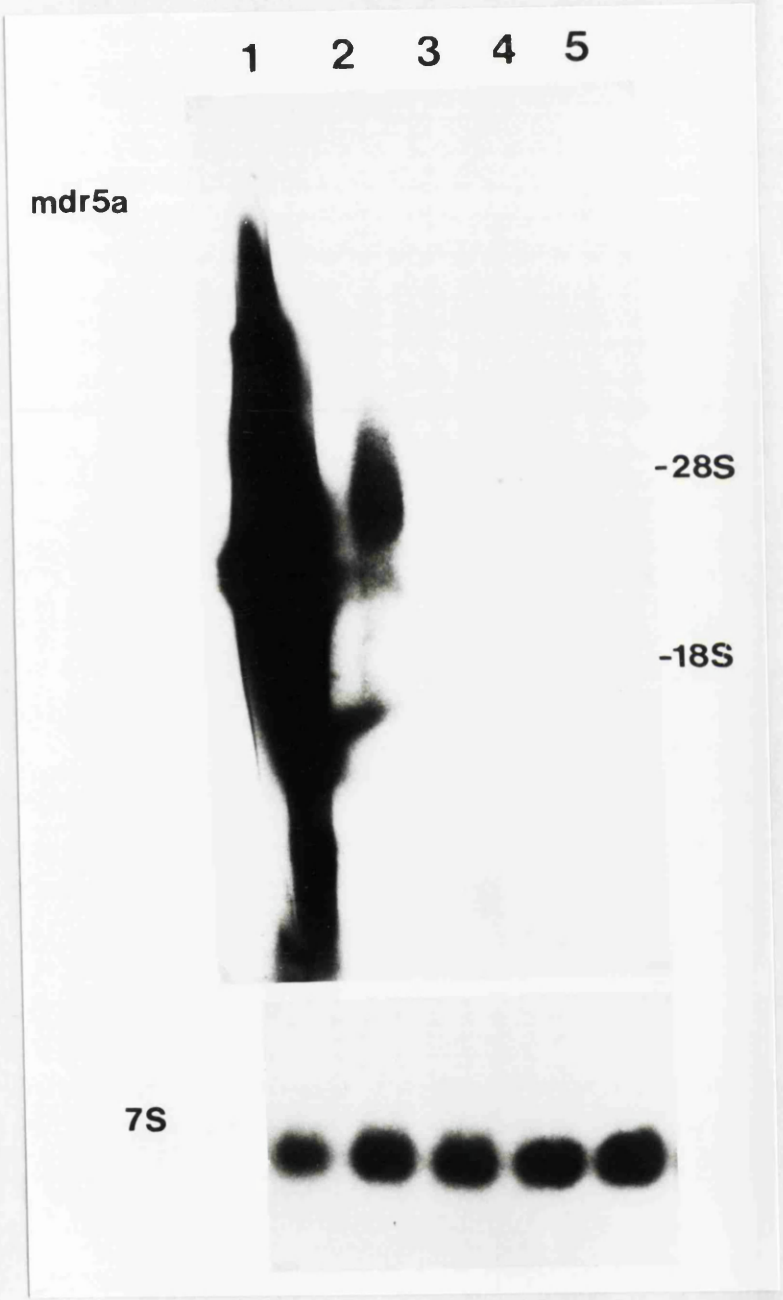
Repeated dotblots of the same batch of RNA from the KB cell lines were performed (data not shown). Following hybridisation to MDR5A, the blots were reprobed with poly(d)T as a measure of poly(A) containing RNA. The *mdr* expression was obtained by dividing the MDR5A densitometric signal by the poly(d)T signal. The ratio of KB8-5 to KB3-1 in repeated experiments (n= 10) varied between 3.45 and 11.3, with a mean of 7.3 and a standard deviation of 2.43. The range obtained is probably a reflection of the imprecision of the technique rather than genuine variations in the *mdr1* content of KB8-5.

The reason for this variation may be related to the weakness of the signal from KB3-1 . It was possible to reduce the KB8-5/KB3-1 ratio from 8.5 to 3.2 merely by using a longer autoradiographic exposure of the same filter. With this sort of technical artefact, it is particularly important to ensure that every clinical sample is assessed at least twice on separate dotblots to minimize artefactual errors.

Irrespective of the *exact* fold difference between the two cell lines, it is apparent that the range obtained here falls short of the 30-40 fold reported by Fojo *et al,* (1987b) and is closer to the figures reported by Shen *et al* (1986c) and Kuwazuru *et al* (1990a) shown in table 5.1. The variances may be largely due to technical differences in factors such as the sensitivity of the densitometry system used.

Figure 5.1 Northern blot hybridised with MDR5A

1. KBV-1
2. KB8-5
3. KB3-1
4. 3132
5 CL-1



The ratio of the KBV-1 to KB3-1 signal obtained from dot-blots was subject to an even greater degree of variation than the KB8-5 results. The ratio ranged from a mere 7.73 to 124.4 with a mean of 37 and a standard deviation of 34.9. This large variation is almost certainly related to the difficulties mentioned earlier i.e. it is virtually impossible to obtain an autoradiograph which has an adequate KB3-1 signal without allowing the KBV-1 signal to reach the saturation plateau of the radiograph film. Again, the fold differences were consistently less than what might have been anticipated from previous published work. However, unavoidable use of these over-exposed KBV-1 radiographs could account for the low readings obtained in this study. The exposures were deliberately aimed to provide readable signals from KB3.1 and KB8.5 and consequently KBV.1 accuracy was jeopardized.

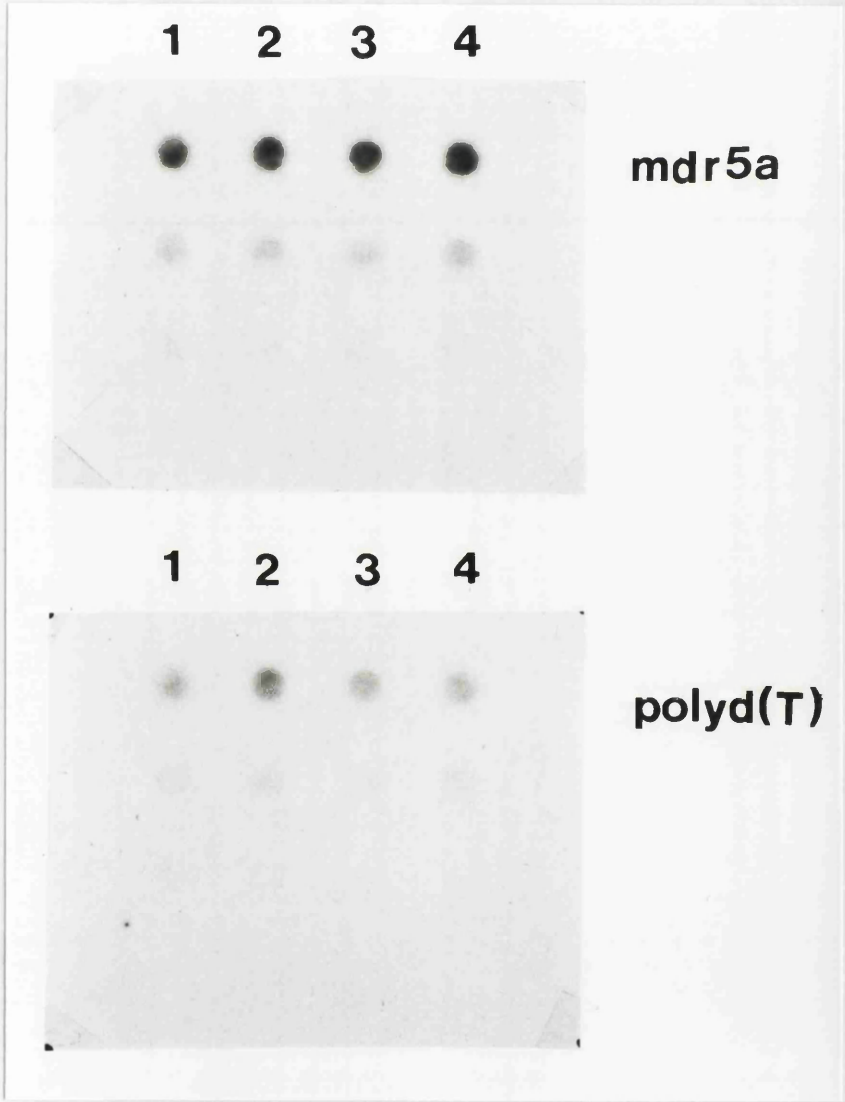
The control cell line RNA is of very good quality as determined by visualization on agarose gels and performance in RNAse protection assays. Unfortunately, the RNA obtained from clinical samples is of mixed quality and not infrequently partially degraded. The impossibility of obtaining consistently good quality RNA determined the need to use dotblots rather then Northern blots in the analysis of these samples.

The effect on *mdr1* signal intensity of using partially degraded RNA in dotblots is seldom addressed. Marie *et al* (1991) reported (without showing data) that controlled degradation of mRNA using RNAase A did not alter the subsequent signal obtained after *mdr1* hybridisation. This was performed using a sensitive parental cell line which had a negligible *mdr1* signal under normal conditions. The effects of degradation on an *mdr1* positive cell line was not examined. To determine the likely consequences of using degraded RNA in this system, 20ug of KB8-5 RNA was incubated with 0.25ng/ml, 2.5ng/ml and 25ng/ml of RNAse A (lanes 2-4 in figure 5.2) for 15 minutes at 37^{o}C. The result of MDR5A hybridisation of the RNAsed KB8.5 samples is shown in figure 5.2. Lane 1 has not had RNAse added. Visually, there is no easily discernible difference between the lanes. However, the computer densitometer could detect a 2.4 fold increase in signal intensity in lane 4 (the lane which received the most RNAse) relative to the control lane 1, whereas there was a negligible variation in the poly(d)T signal. This suggests that under these conditions, dotblot assessment of partially degraded RNA would have the tendency to over-estimate *mdr1* content by about twofold.

Figure 5.2 Effect of controlled RNA degradation on dot-blot hybridisation

All lanes contain KB8-5 RNA. Dotblot methodology is given in section 2.4.4. All dotblot hybridisations were in non-formamide conditions (2.4.6). Upper panel, MDR5A hybridisation. Lower panel, rehybridisation with poly(d)T. Amount of RNAse A used is given below.

1. 0
2. 0.25ng/ml
3. 2.5ng/ml
4. 25ng/ml



5.2 IMMUNOHISTOCHEMICAL DETECTION OF P-GP: SENSITIVITY AND LIMITATIONS OF THE TECHNIQUE.

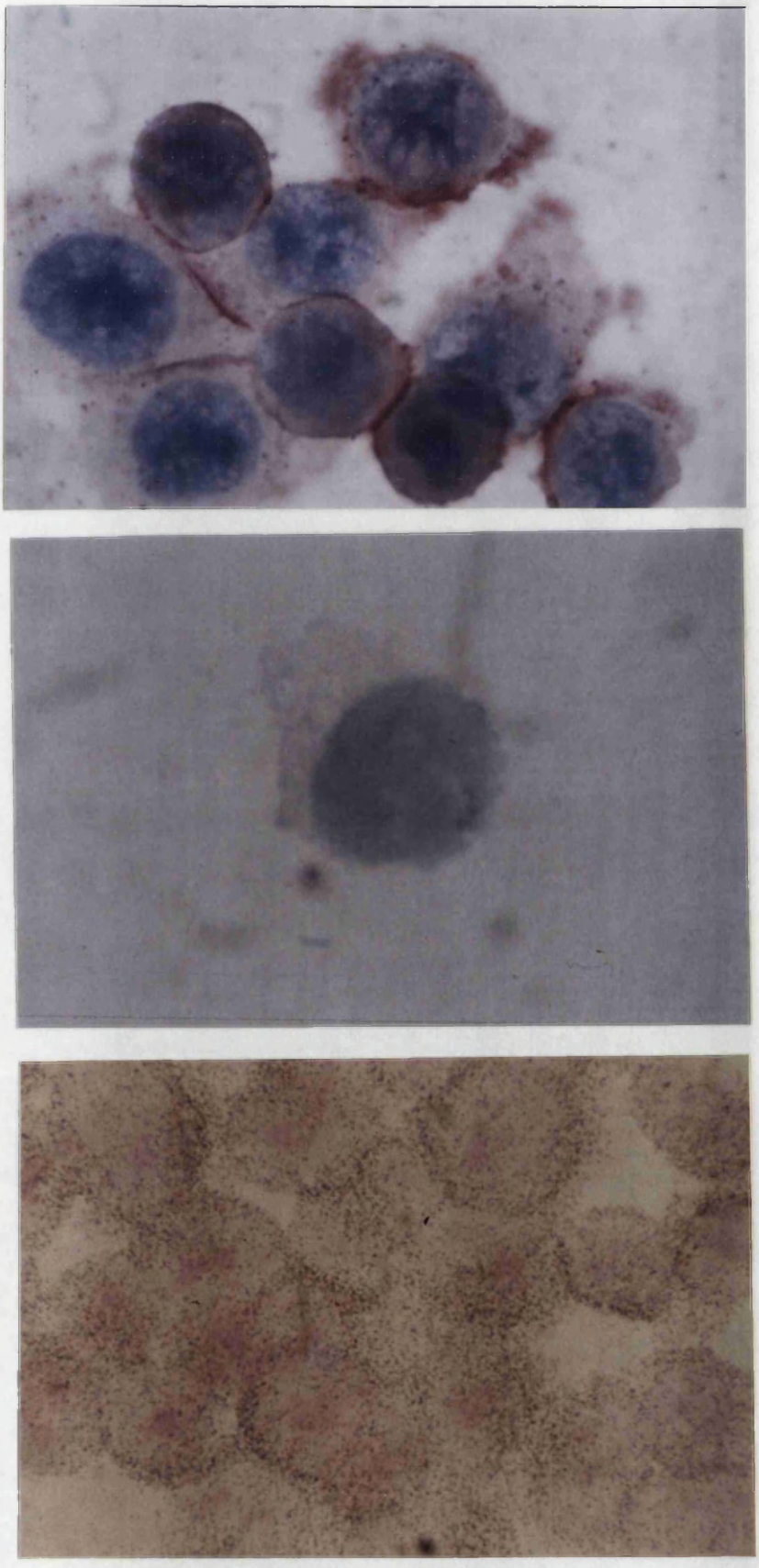
The same KB cell line series was used to determine the sensitivity of the alkaline phosphatase immunohistochemical technique described in section 2.6.2 and used in chapter 3. Figure 5.3 shows KB8-5, and KB3-1 stained with C219 (KBV-1 is shown in figure 3.4a).

KB3-1 does not have any staining whereas KB8-5 has discreet surface staining. Detection of P-gp in KB8-5 appeared to be at the limits of the sensitivity of this immunohistochemical technique in that if multiple KB8-5 coated coverslips were stained in parallel, not all the coverslips would yield positive cells. Thus the cells shown in figure 5.3a were on one of the four out of six positive coverslips on that day. The sensitivity of the system is good, especially considering it is a relatively simple protocol. Chan *et al* (1988) reported a sensitive IHC technique which is considerably more complex than this one which could detect P-gp in an ovarian carcinoma cell line which was only 8 fold resistant to vincristine and was P-gp negative on a Western blot. Both Chan *et al* (1988) and Grogan *et al* (1990) concluded that carefully optimized IHC was more sensitive than western blotting and probably at least as sensitive as Northern blotting. Grogan *et al* (1990) investigated different fixation techniques to use with C219 and concluded that acetone fixation was the most sensitive. Acetone fixation was used throughout this study.

KBV-1 stains very intensely with C219 to the extent that it is not possible determine that the staining is limited to the cell membrane (figure 3.4a). Read-out systems based on the enzymatic production of a coloured substrate are not ideal for providing accurate localisation of the antigen. To help determine if the cytoplasmic component of the staining in KBV-1 was an artefact created by the combination of gross P-gp overexpression and an enzymatic read-out system, KBV-1 was stained with a gold bead system (see section 2.6.4) using identical primary antibody conditions to that used in the alkaline phosphatase based methodology. Figure 5.3c shows KBV-1 stained using Aurogold^{Tm} then counterstained with the cytoplasmic stain saffrenin (it proved difficult to visualise the gold beads against a blue haematoxylin background). This system shows slightly better localisation to the cell membrane suggesting that some of the apparently cytoplasmic staining in KBV-1 using alkaline phosphatase may be a technical artefact. However, many of the cells still appeared to have a generalised scatter of gold beads across the cell. Further investigation of P-gp distribution in KBV-1 was not attempted but would benefit from confocal microscopy. The Aurogold system had problems with sensitivity and visualisation which made it unsuitable for use with clinical material.

Figure 5.3 C219 IHC with the KB cell line series

5.3a KB8-5 using alkaline phosphatase technique, x50 *(Top)*
5.3b KB3.1 using alkaline phosphatase technique, x100 *(Middle)*
5.3c KBV-1 using Aurogold system, x50 *(Bottom)*



5.3 STUDIES WITH CANINE LYMPHOMA CELL LINES

Several basic experiments were performed with the two canine lymphoma cell lines 3132 and CL-1. 3132, has surface Ig (Holmes, 1989) and is therefore a B cell whereas CL-1, has an immature T-cell phenotype, described in section 2.1.7. Dogs with T cells tumours perform poorly following chemotherapy compared to B cell tumours and thus examining these two cell lines provided a means of determining whether this difference in performance was related to the intrinsic chemosensitivity of the cell type or due to the presence P-gp in T cell tumours but not B cell tumours.

5.3.1 IN VITRO CHEMOSENSITIVITY OF CANINE LYMPHOMA CELL LINES.

The *in vitro* chemosensitivity of canine lymphoma cells has not been studied, probably because of the difficulties of growing canine lymphoma cells in culture. The availability of the two canine cell lines allowed the *in vitro* chemosensitivity of these two lines to be established.

The *in vitro* chemosensitivity of these two canine lymphoma cell lines was determined by MTT assay (described in section 2.7.1). The chemosensitivity of 3132 was also determined by a soft agar clonogenic assay (see section 2.7.2). CL-1 did not form colonies in soft agar or methocell. Conditioned media prepared from 3132 and CL-1 (section 2.7.4) was added to the methocell or soft agar at a 10-30% concentration in an attempt to encourage colony growth in CL-1. The conditioned media failed to encourage CL-1 colonies. The chemosensitivity of CL-1 was therefore only determined using the MTT assay.

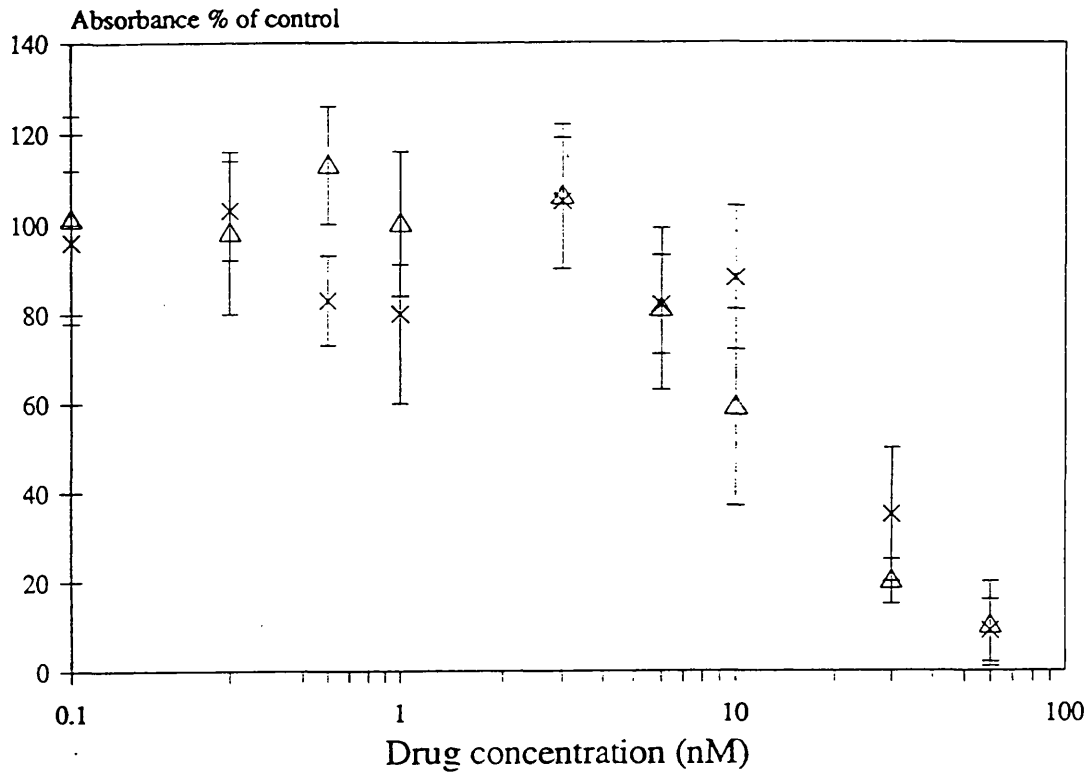
Chemosensitivities were determined for doxorubicin and epirubicin in both cell lines; the cells were exposed to drug for 24 hours and then allowed to recover & grow for a further 3 days (which is approximately three doubling times for these cell lines). The resultant graphs are shown in figure 5.4a and 5.4b. These graphs were obtained by combining the results of two independent MTT assays. Each assay produces eight determinations for each drug concentration so the points in figure 5.4a and 5.4b represent 16 independent observations.

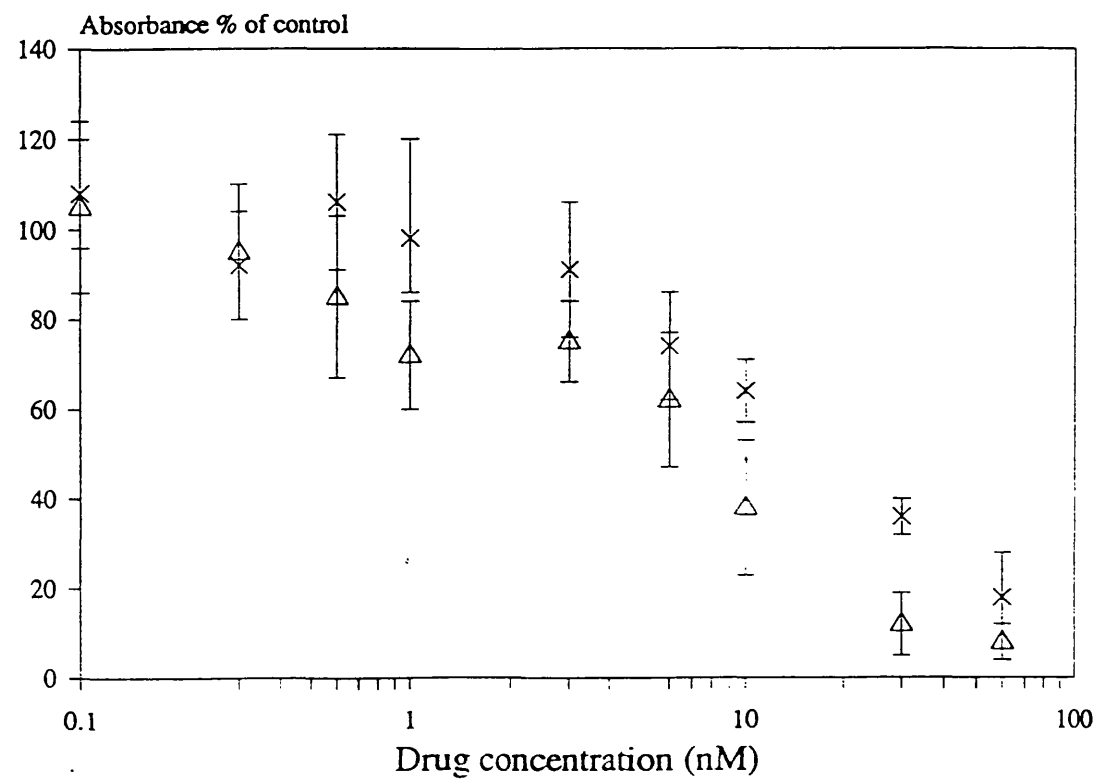
Figures 5.4a and 5.4b show that the putative T cell line is not more resistant to doxorubicin or epirubicin than the B cell line. In fact, CL-1 may even be more sensitive than 3132. The D_{50} value (being the drug concentration required to reduce the absorbence to 50% that of the control untreated cells) for the two drugs is given in Table 5.2. The standard errors on the results of the two independent experiments are sufficiently large that it is impossible to give an accurate D_{50}. However it would be prudent to conclude there are no significant differences between the two cell lines nor is there any significant difference in the cytotoxicity of the two anthracyclines.

Figure 5.4 Chemosensitivity of canine lymphoma lines.
5.4a Doxorubicin chemosensitivity by MTT assay (Top)
5.4b Epirubicin chemosensitivity by MTT assay (Bottom)
5.4c 3132 epirubicin chemosensitivity by clonogenic assay (over page)

Figures 4a and 4b; Graphs of percentage viability as a function of drug concentration for 3132 and CL-1. Mean values are shown plus error bars representing standard errors of the mean (for 16 observations).

X = 3132 cell line Triangle = CL-1 cell line





5.4c Epirubicin chemosensitivity of 3132 determined by soft agar clonogenic assay.

Figure 5.4c: Graph of percentage colony numbers compared to controls. Mean values are shown plus error bars representing the standard error of the mean (for 10 observations)

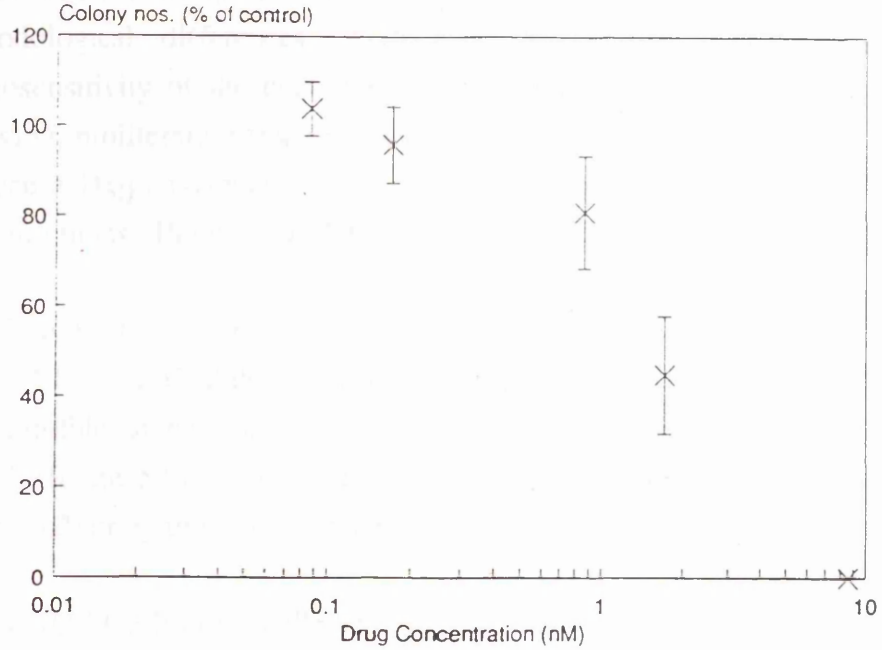


Table 5.2 D_{50} values for canine cell lines.

Cell line	Doxorubicin	Epirubicin	Method
3132	18nM	25nM	MTT
Cl-1	9nM	14nM	MTT
3132	*not done*	2nM	soft agar

The D_{50} of these cell lines are similar to other unselected cell lines. The D_{50} of H69 (a small cell lung cancer cell line) to doxorubicin and epirubicin is approximately 30nM and less than 10nM respectively under similar conditions (Mirski *et al*, 1987). A2780 (an ovarian carcinoma line) has a D_{50} between 1 and 5nM following a 24 hour exposure to drug (Luo, 1992). These cell lines with chemosensitivities in the same range as 3132 and Cl-1 do not express P-gp as determined by a variety of techniques (Luo, 1992; Plumb *et al*, 1990).

The difference between the 3132 D_{50} generated by the clonogenic assay (approximately 2nM) compared to the MTT assay (approximately 25nM) may be

related to the difference in the length of the drug exposure. In the soft agar cloning assay, the drug was incorporated into the agar whereas the MTT assay used a discrete 24 drug hour exposure. The half life of doxorubicin in culture is about 15 hours (Beijnen *et al,* 1986) and so there was longer exposure to the active drug in the clonogenic assay compared to the MTT assay. However, excluding the methodological differences, there may be genuine differences between the chemosensitivity of the clonogenic cells measured by the soft agar assay and the surviving, proliferating fraction measured by the MTT assay. Clonogenic assays can produce a D_{50} concentration that is half that of MTT assays, using identical drug exposure times, (Plumb *et al,* 1989).

5.3.2 LACK OF P-GP EXPRESSION IN CANINE CELL LINES.

Cl-1 and 3132 do not express *mdr* mRNA on Northern blots (figure 5.1) and give a dotblot signal which is less intense even than the sensitive parental cell line KB3-1 (figure 5.8). Neither cell line has P-gp as determined by western blotting (figure 3.2) or by immunocytochemistry with C219 (data not shown).

5.3.3 LACK OF P-GP EXPRESSION FOLLOWING DRUG EXPOSURE

The canine cell lines were also used to assess if P-gp expression could (temporarily) increase following exposure to epirubicin. Normal liver increases *mdr* transcription following surgically or chemically induced damage within a few hours, and levels remain elevated for up to 3 days (Fairchild *et al,* 1987). Under physiological conditions, the P-gp content in the female reproductive tract can alter during pregnancy (Bradley *et al,* 1990; Sugawara *et al,* 1988; Huang *et al,* 1989). Thus certain tissues have the capability of inducing P-gp in response to various stimuli.

It is unknown if normal or neoplastic lymphocytes can undergo fluctuations in their P-gp content in response to the acute effects of chemotherapy. If increases in P-gp do occur within a few days of receiving chemotherapy, then this could affect the interpretation of the results of the *in vivo* P-gp study where dogs may have received chemotherapy only a few days before a relapse sample was collected. To ascertain whether discernible fluctuations would occur, the *mdr1* expression in both CL-1 and 3132 was measured for 5 days following drug exposure.

Cl-1 and 3132 were split in fresh media to give a cell concentration of 10^6/ml. Epirubicin was then added at either 10nM or 1nM (approximately D_{60} and D_{10} respectively). Drug was removed after 24 hours and replaced with fresh media. RNA for analysis was collected at time O, 24hrs, 48hrs, 72hrs and at 5 days. The daily percentage viability of the cells was monitored. The lowest viabilities recorded

were 38% and 43% for CL-1 and 3132 immediately upon termination of the 10nM drug exposure.

The RNA was dot-blotted and electrophoresed on a northern gel . Following MDR5A hybridisation no *mdr1* mRNA was seen in any of the canine cell line samples in the five day period following drug exposure (figure 5.5).

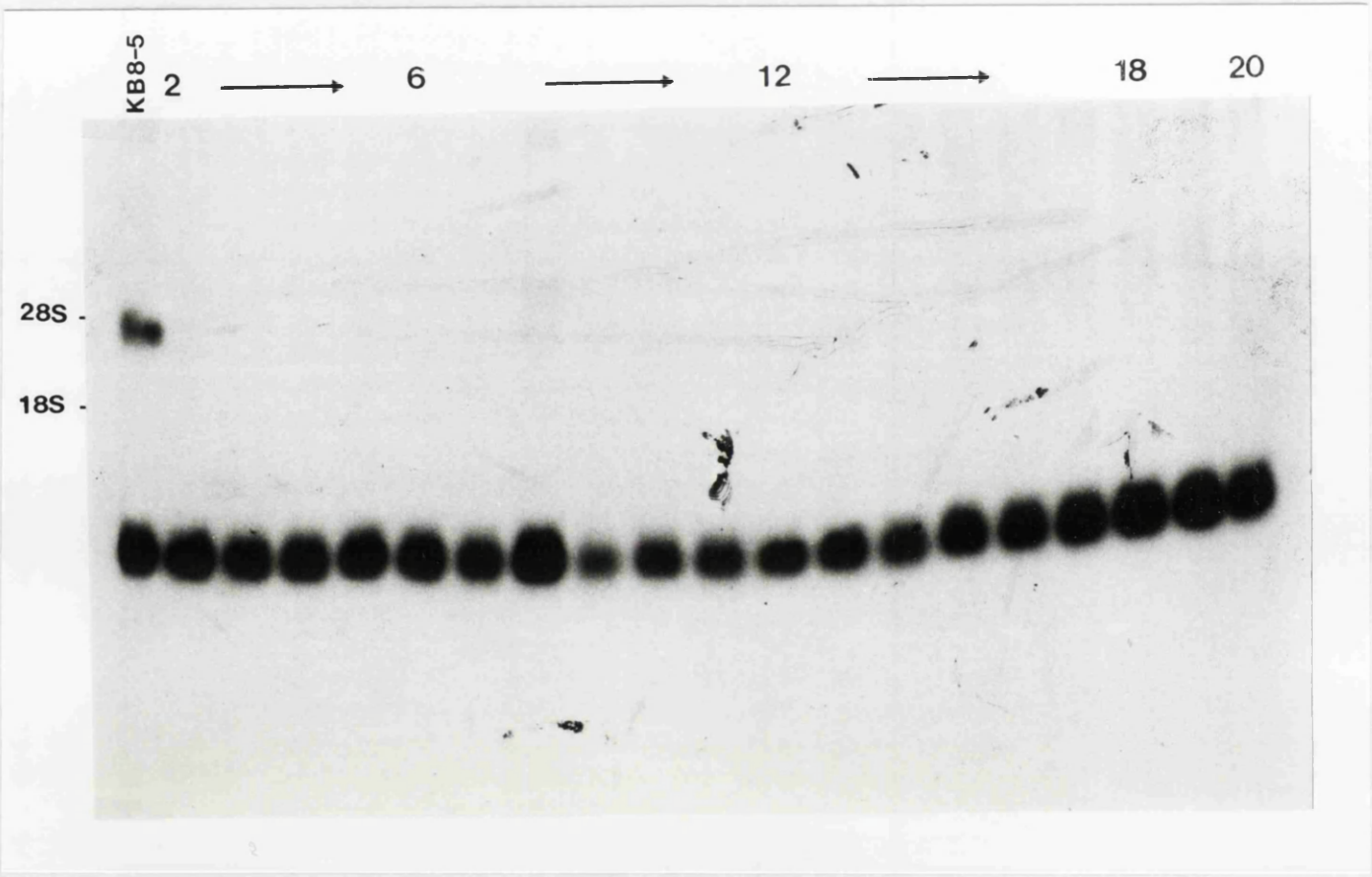
The experiment was only performed once although the RNA recovered from the cell lines was Northern blotted four times to repeat the negative result. This result suggests that significant fluctuations in P-gp do not occur in either the T or B cell line following anthracycline exposure. By analogy, acute P-gp fluctuations in MLSA cells due to induction following chemotherapy may also be absent (or at least below the limits of detection).

