FLOW CYTOMETRIC ANALYSIS OF UVEAL MELANOMA

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ABBREVIATIONS

The abbreviations used in this thesis are those recommended in the Biochemical Journal with the following additions:

APC	Antigen Presenting Cell
BdUR	Bromodeoxyuridine
C region	Constant region
CD	Cluster of Differentiation
CD25	Low affinity receptor for IL-2 receptor (Tac)
Con A	Concanavalin A
CRBC	Chicken Red Blood Cells
CTL	Cytotoxic T Lymphocytes
D region	Diversity region
ECM	Extracellular matrix
EDTA	Ethylene Diaminetetra acetic acid
ER	Endoplasmic Reticulum
FACScan	Fluorescence Activated Cell Scanner
FITC	Fluoroisothiocyanate
FL1,2,3	Fluorescence channels 1,2,and 3
FSc	Forward Scatter of light
G0,1,2	Gap phase 0,1,2 of the DNA cell cycle
HLA	Human Leucocyte Antigen
HPF	High Power Field

ICAM-1	Intercellular adhesion molecule -1
IEL	Intraepithelial lymphocyte
IFN	Interferon
Ii	Invariant chain
IL	Interleukin
J region	Joining region of TCR
LAK cell	Lymphokine activated Killer cell
LFA-1	Leucocyte function associated antigen-1
LPS	Lipopolysaccharide
M	Mitotic phase of the DNA cell cycle
MHC	Major Histocompatibility Antigen
MI	Mitotic Index
PBL	Peripheral Blood Lymphocytes
PBS	Phosphate Buffered Saline
PE	Phycoerytherin
PI	Propidium Iodide
rh IL-2	recombinant human interleukin 2
RPMI	Roswell Park Memorial Institute
S	Synthetic phase of the DNA cell cycle
SSc	Side Scatter of light
Tc	T cytotoxic cell
TCR	T Cell Receptor
Th	T helper cell
TIL	Tumour Infiltrating Lymphocytes
TNF	Tumour Necrosis Factor
V region	Variable region of TCR

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SUMMARY

Uveal melanoma, although a comparatively rare tumour of man, is the commonest malignant tumour of the eye. The tumours can be composed of varying cell types; pure spindle, pure epithelioid and a mixture of spindle and epithelioid cells, with a worse prognosis being associated with the pure epithelioid cells. Approximately 5-12% of uveal melanomas contain a lymphocytic infiltrate amenable to study. The eye has classically been referred to as an immunologically privileged site of the body lacking interaction of cells of the immune system. Such tumours and their associated infiltrates have previously only been studied by immunocytochemical techniques which is a non- quantitative and very subjective method of analysing cell surface marker expression. However, flow cytometry is a modern method of studying cell surface marker expression which makes use of fluorescently labelled antibodies specific for these molecules. It gives quantitative analysis of a large number of cells from a substantial section of the sample in a short period of time and allows small changes in fluorescence to be quantified.

The work in this thesis describes a flow cytometric analysis of not only the cell surface markers of the uveal melanoma cells themselves but also the lymphocytes which infiltrate them. In addition, the cell cycle of the tumour cells was analysed. Among the questions of interest are the immunological status of tumours within a privileged site, such as the eye, and the comparison with extra-ocular tumours, in particular cutaneous melanoma which has been treated by various immunological therapeutic strategies. The basic questions addressed were the origins of the tumours infiltrating lymphocytes. Either they represent a small population of cells which invade the tumour and then expand in this site, or they represent a population which

are expanded elsewhere and activated on site. The data from this work indicates that the latter option is the more likely.

In particular it was proposed to study the tumour infiltrating lymphocytes (TILs) in terms of their activation status compared to peripheral blood lymphocyte (PBLs). Among 27 uveal melanomas, 5 were found to contain TILs. Four had high levels of lymphocytes and the 5th had comparatively low levels but adequate numbers for comprehensive analysis. The TILs were analysed to determine the relative proportions of lymphocyte subsets and markers of activation. The results show the predominance of $CD8^+$ T cells and insignificant levels of B cells present within the infiltrate. The TILs were found to be activated to a higher degree when assayed for levels of histocompatibility antigen, HLA DR, possibly indicating the antigen presenting capabilities of the T cells. The $CD4^+$ T cells expressed more of the activation marker CD25 (Tac, IL-2R) which has relevance to the modern methods of immunotherapy of malignant neoplasms, especially malignant cutaneous melanoma.

It was then decided to examine the TILs in terms of the expression of specific Vβ regions of the T cell receptor (TCR) indicating whether the cells were clonal in the site of the tumour.This was done by using a panel of fluorescently labelled monoclonal antibodies to subfamilies of the TCR.

While the TILs have the ability to locate selectively within the tumour it was found that in the 6 uveal melanomas and the 4 breast carcinomas studied, the TILs comprised a population expressing a diversity of TCR Vβ genes. No probe was highly dominant or unrepresented in any of the tumour samples or PBL samples indicating that within the limitations of the monoclonal antibodies used the cells are not responding to a defined antigen or superantigen in a clonal manner.

In the light of many early reports of a systemic immune response to tumours, a study was performed to identify if such a response could be demonstrated for uveal melanoma. Patient PBL samples, pre-operatively and 1 week post-operatively, were prepared and analysed in terms of the lymphocyte phenotype activation status. There was no significant difference between the PBL samples in the same patient for the time points measured, indicating a lack of systemic immune response.

The relevance of adhesion molecules expressed on the tumour cells was studied to investigate if these correlate to relevant parameters on the lymphocytes within the tumour. Twenty uveal melanomas were studied for the expression of the intercellular adhesion molecule -1 (ICAM-1), and also Class I and II (HLA DR) MHC antigens. It was found that ICAM-1 was present on a high percentage of tumour cells and there appeared to be a negative correlation between the presence of ICAM-1 and HLA DR. There was no correlation between the levels of TILs and the expression of Class I or ICAM- 1 on the tumour cells, but there was a correlation between the levels of TILs and HLA DR present on the tumour cells. Class I was present on the mixed spindle/ epithelioid cell populations to a higher degree and HLA DR was detected on a slightly higher percentage of the pure spindle cells than the other cell types. This indicates the difference in the cell types of the tumours. However, ICAM-1 is expressed on all 3 cell types at similar levels.

Further studies were performed by analysing ICAM-1, Class I MHC and HLA DR on the TILs of 5 patients with uveal melanoma. ICAM-1 was found on higher percentages of the TILs than PBLs. $CD8^+$ T cells also had a higher level of expression than the $CD4^+$ T cells on both the TILs and PBLs. Class I MHC was found on a high percent of the lymphocyte populations whatever the source, as expected, and HLA DR was expressed on the TILs to a greater extent than on the PBL. In 3 of the 5 TIL samples there was also

data on the relevant tumour samples. Within this small sample number there appeared to be a correlation between the expression of HLA DR bearing $CD4^+$ TILs and Class I expressing tumour cells, and also between the HLA DR bearing $CD8^+$ TILs and HLA DR expressing tumour cells.

Finally it was decided to study the tumour cells at a nuclear level. In other studies, immune response has been related to abnormal DNA content (aneuploidy). Flow cytometry has often been used to perform ploidy analysis of primary tumour cells since epithelial tumours such as those of the breast and colon contain a high percentage of cells with abnormal DNA.

In this study 32 uveal melanoma tumours were prepared for FACScan analysis to determine the percentage of cells in each phase of the cell cycle. It was found that only 1 tumour contained abnormal (aneuploid) DNA and the remainder contained normal diploid DNA similar to the patient PBL samples. The tumour that contained the abnormal DNA was identified as being an epithelioid tumour. The majority of the diploid DNA was in the Go/G1 phase of the cell cycle. A correlation was performed between the tumour cell type and the proliferation index (G2/M/S) and it was found that the mixed spindle/ epithelioid cells were proliferating to a higher extent than the pure epithelioid or spindle cells. This finding seemed simply to be due to the small number of epithelioid tumour obtained since in contrast, pathology reports state the mitotic index (MI), of the tumour, another indicator of proliferation, and it was found that the pure epithelioid cell had a greater mean MI than the other cell types.

In all of these studies other than the cell cycle, considerable inter- patient and inter- tumour variation was apparent. It seems very probable that immunological or other therapeutic strategies will have to take this factor into account.

CHAPTER 1

INTRODUCTION

1.1 THE EYE

1.1.1 General structure of the eye

The eyeball is situated in a bony cavity of the skull called the orbit (Figure 1.1). In man, it is approximately spherical having a mean diameter of about 24mm and a weight of about 7g. Except at the anterior pole, the eye has 3 distinct layers: the outermost protective coat, the sclera (from the Greek, scleros meaning hard); the middle nutrative coat, the uveal tunic (from the Latin, uva, meaning grape due to similarity in shape and size to a grape skin); the innermost coat, the retina (from the Latin word translated as a "net like structure").

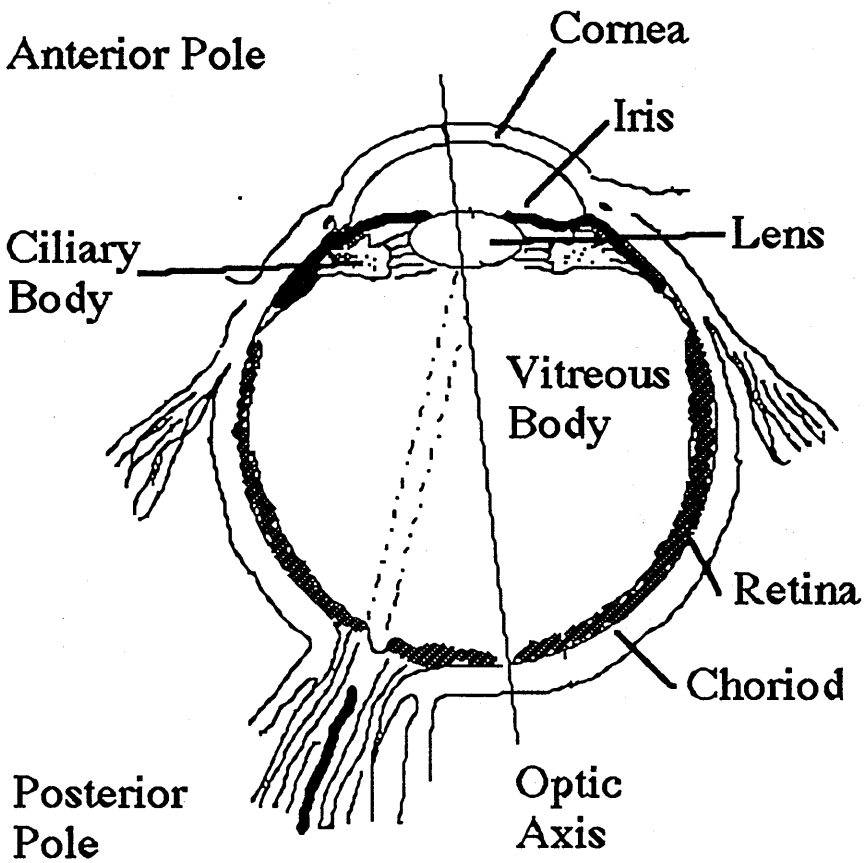


Figure 1.1: Schematic diagram of the eye

1.1.2 The uveal tunic

The sclera is replaced by the transparent cornea at the anterior pole of the eye. The uveal tunic is divided into 3 parts, the choroid, the ciliary body and the iris (Figure 1.1) which are highly vascularised and pigmented structures and together play a nutritive role. The choroid lies beneath the retina and provides the outer retina with its metabolic requirements. The ciliary body alters the refractive power of the lens and produces the aqueous humour. Finally, the iris regulates the amount of light that reaches the retina. Both the ciliary body and the iris exist in the anterior portion of the eye. The retina not only contains the light receptors but also nerve cells, including the fibres which converge at the optic disc to form the optic nerve and blood vessels.

1.1.3 The inner compartments

The interior of the eye contains the lens, the aqueous humour, and the vitreous humour. The aqueous humour is in two compartments: the anterior chamber is contained between the cornea, the anterior surface of the iris and the central portion of the anterior surface of the lens; the posterior chamber is contained between the surface of the iris, the ciliary body, suspensory ligaments and the edges of the lens.

1.1.4 Immunologic privilege in the eye

The anterior chamber of the eye is considered the classic example of a privileged site in which histocompatible allografts escape immunological recognition and undergo prolonged and sometimes permanent residence (Head and Billingham,1985). The immunological privilege has been attributed to the isolation of these sites from the lymphoblastic system, thus preventing these cells from interacting with the immune system. Other areas of the body

that exhibit this phenomenon include the brain (Head and Griffin, 1985) and the pregnant uterus (Beer *et al.*, 1976).

Corneal transplantation has been performed on animal subjects for over 150 years and on human patients for over 80 years (Trevor- Roper, 1972). In the United States, 30,000 corneal transplants are performed each year, with a success rate of well over 90%. Thus along with the anterior chamber, the cornea is a major area of immunologic privilege in the eye. It would thus seem that the cornea, the corneal graft bed and the anterior chamber exist in an environment free of immune- mediated diseases. However, this is an oversimplification, as the eye is apparently vulnerable to a wide range of autoimmune diseases.

Historically, 3 basic hypotheses have been offered to account for the privileged existence of corneal allografts (Silverstein and Khoudadoust, 1973).

(1) The simplest is that the cornea similar to some neuronal tissue, is devoid of major histocompatibility complex (MHC) antigens (Section 1.4.1). Thus alloreactive T cells would not recognise corneal cells. The absence of MHC antigens would not only prevent the arousal of an alloreactive response, but if a response were initiated, the grafts would be immunologically invisible. This theory has been unequivocally disproved, since some findings indicate not only that the cornea is vulnerable to immunological attack, but that it is capable of eliciting an alloimmune response that results in graft rejection. Studies in rats, rabbits and mice indicate that heterotopic transplantation of corneas to subdermal graft beds leads to rapid sensitization and swift rejection of the allografts (Billingham and Boswell, 1953; Khoudadoust and Silverstein, 1966; Chandler *et al.*, 1983; Streilein *et al.*, 1982).
MHC antigens (Section 1.4.1) have been found in all three layers of cornea

(Mayer *et al.*, 1983; Treseler *et al.*, 1984). The density of antigens differs between the cells of the corneal epithelium, stroma, and endothelium and in fact in rat corneal endothelium cells express small quantities of MHC Class I antigens and no detectable MHC Class II antigens (Treseler and Sanfilippo, 1986).

Corneal endothelium grafts stimulate robust cytotoxic T lymphocyte (CTL) responses following heterotopic grafting in the rat (Treseler *et al.*, 1986). Although MHC Class I antigen expression is greater in the epithelium than the endothelium, heterotopic transplantation of isolated allogeneic corneal epithelium fails to induce detectable anti- MHC class I CTL responses in rat (Treseler *et al.*, 1986). The mere expression of MHC antigens on corneal cells does not ensure the induction of alloimmune response, but corneal allografts still have the potential to be immunogenic.

(2) The second hypothesis suggests that the donor cells are rapidly replaced by host cellular components in the graft bed, so that the cellular elements of the graft are replaced before the host's immune system can be aroused.

Again this idea has been refuted by such findings using tritiated thymidine to distinguish donor cells from recipient cells where by it has been demonstrated that long term survival of donor cells occurs (Chi *et al.*, 1965). Clinical findings support this idea, since immunological rejection can occur over a decade after corneal transplantation (Silverstein and Khoudadoust, 1973).

(3) The third and perhaps the most widely acceptable hypothesis relates to the nature of the avascular corneal graft bed. The absence of blood and lymph vessels at the interface of the graft and the graft bed is thought to prevent the escape of alloantigen to the regional lymphoid tissues.

Thus the avascular graft, and the graft bed conspire to produce a state of immunological ignorance. To study this, corneal allografts were transplanted heterotopically (i.e. to an abnormal anatomical site) into subdermal graft beds, richly endowed with blood vessels and lymphatics, forcing the induction of alloimmunity. These grafts were compared with orthotopic grafts (i.e. into the normal anatomical site). If the avascular graft bed contributes to the survival of the allograft, it could be predicted that heterotopic corneal grafts would suffer significantly higher rejection rates than the orthotopic counterparts. This indeed was the case, as 100% of the fully allogeneic heterotypic corneal allografts were rejected in the mouse model (Chandler *et al.*, 1983; Streilein *et al.*, 1982; Matoba *et al.*, 1986). In contrast, only 55-57% of fully allogeneic grafts failed when grafted orthotopically (Callanan *et al.*, 1988; Williams and Coster, 1985).

Maumenee (1951) performed studies showing that rabbits bearing long- term orthotopic corneal allografts rejected skin grafts from donors who provided their corneal grafts. Skin graft rejection indicated that the initial corneal allograft failed to stimulate alloimmunity and that the graft bed produced an afferent blockade of the immune response. However, skin graft rejection led to the rejection of 90% of the previously clear corneal grafts. Thus, the corneal grafts initially displayed immunological privilege, but were antigenically vulnerable to an ongoing systemic immune response.

The high incidence of spontaneous neoplasms that occur in patients suffering from immunological disorders and in transplant recipients subjected to prolonged immunosuppression were earlier cited as evidence in support of the immune surveillance theory (Burnet ,1970) (Section 1.5.1). It is thus reasonable to suspect that the same situation occurs with immunological privilege sites. This is not the case (Head and Billingham, 1985), since the incidence of spontaneous neoplasms in immunologically privileged sites is not

higher than that occurring at other anatomical regions. In the case of the eye, tumours of the cornea are virtually unknown and the most common intraocular tumours occur in the posterior compartments of the eye.
An important question to be asked is: are highly immunogenic tumours that undergo spontaneous immunological rejection at extraocular sites exempt from immunologic rejection within the immunologically privileged confines of the anterior chamber ? This has been studied using P91 mastocytoma, a highly immunogenic mutant of the P815 mastocytoma, which undergoes immunological rejection following subcutaneous transplantation in syngeneic hosts (Niederkorn and Meunier, 1985). Rejection is T cell mediated. Following anterior chamber transplantation, the tumours grow progressively for 3 weeks, but during the 3rd and 4th week post transplantation, an intense inflammatory response is elicited and culminates in ischemic tumour necrosis (Niederkorn and Meunier, 1985). The eye is left irreparably damaged after another 5-7 days (phthisis bulbi). Immunologic findings support the proposition that rejection is predominantly a DTH (Delayed Type Hypersensitivity)- mediated process (Niederkorn and Knisely, 1988). The host also develops tumour- specific CTLs and antibodies, but neither is involved in the rejection of the tumour.

1.2. NEOPLASTIC DISEASE

1.2.1 Malignancy

The size of an organ and the number of cells it contains are both normally maintained at constant and optimal values by the mechanisms which control the mitotic activity of the cells. Neoplasia is a state in which the control mechanisms become deficient and an excessive proliferation of the cells continues indefinitely without relation to normal growth and tissue repair. This gives rise to an neoplasm, an abnormal tissue mass, or tumour

(from the Latin meaning a swelling). Tumours are conveniently classified into 2 main types: benign and malignant. Benign tumours proliferate locally and are composed of differentiated cells resembling the tissue of origin. These structures are usually encapsulated, and readily removed by surgical procedures. Malignant tumours are not encapsulated and their edges are ill-defined. The cells are less well differentiated than the cells of origin and if left untreated the cells are capable of spreading into surrounding tissue. If the tumour invades blood vessels or lymphatics, the cells carried to other parts of the body may proliferate to form secondary tumours in a process referred to as metastasis (Section 1.2.3).