Legend for figure 5.5

1. KB8.5
2. 3132, time 0
3. Cl-1, time 0
4. 3132 + 1nM epirubicin, 24hrs
5. as above, 48 hrs
6. as above, 72 hrs
7. as above, 5 days
8. CL-1 + 1nM epirubicin,24hrs
9. as above, 48 hrs
10. as above, 72 hrs
11. as above, 5 days
12. 3132 + 10nM epirubicin, 24hrs
13. as above, 48 hrs
14. as above, 72 hrs
15. as above, 5 days
16. CL-1 + 10nM epirubicin, 24 hrs
17. as above, 48 hrs
18. as above, 72 hrs
19. as above, 5 days
20. KB3-1

Figure 5.5 Northern blot: Time course of *mdr1* expression in canine lymphoma cell lines after drug exposure

Content of lanes 1-20 is on previous page.



5.4 DETECTION OF P-GP IN NORMAL, REACTIVE & LYMPHOMATOUS NODES

5.4.1 ACQUISITION OF CLINICAL SAMPLES

MLSA samples were obtained from clinical cases which were referred to Glasgow University Veterinary College between September 1988 and March 1992. A total of 62 cases were accrued in this time; the breakdown of these samples in terms of chemotherapy exposure is given in table 5-3.

Lymph nodes were examined both immunohistochemically (methodology in 2.6.2) and by dot-blot hybridisation (2.4.4) for the presence of P-gp and *mdr1* mRNA respectively. All of the 62 cases (with the exception of one relapse sample), were assessed by dotblot hybridisation whereas only 44 of the cases were also examined immunohistochemically. This shortfall in the immunohistochemistry is due to the fact that for the first year of the project, clinical samples were not consistently collected in a manner suitable for subsequent immunohistochemical work.

Table 5.3 GUVC MLSA Patient Sample Collection

Number	Sample Type
29	Pre-chemotherapy only
9	Post-chemotherapy only
2	Unknown status
22	Pre-chemotherapy plus follow-up post-chemotherapy

The majority of the pre-chemotherapy samples were obtained by excisional lymph node biopsy. Most of these dogs went on to receive chemotherapy described in the next section. The post-chemotherapy samples were obtained either from a second surgical biopsy or more commonly at time of euthanasia due to tumour progression. Twenty-eight of the thirty-one post-chemotherapy samples were obtained from dogs with clinically resistant disease; the other three dogs died in remission for reasons unrelated to tumour progression. These three samples are indicated in the text and tables as required.

A list of the sixty-two dogs plus their signalment is provided in table 5.4. Further clinical detail of the GUVC cases is given in chapter 7. MLSA samples were also received from other veterinary institutions. The clinical information on these cases is sparse and the treated dogs received different multi-drug protocols. In total, there were 17 pre-chemotherapy samples, 4 relapse samples and 3 unknowns from other veterinary institutes.

Table 5.4 GUVC MLSA Case Signalment

Name	Case #	Signalment
JAW	108557	9yr M GSD
CRS	107311	2yr F Golden retriever
107	107235	Adult M Golden retriever
KMD	107190	7yr F Bull Terrier
MOK	108548	5yr FN Cross breed
LUJ	109456	9yr M Labrador
LET	109341	5yr FN Great Dane
ROB	109342	10yr M Cross breed
MMG	109533	3yr F WHWT
SMM	109393	13yr M Labrador
PAM	110488	9yr M Border C Collie
BRM	109969	10yr FN Labrador
BMC	109542	5yr FN Labrador
RMD	111055	9yr M Scottie
RIC	111692	5yr M CKCS
HOG	111352	8yr FN Golden Retriever
TOB	111864	13yr M CKCS
PES	111925	6yr M Collie
GOF	102612	12yr F Labrador
MIG	111979	5yr F CKCS
RMI	111961	5yr F Golden Retriever
BOA	112230	5yr MN Bull Mastiff
SAO	112305	9yr M Labrador
CHP	112379	5yr F Bull Mastiff
CIS	112955	5yr FN Cross breed
SAD	112908	3yr M Cocker Spaniel
LAQ	113168	11yr M Cross Breed
RMC	113310	7yr MN Cross Breed
TIT	112539	yr FN Cross breed
SAC	113294	6yr M Golden Retriever
TIH	113617	3yr FN Springer Spaniel

M, male; MN, male neuter; F, female; FN, female neuter; GSD, German Shepherd dog; CKCS Cavalier King Charles spaniel; WHWT, West Highland White terrier.

Table 5.4 (continued) *legend on previous page.*

Name	Case #	Signalment
LOK	113070	6yr M GSD
MAK	113069	6yr M GSD
BRP	113419	11yr M Elkhound
BIP	113579	13yr MN Cross breed
TAB	112614	6yr F Airedale
ZAK	114133	5yr F GSD
CMD	114189	5yr FN Bull Mastiff
SAB	114195	8yr M Cross breed
BEM	114768	5yr MN Golden Retriever
KEH	114800	10yr FN Old English Sheepdog
BEH	114887	4yr M Springer Spaniel
BRC	115029	8yr M Labrador
KEB	115069	5yr F Irish Setter
MMK	115227	8yr FN Bearded Collie
LEB	115251	9yr M Cross breed
TRC	115074	7yr M Old English Sheepdog
BOS	115498	5yr M Collie cross
SPC	115616	4yr M Cross breed
BMD	115824	4yr M Lhaso Apso
BEA	115827	6yr M Great Dane cross
SMD	115839	7yr FN Bull Mastiff
SAS	104529	4yr F Boxer
RMM	116793	3yr M Bull Mastiff
CIP	117240	11yr FN Cross breed
BOB	116202	6yr M Labrador
SAB	116826	9yr M Old English Sheepdog
HEH	117986	3yr M Tibetan Terrier
THH	109400	7yr M Dobermann
SAP	N.A.	11yr FN Miniature Schnauzer
ZAS	116674	7yr FN Cocker Spaniel
DHS	115030	8yr MN Labrador

In addition to the lymphomatous nodes , six normal nodes from three dogs (a submandibular and popliteal node from each dog) plus samples from four dogs with non-neoplastic lymphadenopathy were also included in this study. The case numbers and the histopathologic diagnoses of the four lymphadenopathies is listed in table 5.5. These nodes were surgically biopsied as part of the routine investigation of chronic lymphadenopathy.

Table 5.5 Signalment of lymphadenopathy cases

Code	Number	Signalment
OLS	113813	1.5yr M Bullmastiff Pyogranulomatous Lymphadenitis
GEG	111688	3yr F Border Collie Granulomatous lymphadenitis
MUS	111953	15yr FN English Springer Reactive Hyperplasia
LAM	112275	2yr FN West Highland White Reactive hyperplasia

Abbreviations as for table 5.4

Chemotherapeutic Treatment of MLSA

The main aim of this project was to investigate the role of P-gp in contributing to acquired anthracycline resistance in a naturally occurring chemosensitive tumour. To serve this aim, MLSA cases were given a protocol dominated by the MDR-type anthracycline, epirubicin. The exact protocol is detailed in chapter 1, table 1.14.

Epirubicin was given in preference to doxorubicin because of its reduced cardiotoxicity . It was anticipated from previous work with single agent doxorubicin (Postorino *et al,* 1989) that the majority of dogs would relapse with clinically resistant disease prior to reaching a dangerous cumulative dose.

A total of forty-nine dogs were treated with chemotherapy; the remaining thirteen dogs were euthanased at the owners request at the time of diagnosis. The majority of the forty-nine treated dogs received the epirubicin-based protocol. The epirubicin was provided free of charge and administered under the informed consent of the owners.

Two dogs (BMM and SMD) were not considered suitable for the epirubicin protocol because of pre-existant myelosuppression and cardiomyopathy respectively. These two dogs received a standard vincristine and cyclophosphamide and prednisolone (COP) protocol (Carter *et al,* 1987). Samples were obtained from another six dogs which received COP with or without cytosine arabinoside in addition to the COP. These dogs did not receive epirubicin merely because they were referred to GUVC after the withdrawal of the subsidised epirubicin protocol. Those dogs which did not receive the standard protocol are indicated where appropriate.

Forty-one dogs received the epirubicin/prednisolone protocol until time of first relapse. Once clinical relapse was apparent, the management of the dogs was not standardised. If owners elected to try further treatment, various options were used

which depended on the patient parameters and on clinician preference. Two main approaches were used. The first was increasing the epirubicin dose to 30mg/m^2 or above plus the addition of oral cyclophosphamide (50mg/m^2) every other day and using induction doses of prednisolone. The alternate approach involves substituting weekly vincristine (0.7 mg/m^2) for the augmented epirubicin. If either of these failed to induce a response, L-asparaginase was used in a limited number of patients. 14/31 of the relapse samples were obtained from the dogs following the failure of rescue therapy so it cannot be ruled out that these other drugs may have influenced the expression of P-gp in the lymphomatous nodes. However, for reasons discussed in chapter 7, it was felt that the contribution of the non-epirubicin cytotoxic drugs to the drug resistance profile of the tumour was minimal in comparison to the drug resistance mechanisms already present in the resistant tumours at the onset of relapse. The clinical performance of the protocol is discussed in chapter 7.

5.4.3 P-GP DETECTION IN A DENDRITIC CELL POPULATION AND ENDOTHELIUM OF LYMPH NODES

5.4.3 (i) P-gp in normal, reactive and lymphomatous nodes

The alkaline phosphatase based immunohistochemical technique outlined in 2.6.2 was used to stain all lymph nodes. Two sections from each block were stained in parallel; one with C219 and the other with C219 which had been preincubated with a 1000M excess of 15 amino acid blocking peptide for 1 hour at room temperature, as described in chapter 3.

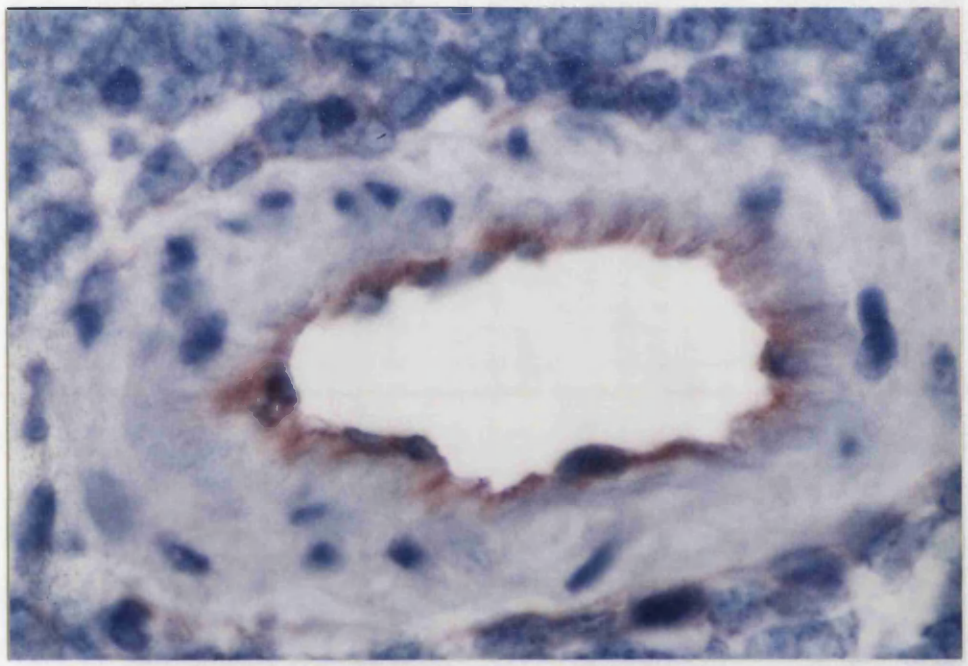
In normal, reactive and lymphomatous nodes, C219 stained the luminal surface of blood vessel endothelium (Figure 5.6a). This endothelial staining was present in virtually all sections. In fact the Fast Red substrate helped in the detection of collapsed small blood vessels which may otherwise have avoided detection. Occasionally larger vessels would be included in the sections and in some of these vessels, P-gp appeared to be within the muscular layer of the vessel wall rather than on the endothelial surface (Figure 5.6b and c); P-gp has been reported in the cytoplasm of smooth muscle in other tissues (Van der Valk, 1990). Endothelial P-gp has been documented in other organs such as the CNS and testes (Theibaut *et al*, 1989; Cordon-Cardo *et al*, 1989; Bradley *et al*, 1990). The physiological significance of P-gp in endothelium is unknown but is unlikely to be involved in the exchange of nutrients between tissue and blood because endothelial P-gp is not limited to capillaries but is also found in small arterioles and venules (Bradley *et al*, 1990) .

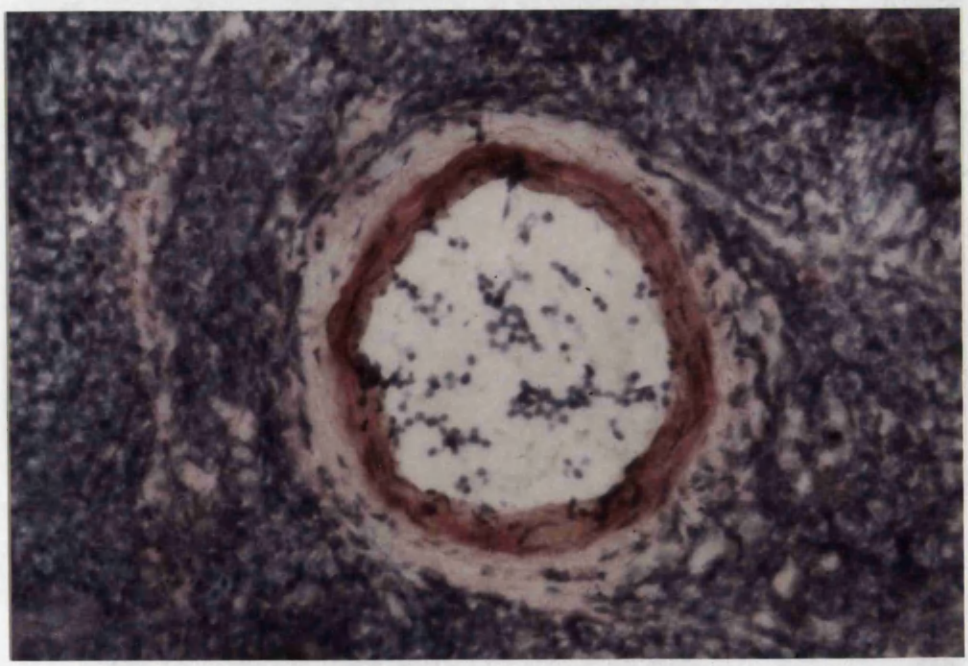
Figure 5.6 P-gp in Blood vessels from lymphomatous nodes
(AP-IHC with C219)

5.6a Endothelial surface staining (x 40) *Top*

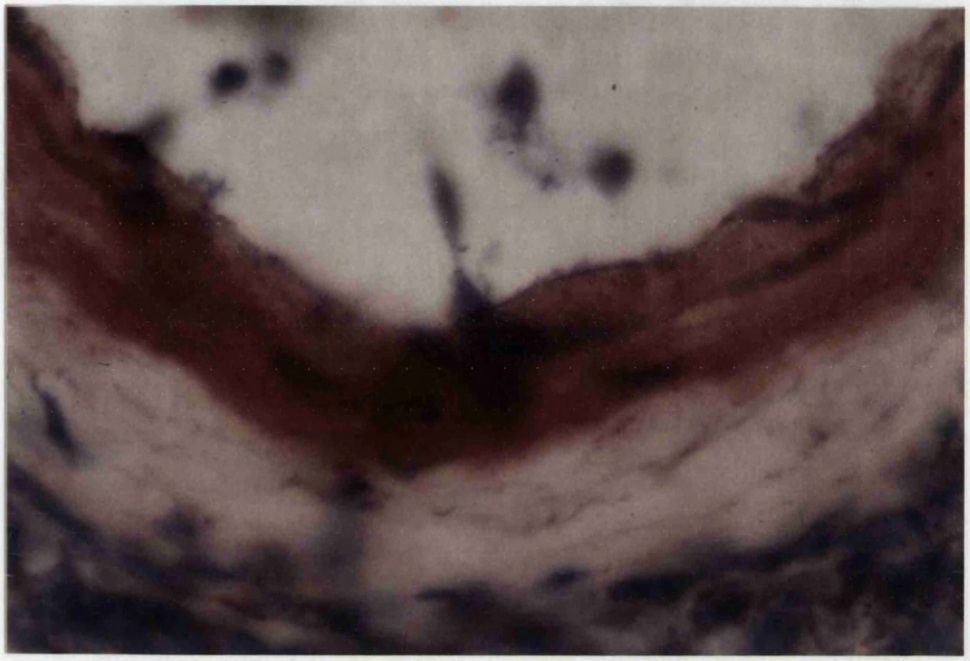
5.6b Muscular layer staining.(x 20) *Bottom*

5.6c Muscular layer staining.(x 50) *Next page*





5.6c Muscular layer staining in blood vessel. (x50)

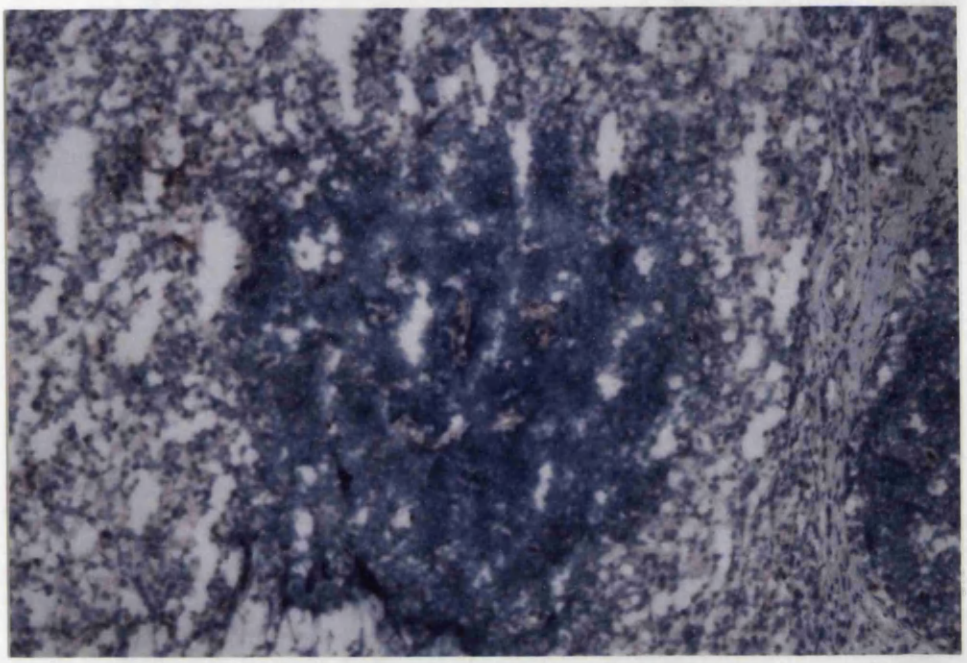


Diffuse staining was also commonly seen in a dendritic type cell (figure 5.7a). These cells were found in the vast majority of lymph nodes examined (Table 5.6). These cells were not frequent in lymphomatous nodes; within an average high power field (x40) less than 5 cells would be identified (figure 5.7b). Two large lymphomatous nodes were dissected into separate blocks and each block examined individually; the distribution of these cells within each block was haphazard but there was no large variation between the blocks (data not shown).

Most lymphomatous nodes do not retain noticeable normal lymph node architecture so it was not possible to ascertain if these dendritic cells were associated with a particular area of the node. However, in the normal and reactive nodes, the distribution of these P-gp positive cells was more orderly. P-gp positive cells were identified in both the B cell follicular areas and in the T cell paracortical areas but only rarely in the lymph node medulla (figure 5.7b and c). Even in the follicles and paracortical areas, the positive dendritic cells were still a minority population compared to the lymphocytes. Figure 5.7c shows a positive cell within a follicle; the block is from case "GEG" which was diagnosed as a reactive process.

Figure 5.7 P-gp in Dendritic cells

5.7a Normal node (x10) *Top*
5.7b Reactive node "GEG" (x20) *Bottom*



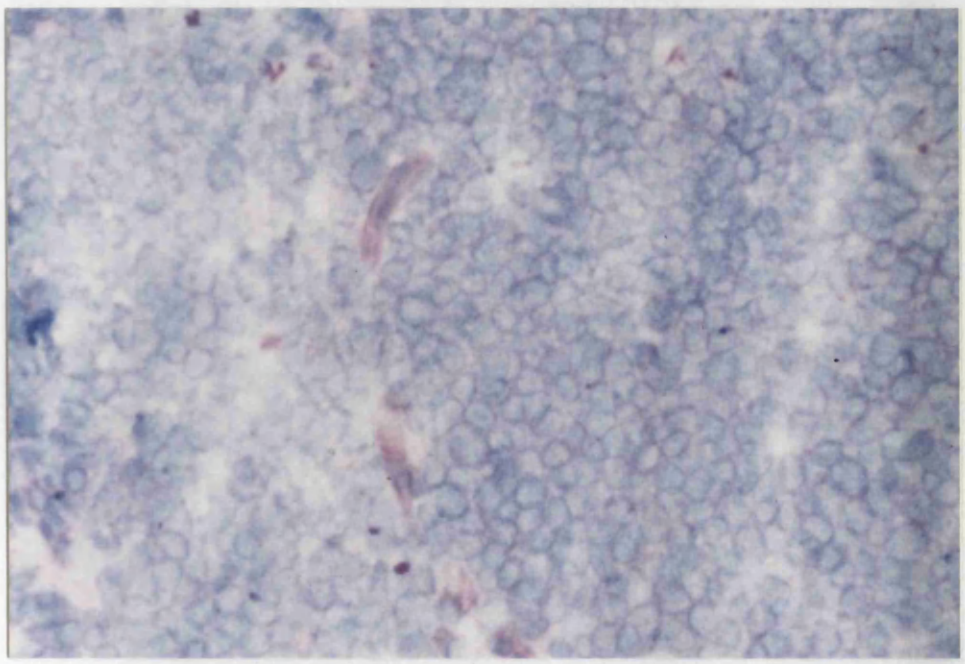
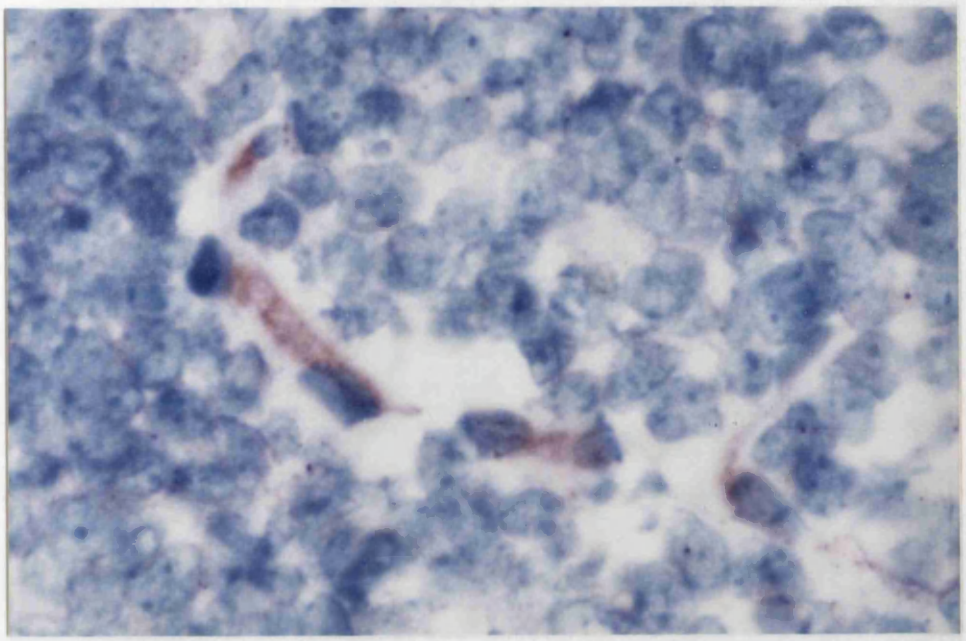
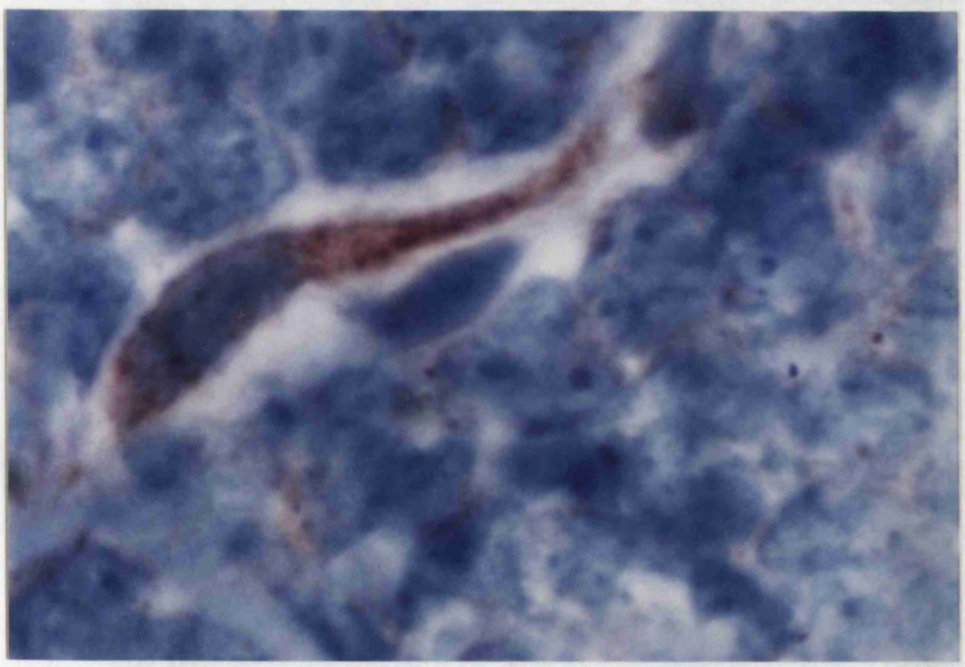


Figure 5.7 P-gp in Dendritic cells (continued)

5.7c Lymphomatous node "KEH" (x40) *Top*
5.7d Lymphomatous node "SNB" (x100) *Bottom*





Interestingly, this "GEG" sample also shows C219 staining in the B cells of the reactive follicles, but not in the T cell areas. This result was repeated in this sample but was not seen in normal nodes. However normal nodes occasionally gave unrepeatable faint staining of B cells; the significance of this is discussed further in section 5.4 and chapter 8.

Two tumours were identified (BMD and KEB) which had an over-representation of the dendritic cells within the lymphomatous node; more than 10 cells per high power field could be detected. These two cases will be discussed in more detail later.

Table 5.6 P-gp Staining in Lymph Node Dendritic cells

Lymph node Type	Number of samples with P-gp in stromal cells
Normal	6/6
Reactive	4/4
GUVC[1] Lymphomas	40/44
NCSU-CVM[2] Lymphomas	18/18
CSU-CVM[3] Lymphomas	3/3
CUVC[4] Lymphomas	2/3

1. Glasgow University Veterinary College
2. North Carolina State University College of Veterinary Medicine
3. Colorado State University College of Veterinary Medicine
4. Cambridge University Veterinary College

The dendritic cells were intimately associated with the lymphocytes and appeared to extend processes between adjacent lymphocytes; occasionally the cells appeared multinucleate. The staining pattern was not obviously localisable to the cell membrane alone but appeared as a rather granular pattern within the cytoplasm (figure 5.7a). Section 5.1 discussed the role of the enzymatic substrate reaction in contributing to the cytoplasmic staining of KBV.1. However these stromal cells do not stain as intensely as the *mdr1* gene amplified cell line and so it may be that the cytoplasmic component may be a true representation of the P-gp localisation and not merely a technical artefact. The Aurogold^{TM} bead system was used to try to confirm the cellular localisation but unfortunately the technique was not sensitive enough for detection in the lymph node sections.

P-gp localisation at sites other than the cell membrane is a contentious issue (Battifora, 1991). P-gp localisation within the Golgi region of cells has been documented repeatedly using immunohistochemistry and has become accepted as a

genuine phenomenon (Willingham *et al,* 1987; Chan *et al,* 1988; Grogan *et al,* 1990; Salmon *et al,* 1989 and 1991). The exact physiological role of the protein at this site is unknown but is presumed to function in some transport capacity.

P-gp has also been reported in the cell cytoplasm in numerous papers (Broxterman *et al,* 1989; Wishart *et al,* 1990; Weinstein *et al,* 1991). This is generally regarded with some scepticism because most background problems manifest themselves as cytoplasmic staining (Battifora, 1991) and in some papers, the controls run are not adequate to identify artefactual staining (Wishart *et al* 1990). Cytoplasmic staining has been reported in tumours using both C219 and the *mdr1* specific monoclonals MRK16 and JSB-1 (Weinstein *et al,* 1991; Wishart *et al,* 1990; Van der Valk *et al,* 1990). When multiple monoclonals to different epitopes on the same protein give the same cytoplasmic localisation and the controls appear to be adequate, it is difficult to dismiss the results as spurious merely because they do not "fit" the currently accepted view of P-gp physiology.

However, in defence of the sceptics, studies which used C219 without a peptide competition block have to be reevaluated more thoroughly since the revelation that certain batches of C219, and JSB-1, have in the past been contaminated with reagents which react with blood-group antigens (Finstad *et al,* 1991). Epithelial tissues such as gastrointestinal tract and genitourinary tract can express blood group antigens and hence spurious staining with C219 in these tissues may have occurred due to contaminated C219 batches (Weinstein *et al,* 1990a). This problem with the quality control of C219 will not affect this canine work firstly because canines do not share blood group antigens with humans and secondly because the use of a competitive peptide block technique would reveal spurious reactivity.

Although the canine lymph nodes will not be affected by blood-group reactive species which may contaminate C219, lymph nodes present their own problems with quality control. Different cell types within normal lymph nodes express receptors for the Fc component of immunoglobulins and hence they have a propensity to bind Ig in a non-specific way. In particular, some of the macrophage/monocyte population can give problems in immunohistochemistry in this regard. This potential problem is normally avoided by exposing the sections to sera or purified immunoglobulin prior to applying the primary antibody so that all Fc receptors will already be occupied. In this study, 3% rabbit sera and 5% skimmed milk were both tried as the initial blocking solution , however general background was lowest when a mixture of 1.5% canine and 1.5% rabbit sera was used inferring that cell populations within the lymph nodes may possess Fc receptors which bind canine Ig in preference to rabbit Ig. The rabbit/canine sera mixture was therefore

adopted as the block solution for all lymph node IHC. However, the stringency of the peptide competition as a control negates the risk of misinterpretation of C219 binding through Fc receptors because this would be seen as background.

5.4.3 (ii) Characterisation of the P-gp positive dendritic cells

P-gp positivity has been reported in non-lymphocytic cells in human lymphomas. Miller *et al,* (1991) reported P-gp within Reed-Sternberg cells in Hodgkins lymphoma using JSB-1 and C219. Schlaifer *et al* (1990a and b) have also reported P-gp expression in a stromal cell population within lymphomatous nodes using both C219 and MRK16. Neither group examined normal lymph nodes but Schlaifer *et al* (1990b) used a CD68 monoclonal to identify certain macrophage/monocyte lineages (Kelly *et al* 1988) which they claimed were morphologically identical to the P-gp positive cell population, however dual staining was not performed. The authors remarked that the CD68 positive cells were more frequent than the P-gp positive cells and suggested that P-gp was only present in a subpopulation of the CD68 positive cells.

P-gp positivity is also reported in macrophage like cells in other tissues. Van der Valk *et al* (1990) used the *mdr1* specific MRK16 and described P-gp positive cells "that were difficult to characterize; they were usually large cells with ample cytoplasm and showed a coarsely granular staining with MRK16. On the basis of their morphologic characteristics and their distribution, they were considered to be macrophages." These putative macrophages were found in the lung, spleen, skin, breast and prostate. The same paper also reported that JSB-1 stained a few cells in the brain with a dendritic morphology and that this staining was granular.

Thus there is precedence for cytoplasmic P-gp in macrophage/monocyte type cells. The morphology of the P-gp positive non-tumour cells in the canine lymph nodes is not typical for a phagocytic type cell. Instead their dendritic morphology and their localisation in normal and reactive canine nodes would suggest that they may be accessory cells involved with antigen processing and presentation. In B cell areas, follicular dendritic cells (FDC) present antigen whereas in T cell areas interdigitating cells (IDC) have a similar function (Steinman, 1991; Heinan *et al,* 1988). The canine cells sometimes appeared to be bi- or multi-nucleate. FDC in other species have been reported to be multi-nucleate (Pallesen and Myrhe-Jensen, 1987; Gerdes *et al,* 1983).

Precise identification of FDC and IDC is not simple. In mice and humans they are usually identified based on the pattern of reaction with a panel of monoclonals against a variety of haemopoietic determinants, plus enzyme histochemistry. Certain monoclonals such as Tul, R4/23, To5 and Ki-M4 were once considered fairly FDC specific but as more information became available on the

antigens recognised by these monoclonals, it has become apparent that they are not FDC specific. For instance Tul recognizes CD23 which is present on B cells as well as FDC (Pallesen, 1987) and To5 recognizes the C3b receptor (CD35) which although is present in FDC, is also present on other macrophages populations. The R4/23 antigen has not yet been characterized but the manufacturers state that R4/23 reacts (weakly) with splenic and follicular B cells (Dako product sheet code no. M709). In a rare case of FDC sarcoma investigated by Pallesen and Myhre-Jensen (1987), the FDC tumour cells did express most of the expected markers but were negative for R4/23. This illustrates the importance of using multiple monoclonal antibodies to different antigens to identify this cell type.

Monoclonal antibodies against murine or human blood cell CD groups do not usually cross-react with canine cells (discussed by Holmes, 1989). It is therefore difficult to take advantage of the commercially available reagents. In an attempt to characterize the canine dendritic cell population further, lymph nodes were stained using the MAC387 monoclonal which identifies a "histiocytic" type of macrophage (Flavell *et al* , 1987). MAC387 is one of the few commercially available monoclonals against macrophage or monocytes which cross-reacts with canine determinants. MAC387 did identify a cell population within lymph nodes but these cells were found predominantly in the medullary region and were more globular than the cells identified with C219 (data not shown). It was concluded that MAC 387 did not identify the same population as C219. However, MAC387 is not reported to react with FDC nor IDC (Dako Product Sheet Code No.M747). Thus from the outset, this monoclonal was not anticipated to react with the same population as C219.

As an alternative to species specific monoclonal reagents, FDC and IDC are reported to possess S-100, which is an acidic protein originally identified in neural tissue (Takahashi *et al,* 1984) and which is conserved between species. S-100 has two subunits, α and β; FDC differentially react with α subunit monoclonals and IDC with β subunit monoclonals (Takahashi *et al*, 1984; Tanaka, 1986). The use of a polyclonal anti-S-100 sera with activity against both subunits should identify both populations of cells. In fact, Fondevila *et al* (1989) used an S-100 polyclonal sera to identify putative FDC and IDC within formalin fixed canine lymph nodes. They documented S-100 positive cells in 13 out of 24 lymphomas examined, either scattered throughout the tumour or in follicles.