1.2.2 Classification of malignant tumours

Tumours are classified according to their tissue of origin. A malignant tumour of the epithelial tissue is referred to as a carcinoma (the suffix "-oma" meaning a mass or swelling) if it is derived from the surface epithelium or an adenocarcinoma if it is derived from the glandular epithelium. A malignant tumour of the connective cells of the muscle is called a sarcoma and tumours of the nerve cells are referred to as neuroblastomas and gliomas. Of particular interest to this thesis are tumours of the pigmented tissue. These malignant tumours are called melanomas and usually refer to lesions of the skin, cutaneous melanoma, but melanoma can also be associated with the certain areas of the eye (Section 1.3).

1.2.3 Metastasis of malignant neoplasms

As mentioned previously (Section 1.2.1) tumour cells have the ability to spread and grow not only in the neighbouring tissue but also in other organs far removed from the original tumour site. The importance of metastatic spread in determining clinical outcome in cancer patients has initiated efforts to increase understanding of the pathobiology of this process.

Considerable importance has been given to the demonstration that populations of metastatic and non- metastatic cells could pre- exist within the same primary tumour (Fidler and Kripke, 1977). Selection of particular cells during the complex process of cancer spread may result in the cellular composition of the secondary tumour being different from that of the primary tumour (Poste and Fidler, 1980). This was studied by comparing primary and secondary growths or by comparing variant lines differing only in their malignant behaviour (Hart *et al.*, 1989). Recent efforts have been made to study genes controlling the metastatic behaviour utilising primary and secondary growths (Section 1.6.1). Gain and loss of a wide variety of different gene products has been documented during the progression of various cancers (Klein and Klein, 1985). Increased expression of specific adhesion molecules (Section 1.4.6) has been implicated in the increased spread of malignant melanoma (Johnson *et al.*, 1989). In contrast with these results in melanoma are the findings from colorectal cancer where allelic deletions involving chromosome 18q, which occur relatively late in progression, have been determined to involve a gene specifying a protein with sequence similarity to neural cell adhesion molecules (Fearon *et al.*, 1990). Whether these changes in genes encoding in cell- cell adhesion molecules is involved in controlling the metastatic potential of tumours is yet to be determined.

Conversion of non metastatic tumour cells to fully metastatic phenotype has been achieved in a number of experimental tumour systems by transfecting the benign population with an activated oncogene, generally of the ras family (Section 1.6.1) (Kyprianou and Isaacs, 1990).

A possible candidate for a metastasis- regulating gene, nm 23, has been identified, and is expressed to different degrees in cells of differing metastatic capacity (Steeg *et al.*, 1988). It was originally identified and isolated from variant lines of a murine melanoma cell line, but the human homologue of this gene has now been cloned (Steeg and Liotta, 1990).

Expression of nm23 RNA was found to be down regulated in metastatic variants in a wide variety of experimental tumour models.

Thus, work is being performed to identify genes controlling the metastatic potential of tumours. In addition it would be interesting to ask why tumours metastasise to one area of the body in preference to another.

1.3 UVEAL MELANOMA

1.3.1 Introduction

Uveal melanoma is a malignant neoplasm that occurs in the iris, ciliary body, or the choroid (Section 1.1) (Figure 1.1), and is derived from melanocytes of the neural crest origin (Rawles, 1947). They contain the pigment melanin, synthesised from tyrosine via DOPA (3,4-dihydroxyphenylalanine) and dopaquinone, using tyrosine as a catalyst. Although apparently amelanotic tumours are well recognised, they usually contain small amounts of melanin. Metastatic secondary tumours may lose their ability to secrete melanin. The neural crest -derived melanocytes and the tumours that arise from them are more like those found in the skin and much research performed into ocular melanomas shadows that into cutaneous melanomas.

Intraocular melanomas, like their cutaneous counterparts, are considered to be relatively antigenic, and studies using animal models suggests immunological responses elicited by uveal melanomas may influence the development of systemic metastases (Niederkorn, 1987).

1.3.2 Epidemiology

Malignant melanomas are the most common primary tumours of the iris; their reported incidence varies between 49% and 72.4% (Heath, 1964; Duke *et al* .,1958; Ashton, 1964). The incidence of malignant melanomas of the iris compared with the incidence of melanomas of the rest of the uvea is

low, but the ratio varies in the different countries, for example, 1:6 in Germany (Holland, 1967), 1:15 in the United States (Ranes *et al* .,1958), 1:19 in Finland (Raivio, 1977), and 1:30 in Denmark (Jensen, 1963). In general the incidence is approximately 6 per million per year in caucasians (Jensen, 1963) and much lower in the pigmented races (Apple *et al* .,1983). The sex ratio is approximately equal and although there have been reports of a higher incidence of melanoma of the iris in women (Martin- Jones ,1946), there are also reports of the opposite to this (Cleasby, 1958; Callender *et al* ., 1942) The mean age of presentation of uveal melanomas is 53 years (Benjamin *et al*., 1948).

1.3.3 Histology

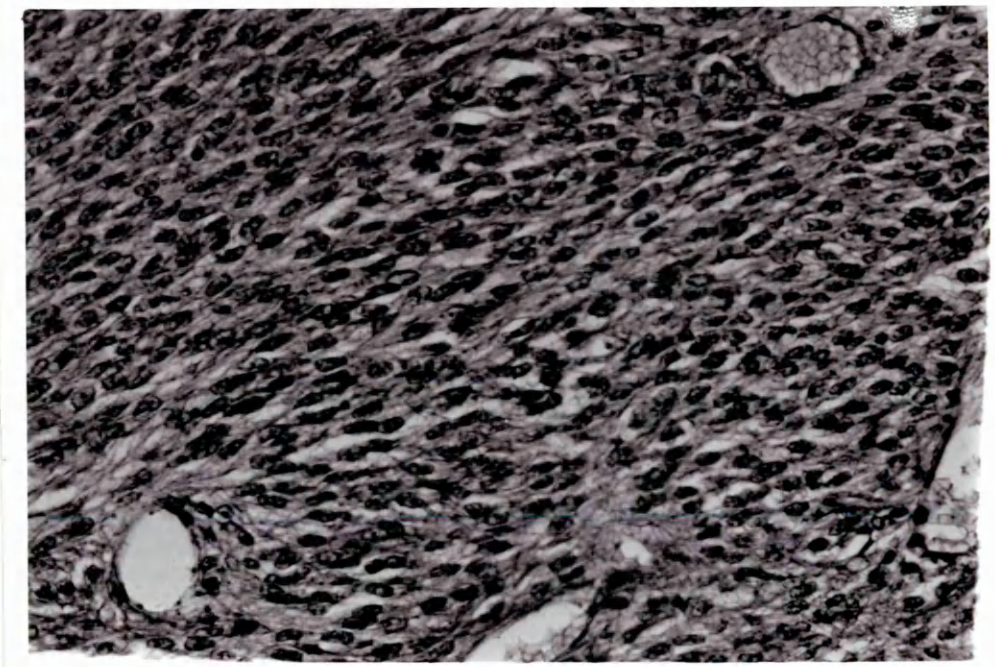
Uveal melanomas consist of pure spindle cells (Figure 1.2), pure epithelioid cells (Figure 1.3) but more commonly mixed spindle/ epithelioid cell populations (Figure 1.4). The cell type determination is a useful prognostic indicator for the survival of the patient after enucleation. Evidence of epithelioid cells is associated with a poor outlook (Callender *et al* 1942). Histologically, aggregates of lymphocytic infiltration are seen in about 5-12 % of uveal melanomas (Lang *et al* .,1977; Durie *et al*., 1990) (Figure 1.4).

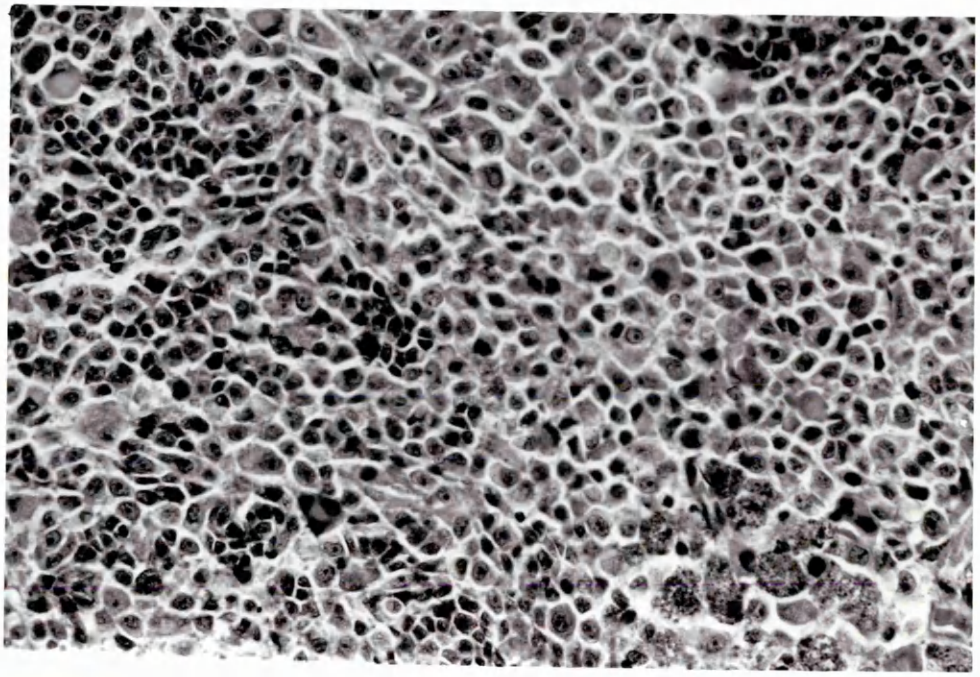
1.3.4 Diagnosis and treatment

Most patients with intraocular melanoma initially complain of painless deterioration of their vision. The tumour can be diagnosed sometimes only during routine ocular examination or it can be identified when the painful eye is removed (enucleation), upon loss of sight. In the latter, the media is opaque (Makely and Tweed, 1958) and thus the tumour cannot be identified during examination of the intact eye. Examination is by ophthalmoscopy (Norton, 1965), transillumination (Coleman *et al*., 1974), computerised tomography (Mafee *et al*., 1985), and magnetic resonance imaging (Chambers *et al*.,

Figure 1.2: Photograph (x25) of tumour section (stained with haematoxylin and eosin) containing pure spindle cells.

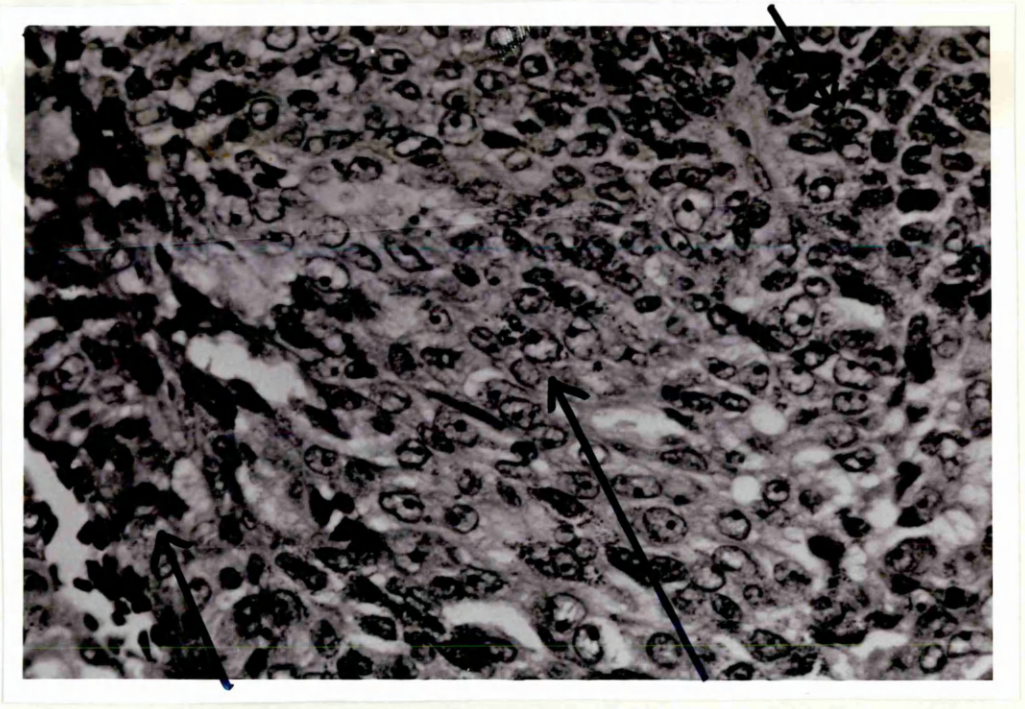
Figure 1.3: Photograph (x25) of tumour section (stained with haematoxylin and eosin) containing pure epithelioid cells.





EPITHELIOID

CELLS



LYMPHOCYTIC AGGREGATES

SPINDLE

CELLS

Figure 1.4: Photograph (X40) of tumour tissue section (stained with haematoxylin and eosin) containing mixed spindle and epithelioid cell populations. Lymphocytic aggregates can also be detected in this tissue section

1987). The uptake of the radioisotope, ^{32}P, or other radioactive tracers (Packer, 1984) can also give useful information about the tumour, but one of the best method for diagnosis is by histological examination of the tissue samples obtained by biopsy (Foulds, 1985).

Until recently the standard method for the treatment of uveal melanomas has been enucleation, except for iris melanomas which can be easily excised. This has lead to mixed reports that removal of the eye actually results in an increase in the mortality rate of patients with uveal melanoma (Zimmerman *et al.*, 1978). Other workers have challenged the statistical methods on which such impressions are based (Seigal *et al.*,1979) and thus consider prompt enucleation to be life- saving (Manschot *et al.*, 1980). The latter hypothesis is supported by reports of a very high mortality from metastatic disease in patients with uveal melanoma who refuse treatment. (Raivio, 1977). Some workers have suggested that removal of the primary tumour interferes with protective immunological mechanisms (Zimmerman *et al.*, 1979), but these results were obtained by the use of animal models.

Thus conservative forms of therapy aimed at eradicating the tumour whilst preserving a functional eye have been developed. These include photocoagulation (Meyer- Schwickerath and Bornfeld, 1983; Foulds and Damato, 1986), photochemotherapy using photosensitising agents (Tse *et al.*, 1984), radiotherapy (Fairchild, 1984), and surgical resection (Foulds, 1978). These methods used either alone or in combination have proved successful in over 80% of cases, and seem to prevent metastatic disease as effectively as enucleation

1.3.5 Prognosis

Following enucleation, orbital recurrence occurs in 2.5-12% of cases and metastatic disease ultimately arises in about 50% of all patients, usually within the first 5 post- operative years (Jensen 1982).

The risk of metastatic disease has been studied by a number of workers (Davidorf and Lang, 1974; Shammos and Blodi, 1977; Packard, 1980; Seddon *et al.*, 1983) and they have shown that poor prognosis is associated with tumour size, anterior location, extraocular extension, epithelial cellularity (Section 1.3.3) and nucleolar pleomorphism. Metastases first tend to appear in the liver and cause death within a few months (Char, 1978). High mortality is also associated with a rare subtype of uveal melanoma, consisting of flat diffuse tumours (Font *et al.*, 1986). Liver disease (Pascal *et al.*, 1988), intercurrent illness (Leff *et al.*, 1985), pregnancy (Apple *et al.*, 1983) and smoking (Keeney *et al.*, 1982) all have been considered to have adverse effects on survival.

1.4 GENERAL IMMUNOLOGY

1.4.1 Major Histocompatibility Antigens

The MHC is a genetic region found in all mammals whose products are primarily responsible for rapid rejection of grafts between individuals. In man the MHC is the HLA gene cluster present on chromosome 6, and occupies about 1/3000 of the total genome. Three major sets of molecules are encoded within this region: Class I,II, and III antigens. Class I and II genes encode molecules involved in immunological recognition. Class III genes encode some of the complement components concerned in the cleavage of C3, a central event in the generation of a B cell inflammatory response.

The products of the Class I gene (Class I antigens) comprise a transmembrane glycoprotein, encoded by the HLA-A, -B, and -C genes, that is non- covalently associated with the polypeptide β2- microglobulin which is encoded outside the MHC (Bjorkman *et al.*, 1987). Class II proteins consist of 2 non- covalently associated peptides, referred to as α and β chains, both of which are encoded by the MHC. The class II αβ heterodimer assembles

transiently in the endoplasmic reticulum (ER) with a third chain, the invariant chain, to form a heterotrimeric complex.

1.4.1.1 Structure of the Class I MHC

The structure of the Class I antigens differs from that of the Class II antigens (Section 1.4.1.2). Each Class I antigen consists of one glycosylated polypeptide chain (Mr 45,000) non- covalently associated with non-glycosylated peptide (β2- microglobulin Mr 12,000) (Figure 1.5). β2-microglobulin also occurs free in serum or urine as a small globular peptide which has a similar tertiary structure to an immunoglobulin constant region domain. It is non-covalently bound to the α3 domain of the Class I heavy chain on the outer side of the plasma membrane. The highly conserved α3 domain does not directly interact with antigen or the T Cell Receptor (TCR) (Section 1.4.3) but can influence recognition of antigenic determinants located within the α1 and α2 domains (Maziarz *et al*, 1988). Three Class I loci have been localised to the human MHC: HLA-A, HLA-B and HLA-C. Using X-ray crystallography Bjorkmann *et al* (1987) have shown that antigen peptides bind to a cleft between α1 and α2 domains (Section 1.4.4). With few exceptions, Class I heavy chains seem to need β2- microglobulin and a tightly bound peptide for their conformational integrity (Cresswell *et al.*, 1987; Townsend *et al.*, 1989; Schumacher *et al.*, 1990; Townsend *et al.*, 1990).

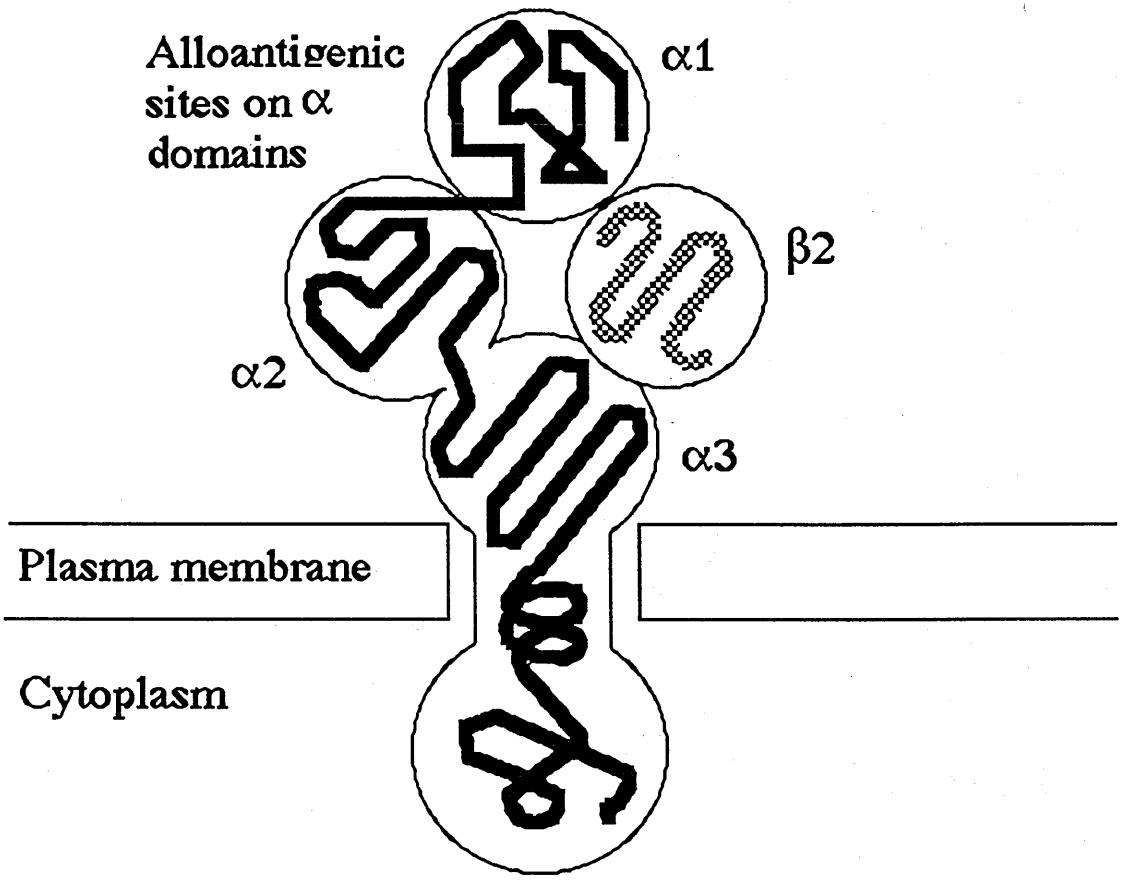


Figure 1.5: A schematic diagram of the structure of the Class I MHC showing the three domains and the β_2 microglobulin.

1.4.1.2 Structure of the Class II MHC

The Class II antigen consists of 2 distinct polypeptide chains (Figure 1.6), the α chain and the β chain. Both chains traverse the membrane and are held together by non- covalent forces. They each carry carbohydrate and the shorter β chain (Mr 28,000) contains the alloantigenic sites. Both chains also have 2 globular domains and all except the α1 domain are stabilised by disulphide bonds. Three families of Class II antigens have been characterised in man, HLA-DR, -DQ and -DP. Class I and Class II contain element that have conserved primary sequences, suggesting Class II molecules may be structurally similar to Class I.

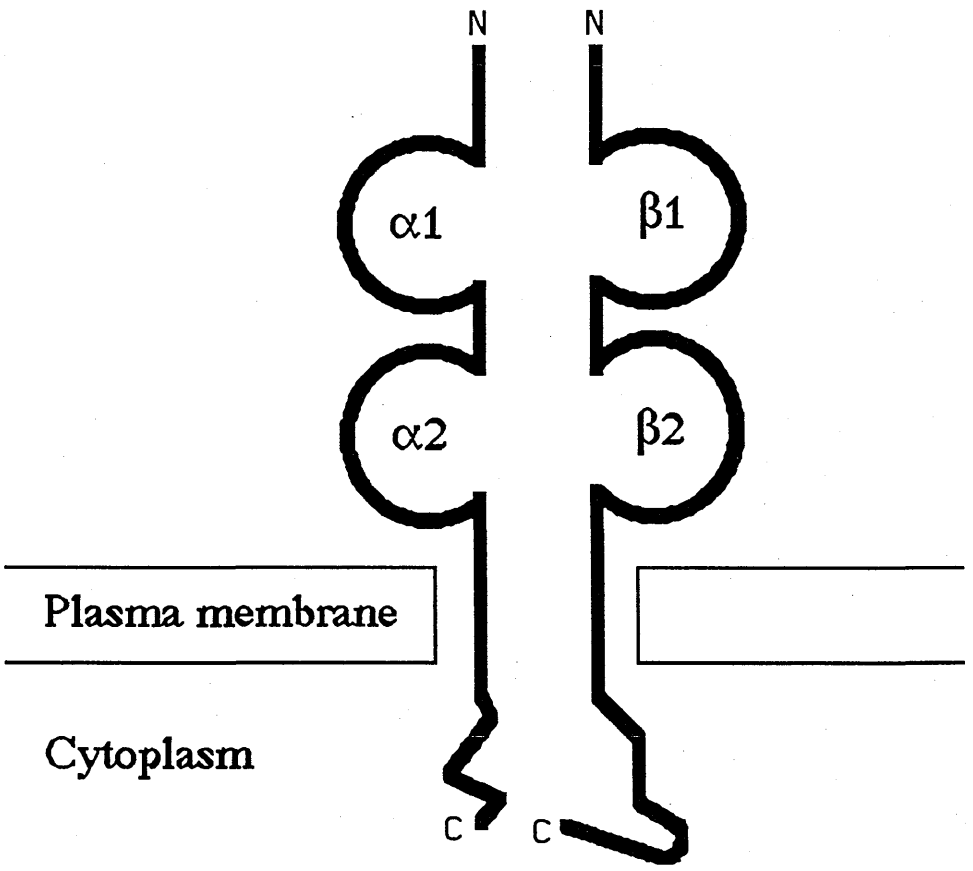


Figure 1.6: Schematic representation of the Class II MHC molecule.

1.4.1.3 Function of the MHC

T cells recognise short peptides from processed antigens in the framework of MHC Class I and Class II products (Townsend *et al* 1985). CTLs involved in recognition and rejection of virally infected cells and foreign tissue grafts recognise HLA-A and -B on the foreign cells, and in cooperation with T- helper (Th) cells, cause the destruction of the foreign cell. CTL of a particular haplotype from an animal infected with a virus are primed to kill cells infected with that virus. It is found that they will not kill cells of a different haplotype infected with the same virus. Similar principles of

haplotype restriction apply to T- h cells which recognise antigen on macrophages and B cells.

In general, T cells do not recognise free antigen. They recognise it on the surface of other cells, such as specialised antigen presenting cells (APC), including macrophages, bone- marrow- derived dendritic cells and vascular endothelial cells, or on virally infected cells as mentioned previously. APC actually take up the antigen that they will present. The antigen is internalised by APC and are then degraded by proteolytic enzymes in the phagolysosomes. Some of the material is only partly degraded and is re-expressed at the cell surface, where it comes to be physically associated with MHC molecules (Section 1.4.4)

Proteins must be processed, and presented to T cells. For viral and endogenous proteins this involves intracellular degradation to an oligopeptide, binding to a Class I MHC molecule, transportation to the cell surface and finally engagement with the TCR. *In vivo*, peptide binding to class I MHC molecules is restricted to the ER (Section 1.4.4.1). The source of the peptide can be protein found in a variety of cellular locations including the cytoplasm, nucleus and mitochondria. The fact that intracellular membranes prevent simple diffusion of other intact or degraded proteins from these compartments to the ER, indicated the involvement of a specific transport mechanism (Townsend *et al,* 1985).

In addition to MHC antigen and TCR interactions, it has been shown that CD8 and CD4 on T cell subsets appear to function as cellular adhesion molecules in the binding of antigen- bearing cells (Bierer and Burakoff, 1988) (Section 1.4.2). It has been demonstrated that CD8 interacts with the $\alpha 3$ domain of Class I (Ratnofsky *et al.*, 1987), and that CD4 interacts with Class II (Sleckman *et al.*, 1987). Class I- CD8 binding can occur on non- T cells (Norment *et al.*, 1988) and can be inhibited by either antibodies to CD8 or

Class I MHC indicating that CD8 can interact with class I MHC molecules independently from the TCR.

1.4.1.4 HLA disease association

Various HLA molecules have been found to be associated with a long list of diseases including multiple sclerosis, psoriasis, haemachromatosis, coeliac disease and diabetes mellitis, myasthenius gravis, ankylosing spondilitis and thyrotoxicosis (Tiwari and Terasaki 1985). Of these diseases multiple sclerosis, diabetes mellitis, myasthenia gravis, and thyrotoxicosis have implications for ophthalmology.

In ocular tissues, Class I HLA molecules are expressed in the vascular endothelium. Opinions differ as to whether other ocular tissues express Class I or Class II HLA molecules in the absence of disease. (Bakker and Kijlstra, 1985)

1.4.2 Role of CD4 and CD8

The cell specific CD4 and CD8 glycoproteins which are encoded by genes which are cloned and well characterised, separate most mature T lymphocytes into mutually exclusive subsets. In general, most Th cells express the CD4 surface marker and recognise antigen in association with Class II MHC molecules while cytotoxic cells (Tc) express CD8 on their surface and recognise antigen in association with Class I molecules (Section 1.4.1).

CD4 is single chain polypeptide of Mr 55,000-67,000. CD8 can be expressed as a heterodimer of α (Mr 34,000-38,000) plus β (Mr 28,000-30,000) subunits, or as α–α homodimer (Figure 1.7). CD4 and CD8 appear to stabilise and increase the avidity of the interaction between TCR- CD3 (Section 1.4.3.2.) and the antigen- MHC complex (Section 1.4.1) by binding to monomorphic determinants on MHC Class I and II molecules. This

association may link CD4 and CD8 to the TCR- CD3 complex during antigen presentation (Section 1.4.4.) (Figure 1.8).

CD4 and CD8 possess intracellular domains that physically associate with the T cell specific tyrosine protein kinase, pp56 ^{lck} (Rudd *et al.*, 1988; Veillette *et al.*, 1988). Close association of CD4 and CD8 to the TCR-CD3 complex during T cell activation by antigen or mitogen might allow pp56^{lck} to phosphorylate physiological substrates, such as the ζ chain of the TCR-CD3 complex. (Biniyash *et al*, 1988 a) (Section 1.4.3). Thus CD4 or CD8 may play important role in regulating tyrosine phosphorylation in T cells.

It has also been suggested that CD4 and CD8 have influence in the selection of TCR repertoire in the thymus (Von Boehmer *et al.*, 1989). Signals delivered via CD4 or CD8 in differentiating thymocytes may act together with some yet undefined thymic ligand to influence the negative or positive selection of T cells.

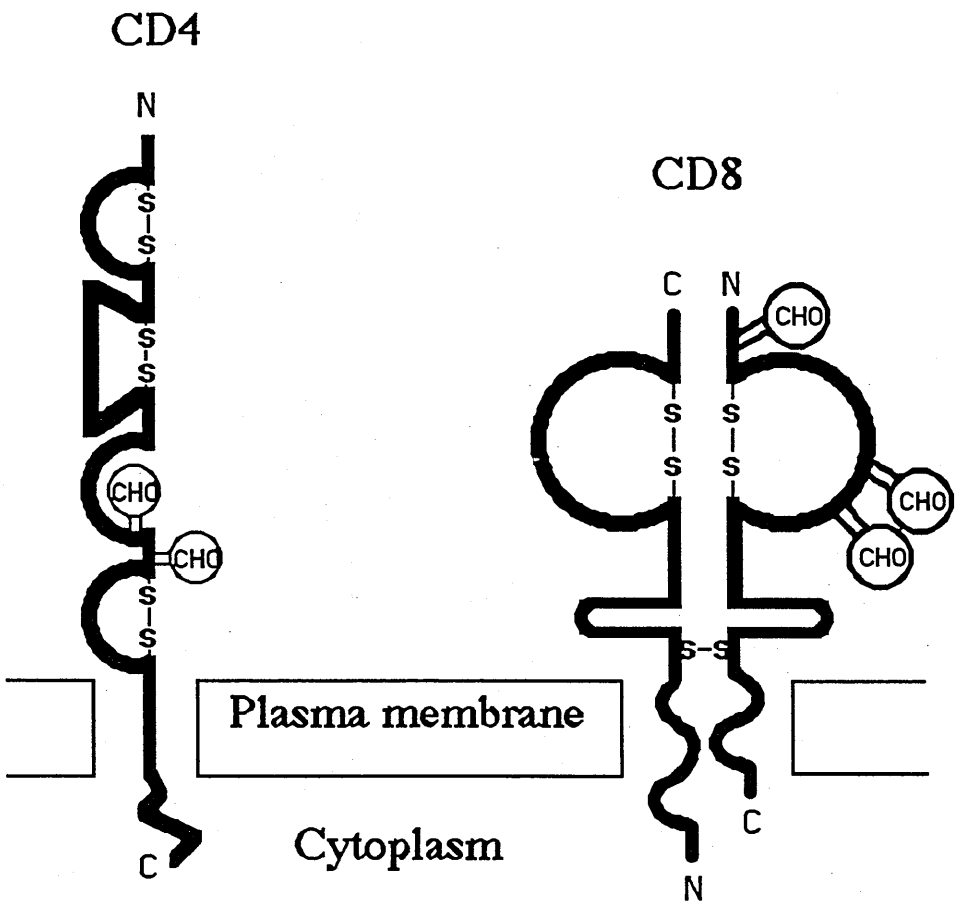


Figure 1.7: Structure of the CD4 and CD8 molecules.

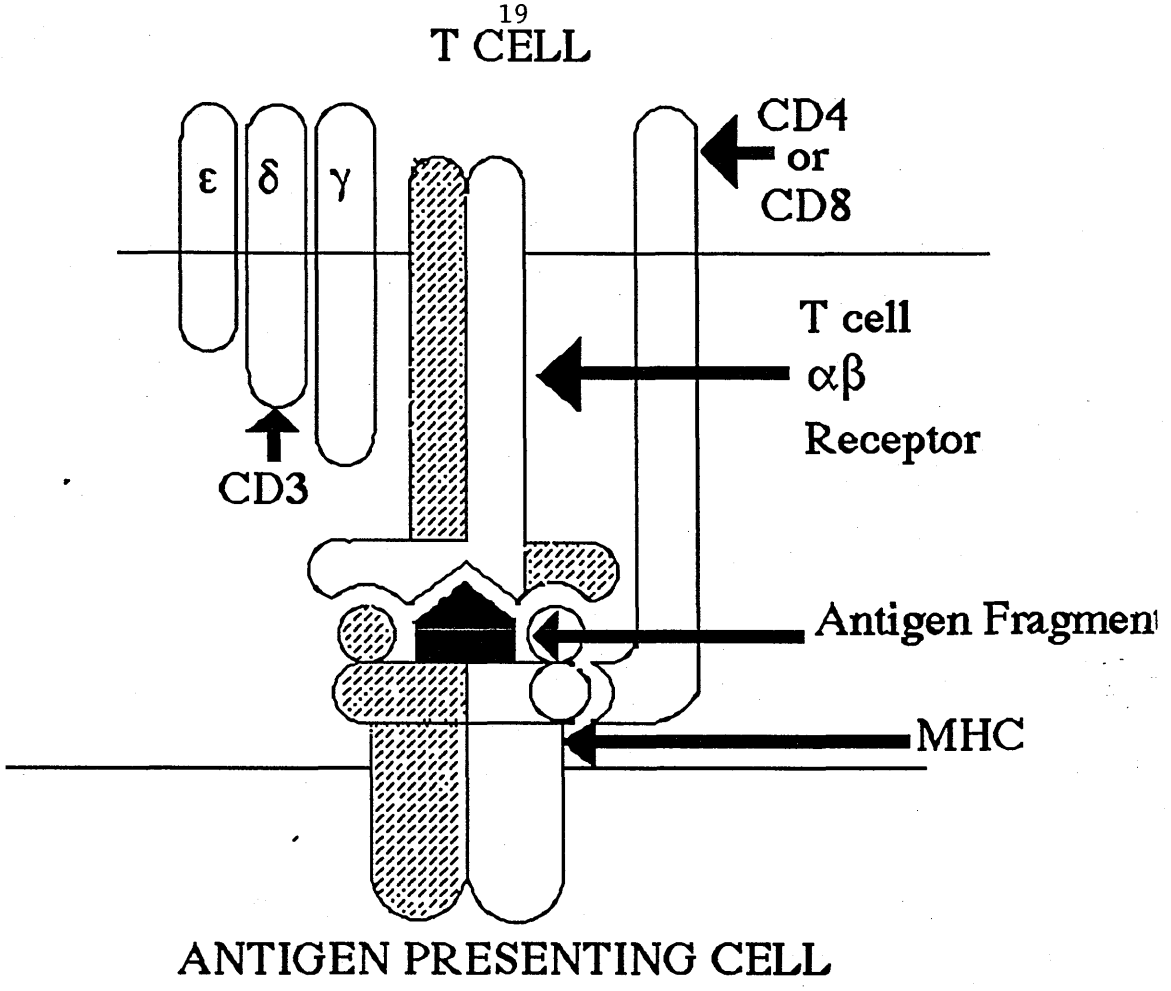


Figure 1.8: Diagram illustrating the interaction between CD4/CD8 with the TCR -CD3 complex and the MHC Class II.

1.4.3 T Cell Antigen Receptor- CD3 Complex

The main recognition and activation element that acts during physiological T cell responses to antigen is a complex receptor. It consists of, first, clonally distributed, highly polymorphic subunits namely the T- cell antigen receptor (TCR) whose function is to recognise and specifically bind antagonistic peptides presented by MHC molecules (Section 1.4.1) and, second, a complex termed "CD3" (formerly T3). CD3 is composed of at least 5 distinct invariant polypeptides some or all of which are believed to function

as the signal transducing unit of the receptor complex. TCR and CD3 associate non- covalently to form the complete and functional receptor.

1.4.3.1 Structure of the CD3 Molecule

The CD3 complex was initially identified by the monoclonal antibody OKT3 (Kung *et al* ., 1979), as a specific T cell marker on human T cells. To date 5 distinct polypeptides termed γ,δ,ε,ζ and η have been identified in non-covalent association with the clonotypic α– β TCR. These proteins range in size between molecular wieghts of 16,000 and Mr 28,000 . The γ and δ subunits contain 1 to 3 extracellular N- glycosylated sites, while ε, ζ and η are non- glycosylated (Samelson *et al* .,1985; Baniyash *et al* .,1988a). About 90% of ζ is found as a homodimer, and the rest forms a heterodimer with the η subunit (Samelson *et al* ,1985; Weissman *et al* 1986). The η chain is not as well characterised as the other subunits. A CD3 subunit, termed p21, which becomes phosphorylated on tyrosine residues in response to antigen stimulation (Samelson *et al* ,1986) was found to represent the phosphorylated form of ζ (Baniyash *et al* 1988b). When T cells are activated by antigen, the γ and ε chains become phosphorylated on some residues and ζ is phosphorylated on tyrosine residues. γ,δ,ε,and ζ components contain a 21 to 22 amino acid signal peptide, and a hydrophobic transmembrane domain consisting of 21- 27 amino acids, in all cases , a centrally located acidic residue (glutamic acid in γ and aspartic acid in δ, ε and ζ). This contrasts with the basic residues found in the corresponding domains of all clonotypic TCR subunits (Yanagi *et al* 1984; Hedrick *et al* 1984). These charged residues could form salt bridges between the TCR and CD3 and may stabilise the non-covalent association of the various components within the hydrophobicity of the T cell membrane. γ, δ, and ε polypeptides have large extracellular domains (79-104 amino acids) with a cytoplasmic domain of 44- 79 amino acids, while ζ has a 9 amino acid extracellular domain and a much larger cytoplasmic

domain of 112- 113 amino acids. The cytoplasmic region of ζ chain has 6 (murine) or 7 (human) tyrosine residues that are potential substrates for tyrosine protein kinases and also possess a consensus ATP binding sequence (Kung *et al* .,1979; Weissman *et al* .,1988a).