S-100 has not been universally successful in detecting canine FDC. Sandusky *et al* (1985) examined canine lymph nodes and reported that S-100 reactivity was restricted to IDC of the paracortex and the same group (Sandusky *et al*, 1987) failed to detect S-100 positive cells in fourteen lymphomas. Failure to detect accessory cells either in normal or B cell tumours may be due to the differential reactivity of the

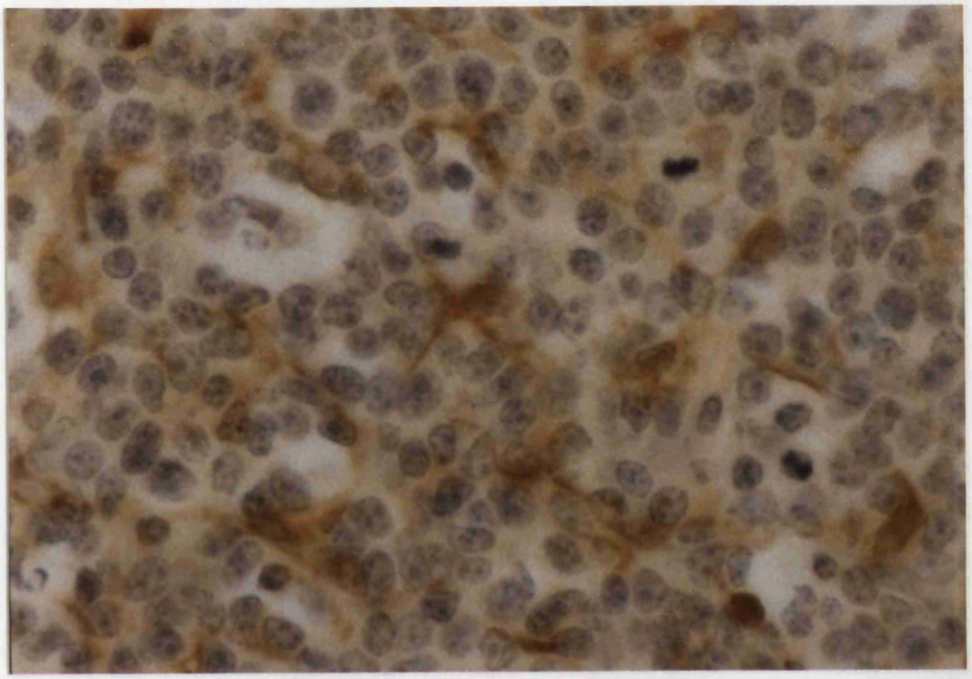
polyclonal sera. Tanaka (1986) suggested that bovine brain (the antigen source for the Dako polyclonal product) is dominated by the β subunit of S-100 and thus there is a propensity for some polyclonal products raised against bovine neural tissue to detect IDC in preference to FDC. In follicular forms of NHL, FDC are known to proliferate in conjunction with the neoplastic B cells, forming a defined meshwork. On the contrary, FDC are relatively rare in T cell NHL and are mainly restricted to peripheral T cell lymphomas such as angioimmunoblastic lymphadenopathies (Gerdes and Flad, 1992). Thus it is possible that a canine B cell tumour may possess numerous FDC but these would not necessarily be detected adequately by S-100 polyclonal sera raised against bovine brain.

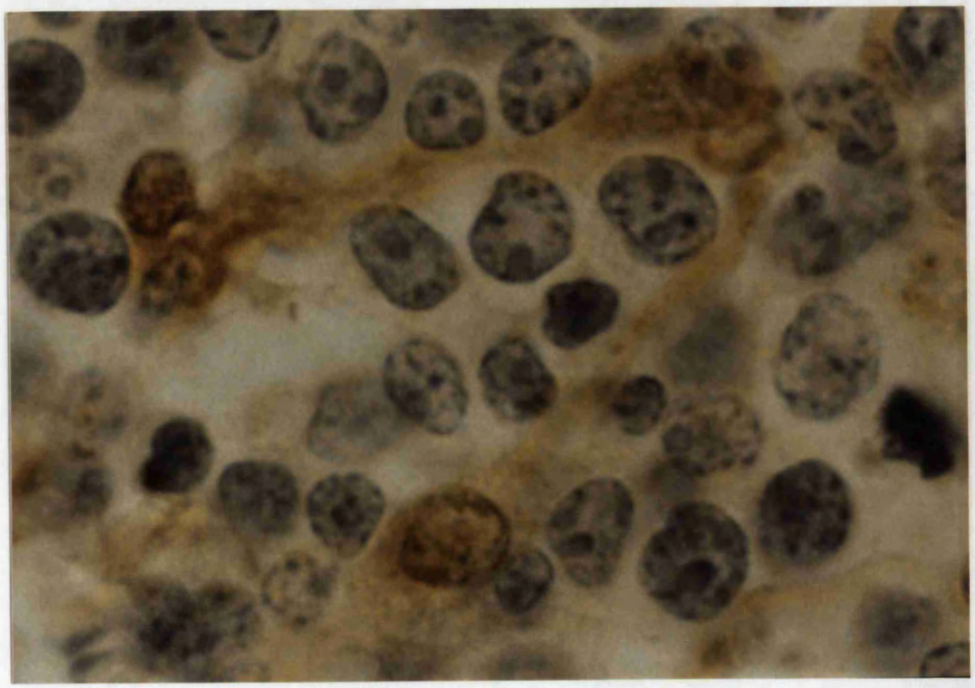
BMD and KEB, the two samples with numerous P-gp positive dendritic cells, are both putative B cell tumours (as determined by their lack of T cell gene rearrangement; see chapter 6) and so they would be expected to contain FDC. Histological reports on these two tumours classed BMD as a diffuse large cell tumour whereas KEB had a mixed follicular/diffuse pattern (histological classification kindly carried out by Sean Callanan of the Dept. Veterinary Pathology). Despite the potential problems with polyclonal S-100 regards the ability to detect FDC, these two tumours were examined using an S-100 polyclonal sera from Dako. This work was kindly performed by members of the Pathology Department at the Royal Infirmary under the guidance of Dr A. McNicol. The S-100 staining failed to identify any cells in frozen sections cut in series with the P-gp positive sections. Formalin fixed blocks and a sensitive ABC system were then tried. Figures 5.8a and 5.8b show dendritic cells stained for S-100, with identical morphology to the P-gp positive cells. These cells are distributed diffusely throughout the BMD sample. The S-100 stained cells are extremely common in this section, more than those identified on the frozen section (data not shown). Unfortunately, the S-100 staining was performed on a paraffin block and not on an adjacent frozen section to the C219 staining, so it was not possible to rule out that this disparity in frequency was merely the result of using different blocks. The KEB sample gave background problems and could not be interpreted.

Figure 5.8 S-100 positive dendritic cells within lymphomatous node

The pre-chemotherapy sample from "BMD" was examined for S-100 using a Dako anti S-100 polyclonal sera and an ABC-peroxidase system on paraffin fixed sections. This was performed by members of the Department of Pathology at the Royal Infirmary.

5.8a "BMD" (x40) (*Top*)
5.8b "BMD" (x100) (*Bottom*)





Without performing dual staining with S-100 and P-gp simultaneously, it is not possible to categorically state that the two monoclonals are identifying the same population or that P-gp is only present in a subset of S-100 positive cells. However the preponderance of both P-gp positive dendritic cells and S-100 positive dendritic cells within the same tumour is highly suggestive that they are the same population. Both C219 and the S-100 antibodies stain cytoplasmic components and so dual staining would be technically difficult. Unfortunately, due to time and resources constraints, it was not possible to extend this work to include S-100 staining of other tumours. It would have been very interesting to look at the distribution of the S-100 cells in normal lymph nodes and in the T cell tumours.

5.4.4 (iii) Do the dendritic cells express *mdr1*?

C219 positivity does not prove that the canine dendritic cells possess *mdr1*. However, as discussed above, macrophage like-cells in human lymph nodes have been identified with *mdr1* specific monoclonal antibodies and hence from cross-species comparison, it is not unlikely that the dog cells also express the *mdr1* isoform. Unfortunately, the dotblot hybridisation results are not useful in confirming *mdr1* expression. Most lymph nodes (the majority of which contain P-gp positive dendritic cells and endothelium) give a signal below that of KB8.5 in a similar range to KB3.1. The relative paucity of the P-gp positive dendritic cells in comparison to the lymphocyte population in most of these tumours is such that, if the lymphoma cells were P-gp negative, it would be unlikely that the dendritic cell population would be able to produce an *mdr1* signal as high as KB8.5.

The two tumours with numerous dendritic cells (BMD and KEB) had positive tumour cells both before epirubicin and at relapse. Despite the frequency of the dendritic cells in these two tumours, lymphoma cells still greatly outnumbered the dendritic cells and made up the bulk of the tumour. The BMD samples gave a signal greater than KB8.5 both before and after chemotherapy but curiously the KEB samples gave a signal similar to the other lymphomatous nodes ie. negative.

Several interpretations can be made of the KEB result. The first would be that there was a technical problem with the dotblot hybridisation such that KEB yielded a false negative. The second (relapse) sample from KEB was collected over twelve hours after death and hence the RNA was very poor quality. From the controlled RNAase experiment (described in section 5.1), degraded RNA may be expected to yield a falsely increased signal. However, the degradation in this clinical sample may have gone beyond that of the experiment and be adversely affecting the hybridisation. However the repetition of the negative result in the first KEB sample in which the RNA quality was excellent would argue against this. The second reason to explain the

negative hybridisation result could be that the dendritic cells did express *mdr1* but the tumour cells expressed an *mdr2* homologue and the dendritic cell *mdr1* mRNA was still not sufficient to boost the entire tumour block to a "significant" signal on the hybridisation. The third could be that neither the stromal cells nor the tumour cells expressed *mdr1*.

The human work suggest that accessory cells in lymph nodes do express *mdr1* and for this and other reasons discussed in the final chapter, the second alternative (*mdr1* positive dendritic cells but *mdr2* homologue positive tumour cells) is favoured.

5.4.4 DETECTION OF P-GP AND *mdr* mRNA IN LYMPHOMA CELLS

Sixty-two MLSA cases from GUVC were examined for *mdr1* mRNA expression using the dotblot hybridisation system described earlier in this chapter. Forty-four of these cases were also examined by IHC, either before and/or after chemotherapy. Both dotblots and IHC was repeated at least twice for each sample.

Positivity in the dotblots was defined as those samples with an *mdr1*/poly(d)T value equal or greater than the KB8.5 value. Samples had to score positive in both dotblots to be ranked as positive. Only one sample gave positive on one dotblot and a negative on the duplicate ("RMM" pre-chemotherapy sample); the IHC on the same sample was consistently negative and so this sample was classed as negative.

Goldstein *et al* (1989) classified tumour positivity into "low" or "high" according to whether the signal was between the values obtained with KB3-1 and KB8-5 or greater than KB8-5. On this basis, almost all the tumours would have been "low-positive" (illustrated in figure 5.10). This low-positive category was felt to be inappropriate for the canine lymphoma samples because the IHC had already revealed that the majority of these "low-positive" samples did not express P-gp in their tumour cells but did have P-gp in a dendritic cell population and endothelia. The problems of RNA degradation potentially increasing the *mdr1* signal also makes interpretation of "low" positives a dubious one.

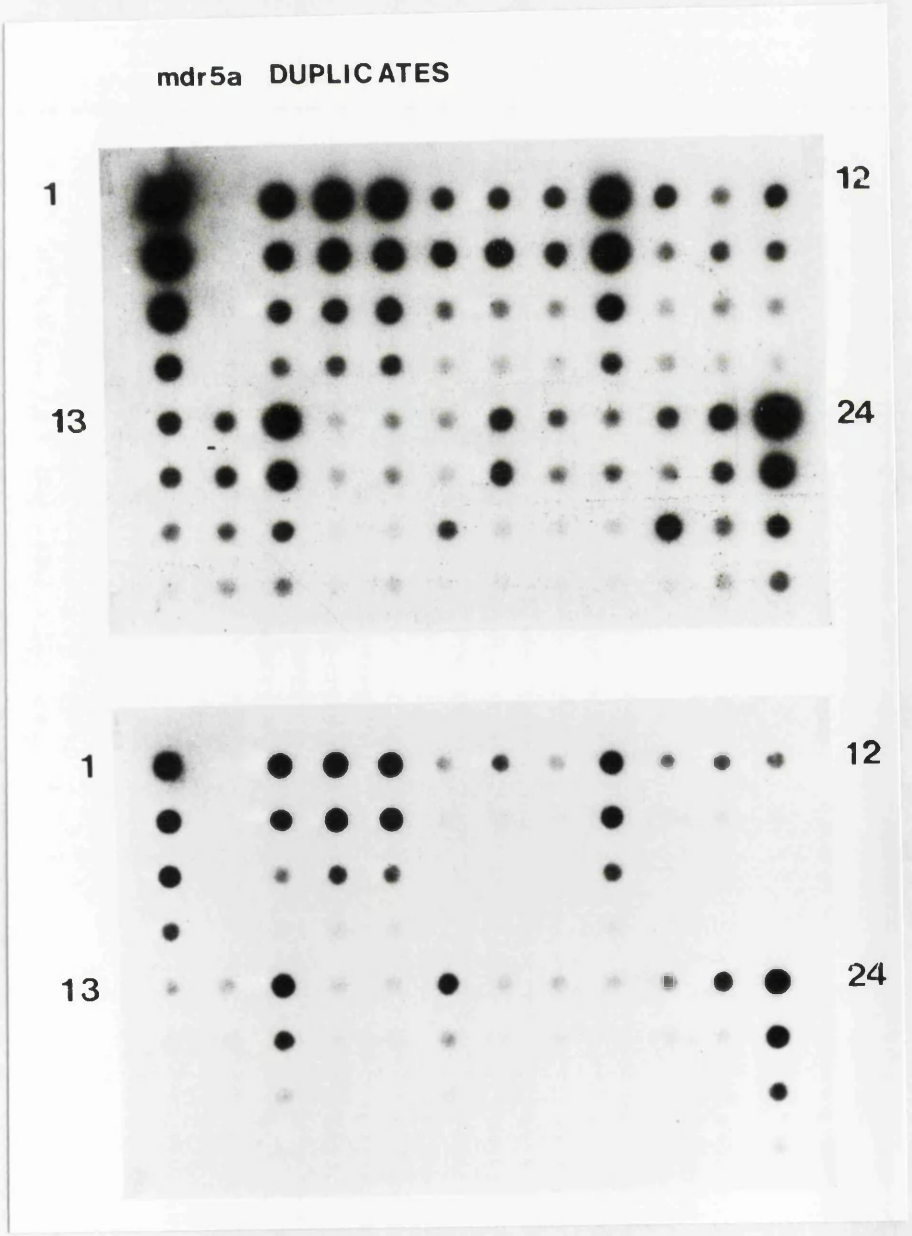
Figure 5.9 illustrates two duplicate dotblots probed with MDR5A containing positive samples from cases BMD, BEH, RMM and CIS. In both dotblots it is possible to visually identify the positive samples. However, the degree of positivity (i.e. the sample MDR5A:poly(d)T value compared to the KB8-5) was not the same in the two dot-blots. For example, in the top panel, the BEH sample in lane 9 gave a signal 2.4 fold greater than KB8-5 but in the lower panel the same sample was only 1.06 fold greater. In general, the clinical samples did not yield values much greater than KB8-5. If KB8-5 is given the same arbitrary value of "30" assigned by Goldstein *et al*, (1989) then the most positive sample was the BMD relapse sample which gave

a value of 86 on one of the dotblots but only 44 on the other. Most of the positive samples gave values in the 30 - 50 unit range.

Figure 5.9 Duplicate dot-blots hybridised with MDR5A

Dotblots have identical samples loaded except lanes 18 and 19 are reversed in the lower panel.
"pre", pre chemotherapy; "post", post chemotherapy

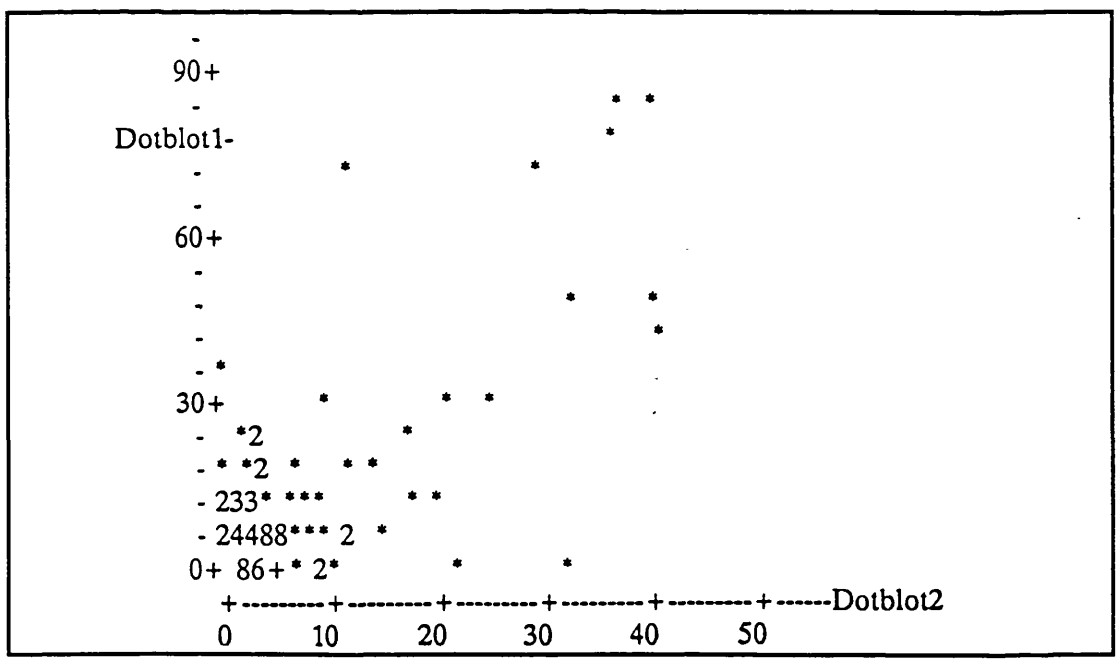
1. KBV-1	7. CMD post	13. KB3-1	19. RMM post
2. empty	8. BEH pre	14. 3132	20. BRC pre
3. KB8.5	9. BEH post	15. mouse kidney	21. BRC post
4. BMD pre	10. KEB pre	16. TIH pre	22. CIS pre
5. BMD post	11 KEB post	17. TIH post	23. CIS 1st post
6. CMD pre	12. LEB pre	18. RMM pre	24. CIS 2nd post



Marie *et al* (1991) examined the repeatability of dotblot readings. Duplicate dotblots of tumour samples were probed with *mdr* probes and they reported correlation coefficients of 0.927. This was calculated by a linear correlation test of the test values relative to the positive and negative controls. Despite considerable effort to standardise the dotblot procedure, correlation coefficients this high could not be achieved in this study.

The value of the MDR5A:poly(d)T ratio was transformed into "units" giving the KB8.5 signal a value of 30 and KB3.1 a value of 1. A correlation test (Pearsons) was performed on the "unit" values. For the samples illustrated in figure 5.9, the correlation coefficient was very good at 0.96. However when all the dotblots were analysed the correlation fell to only 0.77. The graphic representation of this correlation is illustrated in figure 5.10, which shows that the correlation is worst at the lower unit range.

Figure 5.10 Correlation between dotblot duplicates
The value of the MDR5A/poly(d)T ratio was converted into "units" relative to KB8.5 with an arbitrary value of 30.



The original Goldstein paper using KB8-5 on dotblots did not give the exact densitometric value for the four hundred tumour samples in their study. This group divided the tumours into high positive, low positive and negative based on the values from a single dotblot. They expressed no concern over the repeatability of their results; their blots were reprobed with γ actin to standardise for RNA loading but remarked that generally loading was even and did not require standardization. The reader is then left uncertain as to whether all the densitometric-based results

presented in the paper have actually been adjusted for loading using the actin probe. When remarking on the range of values obtained for certain tumour types (e.g colonic tumours gave values ranging from 0 to 90) they ruled out technical artefact as a possible source of error based solely on the fact that the test RNA was intact prior to blotting.

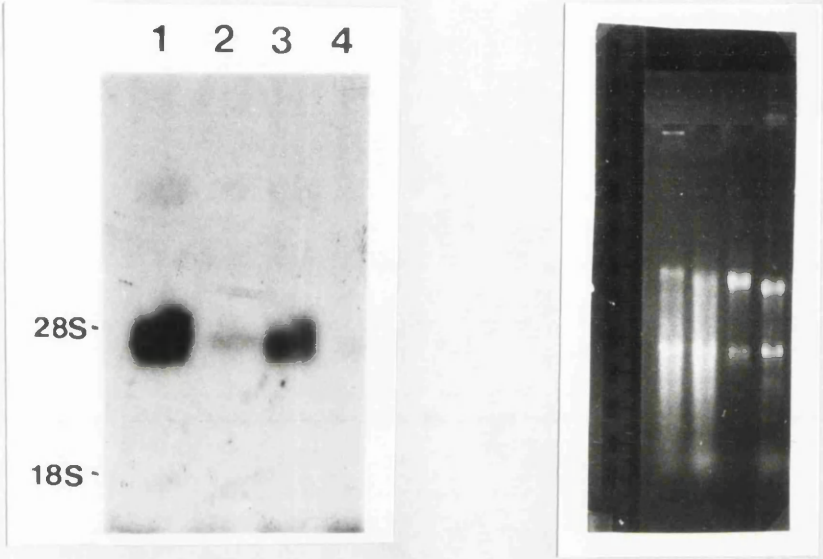
Unfortunately, despite the use of agarose gels and spectrophotometric measurements to quantitate RNA prior to blotting, in this study it was impossible to guarantee even loading of samples accurately enough to give comparable readings on the densitometer. Visually, samples would appear the same but the laser scanner could detect variability which affected the final value generated. The correlation coefficient of 0.77 between dotblot readings is an indicator of the technical artefacts which could not be removed from the methodology. Figure 5.10 plots the correlation between values on duplicate dotblots and illustrates the imperfect correlation, especially at lower spectrophotometric readings.

Because of the inherent problems with the dotblots, in effect, the IHC result was taken as the genuine representation of the presence of P-gp and the dotblot results were used to corroborate the likelihood of this P-gp being an *mdr1* species. This may result in some tumours with P-gp too low to detect by IHC and with a dotblot signal between the KB3.1 and KB8.5 being erroneously classed as negative. The second sample obtained from "CIS" may fall in this category. However, the danger of false negatives was felt to be minor compared to the problems that could be created in adopting the dotblot readings rather than the IHC as the more accurate measure of P-gp/*mdr1*. Figure 5.11 shows a Northern blot of the pre-chemotherapy and relapse samples of case "BEH" probed with MDR5A. By IHC, "BEH" was P-gp negative prior to treatment but positive at relapse. Figure 5.11 illustrates that despite the problems with dotblots, it is possible to confirm the result by Northern blotting in samples with good quality RNA.

Positivity in the IHC was determined over a minimum of two staining session in which the morphology and quality of the staining was considered suitable for interpretation. Staining quality was determined from normal canine liver sections which were stained in parallel with the lymphoma sections. Normal liver was found to provide a more dependable and gradeable reactivity to C219 than the KB8-5 cell line. The KB cell lines had to be grown on coverslips and at least four coverslips used per positive control to allow for problems with cell quality following storage and fixation. It was therefore an impractical and expensive control and was abandoned in favour of the normal liver sections. The liver sections had the added advantage of giving a more accurate representation of the background problems encountered in a tissue section which are not seen with cells grown on coverslips.

Figure 5.11 MDR5A Northern blot with canine lymphoma samples
Left hand side; northern blot hybridised with MDR5A
Right hand side; ethidium bromide stained gel prior to blotting.

1.BEH post chemotherapy
2.BEH pre-chemotherapy
3.KB8.5
4.KB3.1



If a sample yielded divergent results in two runs, then it was stained a third time. Positivity in more than 5% of cells in a section is often used as the cut-off for positivity (Dalton *et al* 1989, Miller *et al* 1991). This was unnecessary in this study because all of the positive samples contained P-gp in over 80% of the cells i.e. virtually all the tumour cells in a given block would be positive. An example of a positive tumour sample, with virtually all the cells positive, is shown in figure 5.12 (c and d).

Other workers emphasize the importance of differentiating cell membrane staining from cytoplasmic staining; Chan *et al* (1990) and Miller *et al* (1991) both exclude cytoplasmic staining from their definition of positive. The previous section discussing the presence of P-gp in a dendritic cell population presented some of the evidence suggesting that P-gp may not be limited to the cell membrane. In the frozen sections prepared from canine lymphomatous nodes and illustrated in figure 5.12, it is not possible to differentiate cell membrane versus cytoplasmic staining of the lymphocytes. This is not just because of the poor morphological detail of frozen sections but also because lymphoma cells have a scanty rim of cytoplasm around a large nucleus and therefore membranous staining by an alkaline phosphatase based system appears virtually identical to cytoplasmic staining. In the dendritic cells it was easy to see that there was a cytoplasmic component to the staining because the

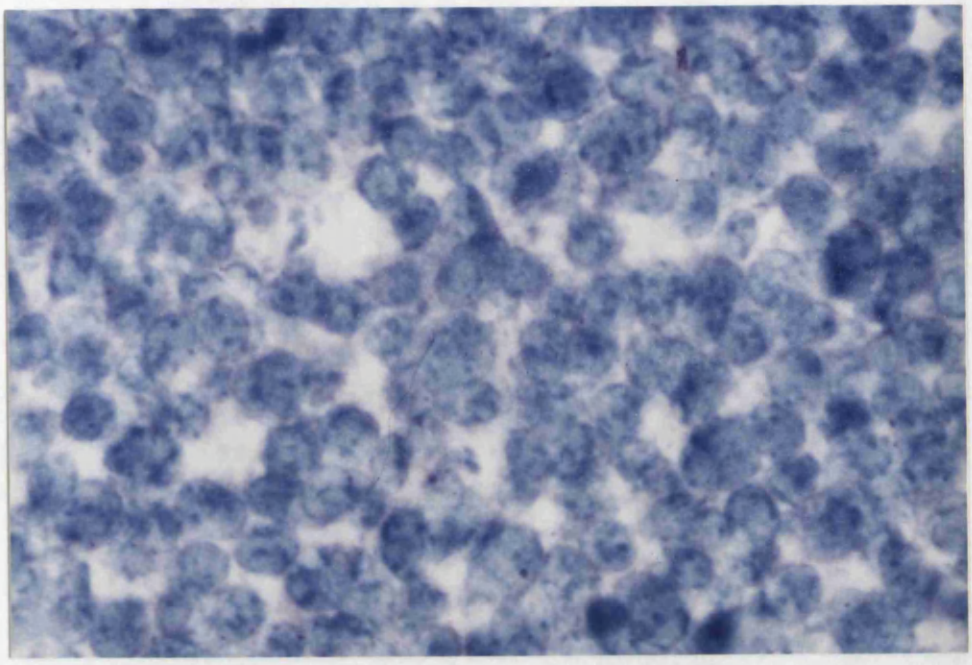
cells are larger than the lymphocytes and had abundant cytoplasm. Positivity in lymphoma cells was therefore by necessity defined as any Fast Red staining in the lymphoma membrane and/or cytoplasm which was repeatable in the competitive IHC assay on at least two occasions.

Two samples required staining more than twice due to difficulties in interpretation; these were SND (figure 5.12 b and c) and CIS (the first relapse sample). SND gave a positive result in 2/3 of the samples and was therefore classed as positive whereas CIS only stained faintly positive in 1/4 samples and was classified as negative. None of the positive samples had an intense P-gp expression. The difference between a negative and a positive tumour sample was often quite subtle (figure 5.12 a and d). Scoring systems based on the intensity of the staining have been adopted for classification of other tumour types (Verrelle *et al*, 1991). Despite the possible merits of this approach (Dalton and Grogan, 1991) the uniformity of staining in the lymphomas made it impossible to adopt a scoring system. The results are therefore given in the same way as the dotblot results as a simple plus or minus.

The results of the dotblots and IHC are given in Tables 5.7 (GUVC cases) and table 5.8 (non-GUVC cases). The two samples of unknown status were both negative on dotblot and are not shown in the tables. The samples from other veterinary institutes were not examined by dot-blot hybridisations.

Figure 5.12 P-gp positive and negative lymphomatous nodes.

5.12a "BEH" pre-chemotherapy: tumour cells P-gp negative (x40)
5.12b "SNB" post-chemotherapy: tumour cells P-gp +/- (x20)



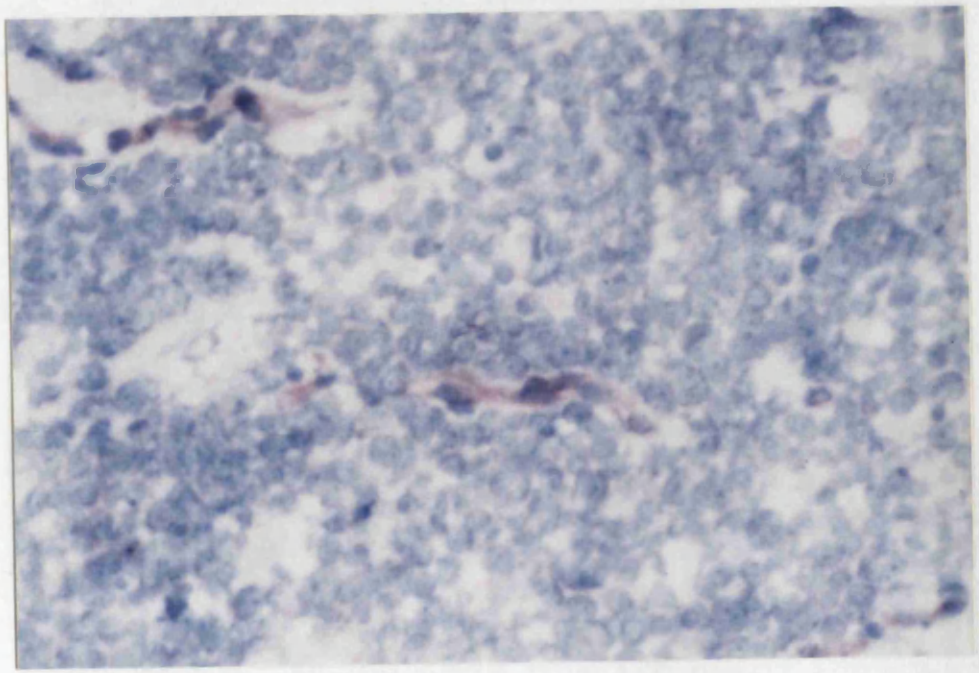
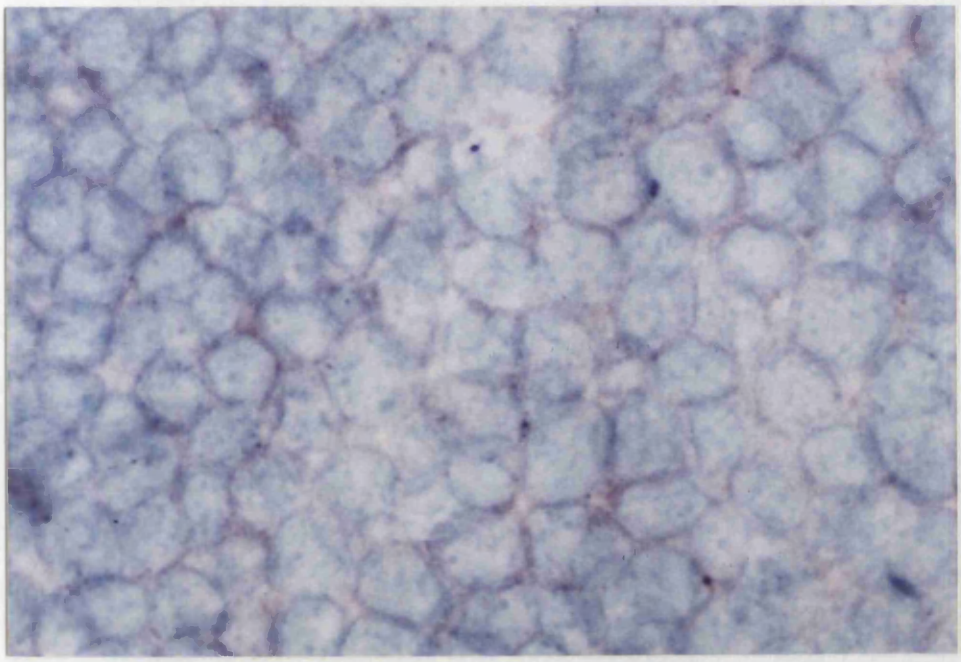
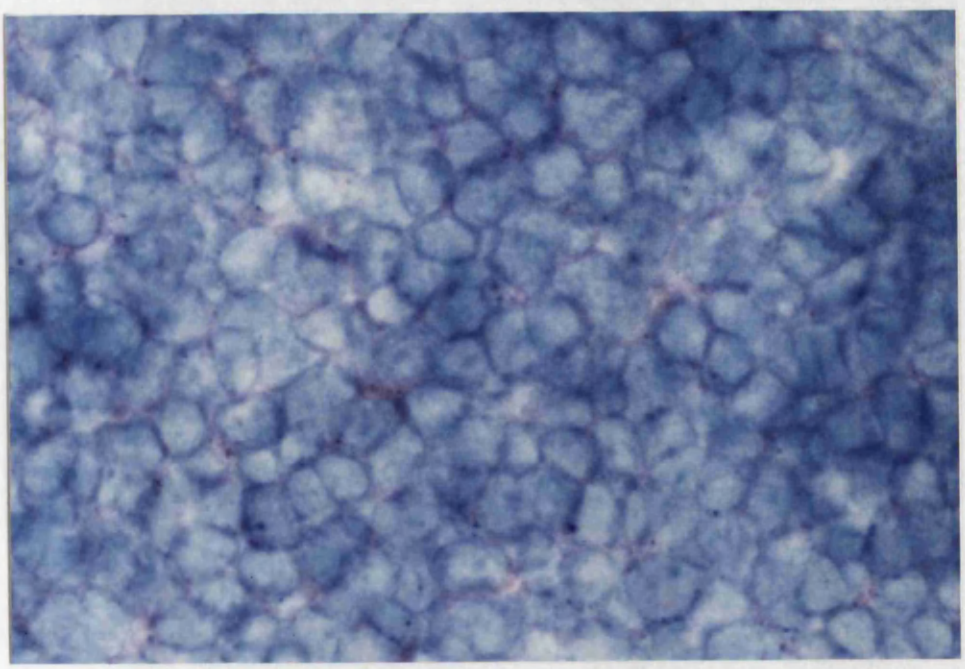


Figure 5.12 (continued)

5.12c "SNB" (repeat staining): tumour cells P-gp positive (x50)
5.12d "JAW" Relapse : tumour cells P-gp positive (x40)





Concentrating on the pre-treatment GUVC samples (upper and middle portions of table 5.7), P-gp expression in tumour cells at the time of diagnosis was a rare occurrence; only 1/33 (3%) pre-chemotherapy samples which were examined by IHC were positive on both IHC and dotblot. An additional one sample (KEB) was positive on IHC but negative on dotblot. Including the GUVC samples which were dotblotted but not examined by IHC, then only 1/51 (1.9%) samples expressed P-gp (of *mdr1*) origin at the time of diagnosis.

The cases from the other institutes yielded similar results; of the 17 pre-chemotherapy samples examined, only 3 were positive, equivalent to 17.6%. One of the positive samples from NCSU, (sample NC5 in table 5.8) was obtained from a dog which subsequently failed the multiple agent induction protocol.

In the post-chemotherapy samples, P-gp expression in the tumour cells was more common. A total of 31 GUVC post-chemotherapy cases were examined by IHC. 10/31 of these samples were P-gp positive but *mdr1* expression could only be confirmed in 7 of these samples by dot-blot hybridisation. One dog (CIS) was sampled immediately on relapse and again at euthanasia less than 7 weeks later. The first relapse sample was positive by IHC on one out of four staining repetitions whereas the sample taken at euthanasia was repeatably positive by IHC. None of the four relapse samples from the other institutes were positive.

In table 5.7, three of the samples were not from dogs with clinically resistant disease. MMK and BEA both died during first remission from complications arising from the chemotherapy. BRC relapsed 44 weeks after induction and was given rescue therapy in the form of an increased dose of epirubicin and oral cyclophosphamide. The dog responded to this treatment but the owners requested euthanasia because of the side effects of the rescue therapy. The lymph nodes were atrophic with no histological evidence of tumour ten days after the epirubicin at the time of euthanasia.

If these dogs are excluded, then there were only 28 dogs with clinically resistant disease examined by IHC and dotblot. This means 7/28 (25%) dogs had evidence of *mdr1* expression at relapse.

The three cases which gave positive signals on IHC but were negative on dotblot shared some common features. All three dogs were unusual in having received chemotherapy within one week of sample collection. Normally, when tumour progression occurred in the face of continued chemotherapy, at each three weekly visit, owners would be offered the option of euthanasia for their pet. If it was felt that the clinical condition was so critical that the dog may actually die between treatments, then for humanitarian reasons, euthanasia was actively encouraged. Thus it was rare for dogs to be treated and then to represent for euthanasia or in a terminal condition shortly after chemotherapy.

KEB was clinically resistant to treatment at time of death but was actually killed in a traffic accident only three days after treatment with an augmented dose of epirubicin (30mg/m^2). There had been no detectable reduction in lymph node size at the time of death. TIH was under the care of the referring veterinary surgeon at the time of euthanasia and had received vincristine injection 5 days previous. (TIH was already completely unresponsive to epirubicin at time of death). BOB received vincristine four days before death at GUVC; he had been induced only twelve days previous but had not responded.

It is interesting to speculate that the positive IHC but negative dotblot results in these three dogs could represent short term induction of an *mdr* species which does not cross react with the MDR5A probe. Chapter 4 provides evidence that the dog may possess four *mdr* genes but only one of these may be recognized by MDR5A. All three dogs lacked β chain TCR gene rearrangements and are therefore putative B cells. Herweijer *et al* (1990) and Nooter *et al* (1990) presented evidence that B cell malignancies, but not T cell malignancies, express the human *mdr3* gene. This provides a precedence for the expression of a non-*mdr1* gene in a B cell tumour.

Figure 5.7b showed a reactive follicle from case "GEG" which apparently had P-gp expression in the reactive follicles: despite this expression in the follicular area, this node gave a low signal on dotblot (less than "10" units on duplicate blots) and was classed as *mdr1* negative. This could represent the expression of the canine equivalent of the human *mdr3* gene in these stimulated B cells. It is also possible that the three B cell tumours with positive P-gp on IHC but no *mdr1* on dotblot are expressing *mdr3*, perhaps related to the chronologic proximity to drug exposure.

However, an alternative explanation for the three dogs results can be offered. Due to the unexpected deaths of all three dogs, all of the samples were collected over 4 hours after death and consequently the RNA quality was not optimal. It was impossible to run adequate Northerns to confirm the negativity of these samples. It cannot be excluded that these three samples may be false negatives due to the RNA degradation.

Legend for Table 5.7 (on next page)
+, positive; -, negative; nd, not done.
**, post-chemotherapy samples from dogs in remission*

Table 5.7 P-gp and *mdr1* detection in lymphoma cells

Name	Pre chemotherapy IHC	Pre chemotherapy Dotblot	Post-chemotherapy IHC	Post-chemotherapy Dotblot
1. CRS	nd	-		
2. MOK	nd	-		
3. LET	nd	-		
4. ROB	nd	-		
5. MMG	nd	-		
6. SMM	nd	-		
7. PAM	nd	-		
8. BRM	nd	-		
9. RMP	nd	-		
10. RIC	nd	-		
11. TOB	-	-		
12. GOF	nd	-		
13. MIG	-	-		
14. SAO	nd	-		
15. DHS	-	-		
16. CHP	nd	-		
17. SAD	nd	-		
18. TIT	nd	-		
19. SAC	-	-		
20. MAK	-	-		
21. BIP	-	-		
22. BEM	-	-		
23. KEH	-	-		
24. LEB	-	-		
25. TRC	-	-		
26. CIP	-	-		
27. HEH	-	-		
28. KMD	nd	-		
29. BRP	-	-		
30. JAW	nd	-	+	+
31. HOG	nd	-	-	-
32. PES	-	-	-	-
33. RMI	-	-	-	-
34. BOA	-	-	-	-
35. CIS	-	-	-	-
(2nd relapse)			+	+
36. LAQ	-	-	-	-
37. RMC	-	-	-	-
38. TIH	-	-	+	-
39. ZAK	-	-	-	-
40. CMD	-	-	-	-
41. BEH*	-	-	+	+
42. BRC*	-	-	-	-
43. KEB*	+	-	+	-
44. MMK*	-	-	-	-
45. BOS	-	-	-	-
46. SPC	-	-	-	nd
47. BMD*	+	+	+	+
48. BEA*	-	-	-	-
49. SMD	-	-	-	-
50. SAS	-	-	-	-
51. RMM	-	-	+	+
52. SNB			+	+
53. BMC			-	-
54. LOK			-	-
55. TAB			-	-
56. BOB			+	-
57. SAB			+	+
58. THH			-	-
59. SAP			-	-
60. ZAS			-	-
Total	2/33	1/51	10/32	7/31

Table 5.8 P-gp in lymphoma cells (IHC only) in non-GUVC cases

Name	Pre-chemotherapy	Name	Pre-chemotherapy
NC1	-	NC9	-
NC2	-	NC10	+
NC3	-	NC11	-
NC4	-	NC12	-
NC5	+	NC13	-
NC6	-	NC14	-
NC7	-	NC15	-
NC8	-	NC16	-
CU1	+		
	Post chemotherapy*		
NC17	-	NC18	-
CU2	-	CU3	-

* *Protocols consisted of multiple agent protocols containing MDR and non MDR drugs. NC, North Carolina State University; CU Cambridge University*

The results presented here are broadly in agreement with the published work with human NHL. The largest study of NHL (Miller *et al,* 1991) found expression at time of diagnosis in only 1 out of 39 tumours (2.5%) by IHC. In table 1.12, the overall incidence of P-gp at time of diagnosis was less than 10%. In the GUVC canine samples examined by IHC at diagnosis, only 1/31 samples contained P-gp positive tumour cells and of the foreign cases, 3/17 were P-gp positive which is an overall incidence of 4/48 (8.3%). Thus the dog seems to mirror human NHL with a low incidence of P-gp at time of presentation.

In clinically resistant NHL, the incidence of P-gp is higher. Miller found 5/9 tumours (55%) were P-gp positive. However, Schlaifer *et al,* (1990) failed to detect P-gp positive tumour cells in fifteen resistant tumours. It is difficult to say whether this discrepancy between the Miller and Schlaifer results is due to differences in the sensitivity of the IHC or merely natural biological variation due to the small number of samples studied. All of the relapse patients in both studies were clinically resistant to combination chemotherapy protocols containing vincristine and doxorubicin plus other non-MDR drugs; the variety of chemotherapy protocols used prior to sample collection excludes the ability to determine the effect of protocol on subsequent P-gp expression in human NHL. The compilation of P-gp expression results in human NHL (table 1.12) gives an overall frequency of P-gp in relapse samples of about 50%.

The frequency of P-gp in these drug resistant dogs is somewhat lower than humans at 25%. It would therefore appear that in canine lymphomas, the acquisition

of P-gp is not a major cause of treatment failure but it may contribute to the drug resistance of approximately one quarter of the tumours.

5.5 SUMMARY

Lymphomatous nodes from dogs before treatment and at time of clinical resistance to epirubicin were examined for P-gp expression using an immunohistochemical technique and dotblot hybridisation for *mdr1* mRNA.

The IHC was a sensitive technique based on its ability to stain the KB8-5 cell line. The *mdr1* mRNA signal from KB8-5 was used as the cut-off for positivity in the dotblot technique. Two canine cell lines (a B cell line and a putative T cell line) were negative for P-gp on both IHC and dotblot. Neither cell line showed evidence of *mdr1* induction following short term exposure to epirubicin.

The IHC revealed that normal, reactive and lymphomatous nodes contained a dendritic cell population which gave a granular cytoplasmic staining with the C219 monoclonal. The morphology and localisation of these cells within normal and reactive nodes suggested that these cells may be antigen presenting cells. One sample which contained numerous dendritic cells also contained numerous S-100 positive cells which supports the hypothesis that the P-gp positive dendritic cells may have some form of antigen presentation function.

P-gp in lymphoma cells was rare at time of diagnosis: only one out of thirty-three samples (3%) examined by both dot-blot and IHC were positive. Of the seventeen pre-chemotherapy samples from dogs referred to other veterinary colleges, only three were positive on IHC (17.6%). The frequency of P-gp in the tumours from clinically resistant dogs was greater: seven out of the twenty-eight (25%) clinically resistant tumours studied by IHC were also positive on dot-blot. Four of these seven dogs were known to have converted from P-gp negative to P-gp positive during the course of their disease.

Three B cell tumours were positive on IHC but negative on dot-blot. A reactive node was identified which showed C219 staining in B cell areas but was negative on dotblot. It is not clear if this represents a subpopulation of B cells which express an *mdr* species not recognised by the MDR5A probe (perhaps as a result of drug induction or stimulation) or if it is an artefact resulting from the use of degraded RNA in the dotblot hybridisation.

CHAPTER 6
GENOTYPING CANINE LYMPHOMAS

It has been feasible to positively identify canine B cell lymphomas for over a decade using anti-immunoglobulin reagents (see section 1.2.3). Unfortunately, it has not been so easy to positively identify phenotypic T cells because of the paucity of suitable monoclonal antibodies and because of unexpected (and perhaps spurious) reactivity of some of these putative T cell antibodies with Ig expressing cells.

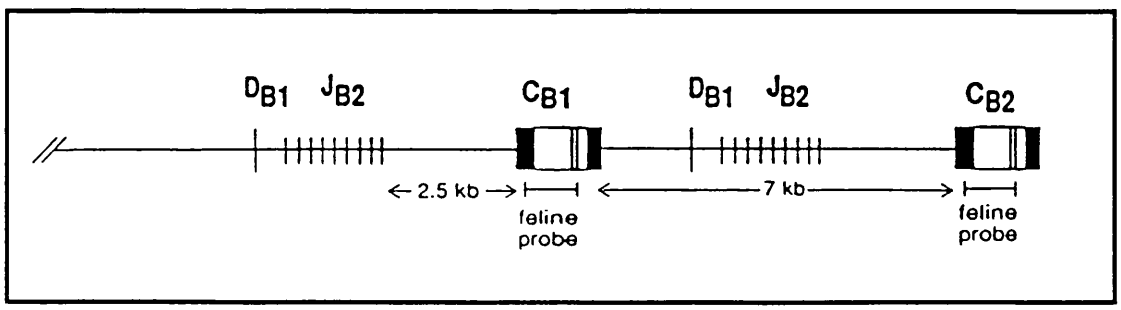
It is accepted that overall, in canine lymphomas, phenotypic T cells perform poorly compared to B cell tumours (Greenlee *et al,* 1990) and one possible explanation for this could be that T cell tumours constitutively express, or acquire P-gp. In an effort to positively identify the T cell tumours within the lymphoma series, genotyping was attempted.

Four T cell genes exist; α, β, γ and δ. Because of the hierarchical rearrangement of the TCR genes, α and β genes are less likely to be rearranged in B cells neoplasms. The α locus is particularly large and without the use of multiple J region probes is not easily screened for rearrangements. Consequently, the β chain was selected as the most suitable indicator gene for T cell genotyping.

6.1 DETECTION OF A TCR GENE REARRANGEMENT IN A CANINE CELL LINE

Genotyping canine MLSA samples was performed by Southern hybridisation analysis using a feline TCR β constant region probe described in section 2.1.9. Figure 6.1 shows the TCR β locus with the position of the feline probe.

Figure 6.1 TCR β chain locus showing position of feline probe



The four constant region exons are shown as filled areas.

The canine TCR β locus is not mapped and consequently prior to starting this study there was no information on the most useful digests to use in the assessment of TCR gene rearrangements. Fortunately, the acquisition of a putative T cell line (Cl-1) from Dr. Tsujimoto allowed initial experiments to be carried out comparing the restriction fragment patterns of ten different restriction enzymes obtained from normal dog germline, 3132 (a canine B cell lymphoma line) and CL-1. Results are

shown in figures 6.2 and 6.3. In each digest (except *Pvu* II) CL-1 has a different pattern from normal dog and 3132.

Figure 6.2 Determining suitable restriction enzymes for TCR β chain locus in canine.

Three canine samples were digested with five restriction enzymes.
Each triplicate is: 1. canine liver 2. 3132 3. CL-1

Lanes 1-3 *EcoR1*
Lanes 4-6 *Hind III*
Lanes 7-9 *BamH1*
Lanes 10-12 *Pvu II*
Lanes 13-15 *Kpn I*

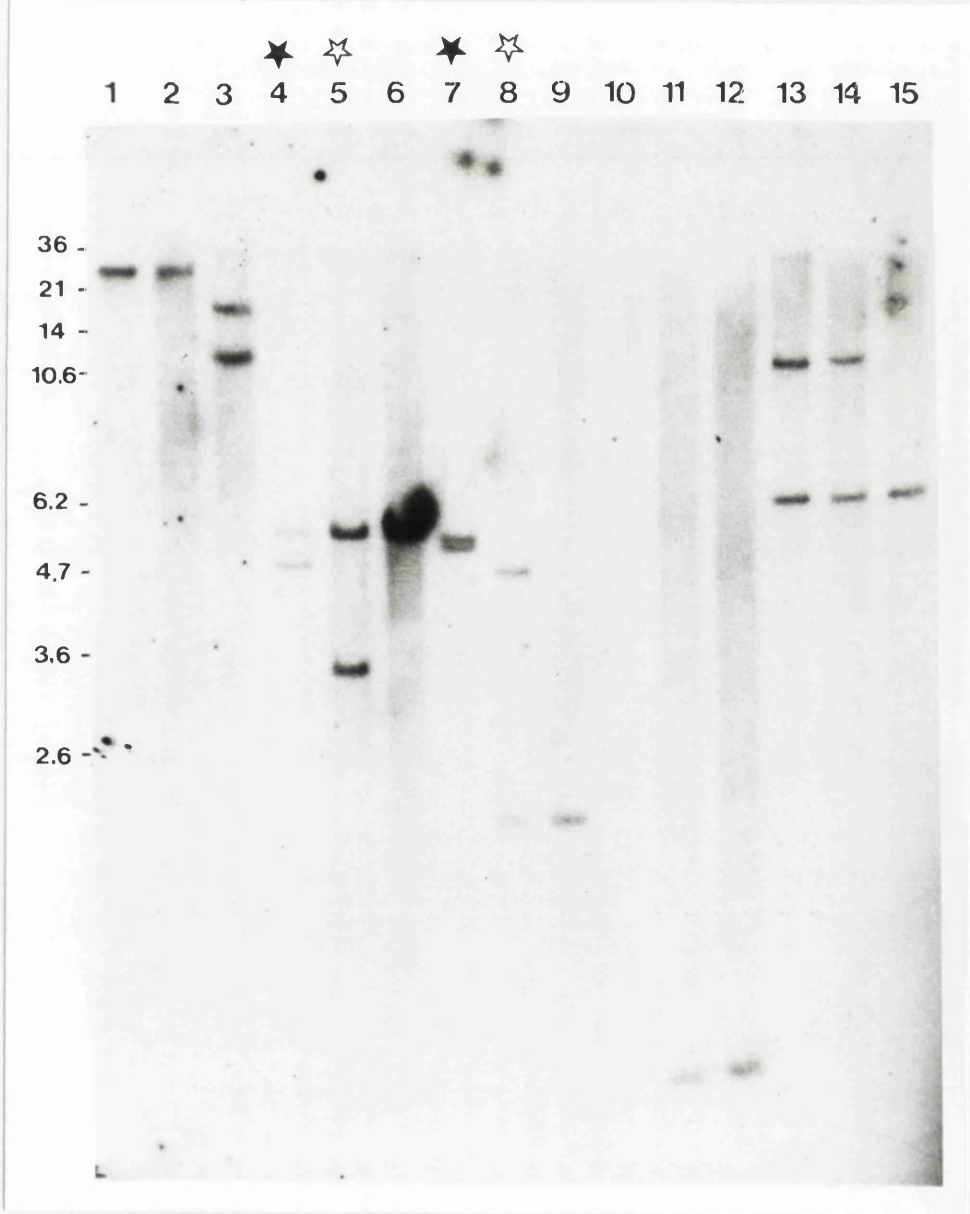
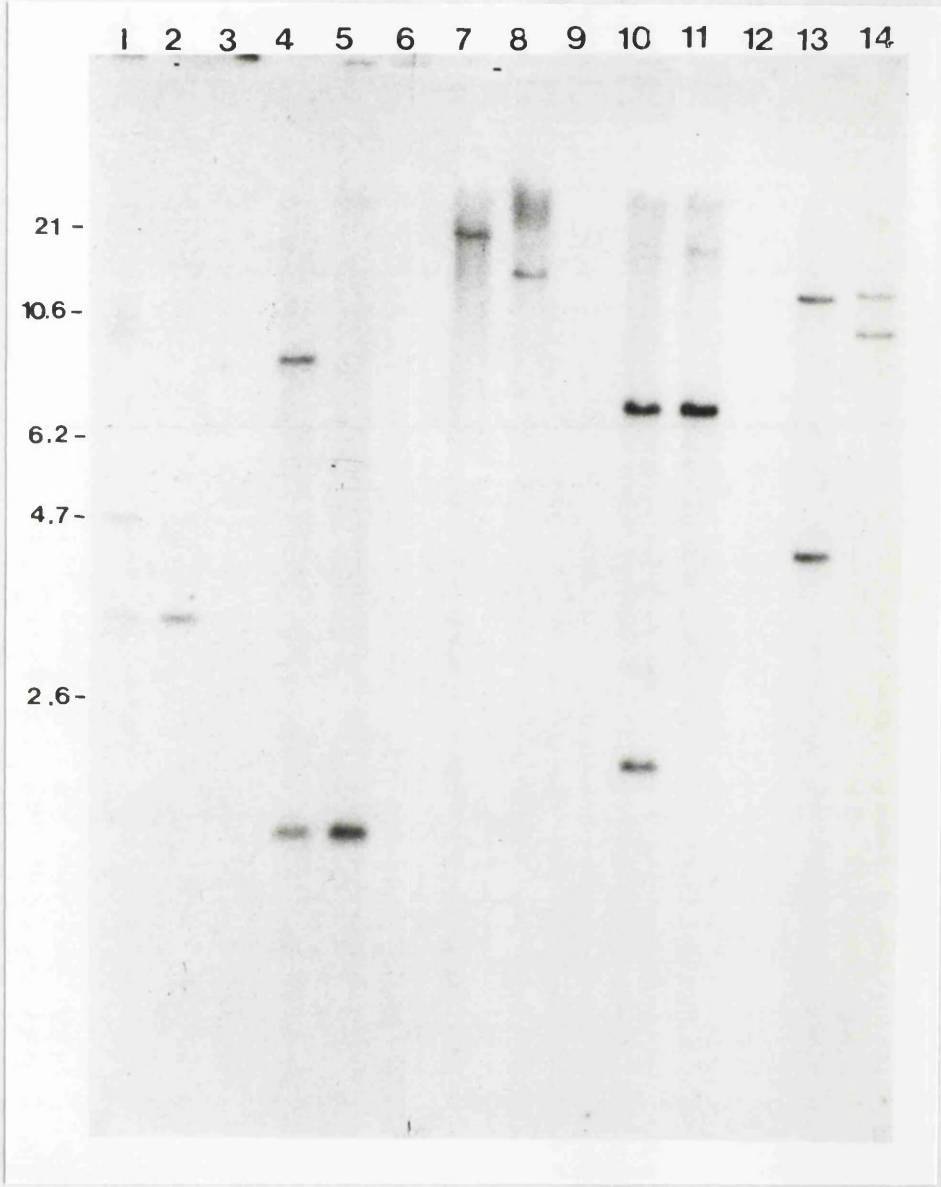


Figure 6.3 Determining suitable restriction enzymes for TCR β chain in canine.

Two canine samples were digested with five restriction enzymes.
Each triplicate is: 1. canine liver 2. CL-1 3. empty

Lanes 1-2 *Sst1* Lanes 10-11 *Sma I*
Lanes 4-5 *Bgl II* Lanes 13-14 *Xba I*
Lanes 7-8 *Xho I*



There is almost complete homology between the two β constant regions in the mouse and humans but relatively poor conservation of the intron sequences. The feline probe used in this study is from a virally transduced TCR β chain which lacks intron sequences (see section 2.1.9). Therefore the expectation would be that this 390bp probe would detect both constant regions in the dog.

In figures 6.2 and 6.3, 7/10 of the enzymes detect two fragments in the normal germline sample and 3132. The simplest interpretation of this finding would be that one of these fragments represents C_{B1} and the other C_{B2}. In each of these seven digests CL-1 lacks one of the germline bands. In T cell neoplasias in humans it is not uncommon that both alleles have undergone rearrangement and for C_{B2} to be used (Rabbits *et al.* 1985). Hence the deletion of one or both C_{B1} regions is expected (Minden *et al,* 1985). Taking this fact into account, the most obvious explanation of the complete loss of germline bands in CL-1 would be that these deleted bands represent the deleted CB_1 regions.

The *EcoR1* digest shown in figure 6.2 and the *Xho*I digest from figure 6.3 both yield a single large fragment which would be large enough to span both CB regions. (In the feline, *EcoR1* also generates a single large fragment on hybridisation with this probe because there is no *EcoR1* site between the two C_B regions; Neil *et al*, 1988). *EcoR1* digested Cl-1 produces two new fragments not evident in the germline tissue. To retain consistency with the previous observation that both C_{B1} alleles are probably deleted in CL-1, these two new *EcoR1* fragments could represent the clonotypic rearrangement of each allele to C_{B2}. On this basis, one would also expect the *Xho*I digest to yield two new fragments but only one is visible. Careful examination of lane 8 in figure 6.3 suggests there may be hybridisation to very large DNA species which is not resolved by routine agarose gels. It may be that the second *XhoI* fragment is lost in these high molecular weight species. There was difficulty digesting canine DNA to completion using *Xho*I and for this quality control reason, *XhoI* digests were not used routinely in the analysis of the canine samples.

The last enzyme which has not been discussed is *Pvu* II which generates a fragment of less than 1kb. This is small enough to be contained completely within the constant region which would explain why CL-1 has the same fragment size as 3132. The strong homology between the two C_B regions means that restriction enzyme sites within exons are likely to be identical in the two regions. Hence the *Pvu* II fragment could represent the superimposition of the two C_B fragments. This digest is uniformative.

In figure 6.2 four lanes are marked with stars. These are above the normal germline DNA and 3132 lanes digested with *HindIII* and *BamH1*. In these two digests, there is discordance between the patterns obtained from the two DNA sources. It would seem unlikely that 3132 should have a TCR β gene rearrangement that would only show up in two out of ten digests. The more likely explanation would be that this discordance is because of RFLP's in the dog population. In support of this a personal communication from Prof F Quimby (Cornell University) revealed that his group had discovered seven different canine RFLP's with a *BamH*1 digest and a

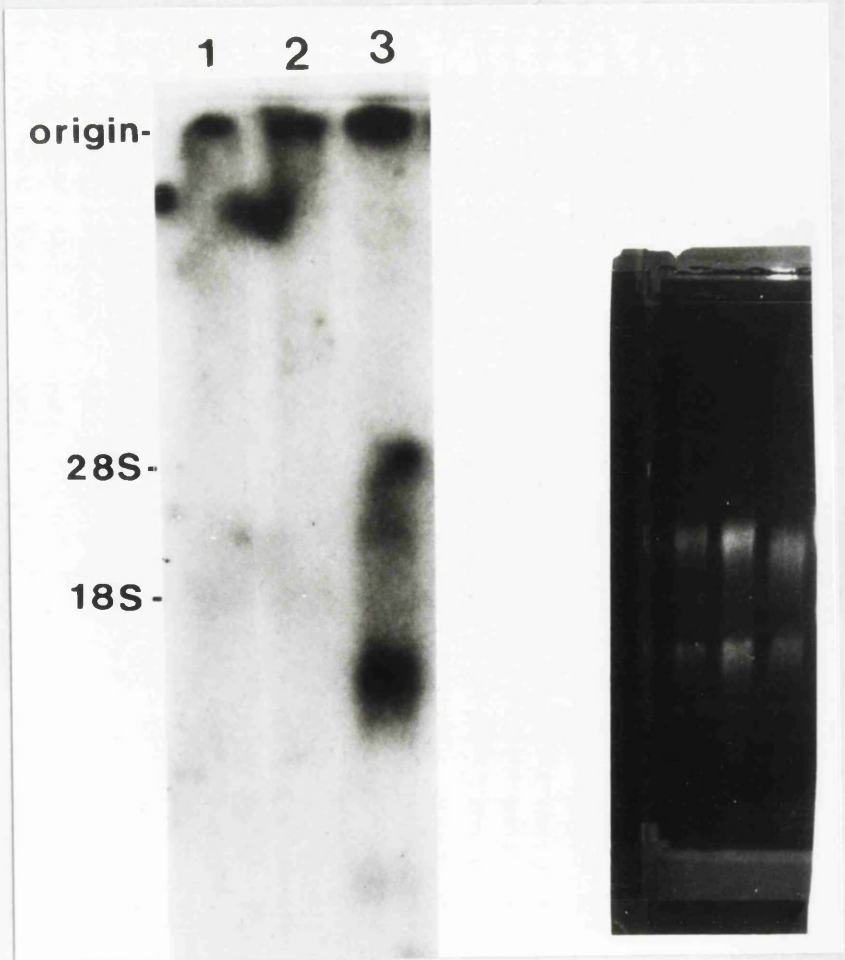
murine constant region probe. When normal dogs expressing different RFLP patterns were mated, the DNA of the offspring showed clear inheritance of the RFLP's. Further work in support of the RFLP nature of the Hind III digest pattern is discussed later in this chapter.

Phenotyping information on CL-1 (detailed in section 2.1.7) suggested CL-1 was a pre-T cell and figures 6.2 and 6.3 confirm that Cl-1 has a T cell genotype. A mature T cell would express a surface TCR heterodimer; 1.3kb TCR β mRNA would be detectable in these cells. Partial rearrangements, characterized by D-J joining without variable region joining, can result in the production of truncated mRNA of 1kb. However Northern analysis of CL-1 does not detect any TCR β message (fig 6.4). This is consistent with CL-1 displaying an immature T cell phenotype. The lack of TCR mRNA could mean that both alleles have aberrant rearranged in a manner that does not permit mRNA production. Confirmation of this would require cloning and sequencing of the two alleles which was beyond the remit of this thesis.

Figure 6.4 Northern blot of putative canine T cell line hybridised with feline TCR_B probe.

Ethidium stained gel prior to blotting is shown on right.

1. 3132 2. Cl-1 3. 3201 (feline T cell line)



6.2 MAPPING THE CANINE TCR LOCUS

From the information obtained from figures 6.2 and 6.3, *EcoR1, KpnI, XbaI and Hind III* digests were selected for use in the clinical samples. Mapping of the restriction fragment sites of these four enzymes was carried out using double digests (figure 6.5). Working on the assumption that the fragment deleted in CL-1 represents the upstream CB_1 region it is possible to orientate the *Hind III, KpnI* and *XbaI* fragments as shown in figure 6.6. The exact positions of these fragments relative to each other and to the TCR locus is not known and hence figure 6.6 is an approximation based on the available information. Lanes 5 & 6 of figure 6.5 illustrate that the *Hind III* and *KpnI* digests are unchanged by digestion with *EcoR1* revealing that the *Hind III* and *KpnI* restriction sites are contained within the large *EcoR1* fragment. The larger (C_{B2}) *XbaI* fragment disappears on digestion with *EcoR1* (lane 8) but a fragment approximately 2kb shorter is generated. This allows one to conclude that the 3' *XbaI* site is downstream to the 3' *EcoR1* site. The *XbaI/HindIII* double digest (lane 10) yields the normal *Hind III* pattern placing each *Hind III* site internal to the *XbaI* sites. The *KpnI/HindIII* digest (lane 7) produces fragments shorter than *Hind* III alone. The constraints imposed by keeping the *KpnI* fragments within the span of the *EcorI* fragment produces the relative position shown in figure 6.3. The *KpnI/XbaI* double digest (lane 9) leaves the C_{B2} *KpnI* fragment unchanged but the C_{B1} fragment is reduced below the length of the normal *XbaI* fragment, this places the 3' *KpnI* site upstream of the 3'*XbaI* site.

Figure 6.6 uses the murine β chain locus as its template. The general homology between the murine and human locus in this region validates the use of the murine locus as a model for the canine locus (Toyonaga *et al,* 1985 and Gascoigne *et al,* 1984). The canine locus is likely to resemble the human & murine loci in terms of the approximate position and extent of its J regions i.e. the J regions are likely to be approximately 2.5kb upstream of the C regions and span 1.5 - 2kb. If this is indeed the case then several predictions can be made regards the use of these digests in detecting gene rearrangements in the MLSA samples.

Predictions:-

EcoR1: fragment spans both J regions and should therefore pick up clonotypic rearrangements to both C_{B1} and C_{B2}.

XbaI: the C_{B1} fragment does not extend into J_{B1} and hence will be uniformative of C_{B1} rearrangements. The C_{B2} fragment is likely to span all of J_{B2} and should pick up most C_{B2} rearrangements.

KpnI: the C_{B1} fragment extends into J_{B1} and should detect C_{B1} rearrangements. The C_{B2} fragment is likely to extend into the 3' end of J_{B2} but may not span all of

J_{B2}; this will depend on the exact extent of the J region in the dog. This detail cannot be fully determined without cloning and sequencing in the region.

***Hind* III**: neither of the fragments extend into J regions and therefore it is unlikely that clonotypic fragments will be generated. However the C_{B1} fragment, like the C_{B1} fragments of the *KpnI* and *XbaI* digests can be monitored for loss of intensity due to loss of one C_{B1} allele, or, as in the case of CL-1, for deletion of both alleles.

Figure 6.5 Mapping canine TCR_B locus using double restriction enzyme digests.

This, and all subsequent figures in this chapter are Southern blots of canine DNA hybridised with the feline TCR β constant region probe using methodology detailed in 2.4.1 and 2.4.6. All lanes in this figure contain 3132 DNA.

1. *Xba I*
2. *Hind III*
3. *Kpn I*
4. *EcoR1*
5. *Hind III plus EcoR1*
6. *Kpn I plus EcoR1*
7. *Hind III plus Kpn I*
8. *Xba I plus EcoR1*
9. *Xba I plus Kpn I*
10. *Xba I plus Hind III*

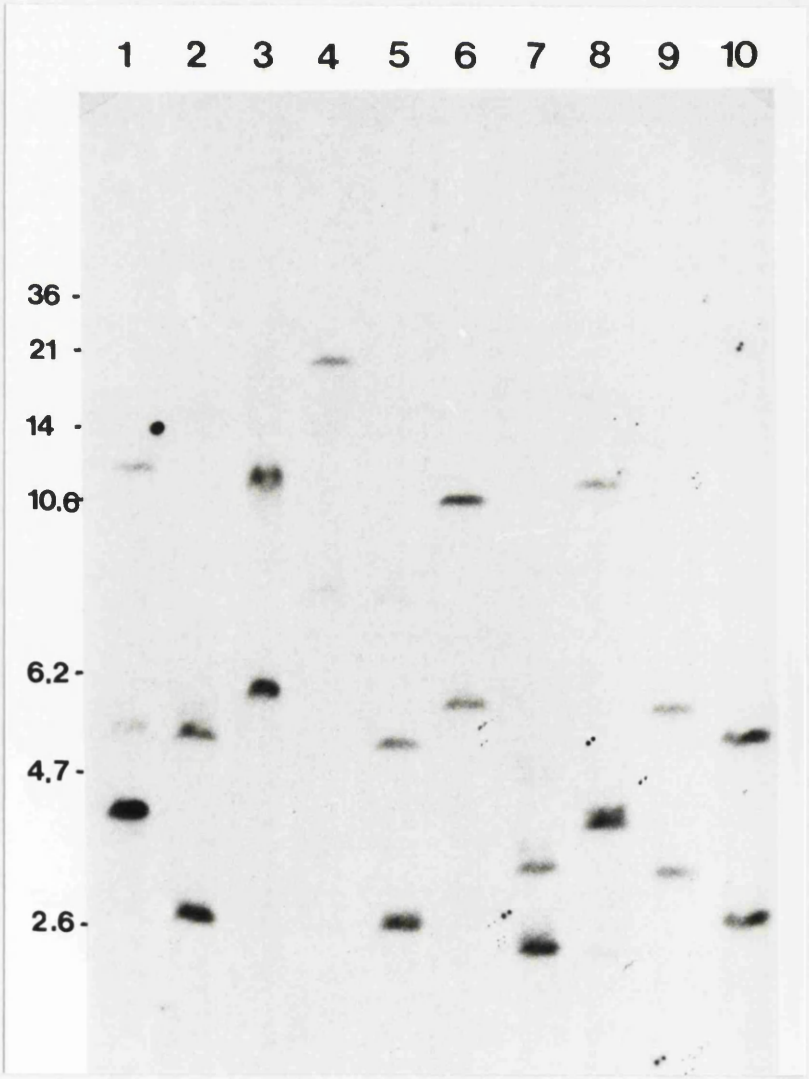
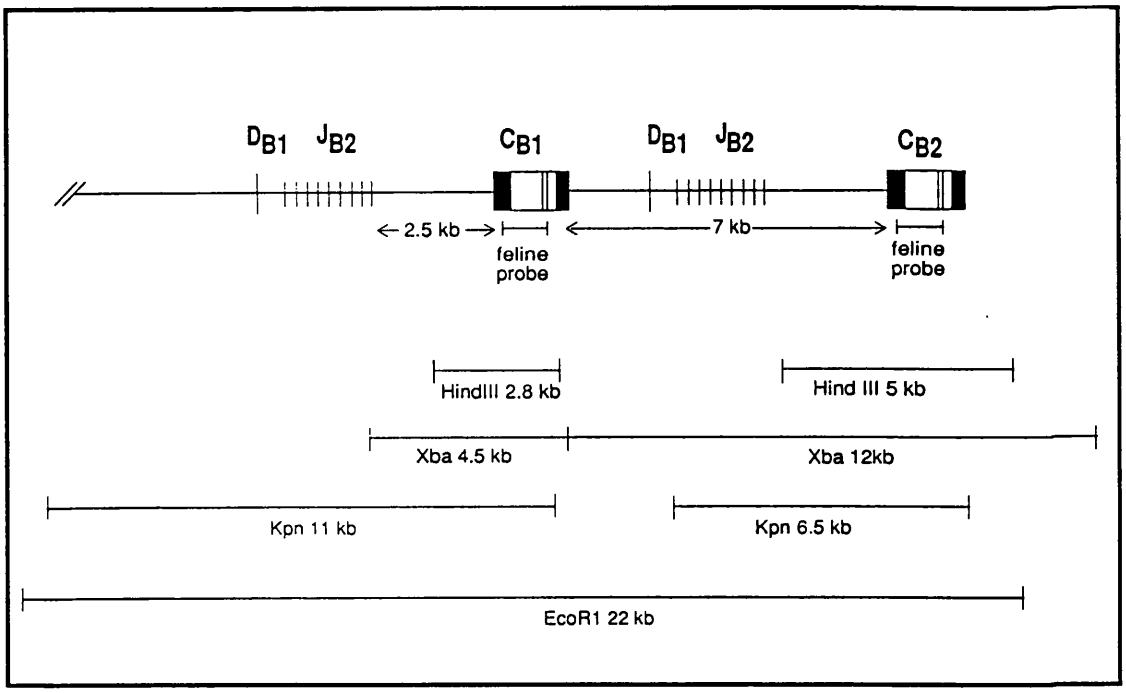


Figure 6.6 Approximate map of the canine TCR β gene



6.3 DETECTION OF TCR GENE REARRANGEMENTS IN CLINICAL SAMPLES

6.3.1 HIND III RESTRICTION FRAGMENT LENGTH POLYMORPHISM IN THE DOG.

The frequency of RFLP for the four chosen digests in the outbred dog population was unknown. Wherever possible, tumour samples were run in parallel with germline tissue samples from the dog. For those tumour samples which are matched with normal DNA from the same dog, RFLP's are easily identified and misinterpretation is avoided. However approximately half of the MLSA samples analysed could not be matched to germline tissue and in this group, RFLP's could present an interpretative dilemma.