Wiessman *et al* .,(1988b) have proposed 2 structural classes of receptor complexes displayed on T cells. One class, the majority, contains only ζ–ζ homodimers while the other classes (10-20%) contains the ζ–η heterodimers. The two subclasses may represent 2 receptor classes coupled to 2 distinct signalling pathways. The ζ chain appears to be the limiting factor for assembly of the mature rceptor complex, emphasising its crucial role in the synthesis and function of the complete receptor

1.4.3.2 Structure of the TCR

The TCR consists of 4 highly polymorphic proteins (α,β,γ and δ) that form two heterodimers (α–β, and γ– δ) similar to immunoglobulins in their overall primary structure gene organisation, and rearrangement patterns. Each of the polypeptides is similar in size (Mr 27,000- 34,000). The α– β heterodimer mediates the specific recognition of antigenic peptides in the context of the MHC molecules. The exact function of the γ– δ TCR is largely unknown. Each of the 4 TCR polypeptides contains a single membrane-spanning region, and notably very short (up to 12 amino acids) cytoplasmic domains (Yanagi *et al* .,1984; Hedrick *et al* .,1984) suggesting that the polymorphic TCR does not play a direct role in signal transduction.

Because the different elements correspond very well to immunoglobulin domains and genes the same nomenclature has been adopted for TCR molecules. Each has a distinct variable (V), joining (J) and constant (C) region element and, in the case of β and δ diversity (D) regions as well (Geliebter *et al*, 1986; Vega *et al*., 1985; Seeman *et al*.,1986; Van Schravendijk *et al*., 1985). It is interesting that polypeptides which contain D

elements appear to pair only with those which do not (for example, TCR α /TCR β and TCR γ / TCR δ).

The vast majority of T cells in normal mice and humans (including all functional Th and CTL lines) bear the α/β type of receptor. Much less well expressed and less numerous is the γ/δ subset which appears early in the course of T cell differentiation (Mellor *et al.*, 1983; Schulze *et al.*, 1983). This subset normally constitutes only 1-5% of mature lymphocytes, but somewhat more in immunodeficient patients (Hammering *et al.*, 1985). Murine dendritic epithelial cells bear exclusively γ/δ receptors (Koning *et al.*, 1987; Kuziel *et al.*, 1987), while the murine intraepithelial lymphocytes (IEL) not only have predominantly CD3- associated γ/δ receptors but also express surface CD8 (Goodman and Lefrancois, 1988). In contrast to murine IEL, human IEL have been reported to have low representations of the γ/δ receptors in the normal individual, with raised levels being observed in coeliac disease (Raulet, 1989; Borst *et al.*, 1988). The discovery that some γ/δ T cells react with bacterial antigens and heat- shock proteins (Spencer *et al.*, 1989) has led to the suggestion that γ/δ T cells may play a role in cytolytic immunosurveillance for infected or transformed autologous cells .

Currently in humans, the V α genomic sequence of the gene segments is estimated to contain approximately 100 members and the V β pool approximately 70 members which can be assembled with D,J, and C regions. The number of permutations created in mature α and β chains by these events, and the association of the different α and β chains into mature heterodimers leads to a considerable potential for diversity. For V γ/δ TCRs, this potential for diversity also exists, but the pool of V γ genes is smaller.

Based on similarities at the nucleic acid level, the V β pool of gene segment has been grouped into 18 "families". Likewise, the V α pool of gene segments has been grouped into 19 "families" (Klein, *et al* .,1987; Toyonaga and Mak, 1987). Each family can contain between 1 and 10 members. Over

the last few years, antibodies to V region determinants of murine and human TCRs have been developed. Some of these antibodies appear to recognise all members of a V region family, some a subset of V regions within a family, and some a particular V region only. These reagents have proven valuable in studying the repertoire of TCRs expressed under many *in vivo* and *in vitro* conditions.

1.4.3.3 Vβ TCR region expression

The use of expanded populations of TILs for therapy (Section 1.5.7) appears promising in animal models of solid tumours and in human clinical trials of melanoma (Rosenberg *et al.*, 1989), although the TCR genes expressed in tumour infiltrating lymphocytes (TILs) are not yet known. Recent evidence from studies in autoimmunity (Zamvil *et al.*, 1985; Oksenborg *et al.*, 1990) and allograft rejection (Miceli and Finn, 1989) indicates that effector cells may utilize a very limited range of TCR genes. Restricted gene expression has been demonstrated in encephalitogenic T cells mediating the autoimmune disease, experimental allergic encephalomyelitis (Acha- Orbea *et al.*, 1988), in T cells in demyelinating plaques from brains of patients with multiple sclerosis (Oksenberg *et al.*, 1990), and on T cell populations on body surfaces such as skin, tongue, and vagina (Itohara *et al.*, 1990). It has been reported that oligoclonality exists for TCR expression in bulk cultures of TILs analysed by southern (DNA) blots (Belldegrun *et al.*, 1989). Studies have also been undertaken to investigate the Vβ product expression on chemically induced murine TIL containing tumours (Karpati *et al.*, 1991). It was found that the TILs expressed all of the measured Vβ's and that TILs cultured in IL-2 initially expressed multiple Vβ product, but rapidly restricted their Vβ expression, frequently expressing a dominant Vβ.

Populations of T cells expressing elevated levels of specific TCR V region epitopes (Section 1.4.3.2) have been identified in sarcoidosis (Moller *et al.*, 1988), Crohn's disease (Schmelkin *et al.*, 1989), cutaneous T cell lymphoma (Jack *et al.*, 1990) and also in human responses to "superantigens" (Kappler *et al.*, 1989). Superantigens were proposed by White *et al* (1989) to describe several structures that stimulate T lymphocytes by novel mechanisms (Figure 1.9). They are distinguished from antigens by the high frequency of responding T cells (approximately 5%- 25%); they are also distinguished from polyclonal T cell mitogens, such as concanavalin A (Con A), by the fact that the stimulus activates the T cell by binding a specific site in the variable portion of the β chain of the specific TCR. At present there are 2 main catagories of superantigens with closely similar properties: the toxin molecules produced by certain bacteria and recently characterised products of mouse MMTV virus (Choi *et al.*, 1991; Reviewed by Acha- Orbea, H., and Palmer, E.,1991). These toxins, including the staphylococcal toxins, do not stimulate all T cells, and each toxin reacts mouse or with human T cells bearing particular Vβ sequences as part of their receptors. It has been suggested that CTLs isolated from tumour draining lymph nodes of pancreatic cancer patients express the α/β heterodimer rather than the γ/δ receptor, which is commonly associated with MHC- unrestricted killing (Borst *et al.*, 1987). Further studies demonstrated that the specific epitope recognised by these CTLs was present on a mucin polypeptide core (Barnd *et al.*, 1989). Thus perhaps these mucins are being recognised as tumour- associated antigen and eliciting a CTL response, and because of the association with α/β TCR expression may be acting as a superantigen (Jerome *et al.*, 1991). Previous work analysing Vβ usage in numerous *in vivo* and *in vitro* systems has provided varying results. In a number of cases (Topalian and Rosenberg, 1990; Weber and Rosenberg, 1990; Posnett *et al.*, 1990; Moebius *et al.*, 1990) selective Vβ usage in response to antigen has been noted.

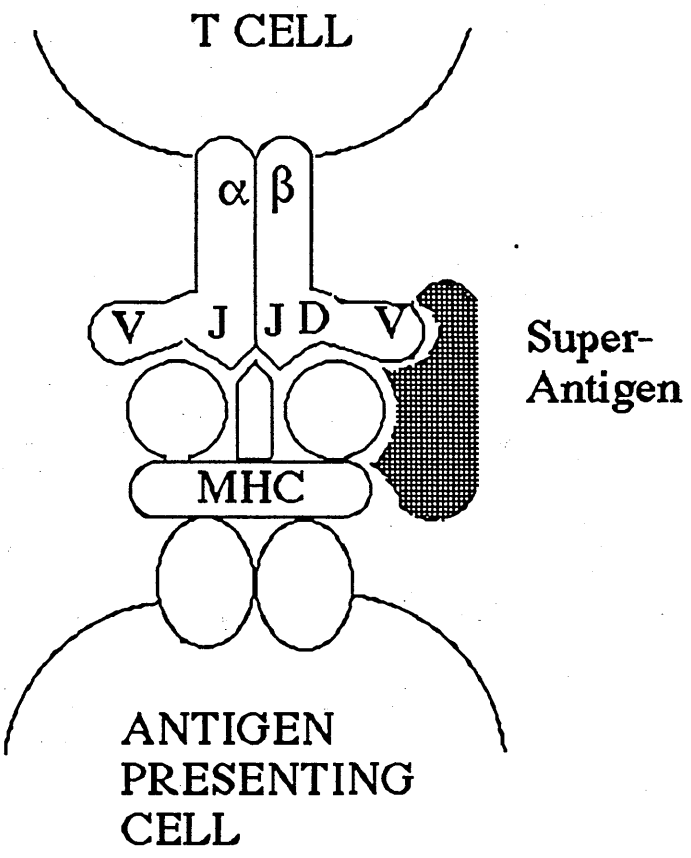


Figure 1.9: Schematic representation of the trimolecular complex of Superantigen- TCR Vβ - MHC Class II.

1.4.4 Antigen presentation

The TCR does not recognise free antigen but recognises small peptide fragments derived from proteins associated with products of the MHC. Fragmentation of viral, bacterial or parasitic polypeptides is carried out by proteases. The resulting fragments are associated with the MHC within the cell (Section 1.4.1) and the complex of peptide and MHC is displayed on the cell surface. MHC- restricted recognition of foreign antigen by T cells,

reflects the specificty of the TCR (Section 1.4.3) for the complex of the MHC molecules and antigen fragments. MHC molecules interact with processed antigen independently of the TCR engagement (Babbitt *et al*, 1985). A peptide of 7-10 residues may normally occupy the MHC Class I cleft (Van Bleek and Nathensen, 1990; Ratzschke *et al.*, 1990).

1.4.4.1 Class I MHC- antigen presentation

For MHC Class I (Section 1.4.1) it is likely that under physiological conditions most Class I molecules arrive at the cell surface complexed with processed antigen fragments. Evidence suggests that peptide charging occurs in the ER, and that peptide association is required for the physiological stabilisation of the Class I- β2- microglobulin complex and subsequent efficient transit out of the ER through the secretory pathway. There is evidence that in normal cells a small fraction of MHC Class I β2-microglobulin dimers do assemble and transit from the ER to the cell surface without peptide (Ljunggen *et al.*, 1990), and it is possible that 'empty' MHC Class I β2- microglobulin dimers might encounter cytosolic peptides in post-ER compartments during transit to the cell surface. The inefficient recognition of endosomally- processed antigen is due to the fact that most Class I molecules that come into contact with this processed antigen are already associated with ER- derived peptide. Newly synthesised MHC Class I molecules do not join with the endocytic pathway on the exit to the cell surface (Neefjes *et al.*, 1990). MHC molecules at the cell surface do not internalise and recycle through an endosomal compartment.

In the absence of peptide, the association of Class I heavy chain (Iα) and β2 microglobulin leads to an unstable complex, which is inefficiently transported out of the ER and empty Iα heavy chains may be degraded in the ER. In the presence of peptide, Iα and β2- microglobulin form stable complexes which are efficiently transported to the cell surface. At the cell

surface empty Iα–β2 microglobulin complexes may dissociate and isolated heavy chains may be degraded. At the cell surface the full complexes dissociate releasing the weakly bound peptides. Empty unstable Iα chains may be charged by exogenous peptide in the presence of exogenous β2-microglobulin and may form stable complexes (Braciale and Braciale 1991).

1.4.4.2 Class II MHC- antigen presentation

The structural constraints on MHC Class II (Section 1.4.1) molecule assembly, transport and stability appear to be less stringent that those of MHC Class I molecule assembly and transport. Cells lacking invariant chain assemble, transport and display normal levels of Class II αβ heterodimers. There is dispute over whether Class II can be charged with peptide in the ER. Nuchtern (1990) reported the contribution to the pool of MHC class II antigen complexes supplied by pre- Golgi compartment processing. Jaraquemada *et al* (1990) have also reported the presence of an endosomal route of antigen presentation. When a gene encoding a pre-processed antigen site recognised by both CD4 and CD8 T cells existing in the form of exogenously added peptide was expressed in the cell cytoplasm, it was found only to charge Class I molecules (Sweetser *et al.*,1989). Thus perhaps Class I, and not Class II molecules appear to be efficiently charged in the ER. Braciale *et al* (1990) have since proposed that newly synthesised glycoprotein antigens must enter a post- Golgi compartment in order to be processed/ presented in association with MHC Class II molecules.

The method of antigen presentation for Class II - antigen presentation may be as follows. The MHC Class IIα (IIα) and Class IIβ (IIβ) assemble in the ER along with the invariant chain (Ii) to form a stable trimolecular complex. Ii is thought to inhibit binding of peptide. Ii is then proteolytically cleaved when the complex is shuttled to the endosome. Loss of a portion of Ii may lead to peptide binding. Binding of peptide may lead to further cleavage

of Ii resulting in exit of the peptide IIα- IIβ complex to the cell surface. IIα and IIβ complexes can assemble in the ER in the absence of Ii and transit to the cell surface.

1.4.5 Interleukin 2 Receptor

The idea that immune responsiveness and immune memory are influenced by interleukins was first introduced when the T cell growth factor, interleukin 2 (IL-2) (Gillis *et al.*, 1978), was found to bind with high affinity to sites expressed only on antigen- or lectin- activated T lymphocytes (Robb *et al.*, 1981).When cells are stimulated with a mitogenic lectin such as Con A, IL-2 appears in the culture medium during the first 24-48hrs but then declines rapidly, so that by 96 hr very low levels of activity are detectable. The decline in activity seemed to be due to some cellular consumption (Gillis, *et al.*, 1978; Baker, *et al.*, 1978). Confirmation of a cell associated removal of IL-2 from the culture medium was obtained when experiments were carried out in which the concentration of IL-2 was found to decrease progressively as the cell concentration increased (Smith, 1980). It has also been shown that only antigen/ lectin -activated T- cells are capable of absorbing IL-2 activity (Smith *et al.*, 1979), as lipopolysaccharide (LPS)- activated spleen cells, which are primarily proliferating B cells , absorb no IL-2 activity. The experiments that were carried out indicated that the disappearance of IL-2 was via its binding to cell surface receptors.

Radiolabelled IL-2 was first used to explore its binding parameters to various target cells. IL-2 seems to bind to a single mass of high affinity sites expressed on antigen- and lectin activated T cells (Robb, *et al.*, 1981), and there was no difference when binding to whole cells is compared to binding to isolated plasma membranes (Smith, 1983). The availability of cloned IL-2-dependent cytolytic T lymphocyte lines (CTLL) (Baker *et al.*, 1979), made it possible to compare the concentrations of IL-2 responsible for promoting T

cell proliferation with those found to bind to high affinity IL-2 receptors. It was found that there was a linear relationship between occupancy of high affinity IL-2 receptors and the concentration of ligand required to effect a half-maximal response (EC 50). A maximal T cell growth response occurs at a concentration of IL-2 that yields 100% receptor occupancy, which indicates that there are no spare receptors.

The picture of the IL-2 receptor is however more complicated than this. A monoclonal antibody was found that was reactive with activated T cells (anti-Tac), and inhibited both radiolabeled IL-2 binding and IL-2 dependent T cell proliferation (Leonard, *et al.*, 1982). This antibody recognised about 10-20 fold more binding sites per cell than could be enumerated by IL-2 binding. A second class of IL-2 binding site was revealed by using 100- fold higher IL-2 concentrations, and this site was found to bind both the antibody and IL-2 (Robb *et al.*, 1984). However this second class of binding sites was characterized by a 1000- fold lower binding than had been detected previously. It was found that a glycoprotein of Mr 55,000 binds IL-2 with a low affinity only (Sabe *et al.*, 1984), and the expression of two IL-2 binding proteins is required to form a high affinity IL-2 receptor (Tsudo *et al.*, 1986; Teshigawara *et al.*, 1987)). Thus high affinity IL-2 receptors are constructed by cooperative binding of IL-2 to both the low affinity (p55 chain) and intermediate affinity (p75 chain) binding sites (Figure 1.10).

Studies into the kinetic binding constants were performed in order to establish the method by which high affinity IL-2 receptors are formed from two distinct lower affinity binding sites (Wang and Smith, 1987)) It was found that IL-2 binds to and dissociates from p55 chains so rapidly that it is difficult to measure (half- times for dissociation $t_{1/2} = 2$ seconds) compared to longer dissociation times for p75 and p55/p75.The combination of a rapid

"on" rate and a slow "off" rate for the high affinity receptor makes for an affinity 100-1000-fold greater than IL-2 binding to either chain alone.