To establish the likelihood of RFLP's being a problem in any of the four digests, PBL DNA was collected from 18 normal dogs. The results of the *EcoR1* and *Hind* III digests are shown in figure 6.7. In the eighteen dogs, no RFLP were detected in *EcoR1*, *KpnI* or *XbaI* digested DNA. In addition to these 18 dogs, germline tissue from a further 26 dogs (23 of them MLSA patients) was analysed for RFLP's. None were found, suggesting that RFLP's at the *EcoR1*, *KpnI* and *XbaI* loci are relatively rare.

However, in the *Hind* III digested DNA, two out of the eighteen dogs (lanes 14 & 18) have an extra band immediately below the upper band as indicated by an

arrow. This is in an equivalent position to the band in the *Hind III* digested germline sample in fig 6.2. The germline sample in Fig 6.2 was obtained from a Boxer dog whereas the dogs represented in lanes 14 & 18 of figure 6.7 were cross-bred dogs of indeterminable origin.

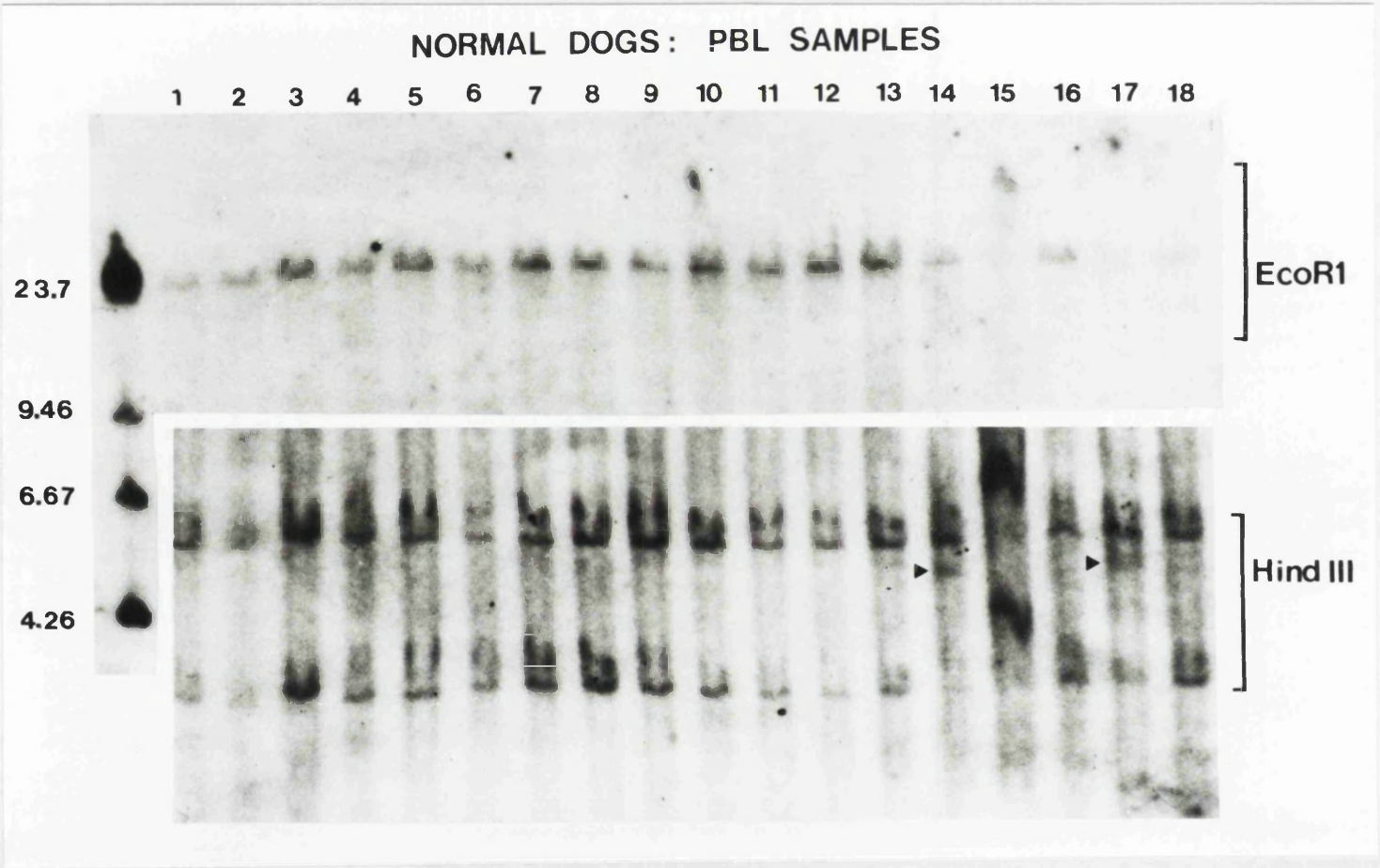
During the course of analysing the clinical samples it became apparent that the 2 band pattern produced by *Hind* III digestion of Boxer dog DNA was found in other boxers and also in another brachycephalic breed, the Bull Mastiff. Figure 6.10 illustrates this pattern in the Boxer dog "SAS" and lane 7 of figure 6.12 shows the same in the Bull Mastiff "BOA". The 3 band pattern shown by the cross-bred dogs was found in 3 other cross-breds (two are shown in figure 6.12) plus two Lhasa Apso's. Lhasa Apso's are extremely short-nosed and are likely to share common ancestry to the other brachycephalic breeds. So although inheritance of these restriction fragment patterns has not been formally proven, the occurrence in distinct breed types is strongly suggestive of heritability. The clinical cases in which *Hind* III RFLP's were found is listed in table 6.1. The Boxer and Bull Mastiffs completely lack the 2.8kb *Hind* III germline fragment and this could be because they are homozygote for this RFLP. The crossbred dogs retain the normal pattern but have the "extra" RFLP band. Thus these dogs are likely to be heterozygotes.

Table 6.1 Breed Disposition of Hind III RFLP

Homozygous[1]		Heterozygous[2]	
Name	Breed	Name	Breed
SAS	Boxer	HEH	Lhasa Apso
SPW	Boxer	BMD	Lhasa Apso
CMD	Bull Mastiff	BIP	Collie X
BOA	Bull Mastiff	CIS	Mongrel
RMD	Bull Mastiff	PES	Collie X
SMD	Bull Mastiff	SAP	M.Schnauzer

1. Homozygosity is based on the complete absence of the lower (2.8kb) Hind III band.
2. Heterozygosity is based on the retention of the 2.8kb Hind III band.

Figure 6.7 Determination of RFLP frequency: germline DNA from outbred dogs.
Peripheral blood samples from 18 normal dogs were digested with restriction enzymes shown.



6.3.2 TCR GENE REARRANGEMENTS IN MLSA

The remainder of this section describes in detail selected cases which exhibit TCR gene rearrangements. In the majority of these cases the *EcoR1*, *KpnI* and *Hind* III digests are shown but the *XbaI* digest is only shown in figure 6.14. Unfortunately, despite the initial results with *XbaI* shown in figure 6.3 (lanes 13 and 14), this restriction enzyme did not always yield the same pattern in the same sample. In particular it was not uncommon to have two or three bands in the 4 - 6 kb range rather than the single band seen in 6.3. For example, the normal DNA run in lane 13 of figure 6.3 is the same DNA that was used in lane 1 of the mapping southern in figure 6.5, yet the latter has an extra fragment at about 5.5kb. In any particular batch of *XbaI* digested samples, all samples digested on the same day would produce identical pattern of fragments of 6kb and less. Some of these bands may have been created through partial digests; certain enzyme aliquots were particularly problematic in this regard. Other workers have also reported difficulty with partial digests using *XbaI* enzyme (Nicol Keith, personal communication; Bremner, 1990). The variation in the exact position and relative intensity of these lower bands made it difficult to judge which one was the equivalent of the 4.5kb C_{B1} band illustrated in the schematic map of the locus (figure 6.6). It was therefore impossible to use it as an accurate measure of C_{B1} loss in the tumours. Fortunately the equivalent information regards the presence of one or two copies of the C_{B1} region could be obtained from the *Hind* III and *KpnI* digests. The larger, 12kb *XbaI* band remained constant throughout the digests but occasionally other bands of higher molecular weight would also be seen. The benefit of retaining the *XbaI* digests in the analysis was that this 12kb fragment, which corresponds to the C_{B2} region, is estimated to span the entire J_{B2} region and should therefore detect most rearrangements involving this region. This proved to be the case; all samples which were judged likely to have a C_{B2} rearrangement based on the other digests did have new bands in the *XbaI* digest. An example of this is shown in figure 6.14.

Figure 6.8 shows the results of genotyping two cases, DHS and ZAK . There is loss of the lower (2.8kb) *Hind* III band, loss of the upper (11kb) *KpnI* band plus loss of the single germline *EcoR1* band and the appearance of two new bands. The interpretation of this is therefore the same as for CL-1. The absent *Hind* III and *KpnI* bands represents the deletion of both C_{B1} alleles. The unique rearrangement of both alleles, presumed to be to CB_2, is represented by the two new *EcoR1* fragments.

The *EcoR1* fragments, both germline and rearranged, are large. The germline *EcoR1* fragment comigrates with the 23.7kb marker and it has not been possible to get an accurate measurement of its size using conventional gels. Attempts to

accurately size this fragment on field inversion gel electrophoresis (kindly performed by Ms J Rose) failed. The *EcoR1* rearranged bands are generally above 12kb. This makes it difficult to compare between gels to assess whether or not these new bands are indeed clonotypic, as would be predicted from the mapping model in the previous section. To ascertain if the rearranged *EcoR1* fragments are unique to an individual tumour, three samples, Cl-1, ZAK and LOK were digested with *EcoR1* and run on a low percentage gel (0.6%) for 18 hours. The results are shown in figure 6.9 and confirm that the fragments appear to be unique.

Figure 6.8 TCR β gene rearrangements: samples "DHS" and "ZAK"

Left hand panel is DHS. Right hand panel is ZAK. Restriction enzymes are indicated in photograph. Germline and tumour samples are run in adjacent lanes; tumour sample is marked with a "T".

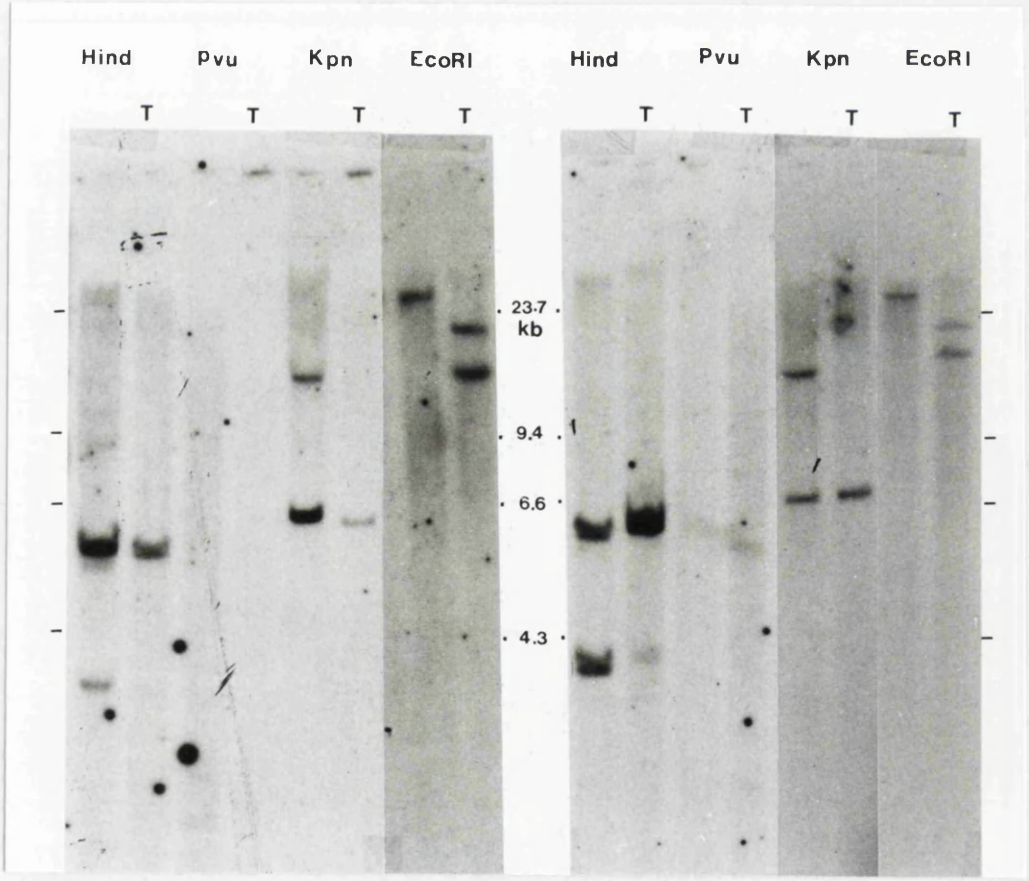


Figure 6.9 Canine samples digested with EcoR1 and separated on low percentage gel show unique fragments.

1. canine germline
2. CL-1
3. ZAK
4. LOK

GL, germline.

The band at 9.4kb was present in all samples and is presumed to represent cross hybridsation to sequences outwith the rearranging TCR β gene.

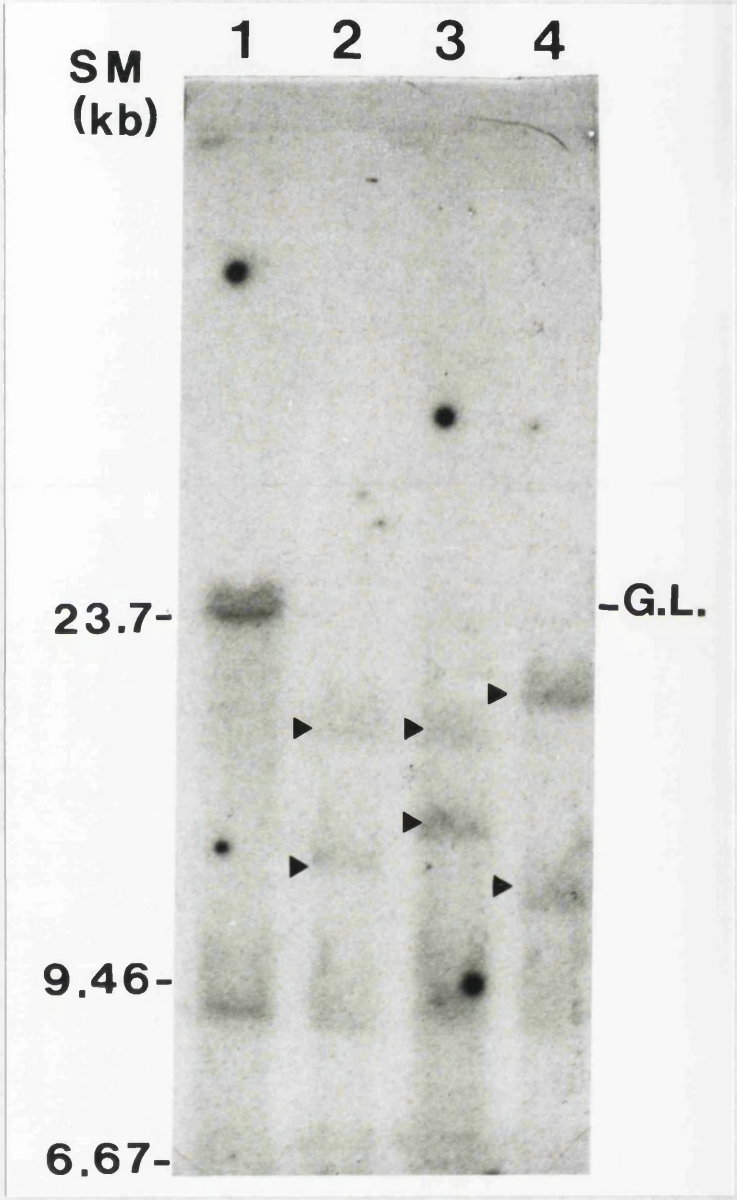
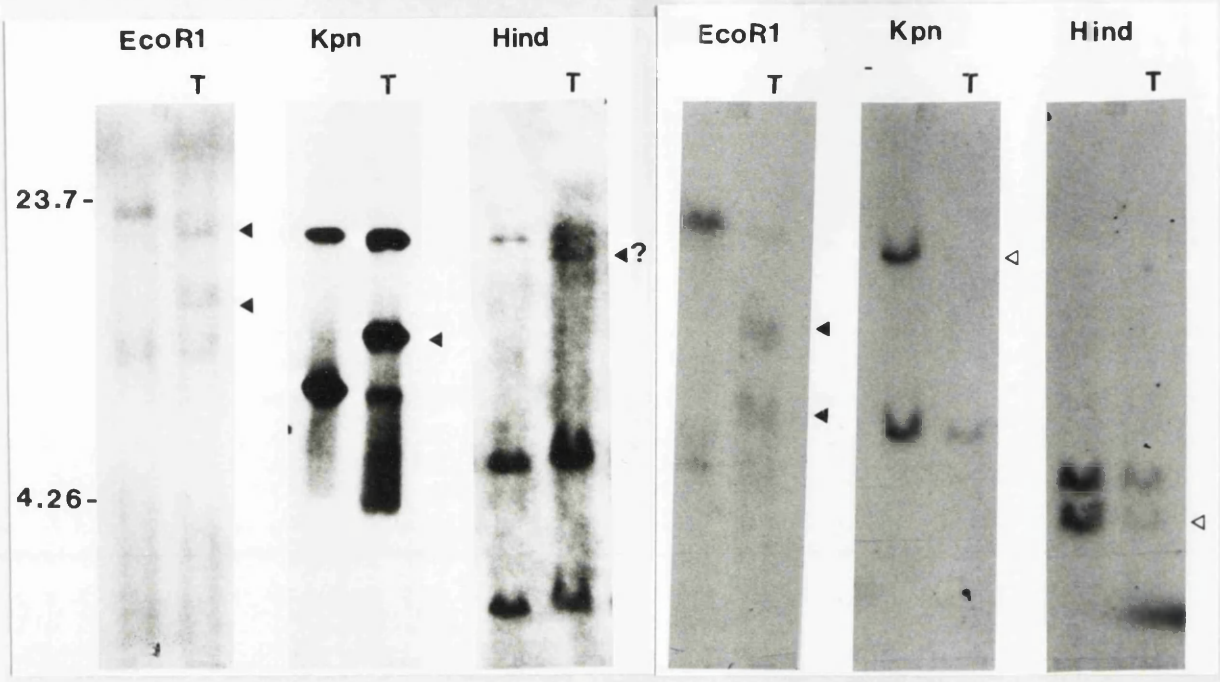


Figure 6.10 shows the rearrangements in the tumours "SAS" and 'LOK". "SAS" has decreased intensity of the germline bands representing the C_{B1} region in all three digests. However there still remains a shadow of a band in the *EcoR1* and *Hind* digests which has been interpreted as contamination with normal lymphocytes. This interpretation is subjective in nature but is taken based on prior experience of other cases plus examination of longer autoradiograph exposures of the same filter.

Figure 6.10 TCR β gene rearrangements: samples "LOK" and "SAS"

Left panel: LOK. Right panel: SAS. Restriction enzymes as indicated in photograph. Germline and tumour samples run in adjacent lanes, tumour sample marked with "T".



"LOK" presents a different pattern. The loss of the germline fragment in the *EcoR1* digest infers both alleles have attempted rearrangements. However examination of the *Hind* and *KpnI* digests to see if there are any deletions of the C_B regions presents a quandary. There is equivocally reduced intensity of the lower (CB_1) *Hind* III fragment but this is not matched by reduced intensity of the upper *KpnI* band. In a similar fashion there is a more obvious reduced intensity of the lower (CB_2) fragment of the *KpnI* digest in comparison with the upper band yet there is no evidence of CB_2 deletion in the *Hind* III digest. In the *KpnI* digest there is a new band, marked with an arrow. The approximate map of the canine locus (figure 6.6), predicts that both *KpnI* fragments may span all or part of the J_B regions and therefore could generate new rearranged fragments. This new *KpnI* fragment in the "LOK" sample could represent a rearrangement to J_{B2}. This would explain why the intensity of the CB_2 germline band is reduced in the *KpnI* digest but not the *Hind* digest. Rearranged *KpnI* bands have not been seen in other samples but this could be because the other cases have used 5' J_{B2} sequences which were outwith the span of the *KpnI* fragment. Neither the exact location and extent of the canine J_{B2} region nor the exact position of the *KpnI* restriction site is known. Placing the 3' end of the J_{B2} region 2.5kb from C_{B2} (equivalent to the mouse locus) would allow the *KpnI* fragment to span the 3' end of the region and make the explanation of the "LOK" *KpnI* pattern a viable hypothesis.

The *Hind* III digest of "LOK" was hybridized with the full length V-D-J-C probe and in addition to the expected bands there is a faint band about 14kb in the normal sample but a doublet in the tumour sample (indicated by an arrow and a question mark). This may represent hybridisation to a rearranging V or J region of the canine loci. However the paucity of information on the use of the full length probe in canine *Hind* III digested samples precludes any firm conclusions.

In the cases discussed so far, the *EcoR1* digests have consistently shown complete loss of the germline fragment plus appearance of two new bands interpreted as rearrangements of both alleles. Figure 6.11 shows 7 samples digested with *EcoR1*. Two of them (lanes 3 and 6) maintain the germline pattern . A further two (lanes 1 and 4) retain the germline band and have a single new band of equal intensity inferring only one allele has attempted rearrangement. However lanes 2, 5 and 7 do not follow the previously described patterns. Lane 2 (RMI) has lost the germline band suggesting both alleles have rearranged yet only one new fragment has been created. Complete deletion of C_{B1} is evident in the *Hind* III digest (figure 6.12, lane 2) and *KpnI* digests (data not shown), confirming that both alleles have rearranged. So why is there only one new *EcoR1* band? It could be that one allele has undergone extensive rearrangements leading to deletion of the entire region including both C_B regions. This could explain why, despite what was sufficient loading of DNA on the gel (shown in figure 6.13), RMI gives a weaker *Hind* III signal compared to the neighbouring lanes.

Lanes 5 and 7 (SAP and SPW) of fig 6.11 are also problematic due to the retention of a faint germline band and the solitary new band. The intensity of the germline band does not seem sufficient to represent the remaining allele in the tumour cells and it could therefore represent normal tissue contamination. In the case of lane 7 "SPW" this is particularly likely because this is actually a *cutaneous* lymphoma case and it is difficult to completely remove normal skin and adnexa from these malignant growths. If both alleles have rearranged then one would expect two new *EcoR1* fragments. The other "SPW" digests do not suggest that complete deletion of one entire allele has occurred so there remains the question of where the missing *EcoR1* fragment has gone. In lane 7, two large fragments are marked with an arrow and a question mark. One of these fragments is *above* the germline fragment. For reasons of its very large size (more than 22kb) the intensity of this band may be poor and hence it could be the missing allele. The interpretation of the "SAP" rearrangements remains a contentious decision. The other three digests could not confirm rearrangements of both alleles. It is therefore classed as uninterpretable in the final assessment of β chain rearrangements in the tumour samples.

Figure 6.11 ***EcoR1*** **digested lymphoma samples**

1. LMG*
2. RMI
3. BRM
4. BRP
5. SAP
6. RMM
7. SPW*

* cutaneous lymphomas

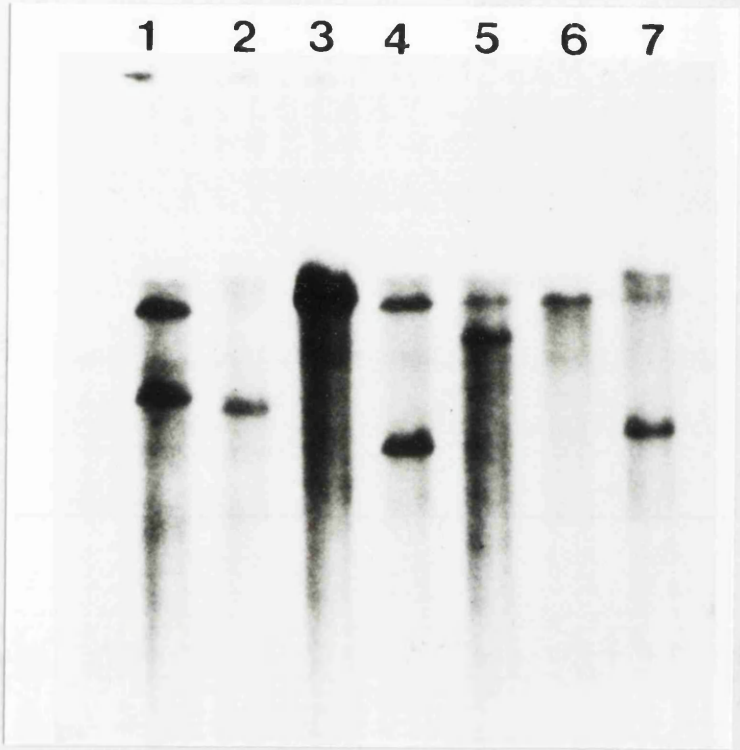


Figure 6.13 Ethidium bromide stained gel of figure 6.12 (next page)
Lane 2 (RMI) is adequately loaded compared to adjacent lanes

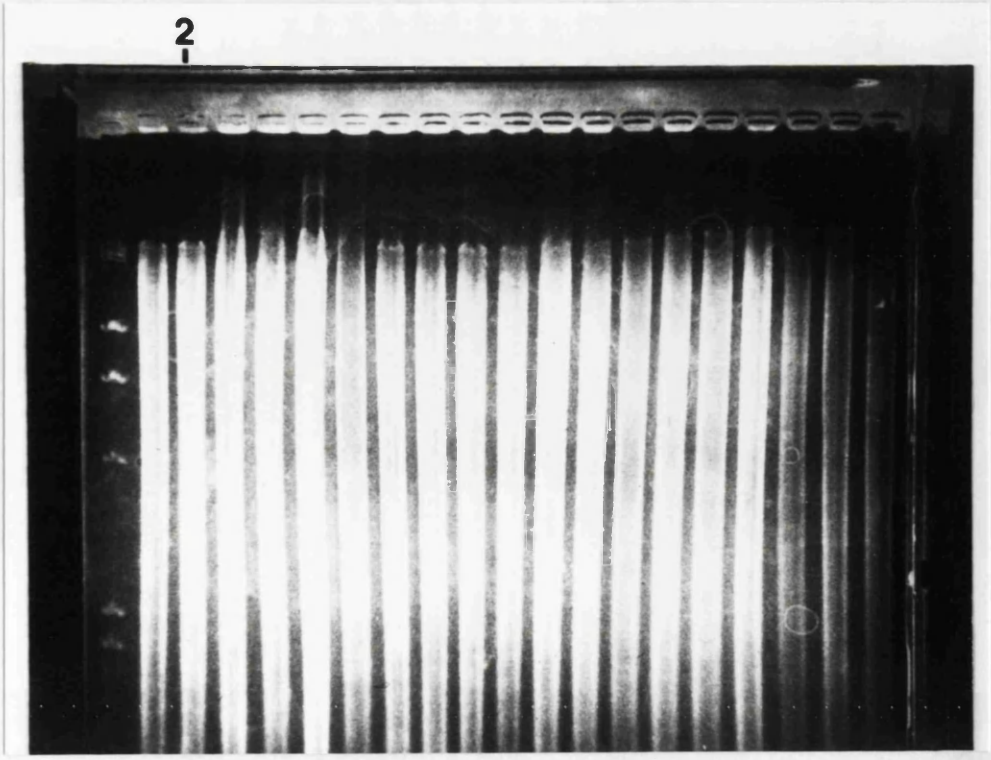
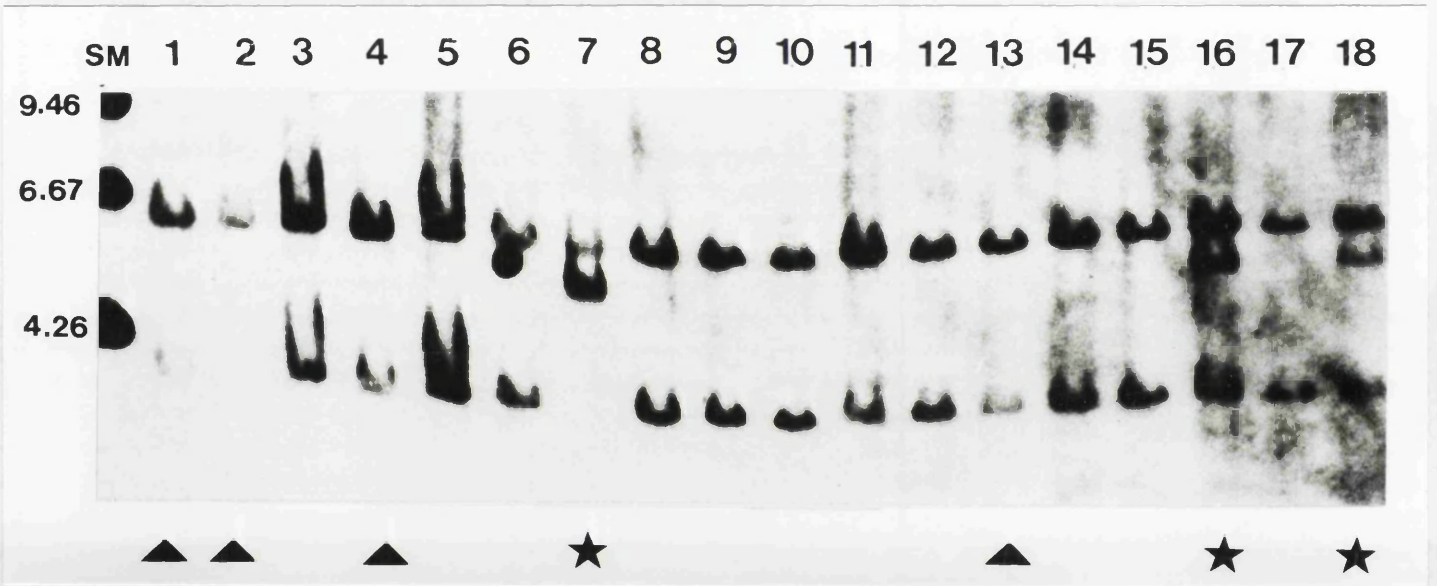


Figure 6.12 Southern blot: Hind III digested lymphoma samples

Arrows indicate a loss of intensity of lower fragment
Lanes marked with stars display an RFLP pattern.

1. LMG*	4. SAD	7. BOA	10. TAB	13. BRP	16. BIP
2. RMI	5. SAB	8. TOB	11. CLL*	14. JAW	17. LAQ
3. TRC	6. BRM	9. SAC	12. HOG	15. RMC	18. CIS

* *LMG is a cutaneous lymphoma. CLL is a peripheral blood sample from a dog with chronic lymphocytic leukaemia.*

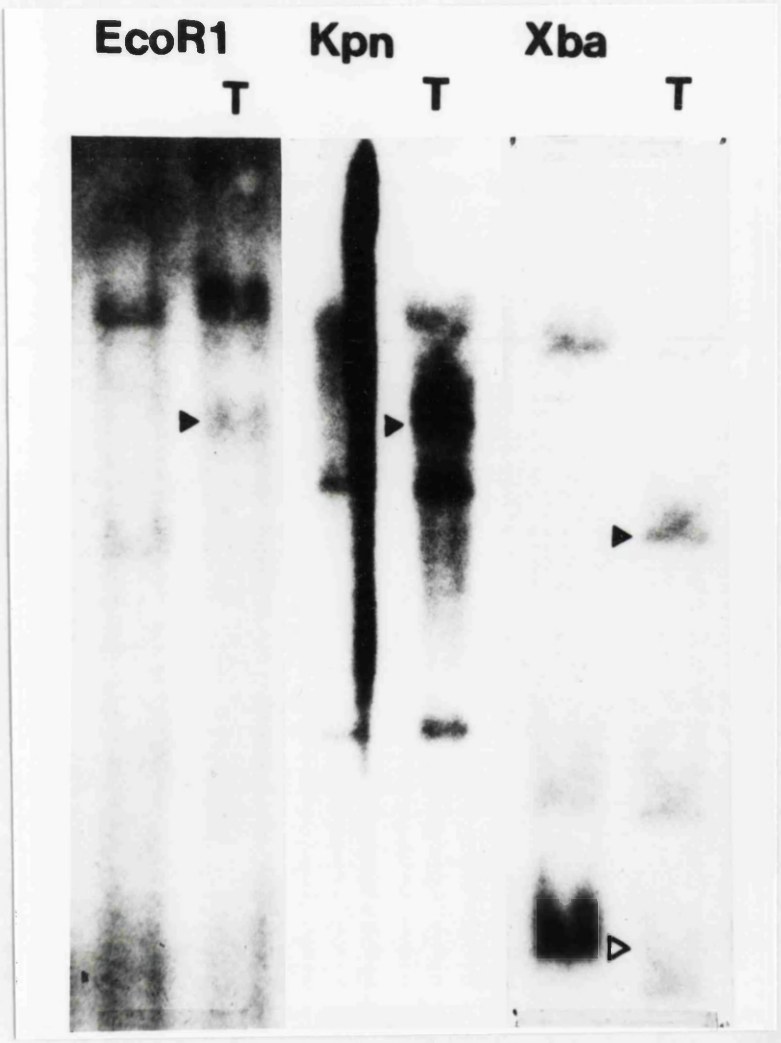


The RFLP pattern in lane 7 of fig. 6.12 (of the bull mastiff BOA) is unusual in that the upper (C_{B2}) band is less intense than the lower band. This could represent loss of one C_{B2} region. Unfortunately this is difficult to confirm this with the other restriction digests (fig 6.14). The *KpnI* digest is overloaded. However, there does appear to be loss of the 4.5kb band in the *Xba* digest (open arrow) which is suggestive of C_{B1} loss. From the *EcoR1* digest it would seem that whatever rearrangement has occurred, one allele remains in germline configuration. In the *Xba I* digest, and probably the *Kpn* digest as well, there is a new fragment in the tumour sample not present in the normal tissue (filled arrow). The C_{B1} *XbaI* fragment is not predicted to enter the junctional region and therefore the *XbaI* fragment is more likely to represent a J_{B2} rearrangement. However, the new *KpnI* fragment could represent either a J_{B1} or a J_{B2} rearrangement (see diagram in fig 6.6). In fact, theoretically, both D_{B1}-J_{B1} and D_{B2}-J_{B2} rearrangements can be detected on the same allele provided the downstream rearrangement has not proceeded to complete V-D-J joining with loss of the intervening sequences. In conclusion, "BOA" has rearranged one allele, probably to D_{B2}-J_{B2}. There is no unequivocal evidence of C_{B1} loss so this would infer that the J_{B2} rearrangement is perhaps non-functional, in which case "BOA" may not be a phenotypic T cell. However, because of the ambiguity of the *Xba I*, *Kpn I* and *Hind III* digest results, it is not possible to rule out a functional rearrangement to C_{B1}.

Figure 6.14 TCR β gene rearrangement: sample BOA.

Restriction enzymes as indicated in photograph. Germline and tumour samples run in adjacent lanes, tumour sample marked with "T".

Open arrow: missing fragment in tumour.
Filled arrow: new fragment in tumour.



6.4 DISCUSSION

The compiled results of the southern analysis of 45 cases of MLSA are given in table 6.2. In total 10/45 cases had evidence of TCR β chain rearrangements. With the available data it is not possible to state if these rearrangements are functional. There are no monoclonal antibodies available against the canine TCR complex hence it is not possible to look at protein production. Northern analysis for evidence of mRNA production is technically possible but could still present interpretative problems if there was a significant non-tumour T cell population present in the sample block. However Northern analysis was not carried out primarily because of time constraints and the availability of adequate quality of RNA in the relevant samples.

Table 6.2 TCR β chain rearrangements in MLSA

Germline Matched Samples		Unmatched samples	
Germline	Rearranged	Germline	Rearranged
LEB	SAS	TRC	RMI
TIH	BEM	SAB	SAD
BEH	DHS	BRM	BOA
BRC	ZAK	TOB	LOK
SMD		SAC	BRP
MMK		TAB	SAP
BEA		HOG	
KEH		JAW	
MAK		RMC	
BMD		BIP	
CMD		LAQ	
BOS		CIS	
BOB		PES	
KEB		ZAS	
CIP		MIG	
SPC		TIT	
RMM			
THH			
HEH			
Total 19	*Total* 4	*Total* 16	*Total* 6

The results of the individual digests in the rearranged samples are presented in table 6.3. This table illustrates that even in the samples where germline tissue was not available, rearrangements were seen in more than one digest. This reduces the likelihood of RFLP's being responsible for the non-germline pattern. Five out of the ten cases have rearranged both alleles. As discussed in the introduction,

rearrangement of both alleles in a B cell tumour has not been documented and thus even without proof of functional β chain rearrangements, it is highly likely that these five samples are genuine T cells.

Table 6.3 TCR Rearrangement Pattern in Putative T cells

Name	Restriction Digest				Interpretation	
	EcoR1	Kpn	Xba	Hind III	No.of alleles rearranged[1]	C_{B1} deleted[2]
SAS	R	L	UI	L	2	both
BEM	R	UI	nd	DI	1	one
RMI	R	L	R	L	2	both
SAD	R	DI	R	DI	1	one
BOA	R	R*	R	DI	1*	none
SAP	R	nd	R	G	1*	none
BRP	R	UI	R	DI	1	one
ZAK	R	L	R	L	2	both
DHS	R	L	R	L	2	both
LOK	R	R	R	G/DI*	2	UI*
				Totals	5/10	2/10[3]

1. Rearrangement of both alleles is based on the loss of the germline band in the EcoR1 digest.
2. Loss of C_{B1} region is based on the relative intensity of the bands in the Hind III and Kpn digest.
3. 2/10 samples retain both copies of C_{B1} .
R - rearranged, L- rearranged with complete loss of one band
DI - decreased intensity of one band relative to the other
UI - uninterpretable G - germline
** interpretative problem discussed in text.*

The majority of the remaining samples, despite having rearrangement on only one allele are also likely to be T cells because, as is shown in table 1.3, the incidence of β chain rearrangements in *mature* B cell malignancies such as NHL is low (6.9%). If the canine lymphoid malignancies are similar to human malignancies then one might expect that, at most, one of the five cases with a single allele rearrangement to be a misidentified B cell.

If there is a bigenotypic B cell tumour within this group of dogs with only single allele rearrangements, then two cases can be suggested as likely candidates. The first is "BOA", whose pattern could be interpreted as an incomplete rearrangement on one allele. The second candidate is "SAP". This sample was obtained from a referring veterinary surgeon at a late date and did not have a matching germline sample. It was not screened with all four enzymes, *KpnI* was omitted. There was no loss of C_{B1} evident on the *Hind* III digest. Neither the *EcoR1*

(figure 6.11) nor the *XbaI* digest (data not shown) produced patterns similar to other rearrangements. Clinically, this dog had a longer remission and survival than any other dog with a T-cell genotype and was not sick at presentation (see chapter 7) which would be consistent with this case being of B cell phenotype.

Confirmation of genotype in these two suspect tumours (and indeed all the tumours) could be achieved by screening concurrently for immunoglobulin gene rearrangements, preferably both heavy and light chain rearrangements. As discussd in section 1.1.4, light chain rearrangements would be the most definitive evidence of B-cell genotype. A human heavy chain junctional region probe, pHJi (Erikson *et al*, 1982), murine kappa constant region probe, pHBC$_K$, (Lewis *et al*, 1982) and a murine heavy chain junctional probe, J11, (Marcu *et al*, 1980) were all used for this purpose. Unfortunately, none of them hybridised adequately to canine DNA and so this part of the work could not be completed.

6.5 SUMMARY

TCR β chain rearrangements were detected in 10/45 MLSA samples using multiple enzyme digests and a feline constant region probe. The percentage of tumours with TCR rearrangements (22%) is in agreement with the phenotyping data produced by Greenlee *et al* (1990).

Five, or possibly six, of the tumours had evidence of rearranging both alleles and are therefore highly likely to be phenotypic T cell. Two samples, "BOA" and "SAP" did not show evidence of deletion of either C_{B1} regions and had atypical rearrangement patterns. One or both may be bigenotypic B cell tumours but due to technical problems this could not be confirmed with the use of Ig gene probes.

CHAPTER 7
STATISTICAL ANALYSIS OF STUDY RESULTS

7.1 INTRODUCTION

The introductory chapter remarked upon the problems with study size and design which has resulted in very few published papers where the significance of P-gp is scrutinized by multivariate analysis. In one of the few longitudinal studies where P-gp status is analysed in conjunction with other patient and tumour characteristics, Chan *et al.* (1990) determined that P-gp was associated with poor prognosis independently of other variables. In haematologic malignancies, this has not yet been demonstrated. In the largest NHL study to date (Miller *et al,* 1991) P-gp was only found in one patient at diagnosis which renders subset analysis of P-gp as a prognostic variable futile. The incidence of P-gp at relapse (5/9) was statistically different but there was no information available on the tumour or patient characteristics of these nine patients.

In the present study, the numbers of cases is greater than the Miller (1991) study; 62 dogs were analysed for P-gp expression either at diagnosis and /or after receiving treatment. 49 of these dogs received chemotherapy, the remaining 13 were euthanased at diagnosis. For the majority of the treated dogs, remission duration and survival times are available and were entered in a database in conjunction with the P-gp status and genotyping information available from this study. Other potentially important prognostic factors entered in the database included stage and "B" symptoms, hypercalcaemia, and administration of oral steroids prior to chemotherapy induction.

Histopathological classification of canine MLSA has not been particularly successful as a prognostic tool (see section 1.2) but more recent papers using Kiel and Working Formulation schemes identified certain high grade tumour types as having a slightly better prognosis than other grades (Greenlee *et al,* 1990; Hahn *et al.* 1992). Hahn *et al,* (1992) identified immunoblastic tumours as having better first remission duration than other tumour types but overall survival was unaffected and there was no difference in performance between intermediate and high grade tumours. (Using the NCI Working Formulation there was only one low grade tumour in Hahn's survey which represented 3% of the population). Despite the poor correlation between histopathology and performance in canine MLSA, it had been hoped to obtain histopathological classification of the cases involved in this study. Unfortunately, this proved to be technically difficult; based on the Kiel classification, differentiating cleaved and non-cleaved populations was not straightforward. Forty-nine of the sixty-two cases in this study were classified but when ten of these tumours were reexamined, the pathologist reclassified all of them to a different subtype. This degree of discordance was felt to be unacceptable so the available histopathological information was excluded from this statistical analysis.

The database was used to identify important prognostic factors in this group of dogs using univariate and multivariate analysis. Table 7.1 shows the information recorded for each of the dogs. The method of defining the entries is described in section 7.2.

7.2 DEFINITIONS OF PATIENT PARAMETERS

Time to Treatment Failure

Time to treatment failure (TTF) is the period of complete remission until time of relapse or death from any cause. Complete remission was defined as the absence of clinical signs of disease and where appropriate, resolution of leukaemia. Duration was calculated from date of induction until the first entry in the medical record indicating the dog had one or more enlarged lymph nodes detected either clinically or radiographically. Times were calculated to the nearest whole week. For the purpose of the database, dogs were divided into complete versus no response. A partial response category was not created because lymph node sizes on a weekly basis were not accurately measured hence the margin of error between partial and no response would be too subjective. Those dogs with a TTF of 0 therefore represents a mixture of partial and no responses.

Survival

Survival times (SURV) were calculated from date of induction until date of death, again adjusted to the nearest whole week. Not all dogs died at GUVC; date of death was found from the referring veterinary surgeon or owner where necessary. Natural death versus euthanasia were not differentiated nor was death from causes other than tumour progression.

Table 7.1a Patient and tumour data (untreated cases)

Abbreviations: M, male; F, female; MN, male neuter; FN, female neuter; Y, yes; n, no; uk, unknown; nd, not done.

Case	Sex	Stage	A/B	Hyper	Steroid	Geno
			Signs	calcaemia	pre-tx	type
PAM	M	IV	B	n	n	nd
MIG	F	IV	B	n	n	B
LEB	M	IV	A	n	n	B
SMM	M	III	A	n	n	nd
BEM	MN	IV	B	Y	n	T
HEH	M	IV	B	n	n	B
DHS	MN	IV	B	n	n	T
TOB	M	III	A	n	n	B
CRS	F	III	A	n	n	nd
MMG	F	IV	B	n	n	nd
SAC	M	IV	B	n	n	B
TIT	FN	IV	B	n	n	B
107	M	uk	uk	uk	uk	nd

Table 7.1b Patient and tumour data (treated cases)

CASE	TTF	SURV	SEX	Stage	A / B	Hyper	Steroid	JS	Epi	Geno	PGP
					signs	calcaemia	pre-tx	Case	Chemo	type	
JAW	31	36	44	III	B	n	n	n	y	B	pos
KMD	17	22	F	uk	uk	uk	uk	n	y	nd	nd
MOK	18	27	FN	III	A	n	n	n	y	nd	nd
LUJ	uk	4	M	IV	B	n	n	n	y	nd	nd
LET	uk	8	FN	III	A	n	n	n	y	nd	nd
ROB	16	uk	M	III	A	n	n	n	y	nd	nd
BRM	44	44	FN	III	B	n	n	n	y	B	nd
BMC	16	19	FN	IV	B	n	n	n	y	nd	neg
RMD	8	8	M	III	A	n	n	n	y	nd	nd
RIC	80	82	M	III	A	n	n	n	y	nd	nd
HOG	0	18	FN	III	A	n	Y	n	y	B	neg
PES	0	8	M	III	A	n	Y	n	y	B	neg
GOF	28	uk	F	III	B	n	Y	n	y	nd	nd
RMI	0	9	F	III	B	Y	n	n	y	T	neg
BOA	12	29	MN	III	A	n	Y	n	y	T	neg
SAO	uk	33	M	III	A	n	n	n	y	nd	nd
CHP	21	25	F	IV	A	n	n	n	y	nd	nd
CIS	11	18	FN	III	A	n	n	n	y	B	pos
SAD	0	5	M	IV	B	n	n	n	y	T	nd
LAQ	16	21	M	III	A	n	n	n	y	B	neg
RMC	8	9	MN	III	B	n	Y	n	y	B	neg
TIH	18	25	FN	III	A	n	n	n	y	B	pos
LOK	8	11	M	IV	B	Y	n	n	y	T	neg
MAK	44	66	M	III	A	n	n	Y	y	B	nd
BRP	uk	8	M	III	B	n	n	n	y	T	nd
BIP	7	13	MN	IV	B	n	n	n	y	B	nd
TAB	24	26	F	III	A	n	n	n	y	B	neg
ZAK	7	21	F	III	A	n	n	n	y	T	neg
CMD	23	35	FN	III	A	n	n	Y	y	B	neg
SNB	uk	2	M	IV	B	n	n	n	y	nd	pos
KEH	52	94	FN	III	A	n	n	Y	y	B	nd
BEH	13	20	M	IV	B	n	n	Y	y	B	pos
BRC	44	48	M	III	A	n	n	Y	y	B	neg
KEB	0	11	F	III	A	n	Y	Y	y	B	neg
MMK	30	30	FN	III	A	n	n	Y	y	B	neg
TRC	1	1	M	IV	A	n	n	Y	y	B	nd
BOS	50	60	M	III	A	n	n	Y	y	B	neg
SPC	54	64	M	III	A	n	n	Y	y	B	neg
BMD	16	17	M	IV	B	n	n	Y	N	B	pos
BEA	59	59	M	III	A	n	n	Y	y	B	neg
SMD	35	50	FN	III	A	n	n	Y	N	B	neg
SAS	14	21	F	III	B	Y	n	Y	y	T	neg
RMM	0	6	M	III	A	n	Y	n	N	B	pos
CIP	18	21	FN	III	A	n	Y	n	N	B	nd
BOB	0	2	M	IV	B	n	n	n	N	B	neg
SAB	0	10	M	IV	A	n	n	n	N	B	pos
THH	8	9	M	III	A	n	n	n	N	B	neg
SAP	33	37	FN	uk	uk	uk	uk	n	N	T	neg
ZAS	28	29	FN	uk	uk	Y	uk	n	N	B	neg

Censored information

Some dogs were lost to follow-up or died while in remission; one dog remained alive at the time of analysis. To allow for these cases, all TTF and survival data was censored according to whether or not the dog relapsed or was alive or dead at time of analysis. In order to simplify the table, this censored information is not shown in table 7.1 but pertains only to a few dogs which are listed below:-

Death during first remission:- RMD, BEA, MMK.

Lost to follow-up in first remission:- GOF, ROB.

Alive in second remission at time of analysis: KEH.

Genotyping and P-glycoprotein status

Genotyping was obtained from the 45 cases which were analysed for TCR β chain rearrangements. Dogs which had detectable TCR rearrangements were defined as T cell tumours and those without were classed as B cell tumours.

Those dogs which were P-gp positive on both IHC and dotblot were recorded as positive in the database. P-gp status (assessed by both IHC and dotblot) was available on 33 pre-chemotherapy samples but because only one dog expressed P-gp at diagnosis, the pre-chemotherapy values did not yield statistically useful results (data not shown). Therefore in table 7.1, the P-gp status is referring to samples taken after treatment.

Hypercalceamia

A calcium concentration above 3mM at any stage of treatment was defined as hypercalcaemic.

Stage

Staging was according to the scheme outlined by Squires *et al,* 1973) which has only four stages. Stage III permits splenomegaly whereas stage IV represents involvement of any other organ including bone-marrow (table 7.2). Animals were retrospectively staged according to the information in the medical records.

Staging was primarily based on the results of survey radiographs, blood cell counts and serum biochemistry parameters including calcium and liver enzyme concentration. Bone marrow biopsies were not routinely performed. In the absence of radiographic signs of organ metastasis or leukaemic blasts in the peripheral blood, dogs were classed as stage III.

Differentiation between "A" and "B" signs was according to clinical signs at presentation, medical history from the owner, and serum biochemistry results.

Table 7.2 Clinical Staging of Canine Lymphoma

Stage	Description
I	Involvement limited to one lymph node or group of nodes in one anatomic region
II	Multiple lymph node involvement limited to one side of diaphragm
III	Generalised involvement but limited to lymphoid tissue, i.e nodes, spleen, tonsils, thymus
IV	Involvement of non-lymphoid tissue, including viscera, bone marrow, central nervous system etc
Clinical signs	
A	slight systemic signs (mild anorexia, lethargy, fever)
B	severe systemic signs; weight loss, vomiting, diarrhoea, abnormal clinical chemistries.

Pre-treatment

This category was created to identify those dogs which had received corticosteroids preceding referral. Most dogs had received some corticosteroids in the weeks preceding referral but only those dogs with evidence of more than ten days of oral steroids or repeated injections of corticosteroids were classed as "pre-treated". It was not possible to define exact steroid doses from the information available.

Chemotherapy protocol and Clinical management

As mentioned in chapter 5, there were some dogs which did not receive the standard epirubicin protocol, either because of clinical contraindications or because the entry onto the epirubicin protocol had ceased. These dogs which received vincristine plus cyclophospamide instead of epirubicin are identified in the database under the column headed "Epi Chemo".

Also recorded are those cases which were managed personally by the author (table heading "JS Case") versus those which were managed by other clinicians at GUVC.

Missing values

Information on stage and calcaemic status etc. were obtained from the medical record. Some records were incomplete or missing (ZAS, KMD) or did not exist because the cases were from outwith GUVC (107 and SAP).

The age and breed of the dogs is available in chapter 5. Age and breed were analysed as potential prognostic factors (see below) but were not found to be significant and so this information is not shown in table 7.1.

7.3 STATISTICAL ANALYSIS

Statistical analysis of the data was performed under the guidance and supervision of Mr. J Paul of the Cancer Research Campaign Clinical Trials office at the Beatson Oncology Centre, Glasgow. Analysis used the computer packages Sysstat (Inc) Evanston, Illinois and Minitab Data Analysis Software (Version 7.1).

TTF and survival curves were created using the Kaplan-Meier method (Kaplan, 1958). The influence of prognostic factors on TTF and survival duration was determined using logrank test. Factors showing an association with death or short TTF by logrank analysis were selected for inclusion in the multivariate analysis. Multivariate analysis used Cox's Proportional Hazards model (Cox, 1972). A step-up procedure was performed where one variable was added at a time until no new variable was statistically significant.

The distribution of prognostic factors between different groups of dogs was compared using Fisher's Exact test.

7.4 POPULATION CHARACTERISTICS

The distribution of the dogs according to stage and symptoms is shown in table 7.4. No dogs presented with disease localised to one side of the diaphragm. As would be expected, the incidence of "B" signs in stage IV dogs was higher than in stage III dogs, and more dogs with advanced disease and severe clinical signs were euthanased at diagnosis.

Table 7.3 Stage at diagnosis of canine lymphoma patients

STAGE	TREATED	TOTAL
IIIA	27	30
IIIB	7	7
IVA	3	4
IVB	9	17
Unclassified	3	4

There were 38% more males than females in the study. Exact numbers were as follows: 33 males, 3 male neuters, 11 females and 15 female neuters . The mean

age of the 62 dogs was 6.9 years, the median was 6 years of age. Patient age was calculated on a whole year basis; parts of years were discounted.

In the 62 dogs, 6 breeds represented 3 or more of the MLSA cases. These were Old English Sheepdog (3 cases), Cavalier King Charles Spaniel (3 cases), German Shepherd Dog (4 cases), Bull Mastiff (5 cases), Golden Retriever (6 cases) and Labrador Retriever (9 cases).

7.5 OVERALL CLINICAL PERFORMANCE

The mean and median TTF and survivals for all the treated dogs versus those dogs which showed a complete response is shown in table 7.4. The complete response rate was 82% (36/44). This response rate excludes those dogs with insufficient information in the medical records to determine response. Numbers are in weeks.

Table 7.4 Clinical Performance of MLSA dogs

	All Cases	Complete Responders
Mean TTF	19.5	24
Median TTF	16	18
Range	0-80	1-80
Mean Survival	25	30.8
Median Survival	21	25
Range	1-94	1-94

7.6 UNIVARIATE ANALYSIS OF PROGNOSTIC FACTORS

The treated dogs were stratified according to the factors listed in table 7.5 and analysed for differences in remission and survival times by log rank test. The resultant "p" values are shown in table 7.5.

From the univariate analysis, two factors are identified as affecting both remission and survival. Stage IV and management by the GUVC service (as apposed to a single clinician) are associated with shorter disease free survival and survival overall. T cell tumours and steroid pretreated tumours are both less likely to achieve a long first remission. P-gp positive tumours (predominantly detected at relapse) was associated with shorter survival but not with short remission. Treatment with the alternate vincristine/cyclophosphamide protocol had no apparent influence on survival.

Sex did not affect remission times or survival times either when neuters were categorised together with entire animals or separately (data not shown for the four

individual categories). The effect of age on clinical performance was calculated by dividing the population into a young and old group. Three different ages were selected as the cut-off point; 5, 7 and 9. Stratification at any of the three ages did not reveal any effect of age on performance.

Table 7.5 Univariate analysis of prognostic variables

Factor	Remission	Survival
Hypercalcaemia/ non-hypercalcaemic	0.265	0.424
Stage IV/ stage III	**0.002**	**0.00005**
"A" signs/ "B" signs	0.086	**0.004**
Steroid pretreatment/ no prolonged steroids	**0.008**	0.068
P-gp positive/ P-gp negative	0.171	**0.044**
T cell genotype/ no TCR rearrangement	**0.037**	0.132
Single clinician (JS)/ Multiple clinicians	**0.013**	**0.004**
Epirubicin protocol/ COP protocol	0.218	0.347
Male or neuter/ female or neuter	0.862	0.532
Age	*see text.*	

Those numbers in bold are below the p=0.05 level of significance.

7.7 MULTIVARIATE ANALYSIS OF PROGNOSTIC FACTORS.

Those factors which were identified as influencing TTF or survival in the univariate analysis were included in the multivariate analysis. The results of a two-factor analysis of performance are shown in table 7.6. The table gives the hazard

functions for each of the variables. The risk ratio represents the increased risk of treatment failure (or death) that one particular factor adds to the pre-existant risk; values greater than two are considered significant.

Table 7.6 Two factor multivariate analysis.

Factor combination	Risk ratio Remission	Survival
Stage IV	1.941	2.703
"B" signs	0.722	1.380
Stage IV	1.126	2.188
P-gp positive	0.528	0.597
Stage IV	3.197	4.189
Steroid pretreatment	**3.179**	**2.869**
Stage IV	2.092	3.377
T cell genotype	**2.068**	1.702
Stage IV	2.319	3.428
Single clinician (JS)	**-2.160**	**-2.518**

Numbers in bold are above the designated significance level.

From the two-factor analysis, it is apparent that stage and pretreatment have the most profound influence on subsequent TTF and survival. The analysis was then "stepped-up" to include a third factor to determine if this would have any influence on clinical performance over and above stage and pretreatment status.

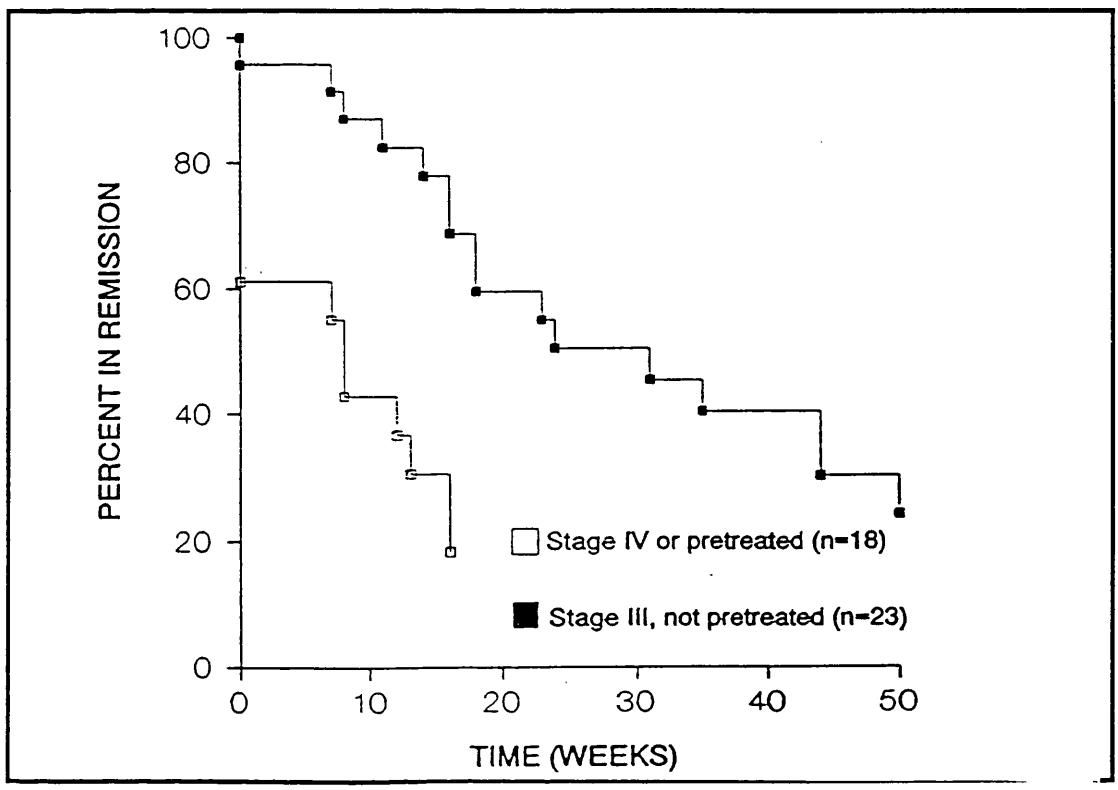
Table 7.7 illustrates that no other variable has a statistically significant effect on TTF after stage and pretreatment status are taken into account. Survival is further influenced by clinician even after allowing for stage and steroid administration; management predominantly by a single clinician (JS) had a positive effect on performance as indicated by the negative risk values. Stage IV and prior treatment with corticosteroids would therefore appear identify a poor prognostic group. The Kaplan-Meier curves for TTF durations in the good prognostic group (stage III and not steroid treated) versus the poor prognostic group is shown in figure 7.1; the difference between the two groups is readily apparent.

Table 7.7 Three factor multivariate analysis

Factor Combination	Risk ratio Remission	Survival
Stage IV	2.876	3.235
Steroid pretreatment	3.045	3.044
"B" signs	-0.004	1.677
Stage IV	1.972	2.712
Steroid pretreatment	3.295	3.028
P-glycoprotein	0.551	0.836
Stage IV	2.962	3.859
Steroid pretreatment	3.127	2.588
T cell genotype	1.729	0.977
Stage IV	2.940	3.830
Steroid pretreatment	2.517	2.281
Clinician-in-charge	-1.425	**-2.066**

Numbers in bold are above the designated significance level.

Figure 7.1 Kaplan-Meier curves of TTF in good and poor prognostic groups.



7.8 COMPARISON OF P-GP POSITIVE VERSUS -NEGATIVE LYMPHOMAS

The low incidence of P-gp at time of diagnosis (only 1/33 positive by IHC) means that it is not possible to perform any meaningful statistics to determine the prognostic significance of P-gp at time of diagnosis. The incidence of P-gp positive tumours was higher in the treated tumours (7/31). If P-gp is assessed in drug-resistant tumours (excluding the dogs who died in remission) then 7/28 samples were P-gp positive, which is a statistically significant difference in frequency compared to the pre-chemotherapy group (p=0.0113). However, by multivariate analysis, the acquisition of P-gp did not seem to have any statistically significant effect on remission and survival over and above the effects of stage and steroid pre-treatment.

To investigate the apparent clinical insignificance of P-gp in treated tumours, the distribution of other prognostic attributes in the P-gp negative and positive tumours was examined by Fisher's Exact test.

Table 7.8 Prognostic factors in P-gp positive drug-treated lymphomas
(missing values not shown)

Factor	P-gp +ve	P-gp -ve	"P" value
Stage III	3	19	
Stage IV	4	3	**0.038**
Steroid pretreatment	6	17	
No pretreatment	1	5	1.00
"A" signs	3	16	
"B" signs	4	6	0.193
T cell genotype	0	6	
B cell genotype	6	17	0.295
Male	6	11	
Female	1	13	0.094
Epirubicin protocol	4	19	
COP protocol	3	5	0.335
Single clinician	2	9	
Multiple clinicians	5	15	1.00

Numbers in bold are below the 0.05 level of significance

Table 7.8 shows that P-gp positivity is associated with stage IV tumours but is not associated with a particular drug protocol. The association of P-gp expression with advanced stage was also apparent in the non-GUVC samples (table 7.9).

Table 7.9 Clinical stage, sex and P-gp status of non-GUVC cases

	Stage III	Stage IV	Stage V	Male	Female
P-gp positive	0	1	2	3	0
P-gp negative	7	9	2	8	10

Curiously, males also have a higher incidence of positive tumours (after treatment) than females but this is not statistically significant (p=0.094). All three P-gp positive dogs from other institutes were also males.

7.9 COMPARISON OF STEROID PRETREATED GROUP VERSUS UNTREATED GROUP

Table 7.8 detailing the distribution of prognostic factors in the P-gp positive and negative groups shows that pre-treatment with corticosteroids is not associated with a greater incidence of P-gp following chemotherapy. The adverse effect that pre-treatment with corticosteroids has on the clinical performance of canine lymphomas would appear to be independent of stage judging from the multivariate analysis. Table 7.10 emphasizes that dogs which have received prolonged corticosteroid pretreatment do not present in a more advanced stage, in fact the exact opposite is true; pretreated dogs are more likely to be stage III.

Table 7.10 Clinical presentation of steroid treated dogs
(missing values are not shown)

Factor	No pretreatment	Steroid pretreatment	"p" value
Stage III	29	8	
Stage IV	21	0	**0.041**
"A" signs	28	6	
"B" signs	22	2	0.449

Numbers in bold represent values below the 0.05 level of significance

7.10 CLINICAL PRESENTATION OF T-CELL GENOTYPE

Univariate analysis showed that T-cell genotype lymphomas had a poorer complete remission time than dogs lacking TCR gene rearrangements. Even after stage was taken into consideration, T-cell genotype had an influence on remission. The clinical information from the T cell tumour dogs was analysed in parallel with the putative B cell tumours to identify any differences in the prognostic factors present at diagnosis in the two populations. The information from the untreated dogs, which included two of T cell genotype, was included in this comparison. The frequency of attributes in the T cell versus the B cell lymphomas were compared using the Fisher Exact test.

Table 7.11 T cell versus B cell presentation
(missing values are not shown)

Factor	T cell group	B cell group	"p" value
"A" signs	2	23	
"B" signs	7	12	**0.027**
Stage III	5	23	
Stage IV	4	12	0.702
Hypercalcaemic	5	35	
Normocalcaemic	4	1	**0.004**
Steroid pretreatment	8	1	
No pretreatment	1	6	1.00

Numbers in bold are below the 0.05 significance level.

From table 7.11 it is apparent that hypercalcaemia is very strongly associated with TCR gene rearrangements and that dogs with T cell tumours do not necessarily present with advanced stage of disease, but they are often systemically ill at time of diagnosis.

7.11 DISCUSSION

This population of MLSA cases showed the expected distribution of stage, age and sex. Compared to humans, most dogs present with more advanced disease and this is reflected in the complete lack of dogs with stage I or II. The mean age of 6.9 years is comparable with other studies (Calvert and Leifert, 1981; Teske *et al.* 1990). A large epidemiological study of lymphoma incidence in the dog detected a 20% greater incidence in males versus females (Schneider *et al,* 1983) whereas this

study had 38% more males than females. However, given the small sample size, this variation is not unexpected. Other small studies have had populations with a large preponderence of males (Calvert and Leifer, 1981; Carter *et al*, 1987) or females (Gray *et al*, 1984). Of the breeds which were most common in the series, Labrador Retriever and Golden Retriever were the most common. However, given the prevalence of this breed in the general population, without performing detailed epidemiological analysis of the referral population, it is not possible to assume that these dogs have a higher incidence rate. However, even without further epidemiological data, five Bull Mastiffs with MLSA would appear to be a greater incidence than other breeds and the extremely high incidence of MLSA in certain Bull Mastiff families has been previously reported (Onions, 1984). The mean TTF and survivals of the Bull Mastiffs or the retriever breeds were not statistically different from the other breeds (data not shown) but numbers were insufficient for detailed subset analysis.

The overall clinical performance of canine lymphoma patients in this study was poor in comparison with published results with some multidrug protocols (Cotter *et al*, 1986; Greenlee *et al*, 1990). However, the comparison between studies at different institutions is difficult for various reasons; staging and data calculations are not standardised and some studies do not necessarily contain equivalent populations. For instance, some published studies do not contain any dogs with advanced stage disease (Gray *et al*, 1984; Weller *et al*,1980) and most do not record the history of steroid intake prior to induction. Price *et al*, (1991) did record steroid intake prior to chemotherapy and found that this did reduce remission duration. In the present study, 20/49 dogs (41%) were either stage IV or had received considerable amounts of corticosteroids prior to referral which has decreased the overall efficacy of the protocol.

Despite the somewhat disappointing overall TTF and survival figures, the results do suggest that epirubicin is considerably less cardiotoxic than doxorubicin when both are given by rapid infusion. In this study, two of the epirubicin treated dogs (BRM and BEA) died of epirubicin induced cardiomyopathy at cumulative doses of over 500mg/m^2 and 550mg/m^2 respectively. In the single agent doxorubicin trial of Postorino *et al*, (1989) which also used a three weekly schedule, 3/37 dogs (8%) died of cardiomyopathy at cumulative doses of only 156, 172 and 271mg/m^2. It would therefore appear that it is possible to give a greater cumulative dose of epirubicin before cardiac damage is dose limiting. The two cardiomyopathic deaths represents 5% of the epirubicin treated dogs and with retrospect it should have been possible to have avoided these fatalities with more frequent monitoring of

cardiac function. One of the dogs, BEA, was phenotypically like a Great Dane and was perhaps at increased risk of cardiomyopathy (Postorino *et al* 1989). From this study, it would appear prudent to perform echocardiograms on treated dogs at each cumulative 50mg/^{2} dose from 350mg/m^2 onwards.

By univariate analysis, quite a few factors were identified as being associated with adverse prognosis. Stage IV and multiple clinicians adversely affected both TTF and survival whereas pretreatment with steroids and the T cell genotype appeared to only affect attainment of a good remission. The presence of P-gp after drug treatment appeared to affect overall survival but not necessarily affect TTF.