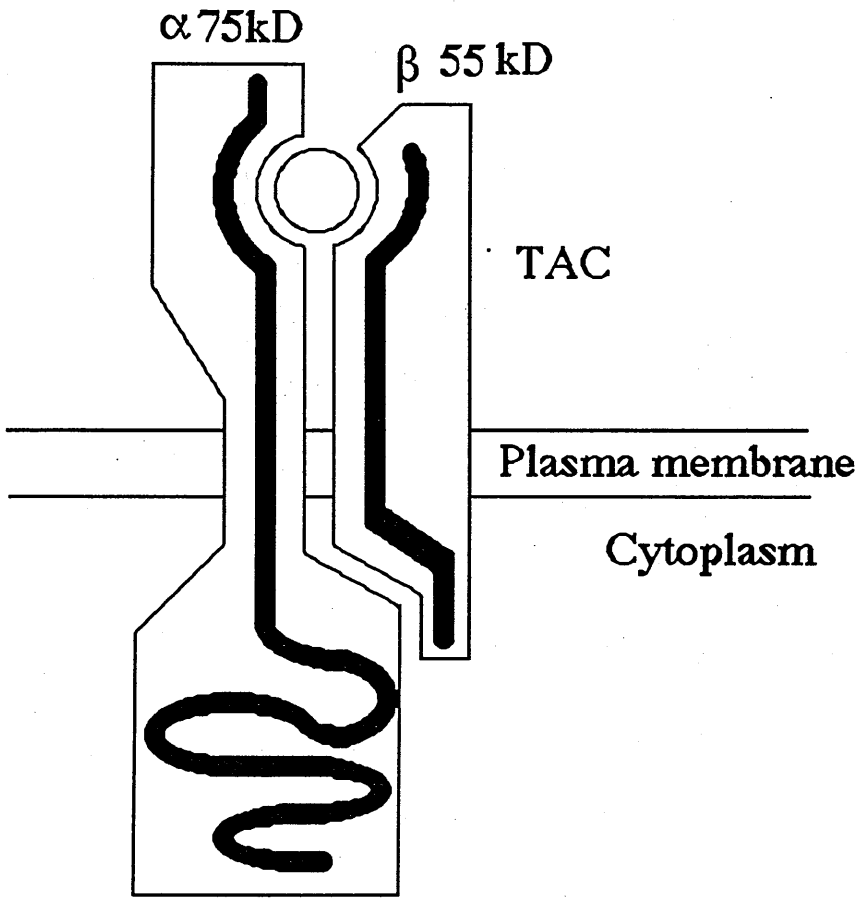


Figure 1.10: Schematic representation of the IL-2 receptor components

1.4.6 Adhesion molecules

The interaction of lymphocytes with other cell types is critical for immune function and provides excellent opportunities to study the cell biology of dynamic cell- cell and cell- extracellular matrix interactions. Lymphocytes rapidly interconvert between a nonadherent state in the circulation and an adherent and highly motile state in lymphoid and other tissues. Mechanisms of lymphocytic adhesion may have a dual function: to provide a foothold for

cell interactions and migration, and also to transmit information across the cell membrane. Rapid transition between adherent and non- adherent states is of key importance to the dual function of immune surveillance and responsiveness.

Three families of adhesion receptors mediate these interactions:

(1)the immunoglobulin superfamily, which include the antigen- specific receptors of T and B lymphocytes (Section 1.4.3),

(2)the integrin family, which is important in dynamic regulation of adhesion and migration, and

(3)the selectins which are prominent in lymphocyte and neutrophil interaction with vascular endothelium.

Among the cell- cell interactions relevant to this thesis are the interaction between 2 specific molecules: namely Lymphocyte function associated antigen (LFA-1), a member of the integrin family, and its counter receptor on the target cell intercellular adhesion molecule (ICAM-1) (Figure 1.11).

1.4.6.1 Structure of LFA-1

LFA-1, like all members of its extended family, the integrins, is a heterodimer of two non- covalently associated transmembrane proteins. It has a unique α chain (CD11a) and a β chain (CD18) (Figure 1.11) which also constitutes part of the related molecules MAC-1 (CD11b) and p150.95 (CD11c). Both the CD18 and CD11a chains are homologous to the chains of other integrins and share their distinctive features such as binding sites for divalent cations, including Ca^{2+}. The most important difference is the inclusion in the CD11a, CD11b and CD11c chains of an 'inserted (1)-domain' of about 200 amino acids near the amino terminus. Sites in this 1-domain might function as binding sites in cell- cell adhesion. The LFA-1

molecule is widely expressed by cells of haematopoietic origin and is used in a variety of lymphocyte, monocyte, natural killer and granulocyte interactions with other cells (Springer *et al.*, 1987; Mattz, 1987).

1.4.6.2 Structure of ICAM-1 (CD54)

ICAM-1 is an integral membrane glycoprotein with 5 immunoglobulin- like domains (Staunton *et al.*, 1988), (Figure 1.11) and particularly strong homology to two immunoglobulin super gene family proteins important in neural cell adhesion: myelin associated glycoprotein (MAG) and neural cell adhesion molecule (NCAM). The possibility that carbohydrate may play a role in ICAM-1 function is highlighted by the 7 possible N- linked oligosaccharides (at least Mr 20,000) and variations in glycosylation between different cell types.

Because it is an important ligand for LFA-1, ICAM-1 is fundamental to many immunologic reactions including antigen- specific recognition and lysis of certain target cells (Makgoba, *et al.*, 1988). ICAM- 1 has also been shown to be associated with the CD25 (Molecular weight 55,000) chain of the IL-2R (Ediden, *et al.*, 1988) (Section 1.4.5) and is identical to a melanoma-associated antigen (Holzmann, *et al.*, 1988; Vogetseder, *et al.*, 1989; Matsui, *et al.*, 1988) P3.58 (Section 1.5.5)

Inflammatory mediators, including LPS, γ - interferon (γ-INF), IL-1 and tumour necrosis factor- α (TNF- α) cause strong induction of ICAM-1 in a wide variety of tissues and greatly increase binding of lymphocytes and monocytes through their surface LFA-1 (Dustin and Springer, 1988). Endothelial, fibroblastic and epithelial cells vary as to which cytokines are capable of inducing ICAM-1 expression, and the types of mediators released may therefore help regulate differing patterns of cell localisation induced by inflammatory stimuli.

1.4.6.3 Regulation of ICAM-1 expression

ICAM-1 is absent from most cells in normal, non lymphoid tissues, except for expression of low levels on endothelial cells. Local immune responses result in a rapid increase in ICAM-1 expression on endothelial cells and induction of ICAM-1 on epithelial cells (Wantzin *et al.*, 1989; Munro *et al.*, 1989). The increase of ICAM-1 expression on malignant melanomas and carcinomas may be secondary to local immune reactions generating cytokines (Temponi *et al.*, 1989).

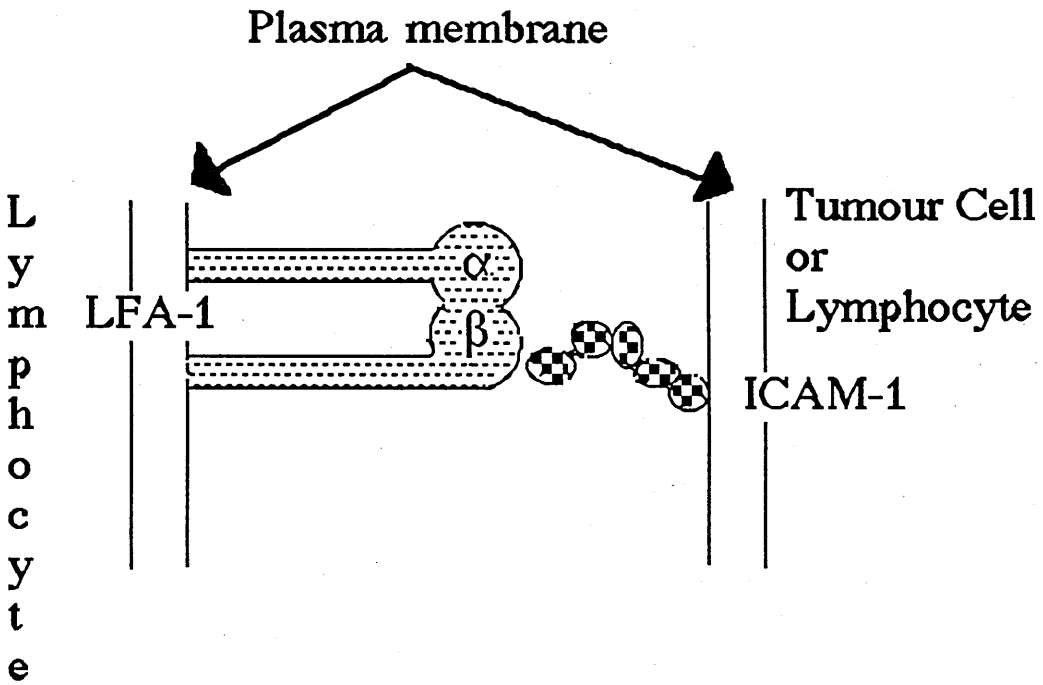


Figure 1.11: Diagram illustrating the interactions between ICAM-1 and LFA-1.

1.5. TUMOUR IMMUNOLOGY

1.5.1 Immunosurveillance theory

This theory states that malignancies express foreign determinants that can be potential targets of the immune system. (Burnet, 1964). T cell mediated immunity is thought to be present to eliminate cells as they arise due to the expression of novel epitopes. These new epitopes are consequently recognised as foreign. Lines of evidence available to support this theory include the increased occurence of malignant tumours in childhood and in old age. This can be explained by the fact that the immune system may not be fully functional at these times. It was found that due to the use of immunosuppressive drugs in transplantation studies in the 1960s, the incidence of malignancy, although only involving a few types of cancer, was found to be higher in immunosuppressed patients. Recently the increased incidence of pathological immunosuppression associated with the human immunodeficiency virus (HIV) has also suggested a relationship between immunosuppression and the development of certain kinds of malignant disease.

There are two main criticisms of the theory:

(1) The first concerns the existence of tumour- specific antigens in human malignancy (Section 1.5.2). Studies in animal models have indicated that spontaneously arising tumours caused in the absence of known carcinogens display no detectable immunogenicity (Foley, 1953; Prehn, 1975). Hewitt (1976) also reported that spontaneously arising tumours when transplanted into syngeneic hosts elicit no detectable immunity. Because transplantation experiments cannot be performed on humans it is not known if human tumours express tumour- specific antigens analogous to experimentally induced animal tumours.

It must be noted that epidemiological evidence clearly shows that the majority of human cancers are induced by physical and chemical carcinogens (Doll, 1980), including benz-α-pyrene in tobacco smoke, and radiation. With some viruses such as hepatitis B virus , the association is direct where as others it is indirect, for example HIV and Kaposi's sarcoma in AIDS patients,where the pathological immunodeficiency caused by the virus probably permits the reactivation of a latent cytomegalovirus infection.

(2) The second involves immunopotentiation of autologous tumour growth during carcinogenesis *in vivo*. Reduced latency of a tumour can be induced by immunostimulation (Prehn, 1977) , thus enhancing tumour growth. This is not predicted by the immunosurveillance theory. Tumours with higher immunogenicity can have shorter latencies (Prehn and Bartlett, 1987).
Two mechanisms have been used to explain this phenomenon:

(a) T h cell subsets may stimulate tumours, followed by local release of lymphokines, promoting tumour cell growth.

(b) Certain antigenic epitopes on tumours might stimulate immunosuppression or tolerance in tumour- bearing hosts.

Consequently immune responses to tumours may not be deleterious toward the neoplastic disease development .

1.5.2 Tumour antigens

An immune response will be elicited in an immunocompetent host by any cell component that is new to the system. When a cell undergoes transformation it expresses molecules which, when recognised immunologically, not necessarily by the host of the tumour, are referred to as tumour- associated antigens. In theory these may be located within the cell or on the surface. The former is not a well studied idea since they are isolated

from any specific antibody or cell-mediated reaction, by the cell membrane. Alternatively the expression of cell surface antigens may elicit some immune reaction, and it is with membrane- bound antigens that tumour immunology is principally concerned.

A method of classifying tumour associated antigens was originally introduced whereby antigens expressed solely by a single tumour were originally catagorised as class 1, those expressed by the same tumour type in different individuals as class 2, and those expressed by a wide range of normal and malignant cells, not necessarily of the human species, as class 3. (Old, 1981)

There is much inter- and intra- tumour heterogeneity (Miller,1982; Natali *et al.*, 1983; Heppner, 1984; Brstryn, 1985), which has obvious significance in the immunological diagnosis and therapy of cancer.

1.5.3.Melanoma associated antigens

Human cutaneous malignant melanoma is one of the best studied solid tumours. Specific Melanoma Associated Antigens (MAA) were originally identified on the surface of melanoma cells (Koprowski *et al.*, 1978). Various immunologically and biologically distinct antigenic structures have been identified along with a variety of less well defined antigens . Examples of these are reviewed by Herlyn and Koprowski (1988). Extracellular matrix proteins such as fibronectin are secreted in large quantities by melanoma cells but are not expressed on the cell surfaces (Herlyn *et al.*, 1987). These extracellular proteins may be involved in adhesion (Section 1.4.6), motility and invasion of the tumours. Gangliosides and high -molecular -weight oncofeotal proteins are expressed on the cell surface and are shed. Cell surface receptors for growth factors and intracellular and extracellular cation transport and binding proteins are involved in the intracellular transport of biologically active material. Histocompatibility antigens (Section 1.4.1) are

expressed and also shed by melanoma cells and they play a part in immune recognition. HLA DR is reported to be expressed on 75% of cultured melanoma lines (Reviewed by Taramelli *et al.*, 1986) and primary tumour cell lesions expressing HLA DR are reported to have high metastatic potential, independent of tumour thickness (Brocker *et al.*, 1985).

Several antigens on melanoma cells are associated with differentiation. Houghton *et al* (1986) and Real *et al* (1985) have proposed a differentiation pathway in melanoma based on morphological and antigenic studies of cultured melanocytes and metastatic melanoma cells.

1.5.4. MHC expression in malignancy

There is a vast literature covering the expression of HLA-A,B,C and D antigens (Section 1.4.1) in human malignancy (Doherty *et al.*, 1984; Tanaka *et al.*, 1988; Bernards, 1987; Hammerling *et al.*, 1987). Many studies have been performed using frozen sections stained by immunofluorescence and immunoperoxidase.

Analysis of MHC levels on certain tumours has suggested a decrease of MHC expression compared to normal tissues. Examples include infiltrating ductal carcinomas (Natali *et al* ., 1986), basal cell carcinomas (Turbitt and Mackie, 1981), colorectal carcinomas (Van den Ingh *et al.*,1987). Loss of Class I has been interpreted as the result of tumours escaping from T- cell immune attack, since a cell that does not express Class I determinants is protected from cytotoxic T- cell attack (Bodmer, 1987). There is a high frequency of individual Class I allele loss, most notably for HLA-A2 (Smith *et al.*,1989a). Perhaps loss of one individual allele may lead to a tumour having a major advantage in escaping from a particular T- cell immune response.

Locus- specific Class II (DR, DP, and DQ) molecules can be readily distinguished by monoclonal antibodies against non- polymorphic

determinants. In certain cases, such as colorectal carcinomas (Momburg *et al.*, 1986) and lung carcinomas (Natali *et al.*,1986), *de novo* expression of Class II MHC antigens was observed. An extensive study of primary and metastatic melanomas cells was performed by Van Vreeswijk *et al*, (1988) who found differential expression of DR,DP, and DQ molecules in primary metastatic disease. HLA-DR and DP antigens were expressed in a higher percentage of metastatic than primary melanomas and there was no marked difference in HLA DQ antigens (Van Duinen *et al.*, 1988). Changes in the inducibility of HLA- class II determinants may be interpreted as the result of escape from T-cell immune recognition, either in connection with antigen presentation or Class II restricted cytotoxicity (Smith *et al.*, 1989b). These changes in HLA expression identify those tumours which have definitely been subjected to T-cell attack, and thus express novel determinants that can be recognised by T cells.

Thus there are some changes in expression of MHC antigens in some malignancies, but there is no defined correlation between MHC expression on malignant transformed cells.

1.5.5 Theories on invasion and metastasis

Metastatic tumour cells (Section 1.2.3) disseminating through the blood stream must penetrate capillary basement membranes as they enter and exit the vascular compartment. However, this extracellular matrix (ECM) represents a significant mechanical barrier to invasion.

There is a 3 step hypothesis for invasion of ECM (Figure 1.12):

(1) The tumour cell attaches to the ECM through cell surface receptors that bind to specific adhesion molecules (Section 1.4.6) in the matrix, such as laminin, Type IV collagen, heparin sulphate, proteoglycan and fibronectin (Black and Horowitz, 1987; Hynes, 1987; Ruoslahti, 1988).

(2) Next the ECM is locally digested by tumour cell associated or host associated hydrolases.

(3) Finally, the tumour cell migrates by active locomotion into the newly created void and forms new attachment sites.

The cycle is then repeated until the tumour cell has penetrated the entire thickness of the basement membrane.

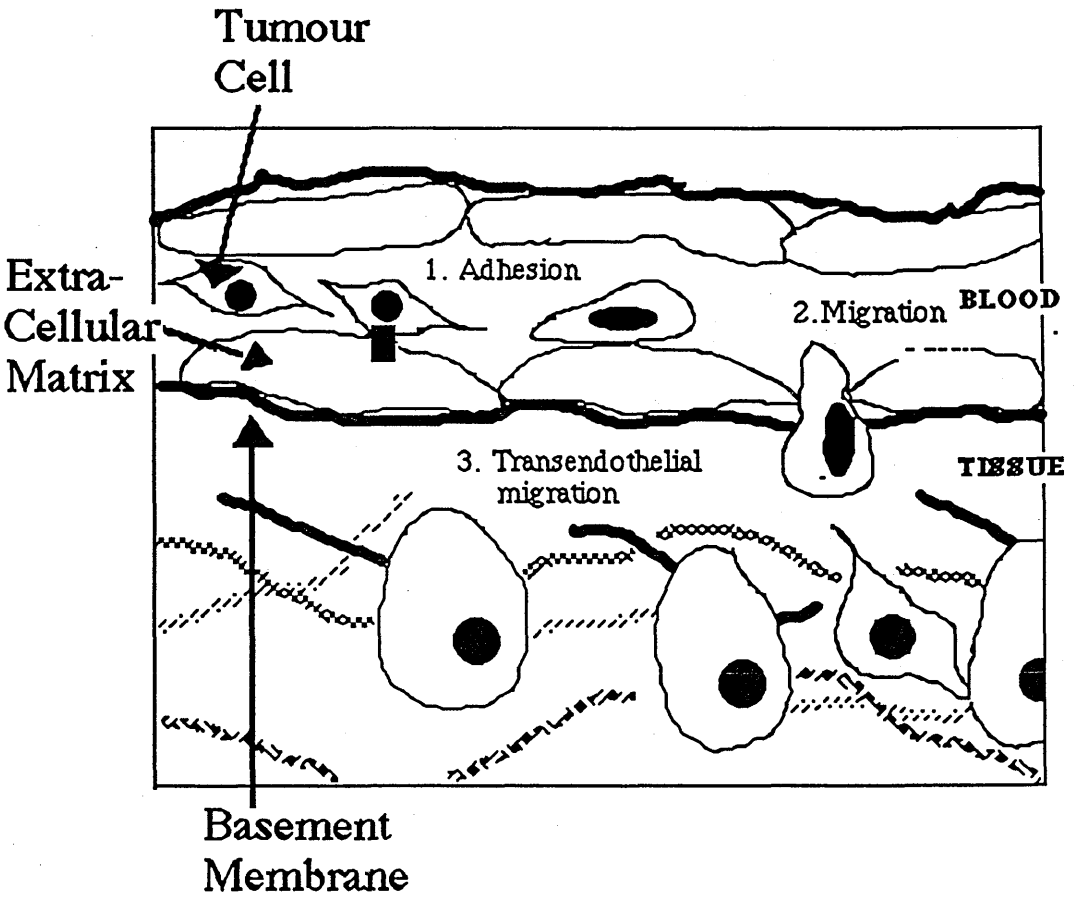


Figure 1.12: Diagram illustrating the invasion of a metastsising tumour cell from the blood stream to the target tissue.