As discussed in the introductory chapter, staging in both human and canine lymphomas has not been universally useful in identifying a poor prognostic group. The three studies which were cited as failing to show a difference between TTF and stage all used a five stage system (Carter *et al,* 1987; MacEwan *et al.* 1987; Greenlee *et al,* 1990) whereas the two studies which did detect a better prognosis for stage III dogs used the simpler four stage system described earlier in this chapter (Squires *et al 1973;* Cotter *et al.* 1983). The difference between stage III and stage IV in the four stage system differentiates tumours which are still contained within the lymphoreticular system (i.e. including spleen) from those which have disseminated to other organs. The five stage system attempts to differentiate those dogs with splenic and/or hepatic involvement from those with other organ involvement. This does not necessarily make biological sense and judging from the results of statistical analysis is not particularly useful.

A second pertinent point to be made about the ability of staging to predict clinical performance in this study was that it was performed almost exclusively without the use of bone marrow biopsies. Severe neutropenia or anaemia automatically classed a dog as stage IV without the need for a bone marrow aspirate to confirm tumour cells in the bone marrow. Because of variations in tumour dissemination within bone marrow, aspirates are less sensitive than core biopsies in detecting tumour spread (Raskin and Krehbiel, 1989). Therefore, unless every case is going to be given a core biopsy, it would seem more sensible to base staging on peripheral blood counts than on aspirates performed in only a few dogs. Inevitably, it is the peripheral blood counts that determine the treatment given and not the aspirate results. The inaccuracies created in differentiating between stage IV and stage V dogs when not all have had bone marrow core biopsies has been remarked on by other workers (Price *et al,* 1991) and they avoided this interpretative problem by analysing stage IV and Stage V dogs as a single group in comparison to stage III. By doing this, they determined that stage IV/V dogs performed worse than stage III dogs. This lends

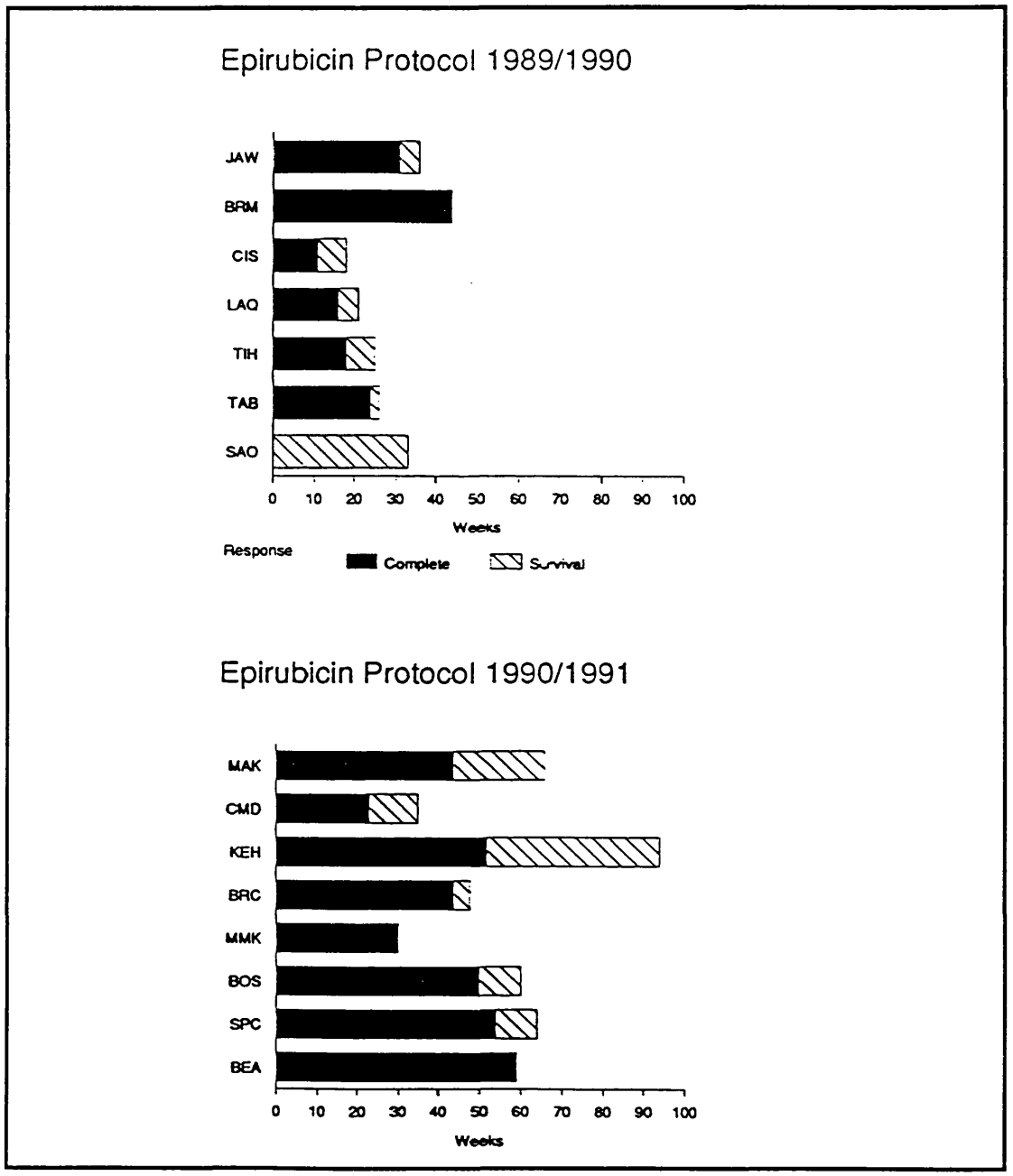
further support to the view that attempting to differentiate the magnitude of tumour spread outwith the lymphoreticular system is not merited as a prognostic exercise.

The influence of clinician on clinical performance is not entirely unexpected. The use of epirubicin as a chemotherapeutic agent for canine lymphoma had not been explored at GUVC prior to this study and management of cases by the author did not occur until the second and third years of the study. This meant that in effect, many of the cases managed by the department in the first year of the study were historical controls for the cases managed by the author in subsequent years, once there was greater experience with the protocol. The problems created by using historical controls in human NHL is discussed elsewhere (Armitage and Cheson, 1988). Figure 7.2 illustrates the difference in performance of the protocol in the first eighteen months versus the second half of the study. The cases in figure 7.2 are all stage III tumours which lacked TCR gene rearrangements and from dogs which had not been pretreated with steroids. The upper panel was managed by GUVC and the lower panel by the author.

Management by the author was associated with prolonged survival over and above the effects of stage but this is profoundly influenced by the attitude of the owners to rescue therapy once their dog had relapsed and probably reflects owner commitment more than biological differences in the tumours.

The effect of P-gp on survival but not remission could be construed as evidence that once a tumour relapses with P-gp positive disease, the animal quickly succumbs to tumour progression due to drug resistance. Unfortunately, due to deficiencies in study design, this cannot be stated categorically. For ethical reasons, it was not always possible to obtain tumour biopsies immediately relapse was clinically apparent. Instead, 14 of the 31 post chemotherapy samples were obtained at euthanasia from dogs which had received, and failed rescue therapy. The time from relapse to death is usually less than 8 weeks and in that time, complete remissions were rare. Only one dog (BRC), which was included in the post-treatment values obtained a complete remission following salvage therapy. This dog was still in complete remission at time of sample collection and was P-gp negative. All other dogs retained residual tumour between onset of relapse and administration of intensified chemotherapy. It is therefore likely, but cannot be proven that P-gp existed at the onset of relapse even before rescue therapy was given.

Figure 7.2 Clinical Performance of the Epirubicin treatment protocol
All cases represent dogs which were stage III at presentation, lacked TCR gene rearrangements and had no history of prolonged steroid administration before referral.



The rescue therapy offered to the epirubicin treated dogs could not be standardised because it was dependant on the clinical condition of the dog, the availability of drugs and the wishes of the owner. Generally dogs were switched from the epirubicin protocol to a vincristine and cyclophosphamide containing protocol, but a few remained on epirubicin with the addition of oral cyclophosphamide and asparaginase. The frequency of tumours with P-gp expression was not statistically

different in those dogs receiving the epirubucin protocol versus a COP based protocol from time of induction. Also, the distribution of P-gp positivity in follow-up samples collected before or after rescue therapy was virtually equal (4/17 versus 3/14) and is illustrated in table 7.12. It would therefore seem unlikely that the exact type of drugs used in the salvage protocols would considerably alter the P-gp status in those dogs which had failed epirubicin treatment after several months.

Table 7.12 P-gp expression in post chemotherapy samples

No prior rescue therapy		Prior Rescue therapy	
P-gp -ve	P-gp +ve	P-gp -ve	P-gp +ve
PES RMI	BMD RMM	BMC HOG	JAW CIS
BOA LAQ	SAB SNB	CMD *BRC*	BEH
RMC LOK		KEB BOS	
TAB ZAK		SPC SMD	
BEA MMK		SAS SAP	
THH ZAS		TIH	
BOB			

Cases in italics were in remission at time of sampling.

It has been suggested that underdosing with chemotherapeutic agents may hasten the onset of clinical drug resistance (Sobrero and Bertino, 1986). Five of the dogs could be identified as chronically receiving epirubicin doses which were below the recommended 25mg/m^2, and instead were in the 18-23mg/m^2 range. This underdosing was either inadvertent (LAQ, BOA, BRP) or because of gastro-intestinal dose-limiting side effects (MAK and BOS). The mean TTF and survival of these dogs was a respectable 30.5 and 36.8 weeks respectively and of the three dogs followed up while in clinical relapse (LAQ, BOA and BOS) none were P-gp positive. Therefore within this small group there was no evidence that a small reduction in dose favoured the selection of P-gp expressing tumour cells or impaired clinical outcome. Hahn *et al* (1992) has shown that 3 cycles of doxorubicin with no further chemotherapy is equally efficacious as chronic COP; this may indicate that chronic maintenance with epirubicin in these dogs is irrelevant to their ultimate clinical performance.

Pretreatment with corticosteroids adversely affected performance but this did not appear to be due to P-gp expression in steroid treated tumours. None of the eight dogs which received considerable amounts of steroids were P-gp positive at diagnosis and only one of the six pre-treated dog (RMM) examined at relapse expressed P-gp.

In conclusion, prior treatment with corticosteroids does adversely affect prognosis but this is not through rapid acquisition of P-gp.

The acquisition of P-gp was associated with tumours which presented in stage IV and hence, in multivariate analysis, P-gp was not a prognostic factor over and above stage. P-gp was also more common in male than female dogs. From the distribution of prognostic factors in the P-gp positive versus negative relapse tumours, stage would appear to be the largest influence on subsequent P-gp expression more than MDR drug dose (either in the epirubicin protocol or in the COP protocol) or steroid pretreatment. The potential significance of these findings are discussed in the final chapter.

One of the aims of this thesis was to determine if the poor prognosis of T cell tumours was due to the rapid expression of P-gp. This does not appear to be the case. None of the T cell tumours examined either at diagnosis or at relapse expressed P-gp. Although only B cell tumours expressed P-gp, the difference in frequency of P-gp expression in the genotyped tumours was not statistically different.

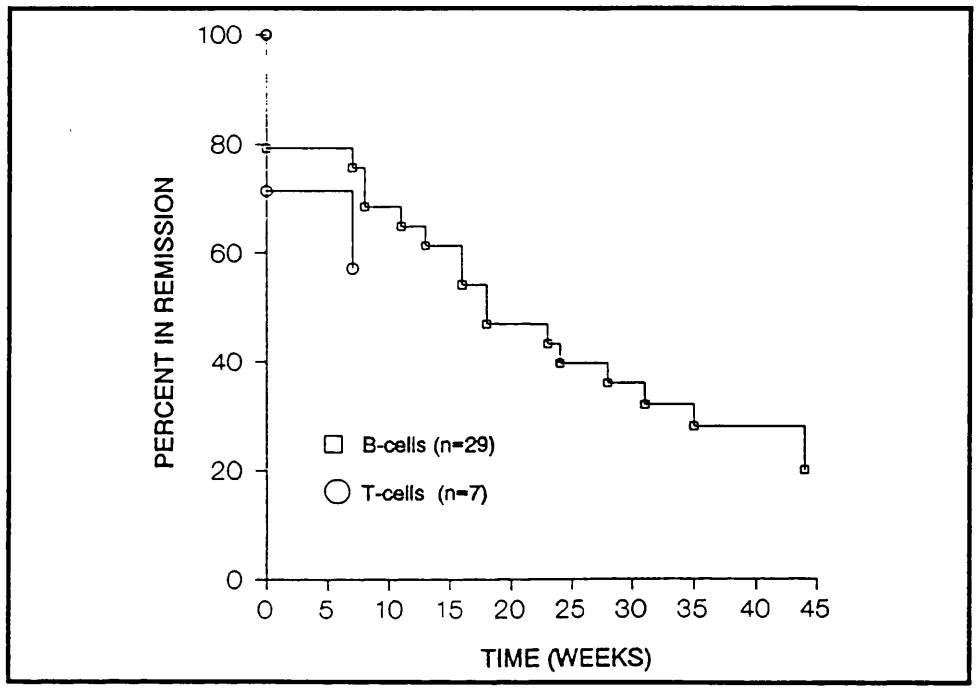
The analysis of presentation of T cell genotype dogs versus those dogs lacking TCR gene rearrangements is in agreement with the findings of Greenlee *et al,* (1990) who found a strong correlation of T cell phenotype with hypercalcaemia. Greenlee *et al.* (1990) also found that T cell phenotype correlated with a shorter remission and survival and secondly that clinical illness was associated with poor survival. The poor prognosis of T cell genotype on outcome is less apparent in this study probably because there were very few treated T cell lymphomas. This is illustrated in the Kaplan-Meier TTF and survival curves of T cell versus B cell tumours shown in figure 7.3. These curves were plotted out until only five dogs remained evaluable. Eight T cell tumours received treatment but only seven T cell lymphomas could be assessed for TTF because of inadequate information in the medical record. The remission curve illustrates that there are too few evaluable cases to determine a statistical difference between the curves.

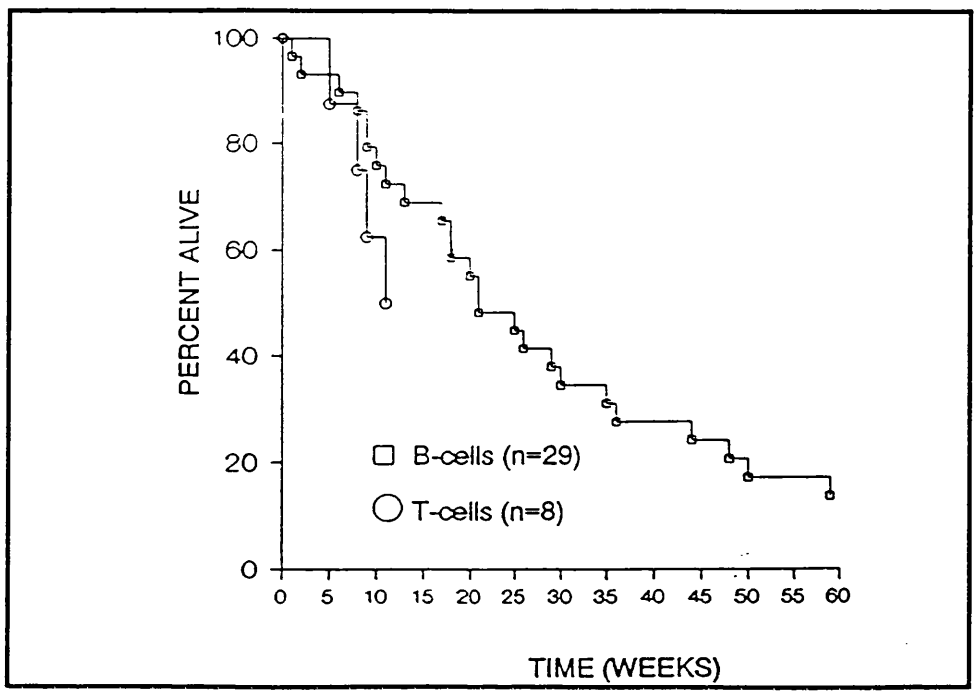
It is not possible to fully account for the poor TTF of T cell tumours from the factors analysed in this study. The dogs did not present in an advanced stage of disease but did commonly present with severe systemic signs. By univariate analysis, severe systemic signs influenced overall survival but not TTF and so it would not be possible to predict poor TTF in the T cell tumours merely from the presence of "B" symptoms. This tendency for T cell tumour dogs to present with less tumour bulk but severe illness has been noted by other authors (Appelbaum *et al,* 1984) and may make a valid, simple method for identification of potential T cell tumours, even in the absence of hypercalcaemia.

Figure 7.3 Kaplan-Meier curves of the clinical performance of genotypic T cell versus B cell lymphoma

7.3a Disease free remission curve (*top*)
7.3b Survival curve (*bottom*)

B cell tumours were defined as those lacking TCR β chain rearrangements.





7.12 SUMMARY

Advanced stage and corticosteroid pretreatment were found to be the most important prognostic factors influencing performance in canine MLSA as determined by multivariate analysis. T cell genotype was also associated with a short disease free period and dogs which presented with systemic signs of disease had a shorter overall survival.

The expression of P-gp at relapse was more prevalent in dogs which presented with advanced disease ($p=0.038$) and hence P-gp as a prognostic factor was not independent of stage. The frequency of P-gp expression at relapse was significantly different from pre-treatment samples ($p=0.0113$) but this expression was not overtly influenced by the drug protocol administered.

Hypercalcaemia and systemic signs were associated with the T cell genotype. The adverse prognosis associated with T cell phenotype and steroid pretreatment was not mediated by P-gp.

CHAPTER 8
FINAL DISCUSSION

8.1 DOES P-GP PREDICT RESPONSE TO CHEMOTHERAPY, AND IF SO, DOES THIS STUDY USE A RELIABLE WAY TO DETECT IT?

8.2 P-GP IN A LYMPHOID TISSUE DENDRITIC CELL POPULATION: A MODEL FOR P-GP FUNCTION

8.3 IS MDR DRUG TREATMENT CRITICAL FOR P-GP ACQUISITION IN LYMPHOMAS?

8.4 CANINE MLSA AS A MODEL FOR NHL: CLINICAL CONCLUSIONS

8.1 DOES P-GP PREDICT RESPONSE TO CHEMOTHERAPY, AND IF SO, DOES THIS STUDY USE A RELIABLE WAY TO DETECT IT?

Since P-gp/*mdr1* was first characterised there has been controversy over the ideal means of detecting its presence in clinical samples (Dalton and Grogan, 1991). It is generally accepted that it is important to exclude non-*mdr1* isoforms when attempting to assess P-gp mediated drug resistance. This is particularly crucial when working in an unknown biological system such as the dog. The human *mdr1* specific probe MDR5A, under the hybridisation conditions used in this study appeared to detect a single RNA species which is presumed to represent the canine *mdr1* homologue. The tissue pattern of expression of the form detected agreed with the pattern observed for *mdr1* in other species. It had been hoped to rule out any remaining ambiguity in the *mdr* mRNA measurement by using a canine specific probe obtained by screening a genomic library with the MDR5A probe. Unfortunately, a *mdr1* homologue was not detected in the canine library screened and so no canine specific *mdr1* probes were obtained.

With retrospect it may have been simpler to take advantage of the sequencing information available to select species conserved primers on either side of the divergent linker region of the *mdr* and use these to amplify *mdr* species present in canine liver or kidney RNA by reverse transcriptase and pcr. This would automatically select that area of the *mdr* gene most likely to be isoform specific.

The monoclonal antibody used in this study was not isoform specific and this represents the most serious technical deficiency of this study. Most of the lymphoma samples which were C219 positive were also positive for *mdr1* based on dotblots analysis. However, three dogs had evidence of P-gp in their lymphoma cells but were negative on MDR5A dotblots hybridisation. No *mdr1* specific monoclonal antibody was available for use in these samples to attempt to confirm *mdr1* P-gp expression. The opposite could not be proven either i.e the definite expression of an MDR2 isoform. A human MDR2 probe was acquired from Herweijer's group in the Netherlands but it did not cross-hybridise with canine sequences either on Southern or Northern hybridisations so could not help in the investigation of these samples. There are no MDR2 specific monoclonals in the human. In the hamster, detection of the *mdr2* homologue is based on the differential reactivity with C219 but not with the antibodies against the *mdr1a/mdr1b* isoforms.

Although not available at the time, the hamster *mdr1* specific monoclonal antibody C494 also reacts with human *mdr1* isoform (Chan *et al*, 1988) and perhaps could cross-react with canine *mdr1*. This monoclonal is not commercially available but probably could be obtained from the originator (Dr. Victor Ling). If the three lymphomas and other canine lymphoma samples were to be re-investigated it would

be worthwhile testing C494 and some of the other monoclonal antibodies which are now commercially available for reactivity in the dog. In this way it may be possible to build up a panel of reagents suitable for use in canine tissue in an analogous manner to Grogan *et al* (1990).

In the introductory chapter it was suggested that preoccupation with improving sensitivity of mRNA detection techniques has at times clouded the fact that heterogeneity of expression of P-gp within normal contaminants and within tumour cells may invalidate some of the results obtained with bulk techniques. The advantage of an IHC technique over slot-blot analysis is well illustrated by this study. The dotblots analysis was shown to be quantitatively difficult to reproduce and prone to artefactual errors created by the densitometric based readout system. After optimisation of the methodology, the IHC was reproducible and more importantly, revealed P-gp expression in non-neoplastic cell types. In this way, measuring P-gp by IHC rather than *mdr1* mRNA by dotblots analysis allows direct visualisation of P-gp in the tumour cell population as apposed to non-tumour elements, and provides a method of determining the extent of P-gp expression within the tumour mass.

Until relatively recently, it has been assumed that the major control of P-gp expression in both tumour and normal tissue was at the transcriptional level and therefore the *mdr1* mRNA content of a tumour would accurately mirror the P-gp content. Some of the work discussed below casts doubts on the validity of this assumption. Regardless of the contribution of post-transcriptional control in determining the final P-gp content of a cell, direct detection of the *mdr* gene protein product in IHC bypasses the inaccuracies that could be created by these mechanisms.

The assumption that transcriptional control is of paramount importance in controlling P-gp concentration was based primarily on early cell line work. The drug selected cell lines which were used in the initial discovery of *mdr*/P-gp contained multiple copies of the *mdr1* gene but it rapidly became apparent that gene amplification did not occur in human tumours (Chabner and Fojo, 1989; Weinstein *et al,* 1990) and so alternative reasons had to be sought for the increased P-gp in some of these tumour samples. Drug resistant cell lines which did not contain *mdr1* amplification showed a correlation between *mdr1* mRNA content and cellular resistance to MDR drugs (Shen *et al,* 1986a). The simplest conclusion at that time was that transcriptional control was the major factor influencing tumour P-gp expression. This conclusion may still be valid for tumours which arise in tissues which do not normally express P-gp in that some event must take place which switches on *mdr1* mRNA transcription. However, in tissues which already have a basal expression of P-gp (such as liver or adrenal cortex), post-transcriptional control

mechanisms may also be relevant and may account for transient increases in P-gp in certain *in vivo* and *in vitro* situations.

Marino *et al* (1990) performed nuclear run-on assays on nuclei isolated from the livers of rats 4-72 hours after partial hepatectomy. Despite documenting a twenty fold increase in *mdr* mRNA, there was little or no increase in *mdr* gene transcription in the nuclear run-on analyses. They remarked that the 3' non-coding region of the *mdr1* mRNA shares instability sequences which are common to other unstable mRNA's and suggested that message stabilisation could account for some of the rise in *mdr* mRNA in these liver nuclei.

In vitro studies of the drug resistant cell lines derived from the macrophage cell line J774.2 have also produced evidence for post-transcriptional control of P-gp expression. Yang *et al* (1990) and Hsu *et al,*(1990) have shown that there may be post-transcriptional control of the *mr1a* isoform which is related to the transcriptional initiation site. It was found that those cells with the mRNA which was initiated at the downstream site expressed more protein than cell lines with initiation from the upstream site.

The relative importance of transcriptional versus post-transcriptional control in tumours is undetermined. With a lack of experimental information on this point, it would seem prudent to verify actual P-gp expression in a tumour, especially when the mRNA content is modest. Another advantage of P-gp rather than *mdr1* mRNA measurement lies in the relative instability of the *mdr1* mRNA compared to the protein. The half life of P-gp has been estimated at between 16 and 24 hours (Cohen *et al* 1990; Mickley *et al.* 1989) or possibly even longer in some chronically drug selected lines (Richert *et al,* 1988; Yoshimura *et al,* 1989) whereas the half life of *mdr1* mRNA (which contains instability sequences) may be less than 3 hours in some transient responses (Mickley *et al.* 1989; Chin *et al,* 1990b). However, the half-life of *mdr1* mRNA may vary according to the mechanism by which it is induced and possibly the cell type. *Mdr1* induced by heat shock in human carcinoma cells has a longer apparent half-life than doxorubicin induced *mdr1* in rodent cells (Chin *et al,* 1990a). *Mdr1* induction by MDR drugs is not a universal phenomenon. Chin *et al,* (1990a) failed to show any induction of *mdr1* in a panel of human cell lines following challenge with a variety of MDR drugs. The two canine cell lines, Cl-1 and 3132, were similar to the human cells in their absence of *mdr1* induction following epirubicin exposure.

The rate of degradation of P-gp and *mdr1* mRNA in tumour cells following the death of the host is likely to be even greater than in normal tissue but it could still be anticipated that P-gp may be retained intact for a longer period *post mortem* than *mdr1* mRNA. The *in vitro* experiments of Chin *et al,* (1990a) showed that the

sustained rise in *mdr1* mRNA following doxorubicin induction in rodent cells required new protein synthesis, perhaps a stabilising protein. The authors postulated that in the absence of a *de novo* synthesised stabilising protein, the *mdr1* mRNA is rapidly degraded. It is not unreasonable to suggest that *mdr1* mRNA degradation *post mortem* (perhaps in the absence of this stabilising protein) may be precipitous in comparison with more stable RNA species such as ribosomal RNA. Ribosomal RNA is in effect used as a marker for RNA quality on gel electrophoresis and may give a false impression of *mdr1* RNA quality. A rapid fall in *mdr1* mRNA could account for the lack of *mdr1* in the P-gp positive lymphoma samples discussed in chapter 5.

From this discussion, it would appear that P-gp protein detection is preferable to mRNA detection for the reasons outlined above. However, even measuring *mdr1* P-gp by IHC does not guarantee that an active pump is being detected. As long ago as 1989, it was apparent that colon carcinoma cell lines which showed a transient 25 fold increase in *mdr1* mRNA and a similar increase in protein following treatment with differentiating agents did not show an MDR phenotype nor did they have decreased drug accumulation (Mickley *et al,* 1989). Similarly, neuroblastoma cell lines which were induced to express P-gp with retinoic acid did not necessarily show any change in vincaalkaloid accumulation (Bates *et al,* 1989). The authors postulated that this paradox could be due to reduced phosphorylation of P-gp at critical residues in these sodium butyrate exposed cell lines.

There is now a growing body of data which shows that P-gp is indeed phosphorylated *in vitro* (Ma *et al,* 1991; Ganapathi *et al,* 1991) and that phosphorylation is associated with the active form of the protein (Fine *et al,* 1988; Chambers, 1990a). This phosphorylation can be through protein kinase C activity (Chambers *et al* 1990b) but transfection of mutant protein kinase A can also alter cellular sensitivity and hence may be implicated in P-gp phosphorylation. Hait and Aftab (1992) present a working hypothesis on how chemotherapeutic drugs induce an MDR phenotype in P-gp positive cells without altering the actual amounts of P-gp in the membrane. This model predicts that amphipathic drugs, such as doxorubicin, can increase the breakdown of phosphatidylinositol (to diacylglycerol and inositol triphosphate) through the activation of phospholipase C. The increased local concentration of diacylglycerol (DAG) leads to the translocation of PKC to the membrane where it phosphorylates P-gp. This then improves P-gp drug efflux hence the initial stimulator of the cascade (doxorubicin) in the membrane is removed. Following DAG metabolism, P-gp can then be dephosphorylated by phosphatases. Much of this is still speculative and the evidence is still somewhat contradictory regards the importance of PKC relative to other kinases (Hait and DeRosa, 1991).

The mechanism by which P-gp phosphorylation increases efflux is completely unknown.

This model has implications for the interpretation of *mdr1*/P-gp studies in normal tissue and tumour. The mere presence of surface P-gp does not equate with a functional drug efflux pump and hence the degree of drug resistance may not exactly match the P-gp protein concentration. Also, the resistance to amphipathic drugs could feasibly be increased without necessarily increasing *mdr1* mRNA or protein concentration.

The pertinence of the phosphorylation studies to this project are that despite the fact that P-gp was primarily detected in MDR drug resistant tumours, it is not possible to firmly conclude that the P-gp is functional in these samples. However, given that most lymphomas were P-gp negative prior to treatment and drug exposure precedes the appearance of P-gp, it is likely that the P-gp is functional as an efflux pump. Proving this was not attempted and would not be easy.

It is as yet extremely difficult to prove that P-gp is functioning as an efflux pump in clinical material. Other workers have attempted to show P-gp function by performing drug accumulation studies on leukaemic cells and determining that accumulation can be abrogated by treatment with MDR modulating agents (Rothenberg *et al,* 1989; Dalton *et al,* 1989; Salmon *et al.* 1991; Solary *et al,* 1991). However even this is not ideal because modulating agents may have effects on cellular drug accumulation and distribution even in the absence of a functional *mdr1* encoded P-gp. For example, Herweijer *et al,* (1990) and Nooter *et al,* (1990) showed that the drug accumulation in *mdr3* expressing leukaemic samples could be increased by cyclosporin treatment. This is in contrast to *mdr3* expressing transfectant cell lines which do not have any demonstrable drug efflux activity (Schinkel *et al,* 1991). There are other examples of MDR drug resistant cell lines and patient tumour cells which can be modulated by MDR modulators even in the absence of P-gp and without altering the total intracellular accumulation (Larsson and Nygren, 1990; Kavallaris *et al,* 1990; Nygren *et al* , 1991; Solary *et al,* 1991). Thus, because of the pleotropic effects of modulating agents (discussed by Twentyman, 1992), decreased drug sensitivity or drug accumulation in the presence of a modulator drug does not construe P-gp mediated resistance.

The use of *in vitro* chemosensitivity can be equally confusing; because of the presence of other resistance mechanisms, an MDR phenotype does not necessarily prove that the *in vitro* resistance is via a P-gp efflux mechanism (Salmon *et al,* 1989; Marie *et al,* 1991; Shen *et al,* 1991).

So unfortunately, there is no easy way of proving *in vivo* resistance in a P-gp positive tumour is actually P-gp mediated. Although the amelioration of resistance

with modulators cannot be taken as proof of P-gp mediated resistance, it may eventually be possible to use monoclonal antibodies against critical residues to more specifically block P-gp activity. The perturbations of drug and ATP binding induced by the C219/C494/C32 series of monoclonals (Georges *et al,* 1991) indicates that this is probably feasible although these particular monoclonal antibodies require cell permeabilisation to work optimally and hence may not be suitable for drug accumulation studies.

In summary, at present the optimal method of detecting P-gp mediated resistance remains immunohistochemistry using *mdr1* specific antibodies. This does not prove P-gp functionality and the likelihood of P-gp mediated efflux has to be judged on the basis of the nature and source of the cell expressing the P-gp.

Using paired samples from lymphomatous nodes before treatment and at relapse, it was possible to document acquisition of P-gp in relapse tumours in four dogs and in another three drug resistant lymphomas with unknown pre-treatment P-gp status. It would seem likely that the P-gp acquired by these lymphomas may well contribute to their clinical drug resistance spectrum. Only one dog had documented P-gp of *mdr1* isoform before treatment. This dog responded to a COP protocol containing MDR and non-MDR drugs but relapsed with drug refractory disease within four months. The three dogs with P-gp, which may not have been *mdr1* isoform, were also drug-refractory to MDR and non-MDR drugs. So it would seem that P-gp in canine lymphomas is predictive of poor response to chemotherapy but this is usually at the relapse stage when all lymphomas, from clinical experience, are only marginally drug responsive. Given that the dogs in relapse (both P-gp positive and -negative) failed MDR and non-MDR drug salvage protocols it is unlikely that P-gp is the only resistance factor in these lymphomas.

8.2 P-GP IN A LYMPHOID TISSUE DENDRITIC CELL POPULATION: A MODEL FOR P-GP FUNCTION

The presence of P-gp in a dendritic cell population in both normal and pathologic nodes does not easily fit with current ideas on *mdr1* efflux of noxious hydrophobic substances (Gottesman and Pastan, 1988): these cells are not part of an excretory epithelial surface. In chapter 5 it was not proven that the canine dendritiform cells expressed *mdr1* but similar cells have been documented in human nodes using *mdr1* specific antibodies (Miler *et al,* 1991; Schlaifer *et al, 1990a* and 1990b). The localisation of the dendritic cells within normal and reactive canine nodes and their morphologic similarities to the S-100 staining cells in one tumour sample supports the view that these dendritic cells may be part of the antigen presenting cell (APC) population of the lymph node. Why would an APC require an

efflux pump? In this section, the presence of P-gp in a potential APC will be discussed in terms of possible functions of a P-gp in this cell type.

The presence of P-gp in this dendritic cell population of lymph nodes is unlikely to have major effects on the drug resistance profile of the lymphoma. It is theoretically possible for a sub-population of resistant cells to enable otherwise sensitive cells to survive drug exposure through cell-cell communication which allows potential resistance factors such as glutathione or drug to move between the resistant and sensitive cells (Frankfurt *et al,* 1991). However, the majority of drug resistant tumours did not contain many P-gp positive dendritic cells and so it would not seem feasible for the dendritic cells to effectively salvage the vast lymphoma cell population. Even in the two examples with numerous dendritic cells, it is not clear whether large moieties such as epirubicin could be transferred from a sensitive P-gp negative lymphoma cell through a structure such as a gap junction to a P-gp positive dendritic cell for efflux to the extracellular space. Lymphoid cells are not a cell type which are normally associated with the formation of gap junctions (Pitts *et al,* 1988). Overall, cooperativity between adjacent P-gp positive dendritic cells and P-glycoprotein negative lymphoma cells to lower the cytotoxic drug concentration in both populations does not seem likely. The presence of numerous dendritic cells in the two tumour samples (BMD and KEB) is more likely to represent the co-proliferation of FDC with B lineage lymphoma cells of germinal centre origin (Gerdes and Flad, 1992).

The purpose of this discussion of P-gp in the putative APC of normal and lymphomatous nodes is not directed at explaining P-gp mediated drug resistance in lymphoma but to use this as a model for exploring the complex biology of P-gp. By doing this it will become apparent that there may be common themes underlying the induced and drug selected expression of P-gp in neoplastic cells and normal lymphoid cells.

The dendritic cells of the lymph node are specialised cells which present antigens which initiate several immune responses including sensitisation of MHC-restricted T cells and the formation of T-dependant antibody responses. Dendritic cells are motile and can efficiently cluster and activate T cells. Distinct populations are recognised in lymph nodes and are often called interdigitating cells (IDC), in the T cell areas and follicular dendritic cells (FDC) in the germinal centres. The biology of these cells has been reviewed recently (Steinman, 1991).