Immunohistochemical analysis of *in situ* phenotypic changes correlating with tumour progression in human cutaneous melanoma has led to the identification of molecules potentially involved in metastasis (Holzmann, *et al.*, 1987). One of these is the melanoma associated antigen P3.58. It is not detectable in quiescent melanocytes and only sporadically found in proliferating benign melanocytic lesions. On melanomas, the frequency with which this antigen is found increases with increasing tumour thickness. This P3.58 is found to be identical with ICAM-1 (Section 1.4.6).

It has been proposed by Johnson *et al* (1989) that the *de novo* expression of ICAM-1 by melanomas leads to heterotypic adhesion between melanoma cells and LFA-1 bearing lymphocytes and thereby causes the dissemination of cells from the primary tumour (Section 1.4.6)

The role of the mononuclear infiltrate in melanomas may be two sided:
(1) Infiltrating leucocytes have been shown to include cells specifically capable of killing tumour cells *in vitro* (Anachini, 1987).
However,
(2) By regulating gene expression in the tumour cells, the mononuclear cell infitrate may also play a direct role in the acquisition that contributes to the progression of the tumour toward metastatic disease (Johnson *et al.*, 1989)

1.5.6 TILs as a prognostic indicator

The immunological role of lymphocytes and plasma cells in malignant neoplasms has been extensively studied in the past 2 decades. Speculations have been made on whether or not the presence of these mononuclear cells in tumours have an effect on patient survival.

Much work has been carried out to identify and characterise the presence of these tumour infiltrating lymphocytes (TILs) within malignant

neoplasms. Such neoplasms include cutaneous malignant melanoma, lung carcinoma, breast carcinomas, lung cancer and medullary carcinomas. The presence of these TILs has been suggested to represent the cancer patient mounting an immune response to their tumours. This was suggested due to early studies carried out by Handley (1907) who reported the spontaneous regression of malignant skin melanomata that contained a "round cell infiltrate" and Da Fano (1912) who used animal models to illustrate that this infiltration might represent the host's tumour defence responding to the tumour. A better prognosis has been associated with lymphocytic infiltration in gastric cancers (MacCartey and Mahle, 1921), breast cancer (Sistrunk, and MacCartey, 1922), colon cancer (MacCartey, 1922) and medullary carcinoma (Moore and Foote, 1949; Cutler and Connelly, 1969; Bloom *et al.*, 1970; Ridolfi *et al.*, 1977). The positive correlation between the presence of infiltrate and prognosis illustrated in some of these early studies was rather weak, but interest into the presence of these TILs lasted with some contradictory consequences. In the case of breast carcinoma there have been reports indicating an improved survival rate due to inflammatory infiltrate (Ridolfi *et al.*, 1977; Black *et al.*, 1975; Underwood, 1974), but there also exists reports that the infiltrate is associated with a poor prognosis (Rosen *et al.*, 1981; Fisher *et al.*, 1983).

This controversy also extends into the field of not only malignant cutaneous melanoma but also malignant uveal melanoma. Some workers (Balch *et al.*,1978; McGovern *et al.*, 1981 and Bernengo, 1983) found that in malignant cutaneous melanoma there was a poor prognosis associated with the presence of lymphocytes. Lang *et al* (1977) also found that in malignant uveal melanoma that survival was better if the tumour contained lymphocytes. De La Cruz *et al* (1990) carried out a retrospective study of 1193 uveal melanomas, and performed histological examination to identify the presence of lymphocytes . It was found that 12.4% contained more that 100

lymphocytes per 20 high power fields (HPF) and this was subsequently identified as the "high" lymphocytic infiltration group. The remaining were classified as containing "low" levels of lymphocytes. When these patients survival rates were followed through it was found that after 15 years 36.5% of the high lymphocyte containing group were alive, but 69.9% of the low lymphocyte group were still living. Thus it was concluded that an increase of lymphocytes per 20 HPF was significantly associated with decreased survival.

1.5.7 Adoptive immunotherapy of cancer

Alone or together, surgery, radiation and chemotherapy can cure or delay cancer in many of the people in whom it develops. However the incidence of cancer, and thus the number of deaths, remains high.

Recent strategies of immunotherapy of cancer patients involve what is termed as adoptive immunotherapy, or cell- transfer therapy. Lymphocytes are removed from a cancer patient and their native ability to kill tumour cells is enhanced by subsequent procedures. The cells are then returned to the patient's blood stream.

This adoptive immunotherapy has been studied by Rosenberg who since the late 1960's has studied over 700 patients with advanced cancer using variations of these methods.

Noting the presence of lymphocytes that invade a high number of tumours, Rosenberg studied methods of removing and expanding these lymphocytes that might have activity against the relevant tumour. The discovery of Interleukin 2 (IL-2) (Gillis *et al.*, 1978)) (Section 1.4.5) and the introduction of methods of growing abundant quantities of T cells in culture with this cytokine provided a new approach. It was found that incubation of unseparated lymphocytes from a variety of sources, including peripheral blood lymphocytes (PBLs), spleen cells, thoracic duct lymphocytes in

recombinant IL-2 for 3-4 days, generated cells referred to as lymphokine activated killer cells (LAK). These cells were capable of *in vitro* lysis of a variety of autologous and allogeneic fresh tumour targets but did not lyse normal tissue (Raynor *et al.*, 1985). LAK cells are non- cytotoxic T cells and are derived from the "null" population that constitutes only 5% of the circulating lymphocytes. They are capable of mediating non- MHC restricted cytotoxicity (Grimm *et al.*, 1985). These positive effector cells are CD3 negative (Section 1.4.3), CD16 negative , and derived from large granular lymphocytes or natural killer cells, together with a minor population of CD3 positive, CD16 positive/ negative cells also possessing MHC unrestricted cytotoxicity (Ortaldo *et al.*, 1986).

When adoptively transferred in conjunction with rIL-2, LAK cells were shown to mediate regression of established pulmonary and hepatic metastases in a variety of murine models (Mule *et al*, 1984), and were also thought to be effective in reducing metastatic tumours in clinical trials (Rosenberg *et al*, 1986). However, it was decided that the result showed limited antitumour activity in patients with malignant melanoma, renal cell and colorectal carcinoma, giving clinical responses rates of 35%, 21% and 17% respectively (Rosenberg *et al.*, 1989)

In an attempt to improve the clinical response of cancer patients to immunotherapy, lymphocytes isolated from the tumour mass were assessed for the ability to promote tumour progression. Preliminary studies in murine systems indicated that these cells were 50- 100 times more effective than LAK cells in mediating tumour regression (Rosenberg, 1986). This methodology was then applied to the human system whereby TILs were obtained by surgically removing a tumour nodule from patients resulting in about 50×10^6 tumour cells which then were cultured in rhIL-2 until the tumour cells regressed, giving extensive expansion of the TILs. Approximately 200×10^9 of these cells were then re- administered intravenously, along with rhIL-2

(Topalian *et al.*, 1987) TILs were successfully expanded on an experimental scale from 24 of 25 consecutive human tumours including 6 melanomas, 10 sarcomas and 8 adenocarcinomas. In a clinical trial started in 1988, there was a 55% "response rate", twice as good than with LAK therapy.

Immunotherapy based on the administration of LAK cells plus IL-2 or in some cases IL-2 alone, can however produce some side- effects. The proliferation of lymphocytes in tissues can interfere with the function of vital organs. The administration of high doses of rhIL-2 leads to leakage of fluid from the blood into tissues, and weight gain from the fluid is common. Less commonly, the accumulating fluid impairs lung function and thereby impedes the delivery of oxygen to tissues. It is claimed that the mortality due to this treatment to be approximately 1% and in the remaining 99% the side effects disappear rapidly once treatment is completed.

Methods to optimise this treatment have been undertaken so as to define the *in vivo* distribution and survival of TILs. This involves using retroviral-mediated gene transduction to introduce the gene coding for resistance to neomycin into human TILs before their infusion into patients (Rosenberg *et al.*, 1990). With the inserted gene as a stable component of the TIL genome, these cells and their offspring can be identified in long term studies even if they represent a tiny fraction of the total number of cells present. Using the polymerase chain reaction , gene modified cells were found to remain in the circulation of all the patients for 3 weeks and for as long as 2 months in 2 of 5 patients. Cells were recovered from further tumour growth as long as 64 days after the initial administration.

This particular study indicated the feasibility of using retroviral gene transduction for human gene therapy and there are consequent implications for

the design of TIL therapy with improved antitumour potency. Among the candidates are the gene for TNF and INF-α or perhaps for IL-2 itself.

1.6 GENETIC FACTORS IN MALIGNANCY

1.6.1 Oncogenes

Although it has long since been proposed that cancer is a disease associated with fundamental alterations of the cellular genome. However it is only within the last decade that it has been possible to identify growth regulating genes participating in the transformation process (reviewed by Spandidos and Anderson, 1989). It appears that 2 classes of genes can be involved: (a) genetic elements which act dominantly to convert normal cells to transformed or malignant cells, termed oncogenes, (b) suppressor oncogenes which physiologically inhibit cell growth by inhibiting any of the cancer related phenotypes of the cell. Mutation leading to loss of function of these leads to loss of control. Such genes are termed "anti- oncogenes" and characterised by "loss of heterozygosity" in DNA blots.

Oncogenes are derived by genetic damage to proto-oncogenes which are normal constituents of cellular DNA. More than 59 oncogenes have been isolated and encode proteins with diverse functions. Some oncogene products are structurally similar to secreted growth factors such as platelet derived growth factor. Oncogene activation may lead to the production of growth factors by the tumours itself, for example TGF α or β. Other oncoproteins are growth factor membrane receptors. *Ras, src, fps* and *abl* proteins are examples of oncopeptides located on the internal cytoplasmic membrane and are thought to act in a mitogenic pathway in the cytoplasm. The proto-oncogene *myc* has been implicated in the pathogenesis of a wide range of neoplasms including breast, lung, stomach, colon carcinomas; leukaemias; neuroblastomas and glioblastomas. The biochemical function of *myc* remains unclear (Cole, 1986; Eisenman, 1989). A number of studies have suggested

that *myc* might regulate transcription (Schweinfest *et al.*, 1988), whereas others have suggested the role of *myc* in DNA replication (Hann *et al.*, 1988). This oncogene encodes a nuclear protein which shares sequence homology with a region of the adenovirus EIA protein and the simian virus SV 40 T antigen that is necessary for the interaction of the retinoblastoma locus, Rb (Figge *et al.*, 1988).

Suppressor genes contribute to transformation only if both alleles are mutationally and functionally inactivated. This can be demonstrated in hereditary retinoblastoma which results from the loss of both alleles of the retinoblastoma gene (Rb-1) (Lee *et al.*, 1987). Studies involving the adenovirus 2 EIA protein have suggested that the Rb-1 protein is a member of a signalling pathway that allows the cell to respond to environmental conditions, since it was found that EIA binds to several host proteins one of which was identified as the Rb-1 gene (Whyte *et al.*, 1988). People with hereditary predisposition to retinoblastoma have a much increased risk of developing other non- ocular primary cancers such as osteosarcoma (Abramson,1984). It was found that as with retinoblastoma, osteosarcoma also involves loss of heterozygosity of a gene on chromosome 13 that includes the Rb-1 locus (Hansen, *et al.*, 1985). Thus, this Rb-1 gene may be more than merely a suppressor of retinoblastoma.

Although there have been several reports about the role of oncogenes in the pathogenesis of melanoma, virtually all such work has been performed on cutaneous melanoma (Albino, 1988; Albino *et al.*, 1984; Price *et al.*,.,1989). The *ras* oncogene has been implicated in approximately 20% of cutaneous melanomas, and aberrations have been identified in chromosomes 1,6 and 7. The majority of mutations are found on the N- *ras* gene located on chromosome 1 (Bos, 1989). *Ras* gene products are known to comprise a family of proteins (Mr 21,000) designated as p21 *ras*. Due to the structural and functional resemblance of the *ras* proteins to the G-proteins controlling

adenylate cyclase it has been proposed that in the normal cell, p21 *ras* proteins are involved in the transduction of external stimuli, most likely induced by growth factors. After transducing the signal to an effector molecule, normal *ras* proteins become inactivated. Thus mutated *ras* proteins lose the ability to become inactivated and growth and differentiation occur in an uncontrolled manner (Bos, 1989).

Another suppressor gene which has been found in association with colorectal carcinomas (Baker *et al.*, 1989), lung cancer (Takahashi *et al.*,1989), brain (James *et al.*, 1989) and breast tumours (Mackay *et al.*, 1988) is the p53 gene located on the short arm of chromosome 17 (Reviewed by Levine *et al.*,1991).

It has been realised that colon cancer, apparently develops by a stepwise accumulation of several mutations including *ras* gene mutations, loss of chromosome 18 involving the DCC gene (deleted in colonic carcinomas gene) and the previously mentioned suppressor gene p53. It has been reported (Kinzler and Vogelstein, 1991) that loss of a chromosome 5 gene seems to appear early in development of colonic cancer, with the normal gene product having tumour suppressive effects functioning via a cytoplasmic signalling pathway involving interaction with a G protein.

It is now apparent that some of the changes that are part of the progression of a tumour toward malignancy give rise to mutations in proteins that are potential targets for the immune system, even when they are internal cellular products such as p53 and *ras*. As mentioned previously (Section 1.5.4) some tumour cells can lack HLA Class I and thus are no longer susceptible to immune T cell cytotoxicity. If a tumour is thought to have lost expression of one or more HLA Class I determinants, this is suggested to be evidence that the tumour has been subjected to immune T cell attack and carries a genetic change that has been recognised by immune T cell (reviewed by Bodmer, 1991). This genetic change may involve such oncogenes or

tumour suppressor genes as *ras* and p53 and these may be used as potential targets for immunodiagnosis.

The role of oncogenes and suppressor genes in the development of uveal melanomas is completely unknown, although, there is evidence for a role of immune surveillance in the control of growth of uveal melanomas (Section 1.5.1). The malignant transformation of cutaneous melanocytes and further neoplastic progression has been shown to be inversely related to the levels of HLA Class 1 antigen on their surface and the amount of Class 1 antigen on the cell surface has been found to be controlled by the c-*fos* proto- oncogene (Feldman, 1988; D' Alessandro *et al.*, 1987). The oncogene c- *myc* has also been implicated in the down regulation of HLA class 1 antigens in cutaneous melanoma (Versloog, 1988).

The involvement of oncogenes in carcinogenesis is well established, but it is only fairly recently that the importance of onco- suppressor genes in this process has been appreciated. The limiting factor at present has been the lack of appropriate systems for identification of as yet unknown oncogenes.and tumour suppressor elements.

1.6.2 Ploidy analysis

1.6.2.1 Cell cycle analysis as an indicator for prognosis.

DNA cell cycle analysis of primary tumour cells gives information on both the nuclear DNA content (DNA ploidy), which is frequently abnormal in major subsets of cells within primary tumours, and the G2/M+S phase fractions of the cell cycle (Figure 1.13), which are considered to be a measure of the proliferative rate of a growing cell population. A number of studies have now suggested that DNA ploidy and cell cycle analysis are of prognostic relevance and predictive value in patients with epithelial tumours of the breast

and colon. Aneuploidy has also been reported in bladder cancer (Murphy *et al.*, 1986; Gustafson *et al.*, 1982) and in stem cell malignancies such as ovarian tumours (Iversen and Skaarland, 1987; Friedlander *et al.*, 1984). Aneuploidy has also been found in pre- malignant disease such as chronic atrophic gastritis (Teodori *et al.*, 1984), and pre- malignant skin lesions such as solar keratoses,(Newton *et al.*, 1987). Normal or reactive tissue is not associated with DNA aneuploidy (Barlogie *et al.*, 1980) and thus DNA aneuploidy may be held to be implicit of neoplasia. Within this group, by far the most extensively studied is breast cancer where 65-74% of excised tumours contain a substantial aneuploid population and very large numbers (>1000 in some studies) of patient samples have been available for statistically relevant analysis. The aneuploid profile of breast tumours differs between any two patients, but is generally similar between primary and secondary growths within the same patient (Auer *et al* 1980a,b; Remvikos *et al.*, 1988). Studies on both recurrences and overall survival, have suggested that patients with diploid, low S-phase tumour DNA profiles have a good prognosis (Eskelinen *et al.*, 1989; Clark *et al.*, 1989; Toikkanen *et al.*, 1989; Cornelisse *et al.*, 1987; Hedley *et al.*,1987; Kallioniemi *et al.*, 1988; Olsz *et al* .1981; Moran *et al.*, 1984). Similar data have been presented for colon carcinoma (Armitage *et al.*, 1985).

Tumour ploidy cannot be used to differentiate between benign and malignant tumours as the latter are frequently diploid but many attempts have been made to correlate the degree of histopathologically defined tumour differentiation with tumour ploidy. In breast and ovarian cancer, these studies have found DNA aneuploidy to be significantly associated with axillary lymph node involvement (Eskelinen *et al.*, 1989; Toikkanen *et al.*, 1989),and histological grade/ steroid receptor status (Toikkanen *et al.*, 1989; O'Reilly *et al.*, 1990; Thorud *et al.*, 1986; Moran *et al.*, 1984; Kute *et al.*, 1985; Kallioniemi *et al.*, 1984; Feichter *et al.*, 1988; Dowle *et al.*, 1987; Thorud *et*

al, 1986;Friedlander, 1984). It is suggested that the bulk of early tumours tend to be diploid while more advanced tumours contain significant populations of aneuploid cells (Iversen and Skaarland, 1987).

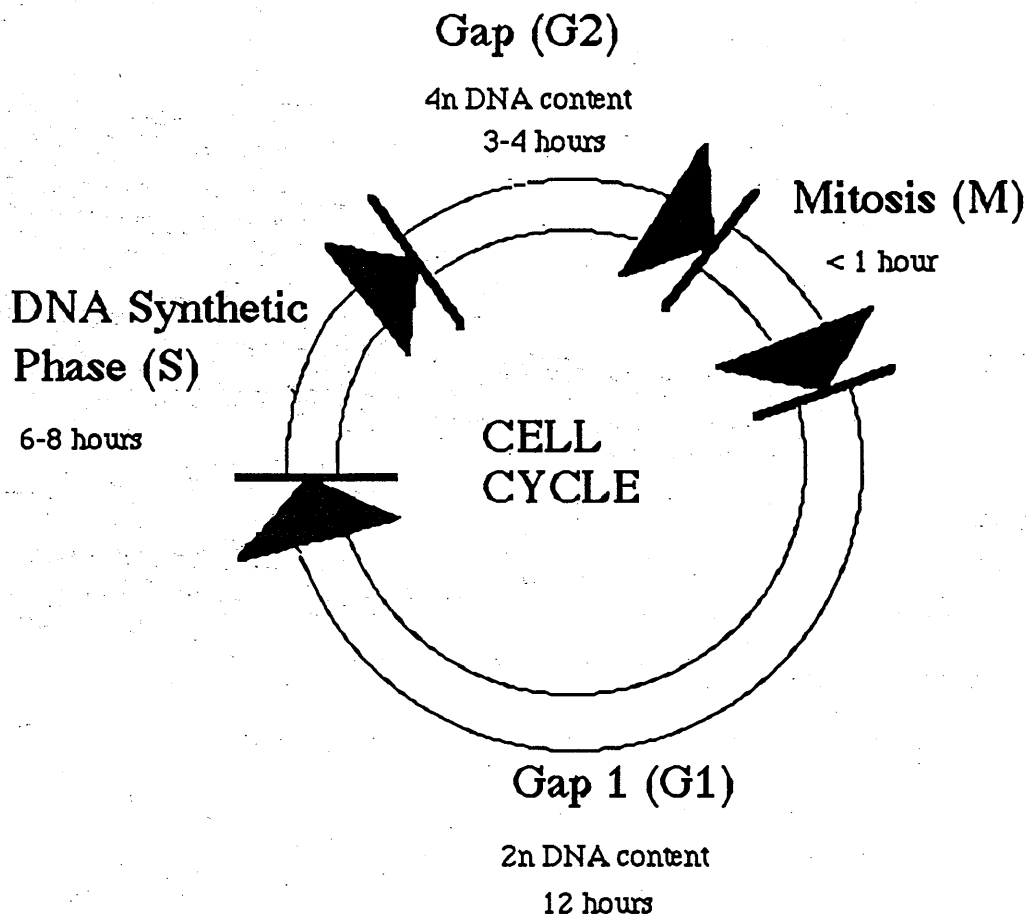


Figure 1.13: Diagram illustrating stages of the cell cycle.