Both of these cell types may express P-gp. Schlaifer *et al* (1990b) reported that the CD68 monoclonal KP-1 (from Dako) stained a cell population morphologically identical to the P-gp positive population. KP-1 is reported to recognise IDC in preference to FDC (Dako product sheet) but this distinction is

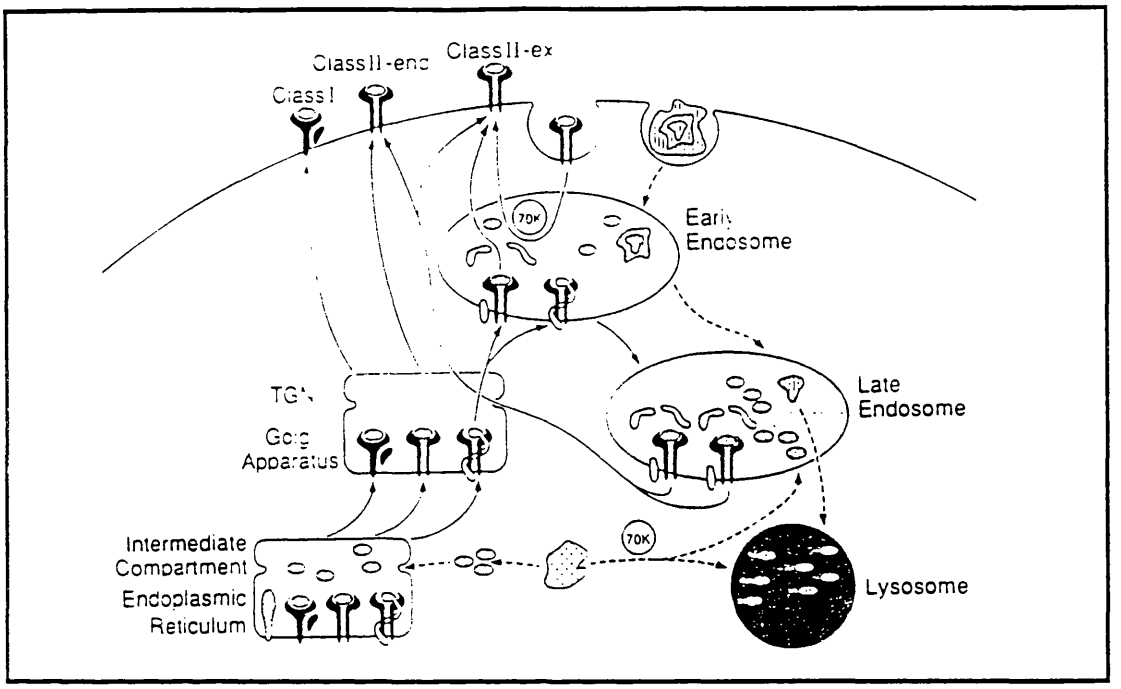
unlikely to be absolute. The association of numerous P-gp positive dendritic cells with canine B cell tumours would suggest that FDC are P-gp positive. So for the purposes of this discussion, IDC and FDC will be considered together as APC. As discussed in chapter 5, Schlaifer *et al,* (1990a and b) and Miller *et al,* (1991) both identified P-gp positive non-lymphoid cells in lymphoma samples with *mdr1* specific monoclonal antibodies and so it would appear that APC may contain the *mdr1* isoform of P-gp. However isoform type was not proven in the dog and so it should not be completely excluded that other isoforms could be expressed in these dendritic cells.

The pathways of antigen processing is an area of intense interest at present. In brief, antigen processing can be divided into two pathways according to whether antigen is presented in association with class II (major histocompatibilty) molecules or class I molecules. Peptides derived from intracellular antigens are generally presented on the APC surface in conjunction with class I molecules whereas antigenic fragments from exogenous antigens are presented with Class II molecules. Some of the most recent developments in the molecular models of antigen processing are discussed by Monaco (1992).

In class II presentation, antigens are acquired through an endocytotic process and are gradually degraded by proteases in the endosomal compartments into small peptides which eventually associate with class II molecules (figure 8.1 and reviewed by Brodsky and Guagliardi, 1991). The effective processing of antigen requires an increasingly acidic endosome environment and can be abrogated by drugs (such as chloroquine and ammonium chloride) which raise the pH of intracellular compartments. Macrophages and B cells can also present antigen acquired through endocytosis via the class II pathway.

Class I presentation is intimately involved with pathways of degradation of endogenous proteins in both cytosol and lysosomal compartments. Class I molecules are assembled and bind class I peptides in lumenal compartments; this would suggest that a mechanism of translocation is required to transport peptides across the endoplasmic reticulum or some pre-Golgi compartment. Recently, a group of genes, renamed the *tap* genes, (old and new nomenclature is given in Monaco, 1992) have been characterised which are members of the same ABC family as P-gp and the yeast peptide transporter STE 6. Transfection of these *tap* genes into mutant murine cells which are defective in peptide transport can restore antigen presentation function. It has therefore been proposed that the *Tap* proteins act as ATP dependant peptide transporters feeding antigenic fragments to the class I pathway.

Figure 8.1 Antigen Presentation Pathways (from Brodsky and Guagliardi, 1991)



Endogenous proteins (dotted) have 3 processing/presentation pathways: (i) cytoplasmic degradation then translocation into ER (ii) from translocated proteins in the intermediate compartment between the ER and Golgi (iii) following translocation into a late endosome/lysosome in a process involving a 70kd heat shock protein.
Exogenous proteins: are internalised via endocytosis and degraded in endosomes. Class II molecules are delivered to endosome and following peptide binding are released either from an early or late endosome. Internalisation and recycled class II molecules may get a second chance to bind peptide with possible involvement of a 70kd heat shock protein.

The *tap* proteins, as members of the ABC superfamily, share homology to P-gp. It can be anticipated that homology will be greatest at or around the ATP-binding sites. C219 reacts with an epitope close to the ATP binding site of P-gp which is highly conserved between P-gp isoforms and between different species. There was therefore some concern that C219 could be cross-reacting with a *tap* gene product in these dendritic cells. However, in a manual sequence search performed by the author, the *tap* proteins do not contain the V-x-x-x-x-D motif considered obligatory for C219 recognition (Georges *et al,* 1990), but more importantly MRK16 can also recognise the P-gp species in human APC so it is unlikely that the C219 reactivity is a cross-reaction with *tap* proteins.

Since peptide transport appears to be crucial for antigen presentation, and P-gp is a transporter for many moieties, it could be speculated that P-gp could contribute to this APC peptide transport capacity. The site of this transport is likely to be in either the endoplasmic reticulum or an early Golgi compartment, both of which are compatible with known P-gp intracellular distribution (Willingham *et al,* 1987).

P-gp would appear capable of peptide transport; MDR cells are often cross-resistant to the peptide antibiotics gramicidin D and valinomycin. In certain cell lines the relative resistance to these peptides greatly exceeds resistance to other MDR drugs (Mirski *et al*, 1987). For instance, the colchicine resistant cell line CH_RC5 is 180 fold resistant to colchicine but 5000 fold resistant to gramicidin D perhaps suggestive that these peptides are a closer approximation of the natural substrate of the P-gp pump (Gerlach *et al*, 1986b). The fundamental nature of this peptide transporting ability of P-gp is illustrated by the fact that expression of human *mdr1* in yeast will still confer resistance to the peptide antibiotic valinomycin (Kuchler and Thorner, 1992). However, although P-gp may be capable of effluxing these highly hydrophobic peptides, it is probably not feasible that P-gp could be a universal peptide transporter for other more hydrophilic peptides. Certainly, the *mdr1* P-gp was incapable of transporting the 12 amino acid lipopeptide yeast mating factor when expressed on the yeast surface (Kuchler and Thorner, 1992).

So an alternative role for P-gp in APC could be postulated. In this study, the P-gp in the dendritic cells appeared as a coarse granular stain throughout the cytoplasm and not confined to a Golgi-like distribution pattern. As described in figure 8.1, Class II presentation involves protein degradation within acidic compartments and an alternate transport system is also postulated for some cytosolic class I peptides which involves translocating peptides directly into a pre-lysosomal compartment. It is not inconceivable that the granular staining pattern in the canine APC (and in human phagocytic cells) could represent localisation in endosomal/lysosomal structures. P-gp expressing Ehrlich ascites cells show an increased rate of endocytosis (Sehested *et al*, 1987) suggesting there may be a connection between the MDR phenotype and endocytosis is some cell lines.

Endosomes become increasingly acidic as they develop into late endosomes/lysosome compartments. The MDR drug daunomycin has been shown to localise in organelles in the Golgi region and in lysosomes in drug resistant cells (Willingham *et al* 1986). Keizer *et al*, (1989) noted that drug sensitive cells retained drug in the nucleus whereas MDR cell lines distributed drug elsewhere (exact localisation was not possible with their system) and that this redistribution could be blocked by raising the extracellular pH. The pH required to return drug distribution to the nuclear pattern of the parental cell line increased in parallel with the P-gp content of the cell lines. This connection between drug distribution, pH and P-gp has not been explained. Could P-gp be involved, either directly or indirectly with acidification of internal organelles? Gervasoni *et al*, (1991) stated "the possibility that a molecule similar to the P-gp may act as an ion channel to acidify and trap drug within the vesicles is now being investigated". Since Gervasoni made this statement, there is

evidence that *mdr1* may act as a volume regulated chloride channel (Valverde *et al,* 1992), but the link between this and pH gradients in organelles remains obscure.

There are some intriguing associations between P-gp like molecules and acidic vacuoles in other biological systems. In malarial parasites, the ABC superfamily transporter protein encoded by the *pfmdr1* gene has been localised to the acidic digestive vacuole of the parasite. This internal compartment is the site of chloroquine accumulation in the parasite and resistant strains of *P.falciparum* accumulate less drug in this compartment. The product of the *pfmdr* gene appears to contribute to the reduced intra-vacuolar accumulation of drug (reviewed by Ginsburg and Krugliak, 1992). This accumulation deficiency can be partially corrected by MDR drugs such as vinblastine and daunomycin and by modulators such as verapamil and so it seemed reasonable to postulate that this accumulation deficit was due to the *pfmdr* product pumping chloroquine out of the food vacuole.

Just like the complexities of *mdr1* efflux and drug accumulation studies in cancer cells, the association of chloroquine efflux and drug concentration in the food vacuole is not straightforward and cannot be explained solely on the basis of a *pfmdr* chloroquine pump. In fact the hypothesis that point mutations in the *pfmdr* protein and *pfmdr* gene amplification lead to chloroquine resistance in malaria have now been discredited (Krogstad *et al*, 1992). Instead, complex changes in the kinetics of influx and efflux into the vacuole suggest that alterations in H^+ pumping and H^+ leak from the vacuole may contribute to the localisation of the drug. As yet, there is no clear understanding of the normal function of the *pfmdr* product in the digestive vacuole or its role in ion or proton fluxes.

It is obvious from analogies to *Plasmodia* that it is important to address the question of P-gp localisation and how this contributes to maintenance of acidic organelle compartments in cells. This is not just of relevance to antigen presenting cells and *plasmodia* but is also of major importance in drug resistance. The introductory chapter discussed some of the evidence suggesting that part of the MDR phenotype may be due to effectively separating the MDR drugs from their targets through re-distribution into alternate cellular compartments. Some of the MDR drugs, such as doxorubicin, are weak bases with a tendency to accumulate in acidic cellular compartments (discussed in review article by Van der Bliek and Borst, 1989). The role of P-gp in facilitating this compartmentalisation has to be studied further.

The antigen presenting cells, or perhaps phagocytic macrophages, may represent a convenient model for these investigations. However the presence of the *tap* gene products will have to be taken into account when assessing experimental results in APC. For instance, cyclosporin A, which is a P-gp modulator, is known to inhibit antigen presentation *in vitro* through an unknown mechanism (Dupuy *et al,*

1991). This mechanism could conceivably involve blocking of P-gp or the *tap* proteins action. More specific methods of addressing physiological and pathological P-gp function are obviously needed; antisense oligonucleotides and function perturbing antibodies are two potentially useful approaches which would have greater specificity than modulator drugs.

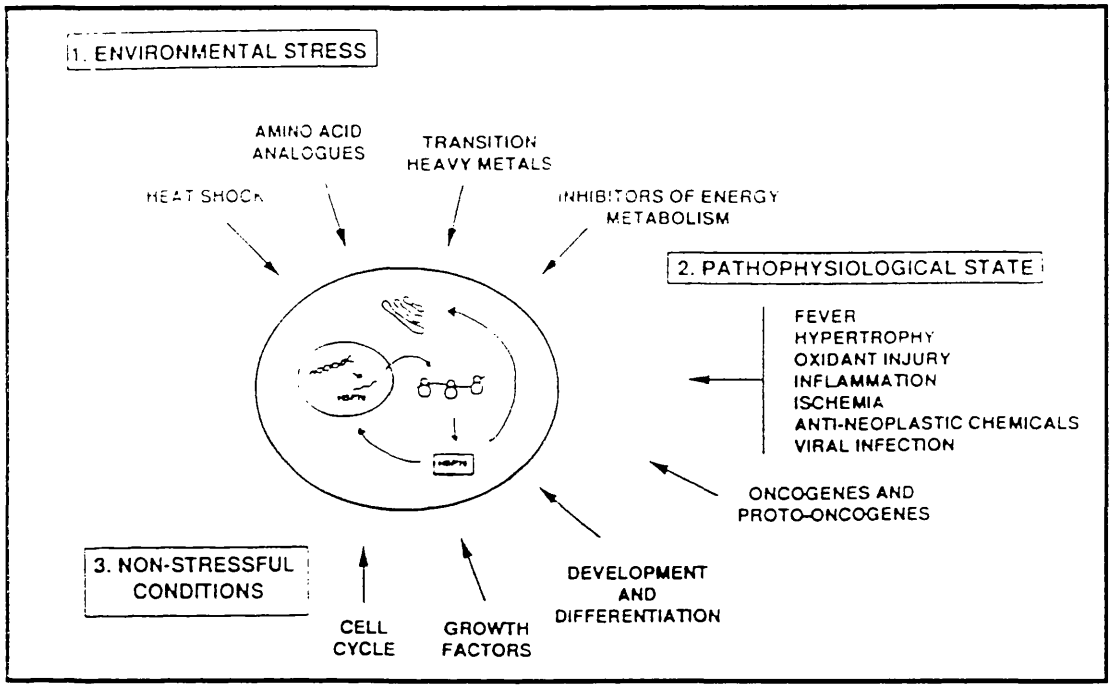
In the canine dendritic cells, P-gp staining was granular rather than diffuse and hence the above discussion focussed on organelle localisation that could give this granular appearance. However cytoplasmic localisation of P-gp in cancer cells has been interpreted in a very different way by other workers.

For example, Weinstein *et al* (1991) have observed a relationship between expression of P-gp in solitary invading cells at the leading edge of colonic tumours and metastasis of these heterogeneously P-gp expressing tumours to the regional lymph nodes. They postulate that the solitary P-gp positive cells may have an increased potential for dissemination. Part of their hypothesis is based on preliminary observations that P-gp may be concentrated on the cell surface at adhesion plaques and that P-gp hyperexpressing cells have enhanced cell locomotion (Weinstein *et al,* 1989). The authors do not speculate as to the function of P-gp at these sites. Dendritic APC are also highly motile cells; in the living state they can extend and retract lamellipodia from the cell body as if "sampling" the surrounding lymphocyte population (Inaba *et al,* 1992).

However, despite the elapse of three years since the meeting abstract was published which showed P-gp at adhesion plaques, these findings have not yet been published in a refereed journal. Although Weinstein's hypothesis is biologically interesting in terms of tumour progression events, there are problems in their argument. In the P-gp positive cells present at the leading edge of the colonic carcinomas, the P-gp in these cells was not proven to be associated with the cell surface or with adhesion plaques. In the authors own words "in P-gp positive invasive carcinoma cells, granular and diffuse cytoplasmic staining was common. Plasma membrane staining was difficult to demonstrate in paraffin sections." It is unfortunate that despite using paraffin sections rather than frozen sections these authors were still unable to show convincing membrane staining. This places their hypothesis about P-gp localisation around adhesion plaques in some doubt. In an earlier review article by the same group (Weinstein *et al,* 1990b), the P-gp positive cells at the invasion front of the colonic carcinomas was not linked to potential metastatic potential but instead likened to the morphologic alterations that can occur in bacteria when placed in impoverished circumstances. The authors postulated that this cytoplasmic *mdr1* expression could be a survival strategy for the cancer cells in a hostile environment.

This latter hypothesis, suggesting a connection between P-gp and environmental stress certainly has more experimental support than the connection with cell motility. Figure 8.2 is taken from an article describing agents which can induce transcription of the hsp70 (heat shock protein) family (Morimoto, 1991). Many of the heat shock family inducers have also been shown to increase *mdr1* gene expression either *in vitro* or *in vivo*; these are listed in the legend of figure 8.2.

Figure 8.2 Factors which increase *mdr1* mRNA



References for figure 8.2
1. Heat shock: Chin *et al*, 1990b
2. Transition heavy metals: Chin *et al*, 1990b
3. Hypertrophy: Fairchild *et al*, 1987; Thorgeirsson *et al*, 1987
4. Inflammation: Marino *et al*,1990; Thorgeirsson *et al*, 1987
5. Anti-neoplastic chemicals: Chin *et al*, 1990a; Licht *et al*, 1991
6. Viral infection: Gollapudi & Gupta, 1990
7. Oncogenes: Burt *et al*, 1988
8. Development and differentiation: Bates *et al* 1989, Meyers *et al*. 1991
9. Growth factors: Chapekar *et al*. 1990

Figure 8.2 illustrates that induction of *mdr1* expression is not limited to agents which are potential MDR substrates. Indeed the connection between some of the factors and the proposed function of P-gp as an efflux pump is obscure. In rat liver *in vivo*, noxious chemicals (which are not P-gp substrates) have been shown to induce liver *mdr1* in conjunction with inciting changes in cytochrome P450 enzymes and glutathione transferases (Fairchild *et al*,, 1987; Burt and Thorgeirsson, 1988). This apparently co-ordinated response suggests that *mdr1* induction can occur as part of a

generalised detoxification reaction in liver cells (Gottesman 1988). The mechanism underlying this coordinated response is not known. Initial experiments indicated that the Ah receptor may be involved in the coordinated response (Burt and Thorgeirsson, 1988) but further work has suggested otherwise (Gant *et al,* 1991).

The presence of a conserved AP-1 site in both human *mdr1* and mouse *mdr1a* promotors (Ueda *et al,* 1987b; Hsu *et al,* 1990) make the *c-fos* and c-*jun* proto-oncogene products contenders for controlling events in *mdr1* induction. The coinduction of glutathione S-transferase and *mdr1* in rat liver supports this role; glutathione S-transferase is known to be induced via AP-1 elements in the placenta (Sakai *et al,* 1988) and c-*jun* and c-*fos* levels are known to be elevated during chemical hepatocarcinogenesis (Sakai *et al,* 1989). The role of the heat shock proteins themselves in *mdr1* induction is unknown.

Returning to the APC model of a P-gp expressing cell, one of the alternate class I presentation pathways which occurs in serum-starved cells involves a 70kd hsp which translocates cytosolic proteins into the lysosomal pathway of degradation. Brodsky and Guagliardi (1991) propose that this association with hsp70 could mean that any stressed cell could upregulate its lysosomal degradative pathway. If P-gp is also needed for some unknown function in these acidic compartments then it would be logical that the hsp70 and P-gp should have coordinate regulation.

8.3 IS MDR DRUG TREATMENT CRITICAL FOR P-GP ACQUISITION IN LYMPHOMAS?

The connection between non-specific cellular stress and P-gp induction returns this discussion to the original aim of this thesis; to investigate the role of P-gp in mediating *in vivo* resistance to anthracyclines. This study has shown that treatment with an anthracycline only protocol was no more likely to result in P-gp positive disease at relapse than a COP protocol in which cyclophosphamide (a non-MDR drug) was the predominant drug. However, the COP protocol does have an MDR component and so it is still feasible that MDR drug selection has allowed the preferential outgrowth of a P-gp positive sub-population.

The experimental evidence from the rat liver carcinogenesis model has shown that certain chemicals (which are not *mdr* substrates) can elicit a detoxification response which includes upregulation of *mdr1* (Gant *et al,* 1991). This would suggest that it could be possible for cyclophosphamide to select lymphoma cells which have multiple resistance mechanisms, one of which is P-gp expression.

The Goldie-Coldman hypothesis proposes that resistant variants arise at a frequency compatible with the spontaneous mutation rate of the cell population. Based on purely stochastic model of drug resistance, the appearance of mutants with

resistance to multiple agents should be a very rare event. However acquisition of multiple agent resistance does not always appear to be stochastic. Cells selected with doxorubicin and methotrexate produce dual resistant variants at a higher rate than would be predicted from the frequency of single drug resistance variants (Rice *et al,* 1987).

In cell lines prior exposure to carcinogens can increase the frequency of drug resistant variants in the population (McLaughlin *et al,* 1991). In the rat liver model, chemical carcinogen treatment led to increased P-gp expression. Hepatocytes isolated from rats exposed to 2-acetylaminofluorine (AAF) and other carcinogens are more resistant to doxorubicin and methotrexate than normal hepatocytes (Carr, 1987). In a similar fashion, mouse skin papillomas which have been promoted by the chronic application of chemical carcinogens are more doxorubicin resistant than virally induced papillomas (Keith *et al,* 1990b). This suggests that exposure to chemical carcinogens results in the development of a population of cells resistant to the cytotoxic effects of the carcinogen (Farber, 1980).

What relevance does this have to canine and human NHL which do not have a strong link to chemical carcinogenesis? The pleotropic effects of the chemical carcinogens in rat liver cells can be mimicked by oncogenic activation events. Burt *et al.* (1988) transformed rat liver epithelial cells with v-H-*ras* or *v-raf* ; this transformation was found to be accompanied by the acquisition of an MDR phenotype and increased *mdr* gene expression. So in this particular model, neoplastic transformation of liver epithelial cells resulted in *mdr1* expression independent of chemical exposure.

That *mdr* expression could arise as part of the neoplastic process would concur with the observations made in the introductory chapter regarding P-gp expression in leukaemias. Mature leucocytes do not seem to express P-gp and yet untreated leukaemias, especially the blast crises of CML are not infrequently P-gp positive. It is also relevant to remember that P-gp expression is more likely to occur in canine lymphomas which present in an advanced stage of disease. These tumours, which have metastasized outwith the lympho-reticular system, are presumed to have undergone more cell doublings to have reached their extensive tumour bulk and to have gained a metastatic potential. The association of P-gp with clinically advanced disease has been noted in other tumour types including neuroblastomas and retinoblastomas (Chan *et al,* 1991a and 1991b).

The association of P-gp expression with advanced stage malignancies is explicable by the Goldie-Coldman hypothesis. The biologically advanced tumours would be presumed to contain a more heterogeneous population upon which drug treatment selects resistant variants. The treatment with mutagenic chemotherapeutic

agents (MDR and non-MDR) may even increase the number of resistant variants in the total population.

It is accepted that NHL is a progressive disease in which phenotypic changes occur. NHL in humans commonly displays histologic and cytogenetic evidence of tumour progression. Histologically, follicular tumours can transform into diffuse tumours both in treated and untreated malignancies (Horning *et al,* 1985). This has also been reported in the dog (Gray *et al,* 1984). The cytogenetic abnormalities which amass in NHL were described in sections 1.5 and 1.6. It was noted that morphologically diverse tumours acquired different genetic abnormalities suggestive of inexorable, defined, tumour progression.

Recent observations are beginning to shed light on the connection between tumour progression events and the frustrating emergence of a resistant phenotype. One of the most common genetic abnormalities associated with human malignancy is the loss or mutation of the p53 tumour suppressor gene on chromosome 17q. Wild-type p53 appears to be potent transcription factor for as yet unidentified genes (Farmer *et al,* 1992) and can prevent the transforming activity of mutant p53 in co-transfection studies. Lane (1992) proposes that the tumour suppressor activity of p53 may be related to its ability to prevent DNA replication in the presence of DNA damage. This is based on observations that DNA damaging agents cause accumulation of wild-type p53 and a concurrent cell cycle block in G1 (Kastan *et al,* 1991). Prolonged elevation of wild type p53, presumably in the face of irrepairable DNA damage, can result in apoptosis (Yonish-Rouach *et al,* 1991) . In this way p53 guards against heritable genetic abnormalities.

Wild type p53 function can be compromised by complexing with mutant p53 and other cellular proteins which are involved in neoplastic progression such as MDM2 (Oliner *et al,* 1992). The net result is that many malignancies do not have normal p53 function and therefore do not effectively shut down DNA replication in the presence of DNA damage. This would allow tumour cells to continue dividing despite the DNA damage imposed by chemotherapeutic agents. Some tumour cells would die because the resultant genetic defects are incompatible with survival but inevitably the tumour cells would accumulate genetic abnormalities which would provide a wealth of variants upon which drug selection could act.

Recently, preliminary observations suggest there may be a direct connection between p53 tumour progression events and the acquisition of P-gp expression. The MDR1 gene promoter is a target for p53 modulating activity. Chin *et al* (1992) examined the effects of wild-type and mutant p53 on the MDR1 promoter activity by cotransfecting MDR1 promoter-CAT constructs with expression vectors containing the oncogene and the tumour suppressor genes. The mutant p53 stimulated

MDRCAT expression in the NIH3T3 transfectants and this enhancement was completely abolished by cotransfection with wild-type p53. Obviously, these findings using promoter constructs have to be confirmed in the intact *mdr1* gene. Essential experiments, which are presumed to be underway, would include the effects of removing normal p53 activity in cell lines with constitutive versus inducible *mdr1* expression.

A fairly direct link between p53 function and P-gp expression may account for the rather inexplicable connection between X-irradiation of cell lines and consequent stable expression of P-gp (Hill *et al,* 1990). Temporary expression of P-gp following DNA damage could occur due to the ill-defined "stress" related induction of *mdr1* but stable expression would require a different mechanism, perhaps mutation of p53. However, even this does not entirely fit all the available information; irradiation led to increased P-gp but no significant alteration in *mdr1* mRNA. So perhaps the mutagenic event involves translational control rather than transcription or message stabilisation.

Abnormalities which could result in loss of one p53 allele have been identified as adversely affecting prognosis of NHL in three studies (Levine *et al,* 1990; Yunis *et al,* 1989; Rodriguez *et al,* 1991). However, the involvement of other cellular proteins that can complex with p53 and prevent normal function means that tumours which have retained normal p53 may still be defective in p53 function due to amplifications at another loci (Oliner *et al.* 1992). These findings suggest that the emergence of *mdr1* drug resistant disease may not simply be due to drug selection of randomly occurring resistant mutants but be an integral part of the neoplastic process in which the emergence of P-gp positive clones is a genetically favoured event due to the lack of normal p53 activity. If this is indeed the case, then drug resistance may be an inevitable consequence of the genetic instability of malignancy and drug treatment merely acts as a catalyst to speed up the process.

8.4 CANINE MLSA AS A MODEL FOR NHL: CLINICAL CONCLUSIONS

The results of this longitudinal study mirror the emerging picture of P-gp in NHL (Miller *et al,* 1991). P-gp is rarely expressed in pre-treatment disease but is considerably more common at relapse, but this expression is definitely not ubiquitous. The relatively small percentage of canine patients who expressed P-gp at relapse confirms the fact that studies with P-gp modulators have to be interpreted following establishment of the P-gp status of the tumour. In human, and especially canine NHL, it cannot be assumed that MDR drug resistant disease will be P-gp positive.

As discussed in the first section of this chapter, modulators can have effects even in P-gp negative cell lines and so apparent amelioration of clinical drug resistance cannot be construed as proof of P-gp activity. Similarly, this study does not prove that the resistance to MDR drugs in the minority of relapse tumours which expressed *mdr1* was actually P-gp mediated. In fact given that the majority (75%) of relapse tumours did not express P-gp and were equally drug refractory, the dominance of other resistance factors has to be proposed. Among the most likely candidates for anthracycline resistance would be alterations in the amount or activity of the topoisomerase II genes which are one of the targets of the anthracyclines (Zhang *et al.* 1990). However, this cannot explain the coincident resistance of these lymphomas to the alkylating agent cyclophosphamide.

Stage of disease had the most profound effect on P-gp expression, more than the dose of MDR drug or the length of treatment. This validates some of the central dogmas of medical oncology. It is often repeated in textbooks that chemotherapy should be instituted at the earliest oppurtunity, that the highest tolerated dose of drugs should be used and that wherever possible the least mutagenic agents should be chosen; this is of course all based on the recognised fact that the best chance of a cure is at first presentation (Sobrero and Bertino, 1986). From the discussion in the previous section, the molecular basis for these clinical guidelines are at last being discovered.

P-gp expression was connected with clinical stage at presentation but several other trends were also evident, which because of low numbers involved, did not reach statistical significance. Among these were a lack of P-gp in all tumours with TCR gene rearrangements and a propensity for P-gp in males rather than females. This latter point may appear trivial, but the hamster provides a precedence for male restricted P-gp expression in the adult adrenal gland (Bradley *et al,* 1990) and so it should not be excluded that sex-specific effects in P-gp expression in tumours could occur.

The lack of P-gp in T cell tumours is definitely worthy of further investigation; earlier chapters have discussed the evidence that P-gp, of different isoforms, can be expressed in normal and malignant B cells and section 8.2 discussed the connection between P-gp and antigen presenting cells. Germinal centre B cells can be very efficient APC (Brodsky and Guagliardi, 1991) and given that the centrocytic histologic class of NHL is presumed to arise from these germinal centre cells (Lennert *et al,* 1975) it could be that certain B cell tumours have a propensity to express P-gp because the oncogenic process is mimicking a normal lymphocyte activation process. The association of P-gp with B cells could be simply and quickly confirmed or refuted in human NHL. Immunophenotyping is routinely carried out in

many centres and could easily be incorporated into study design. It is imperative that human NHL studies start to assess P-gp status in conjunction with basic prognostic factors such as histological subtype and immunophenotype if the biology and relevance of P-gp are to be better understood.

The first chapter noted that human HTLV-I lymphomas had a higher frequency of P-gp expression than other NHL. These are exclusively T cell tumours; this does not nullify the above observations that immunophenotype should be defined in P-gp studies. The connection between specific oncogenic events and P-gp expression in tumours could account for the higher incidence of P-gp in the HTLV lymphomas. Unfortunately, at this stage, it is still impossible to rule out that the differences between NHL and ATL could be due to sample size and methodologic variation.

It is also worthy of note that in the series of five bull mastiffs lymphomas, four cases did not have any TCR β gene rearrangements and only BOA had evidence of one TCR β chain allele rearrangement. There was dubiety about whether BOA had both alleles rearranged and so this sample could quite possibly not be a phenotypic T cell. As mentioned in the introduction, the clustering of lymphomas in three families of Bull Mastiffs could be construed as evidence of a possible infectious aetiology. The leukaemogenic retrovirus' feline leukaemia virus and HTLV-I produce almost exclusively T cell tumours. In the case of HTLV-I, these lymphomas are histologically high grade and clinically aggressive. This does not parallel the clinical picture of Bull Mastiff lymphomas which clinically are often quite indolent. Indeed one of the bull mastiffs, CMD, was the only low grade tumour, according to the Working Formulation, in the entire series of all lymphomas (Sean Callanan, personal communication). This clinical and histopathological discrepancy from HTLV-I lymphomas does not rule out a retroviral aetiology. Bovine leucosis virus, which is related to HTLV-I, produces chronic B-cell proliferation in infected cattle and in a small percentage leads to (pre)-B cell tumour formation (Burny *et al,* 1987). The susceptibility to B cell lymphocytosis is heritable and is related to the bovine lymphocyte antigen (BoLA) haplotype (Lewin and Bernoco, 1986). Non-retrovirus' are also implicated in B cell lymphoproliferative diseases in man. The herpes virus Epstein Barr virus can immortalise B cells (through an undefined mechanism) and it has been proposed, that this immortalisation then allows B cells carrying a random second event (such as chromosomal translocations of *c-myc*) to survive and expand (Thomas *et al* 1991; Rogers *et al,* 1992). Thus there are several natural models upon which to base investigation of an infectious origin of the Bull Mastiff lymphomas.

This study confirms the observations of Price *et al* (1991) in which steroid pretreatment adversely affected clinical performance. Fortunately, humans with

lymphoma will not often have been prescribed steroids prior to the initiation of chemotherapy. However, the association of corticosteroids with poor complete response rate does have some relevance to human oncology. None of the dogs which received considerable corticosteroids prior to chemotherapy were P-gp positive at diagnosis. The promoter region of the murine *mdr1b* has been reported to contain steroid responsive elements (Cohen *et al*, 1991) and in the secretory epithelium of the mouse uterus, *mdr1* expression is reported to be under the control of steroid hormones (Arceci *et al*, 1990). Hence a direct relationship between steroid administration and P-gp induction could be envisaged. This study would suggest that this does not often occur in lymphomas.

The biochemical basis for steroid induced anthracycline resistance is not known. From the clinical presentation of the pre-treated dogs it is not likely to be due to major changes in the cell-cycling status of the tumour population because the referred dogs showed rapid regrowth of the lymphomatous nodes either following cessation of the steroids or while still receiving steroids. Continued tumour growth was usually the underlying reason for the case referral. Corticosteroids induce apoptosis in normal lymphocytes and most lymphomas are steroid-sensitive initially; loss of steroid sensitivity in human lymphomas is usually linked to down regulation of steroid receptors (Norgaard and Poulsen, 1991). But what could be the connection between down-regulation of the steroid receptors and anthracycline resistance? This may be another area where a better understanding of the events which promote and block apoptosis would be highly beneficial.

One of the main suggestions that the preceding section on p53 and tumour progression makes is that tumour cells may not necessarily become drug resistant in the sense of avoiding all damage from the agent. It is perhaps more accurate to describe them as drug-tolerant in that DNA replication of damaged DNA is allowed to proceed. If this is true, to investigate the phenomenon of clinical multiple agent resistance, it may be more worthwhile concentrating efforts on the understanding of DNA repair and apoptosis than on mechanisms peculiar to only a small group of drugs. Topoisomerases (as the targets for etoposide and the anthracyclines) are exceptional in that these enzymes are intimately involved with DNA metabolism and abnormal function of these enzymes probably contributes to the genetic instability of malignancy.

As a natural model for acquired resistance, canine MLSA has proved very apt and could profitably be used again. For instance, the hypothesis that MDR drugs are not a pre-requisite for acquired P-gp positive disease could be confirmed using MLSA cases treated with asparaginase. Single agent treatment with asparaginase (which is not an MDR substrate) gives remission and survivals which are directly

comparable with this study (Teske *et al,* 1990) and so the time-span of treatment would be similar.

It is unfortunate that the chromosomal translocations and cytogenetic abnormalities in canine MLSA are completely unknown. However, as a model of tumour progression, this disease is still worthy of attention. Monoclonal antibodies used to detect wild-type and mutant p53 can work across species barriers and it is feasible that this could be exploited to investigate alterations in p53 which accompany drug resistant relapse.

The power of this longitudinal study was jeopardised by the loss of follow-up samples, the inconsistency of clinical results in the first year and by lack of detailed histopathological reports. Such problems could be minimised in future studies by diligent client communication, use of a single designated clinician and full collaboration with the pathology department. In this way, canine MLSA could continue to be a useful natural tumour model.

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