1.6.2.2 The DNA content of cutaneous melanoma

Newton *et al* (1988) performed DNA flow cytometry on formalin fixed, paraffin embedded melanocytic naevi. They reported aneuploidy especially in those naevi accepted as precursors of malignancy: that is, dysplastic and congenital pigmented hairy naevi. Congenital pigmented hairy naevi (CPHN) present even more of a management problem than do the dysplastic naevi.

They may be small (less than 20 cm in diameter) or large. The giant CPHN can cover large areas of the body, especially the limbs, head and neck regions. All CPHN probably carry a risk of malignant change although the precise risk for the small naevi remains controversial. It has been estimated that for small CPHN a cumulative lifetime risk of 2.6% to 4.9% exists (Rhodes *et al.*, 1985). The lifetime risk of melanoma for large lesions is probably at least 6.3% (Stenzinger *et al.*, 1984). Excision of the majority of moderate to large CPNH in order to prevent malignant change is not a practical proposition because of the area of skin involved and also the considerable depth to which the melanocytes extend. Management is further complicated by the apparent lack of any correlation between histological degree of atypia and the risk of malignant change (Reed *et al.*, 1965; Borges and Lineberger, 1984). A marker of malignancy would therefore be of considerable practical value to physicians who manage these cases.

In the study of Newton *et al* (1988), DNA aneuploidy was found to be higher in the giant CPHN than the small CPHN. This suggests that the risk of malignant melanoma could be higher in the giant naevi and thus the presence of aneuploidy indicates a higher risk of malignant change.

Previous studies, one of fresh tissue and one of formalin fixed tissue, have reported that a small percentage of benign naevi were aneuploid (Sondergaard *et al.*, 1983; Von Roenn *et al.*, 1986). One study of fresh tissue found no aneuploidy in 62 naevi (Stenzinger *et al.*,1984). Thus it is unclear if this indicates that DNA aneuploidy in naevi is not always associated with neoplasia potential or whether some apparent benign naevi do have a possibility of malignant transformation.

1.6.2.3 The DNA content of uveal melanoma

To date, flow cytometric studies of uveal melanomas have been limited to analysis of histological material recovered from paraffin embedded specimens (Meecham and Char, 1986; Shapiro *et al.*, 1986), but many technical difficulties have been encountered including possible formalin induced artefacts, reduced fluorescent intensity and high levels of cellular debris which makes histogram interpretation difficult.(Shapiro *et al.*, 1986) Meecham and Char *et al* (1986)demonstrated aneuploidy in 37% of tumour samples measured and also strong correlation of the DNA index with higher tumour- related mortality. Greatest tumour diameter and cell type also correlated with survival, but less strongly than did the DNA index. Thus it appeared that those patients in this particular study with elevated DNA indices were at greater risk of developing metastatic disease. In contrast, Shapiro *et al* .,1986) found the incidence of aneuploidy to be 77% and there was found to be no correlation between DNA (ploidy) index and death from metastatic disease.

Char *et al* (1989) made a histological study of uveal melanomas treated with surgery, low dose (20Gy) pre-enucleation radiation followed by enucleation, or enucleated melanomas after high- dose(50-80Gy) charged particle beam therapy. In this study the cells were labelled with bromodeoxyuridine (BdUR) (Section 5.1.1.3), stained with an anti- BdUR monoclonal antibody and analysed using the fluorescence microscope. There were more melanoma cells in the DNA synthesis phase in tumours that were not treated with ionising radiation than in irradiated uveal melanomas. Two colour flow cytometry was also employed and gave generally similar results. Samples with few or no cells in S phase had low counts with both methods, and higher BdUR uptake was noted with greater numbers of cells in S phase

by both methods. The lesser BdUR uptake in irradiated versus untreated melanomas, and the reduced ability of irradiated tumours to grow in tissue culture demonstrated that the helium ion irradiated melanomas had lost their reproductive integrity. Although the microscopic data showed little deviation from the flow cytometric data the authors were obliged to rely on the microscopic data since it allowed them to eliminate artefacts caused either by staining of debris or non- tumour cells infiltrating the melanomas (Guntuz, 1985).

In a more recent report Rennie *et al* (1989) used flow cytometry in a study of the cellular DNA content of fresh tumour tissue from 19 uveal melanomas. It was found that 84% of the tumour samples were diploid and 15.8% (3 out of 19) were aneuploid. This was low compared to previous studies (Meecham and Char, 1986; Shapiro *et al*, 1986) and could perhaps be result of sample number. An index of proliferation was also obtained (summation of G2M/S) and was found to be 5.96% (range 2.2-9.8%). It was concluded that spindle neoplasms appeared to have lower rates of proliferation than epithelial cell tumours, as has also been found in an earlier study by Augsberger et al (1984). There was also reported to be no correlation between cell turnover and either tumour size or anatomical location, although it is probable that tumour growth is not uniform with times of relative quiescence and increased activity (Char *et al.*, 1983).

1.7 OBJECTIVES

1.7.1 Uveal melanoma

Uveal melanoma is a tumour which has several interesting features. Firstly its location in the eye, a site of immunological privilege, makes the nature of its interaction with the immune system an area of interest. Secondly, probably the most studied malignancy in tumour immunology is cutaneous melanoma and the relationship between this, and the parallel eye neoplasm, is a relevant area of study. Thirdly, Glasgow has been unusual in having unique access to uveal melanoma tissue via the Tennent Institute of Ophthalmology which a centre noted for its ability to remove the tumour leaving the vision functional.

1.7.2 Flow cytometry

Flow cytometry is a technique which has been extensively used in the study of the immune response, particularly in cells of the blood. The advantages are many in comparison to a single individual making a subjective analysis down a microscope. Flow cytometry can analyse several thousand cells in a short period of time. It is not subjective and small changes within a population can be assessed. In the case of tumour cells, it has largely been used only for retrospective ploidy analysis. It is obviously of interest to extend the application of this technique to analyse fresh, living cells of the tumour and its associated lymphocytes in order to further elucidate the nature of the autologous immune response in uveal melanoma patients.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

All routine chemicals are of the highest grade available and were supplied by Sigma Chemical Company or BDH Chemicals, both of Poole, Dorset, England, except for the following:

2.1.1 Human Tissue

Peripheral blood	Male and female patients with uveal melanoma, breast carcinoma (female only) or retinitis pigmentosa.
Tumour samples	Patients with either uveal melanoma (provided by Dr B Damato) or breast carcinoma (courtesy of Professor W.D. George, Department of Surgery, Western Infirmary, Glasgow)

2.1.2 Tumour cell preparations

RPMI-1640	Gibco Ltd., Paisley, Scotland
DMSO	BDH Chemicals Ltd., Poole
Foetal calf serum	Imperial Laboratories, Andover, Hants.

2.1.3 Peripheral blood lymphocyte preparations

Ficoll- Hypaque	Pharmacia Fine Chemicals Uppsala, Sweden
Plasticware	Sterilin Ltd., Feltham, England

2.1.4 DNA analysis solutions

as stated in section 2.2.2.2

Nonidet P40	BDH Chemicals
Trypsin	Sigma Chemical Co.
Trypsin Inhibitor	"
Ribonuclease A	"
Propidium Iodide	"

2.1.5 Antibodies

All antibodies were obtained from Becton Dickinson, Cowley, Oxford (Table 3.1) with the following exceptions:

Sheep anti- mouse Ig, (whole antibody) FITC conjugated.	Amersham International plc., Amersham, Bucks.
Mouse anti- human Vb region TCR FITC conjugated (Screening panel)	T Cell Sciences, Inc. Cambridge, MA, USA
Mouse anti- human ICAM-1 (CD54) and LFA-1 (CD11a)	Generous gift from Dr D. Haskard, Dept. Medicine, Hammersmith Hospital

2.1.6 Radiochemicals

Sodium Chromate (^{51}Cr) Solution (1mCi/ml) was obtained from Amersham International plc. Amersham, Bucks.

2.1.7 Buffers

Phosphate Buffered Saline (PBS) pH 7.4

170mM NaCl

3.4mM KCl

10mM Na_2HPO_4

1.8mM KH_2PO

Citrate buffer pH 7.6

250mM sucrose

40mM trisodium citrate

5% DMSO (v/v)

Sheath fluid pH 7.2 (FACScan fluid)

1.3mM NaCl

0.02 mM KCl

20mM LiCl

15mM KH_2PO_4

10mM Na_2HPO_4

10mM EDTA

2.1.8 Miscellaneous

FACScan tubes (Falcon 2052) were purchased from Becton Dickinson, Cowley, Oxford.

2.2 METHODS

2.2.1 Preparation and storage of tumour derived samples

2.2.1.1 Collection and storage of uveal melanoma tissue

Tumour tissue was obtained from uveal melanomas which were treated at the Tennent Institute of Ophthalmology either by local surgical resection or by enucleation. The tumour was first transported in saline to the Pathology laboratory. Triangular blocks (2-4 mm) were excised from the apical part of the tumour in such a way that the anatomy of the apex and the clearance margins were unaffected. These specimens were then taken to the Biochemistry laboratory, in a sterile container containing RPMI 1640 tissue culture medium, where further processing was undertaken within 30 minutes of their excision.

2.2.1.2 Preparation of spilled cells

Tumour pieces were transferred into a 60 mm sterile petri dish containing RPMI 1640 tissue culture media. Spilled cells were teased out using a sterile needle and scalpel. The cells were then harvested, washed by centrifugation at 150g for 5 minutes, counted and adjusted to $1x10^6$/ml in RPMI 1640 medium. If these cells were not being used immediately, they were cryopreserved until use (Section 2.2.1.3).

2.2.1.3 Cryopreservation of tumour cell samples

The number of cells available for storage was determined by counting with a Neubauer Haemocytometer. The cell suspension was centrifuged 150g for 5 minutes at room temperature and the pellet was re-suspended in a 10% solution of DMSO in 90% PBS to achieve a final concentration of 0.5-1 x 10^6cells /ml. This cell suspension was transferred in 1ml aliquots to freezing vials which were placed in a -70°C freezer overnight prior to storage in a liquid nitrogen freezer.

On removal from liquid nitrogen, each ampoule of cells was thawed rapidly in a 37°C water bath and immediately transferred to a universal container containing 15 ml of RPMI 1640 medium. This was centrifuged at 150g for 5 minutes and resuspended at the required cell concentration in filtered PBS pH 7.4 ready for FACScan analysis.

2.2.1.4 Preparation of lymphocytes from human peripheral blood

Blood (10ml) was obtained by venipuncture from normal donors, patients with uveal melanoma, or patients with other eye conditions. The blood was stored in potassium - EDTA anti-coagulant until ready for processing, usually within 1 hour of removal from the patient.
10 ml of blood was layered on top of an equal volume of Ficoll- Hypaque density gradient. The tubes were centrifuged at 1400g for 20 minutes, and the lymphocyte layer pipetted off and added to a clean tube . The plasma, ficoll and red blood cells were discarded. The lymphocytes were then centrifuged at 150g for 5 minutes and the supernatant was removed. The pellet was resuspended in 10 ml RPMI and the cells were washed once again.The subsequent lymphocytes were then either used fresh or frozen for later analysis.

2.2.2 Preparation of FACScan Samples

2.2.2.1 General FACS procedures

Cell suspensions of TIL, tumour cell , and PBL were washed and resuspended in PBS at a final cell density of 10^6/ ml . Cells (50µl) were incubated on ice with the appropriate antibody, for 20 minutes. All samples were maintained in the dark to prevent bleaching of the fluorochrome. If a double staining procedure was required then after the initial 20 minute incubation the cells were washed in 1ml of PBS for 5 minutes, the

supernatant was gently removed, and the cells were incubated in the appropriate antibody for 20 minutes. After the final incubation the cells were centrifuged at 150g for 5 minutes, the supernatant was again removed and the cells were resuspended in 0.5 ml of PBS and gently vortexed. Propidium Iodide (PI) was added to each tube to a final concentration of 2ug/ml, so that a live dead discrimination gate could be set (Section 2.2.4.1)

2.2.2.2 Preparation of nuclei for Flow Cytometric DNA analysis

This was performed by the detergent- trypsin method of Vindeløv *et al* (1983). Nuclei were prepared from lymphocyte and tumour cells using the detergent- Trypsin method. The following solutions were used in the preparation:

Citrate buffer:

sucrose 85.5g (250mM)

trisodium citrate $2H_2O$ 11.76g (40mM)

These were dissolved in approximately 800 ml dH_2O. 50ml of dimethylsulphoxide were added and then distilled water added to a final volume of 1000ml with the pH adjusted to 7.6.

Stock solution:

Trisodium citrate, $2H_2O$ 2000mg (3.4 mM)

Nonidet P40 2000µl (0.1% v/v)

Spermine tetrahydrochloride 1044 mg (1.5mM)

Tris (Hydroxymethyl)- aminomethane 121mg (0.5mM)

These were dissolved in dH_2O to make a final volume of 2000ml .

The pH was adjusted to 7.6.

This stock solution was used as the basis for the preparation of the staining solutions.

Staining solutions:

Solution A:

15mg trypsin dissolved in 500ml of stock solution and pH adjusted to 7.6.

Solution B:

250mg trypsin inhibitor

50mg Ribonuclease A

Both were added to 500 ml of stock solution and the pH adjusted to 7.6.

Solution C:

208mg Propidium iodide

580mg Spermine tetrahydrochloride

Both were added to 500 ml of stock solution and the pH was adjusted to 7.6. This solution was protected from light with tinfoil during preparation, storage and staining procedures.

Long term storage of staining solutions:

The solutions were stored in 5ml aliquots in plastic tubes at -70°C. Before use the solutions were thawed in a water bath at 37°C (but not heated to 37°C). Solutions A and B were kept at room temperature until use. Solution C was kept in an ice bath.

Staining procedure:

Solution A (900µl) was added to 200µl of the cell (10^6) suspension in citrate buffer and the tube was inverted to mix the contents gently. After 10 minutes at room temperature, during which time the tubes were inverted 5 or 6 times, 750µl of solution B was added. The tubes were again inverted and after a further 10 minutes at room temperature 750µl of solution C was added. At this stage the samples were stored at 4°C and protected from light. The samples were analysed between 15 minutes and 3 hours after the addition of solution C.

2.2.2.3 Preparation of single cell suspensions from paraffin fixed tissue sections

This was performed by the method described by Hedley *et al* (1983). Tissue sections were dewaxed in xylene for 10 minutes, following which they were rehydrated by sequential immersion in 100%, 95%, 70% and 50% ethanol for 10 minutes each at room temperature and then finally washed twice in distilled water. The tissue was then resuspended in 0.5% pepsin, in 0.9% NaCl, with the pH adjusted to 1.5 with 2N HCl in a water bath at 37°C. During this 30 minutes incubation the cell suspensions were frequently vortexed.

The nuclei were then prepared using the method described in Section 2.2.2.2.

2.2.3 Flow Cytometry

PBLs, TILs and tumour cells themselves were studied for the expression of specific cell surface markers by flow cytometry. The flow cytometer used was a Becton- Dickinson designed FACScan (Fluorescence Activated Cell Scanner), which is a laser - driven, automated cell analyser capable of detecting and analysing cells using 5 optical detectors, consisting of 2 physical parameters and 3 different spectral regions of fluorescence. The machine is dependent on the ability of the immunologist to provide high affinity fluorescently labelled monoclonal antibodies.

The FACScan can also detect 2 physical parameters: the amount of incident light scattered in the forward direction (FSC or forward scatter) which is a measure of the cell volume, and the amount of incident light scattered at 90 degrees. (SSC or side scatter) which gives an indication of the granularity of the cell. Cells can be identified by their specific size and granularity, and it is possible to utilise these parameters to place a gate round a particular cell population so as to distinguish it from other cell types present (Section 2.2.4.2)

2.2.3.1 Fluorochromes used

Such fluorescent dyes include fluorescein-5- isothiocyanate (FITC) ($C_{12}H_{11}NO_5S$) which is a commonly preferred conjugate because it reacts in a covalent manner with immunoglobulin and thus no coupling reagents are required. It produces stable conjugates and gives bright distinctive fluorescence (FL1 channel) absorbing light at 488nm and emitting a strong green fluorescence signal at 525 nm.

A second dye, R- phycoerythrin (PE) is a member of the phycobiliproteins which is a family of highly fluorescent macromolecules (Mr 240, 000). Coupling of PE to immunoglobulin requires thiolated phycoerythrin (FL2 channel) which reacts with a cross- linking reagent such

as N-succinimidyl 3-(2-pyridylthio)- propronate (SPDP) conjugated to the immunoglobulin. PE also absorbs at 488 nm but emits at a wavelength of 575 nm with a strong orange coloured signal.

The third dye is propidium iodide (PI) which is used to perform live / dead discriminations (Section 2.2.4.1). This again absorbs at 488nm and emits light at greater than 650 nm detected in the red part of the spectrum.

Thus the 15mW Argon laser with a fixed wavelength of 488nm excites the cells labelled with these mentioned fluorochromes which then emit in the red - green part of the spectrum.

2.2.3.2 FACScan optical systems

The optical system is based on an air cooled, 15 mW Argon laser set at 488nm. The output beam is transformed into an elliptical shape using a refracting beam expander. This is focused on a stream of cells in the flow chamber by a steering plate, and the behaviour of the cells within the laser beam is recorded (Figure 2.1).

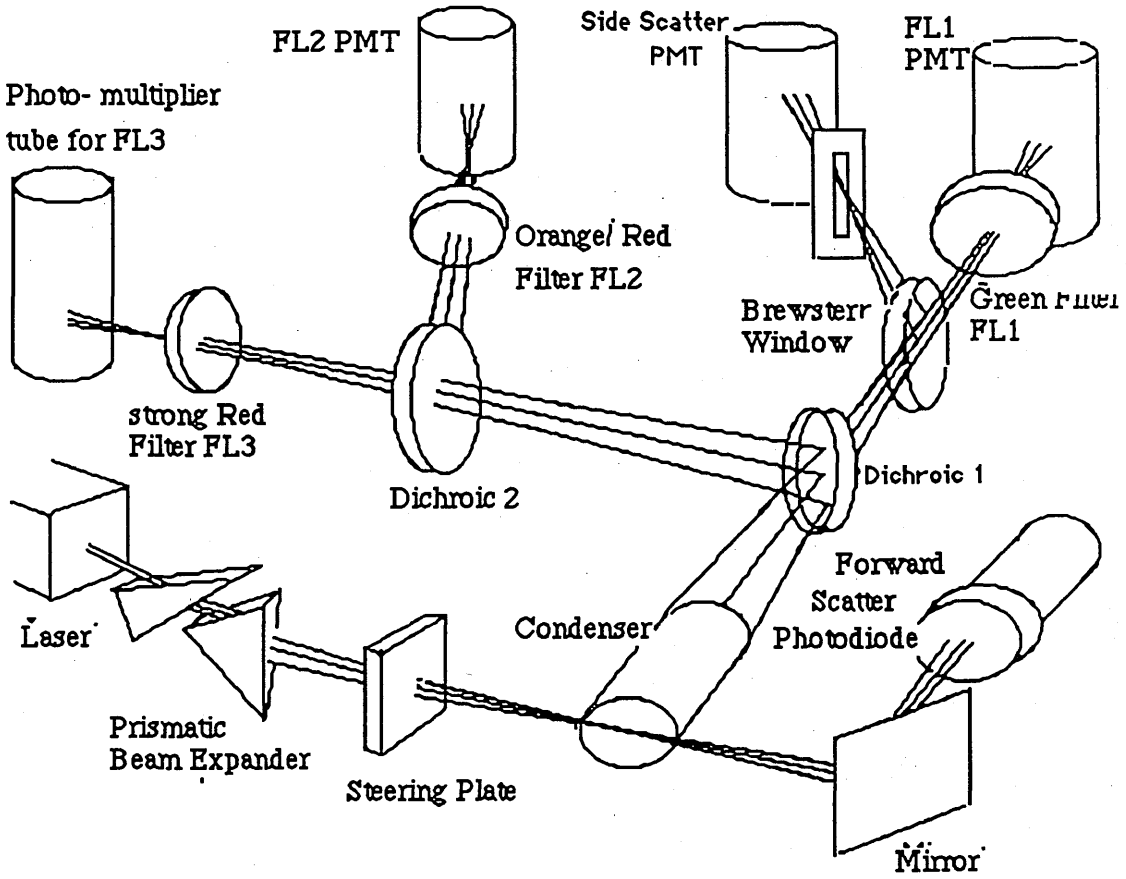


Figure 2.1: Diagram illustrating the optical system of the FACScan

The long wavelength is spectrally filtered from scatter and green fluorescence by a dichroic mirror (dichroic 1). The edge efficiently reflects longer wavelengths and efficiently transmits shorter wavelengths.

The transmitted path scatter is further split from the green fluorescence by a Brewster- angle beam splitter which takes advantage of the polarised nature of 90 degree scatter and the mostly unpolarised state of fluorescence. Meanwhile a 45 degree dichroic mirror (dichroic 2) with a 640nm edge further splits off the orange/ red fluorescence (FL2) from the red (FL3) fluorescence.

Forward scatter light is collected using lenses with makes use of a silicon photodiode with a sensitive area of 11.3 mm diameter. The side scatter channel does not require a filter, but the red fluorescence (FL3) channel uses a emission filter which transmits long wavelength light beams above 650 nm

The filter in front of the the FL1 photomultiplier transmits 525 nm light and has a band width of 30nm, optimised for FITC detection. The filter used in the FL2 channel is optimised for the detection of PE. It transmits at 575nm and has a bandwidth of 42 nm.

Spectral overlap of fluorochrome emission

The FITC emission extends from 500nm to about 600nm with maximum emission at the 525nm The RPE emission bandwidth begins at the 550nm and extends to about 640nm with maximum at 575nm, while the PI emission covers the range from 550nm to over 700nm, and has maximum at approximately 650nm. Thus it can be seen that there exists some spectral overlap between FITC and PE across the region 550nm to 600nm, while PE and PI emission overlap across the region from 550nm to 650 nm. Emission filters in each of the detector channels are selected to minimise this, although there is still some overlap between the FITC and the PE spectra. This is important when considering that these are the fluorochromes conjugated to the monoclonal antibodies being used. This overlap can be compensated for electronically (Figure 2.2)

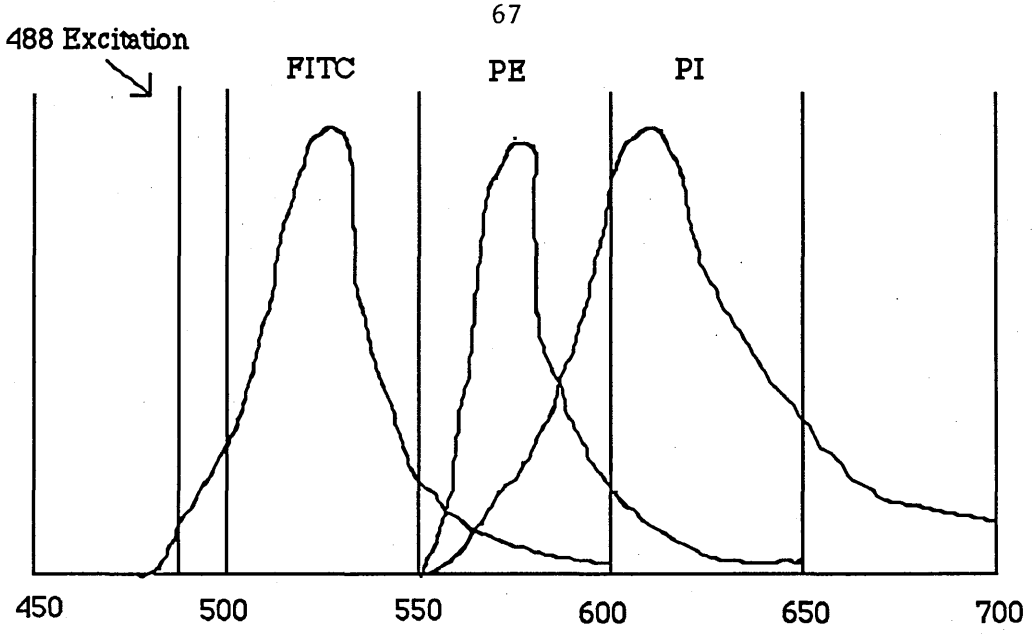


Figure 2.2: Diagram illustrating the spectral overlap of the three fluorochromes used (FITC, PE, PI)

2.2.3.3 The FACScan fluidics system

The function of the fluidics system is to provide a laminar, single file flow of cells through a sensory region, contained within a rectangular volume inside a quartz viewing orifice (Figure 2.3). The air pump is provided with a regulator for the air pressure which drives the isotonic sheath fluid from a reservoir through the viewing orifice. Once a single cell suspension is prepared, the sample is placed into a sample test tube that specifically fits over the sample capillary tube so as to form a tight seal. The sample is driven into the capillary tube by a differential pressure. The flow rate of the sample can be either high (60µl/ min) or low (12µl/min). The high flow rate is suitable for immune monitoring applications, whereas the low flow rate allows more precise cell positioning providing higher resolution in applications such as DNA cell- cycle analysis.

In the flow cell assembly the cells are then exposed to the argon laser beam. To avoid blockage by cell clumps or debris the flow cell of the

FACScan analyser consists of a regulator quartz cuvette with a large internal cross- section of 180μm by 480μm.

The cell sample is then carried to the waste reservoir by the sheath fluid.

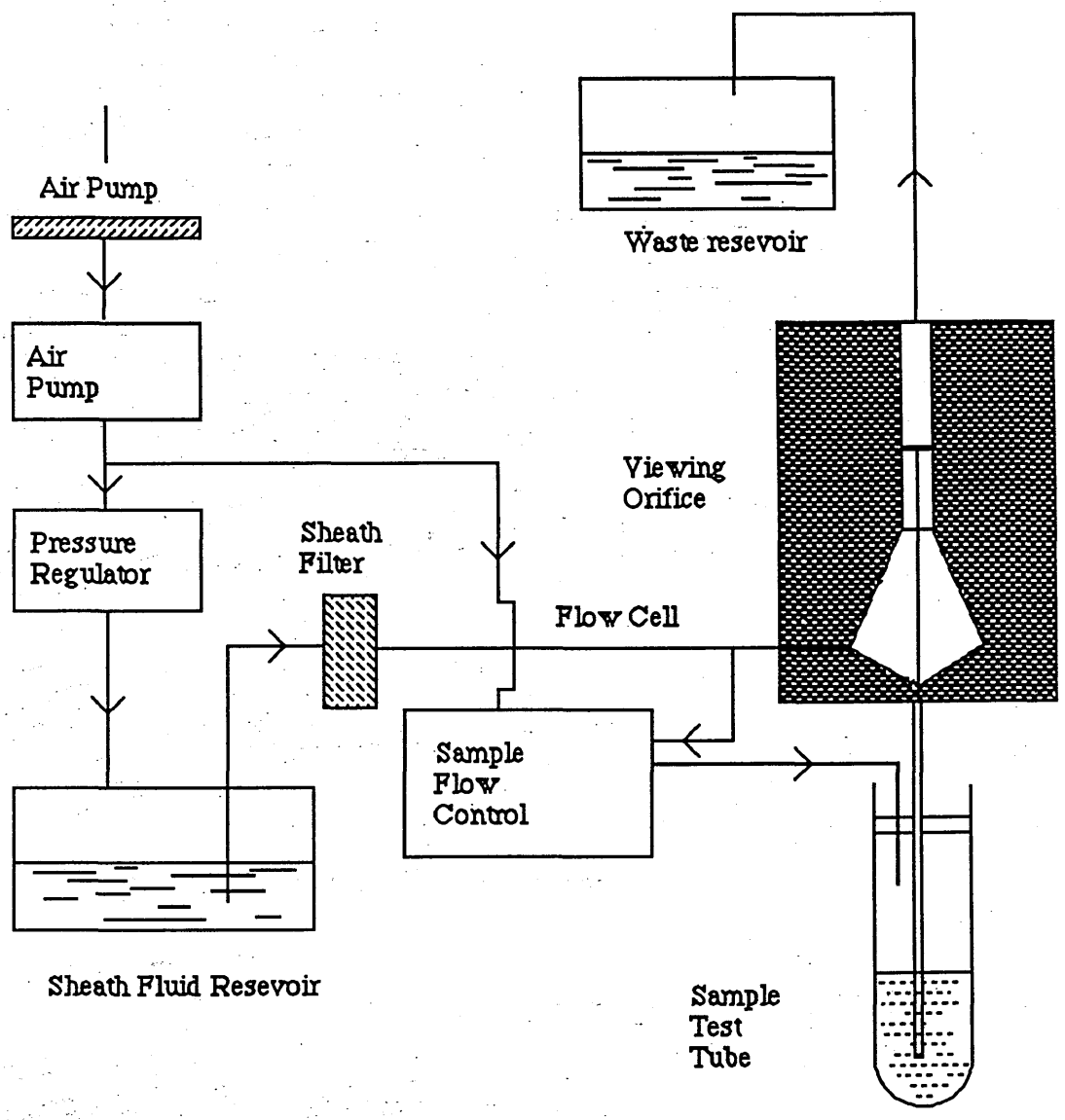


Figure 2.3: A box diagram of the FACScan fluidics system.

2.2.3.4 FACScan associated computer systems

The FACScan computer module consists of the Hewlett- Packard Model 310 computer, a 20 Mbyte hard disk, floppy disk, keyboard, mouse, printer, and monitor. This system displays, stores, gates and statistically analyses data collected.

The software program utilised in this study was FACScan research which allows the collection and storage of raw data from all 5 parameters.

The limit of sensitivity is 1000 FITC equivalent molecules per cell and as the amount of fluorescence on the surface of the cell varies widely.

2.2.4 Analysis of cell surface antigen expression

Once the cells have been prepared and the data collected, the information can be analysed in several ways. There are problems in analysing the data thus a few techniques either prior to the collection of the data, or during the analysis of the data must be put into operation.

2.2.4.1 Discrimination of live from dead cells

When the cells are being prepared for flow cytometric analysis, either during primary tissue cell isolation or antibody staining procedures, 100% viability is never obtained. Cells die during incubation periods, centrifugation steps and more practically while waiting to be collected on the FACScan. Dead cells than lead to high non- specific staining of the antibodies and this must be removed before a reliable result can be obtained. To remove dead, nucleated material from the collection data, a DNA stain called propidium iodide (PI) is employed(Section 2.2.3.1). PI enters a permeable, non- viable cell and intercollates with the DNA and RNA. PI staining of cells can then be negated by setting a gate that excludes cells that stain highly on a FL3 channel(Figure 2.4) This particular gating system however only removes nucleated, non- viable cellular debris. Cells that have disintegrated thus

leaving membranous material, are not included in this gate and must be removed using the FSC versus SSC plot system. This debris will be positioned in the lower left hand portion of the scatter plot (Figure 2.5)

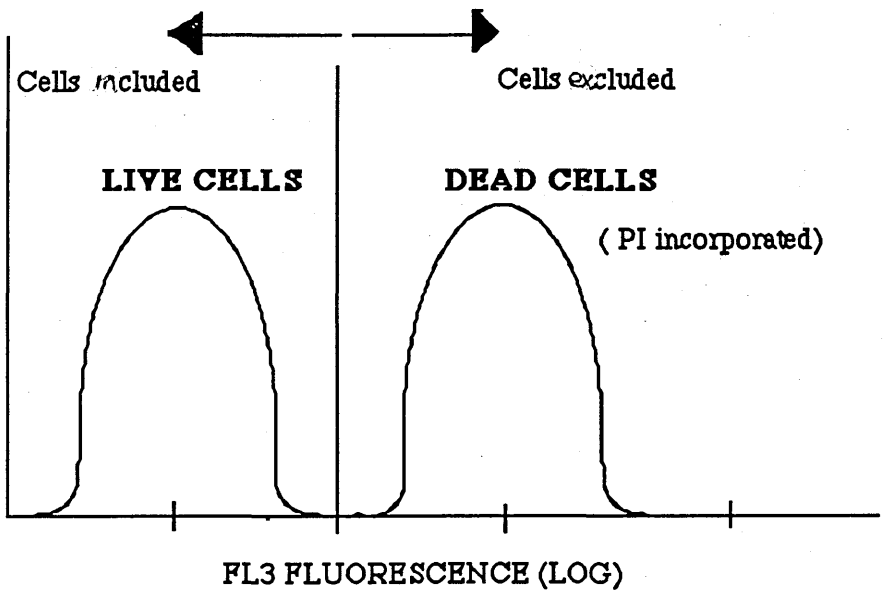


Figure 2.4: Diagram illustrating cells staining with PI, which can be excluded by applying the appropriate gate.

2.2.4.2 Discrimination of individual cell populations

Cells show specific size and granularity, and thus can be identified by viewing a FSC versus SSC plot of the collection data (Figure 2.6). By making use of fluorescently labelled antibodies it is possible to gate the material under examination so as to exclude any unwanted cells. Also with Becton Dickinson software package called "Paint-a-Gate" it is possible to identify a populations of cells in one parameter and then identify the same

cells in another parameter. Gates cannot be set using this program but it can be used to check the validity of the gate applied (Figure 2.6)

This technique was applied when looking at the phenotypic expression of tumour infiltrating lymphocytes within a mixed tumour cell population.(Section 3.1 and 3.2)

2.2.5 DNA Cell cycle analysis

DNA Cell- Cycle Analysis Software provides a quantitative analysis of cell cycle distribution from flow cytometric files (Section 1.6.2). There are many programmes available for this type of study and they often give disturbingly different results. For this work, only two were available although it was also possible to use operator directed measurement on the Lysys software programme. Once the nuclei were stained (Vindeløv *et al* 1983), the data was collected and analysed using one of these two main available DNA Cell- Cycle Analysis Software programs.

(a) The Polynomial Model

(b) The Sum of Broadened Rectangles Model

Both models calculate the percentages, total cells, and other parameters for the G1, G2+M, and S phases of the cell cycle (Figure 2.7)

2.2.5.1 The Polynomial Model (SFIT)

This model is an automated model that searches for the highest peak between channels 60 and 120 in the histogram and assumes this peak is the G1 peak (Dean, 1980). the model then searches for the G2+M peak. After locating the G2+M peak, The model determines a region of "pure" S phase (no G1, G2, or M phase cell present). "Pure" S phase is the region from 3.5 standard deviations to the right of the G1 peak to 2.5 standard deviations to

the left of the G2+M peak. Next, the Polynomial Model divides the calculation region into two equal subregions and sums the number of events, or nuclei, in each subregion. It assumes that the total S phase region runs from the mean of the G1 peak to the mean of the G2+M peak. Using the polynomial equation, the model computes the number of cells in each half of the S phase. The polynomial model then determines the number of cells in the G1 phase by subtracting the number of cells in the first half of the S phase from the total number of cells in the first subregion. A similar operation determines the number of cells in the G2+ M regions. This means that if the G1 or G2/M peaks are slightly broadened, an abnormal s phase figure may be obtained.

2.2.5.2 Sum of Broadened Rectangles Model (SOBR)

This model uses successive approximations to match a computed DNA distribution to the actual cell analysis data. The model uses Gaussian curves to model the G1 and G2+M peaks. It then approximates the S phase region by dividing it into a number of compartments, each are referred to as broadened rectangles, and the computer forms these by summing a series of Gaussian curves, contained within the rectangles.

Unlike the Polynomial model, the SOBR uses initial parameters that the operator specifies. The initial parameters used in this SOBR are the G1 and G2+M peak positions. The model computes a DNA distribution based on the initial estimation of these parameters. It then compares the computed and actual distribution and calculates the goodness- of fit. The model then determines the number of cells within the G1 and the G2+M regions from their respective Gaussian curves. It determines the number of cells in S phase by summing the number of cells in each of the S phase compartments.

Which is the Best Model?

The following criteria must apply when deciding to use the Polynomial Model:

(1) The cell population is nearly asynchronous in growth.

(2) Very little debris is present in the sample

(3) The G1 peak occurs within the channel range of 60 to 120 on the histogram.

(4) The reference peak, if present, occurs in a channel greater than or equal to 10 (CRBC are used because they contain approximately 35% the amount of human DNA.)

Use of the SOBR for cell cycle analysis is appropriate the data meets these criteria:

(1) When an error message appears after an attempt has been made to analyse the data with the Polynomial model.

(2) When it is suspected that the cell population may be partially synchronised.

(3) When the G1 peak is not in the channels that the Polynomial Model requires.

These models can only really be applied if the cells under examination are diploid.(Figure 2.7) The percentage of cells in the S phase of an diploid tumour, that also contains some aneuploid cells, cannot be measured because the G0/G1 peak of the aneuploid tumour is situated in the area corresponding to the S phase of the diploid tumour (Figure 2.8).

Certain profiles are also unintelligable because they contain too much debris

In this situation the sample must be discarded.

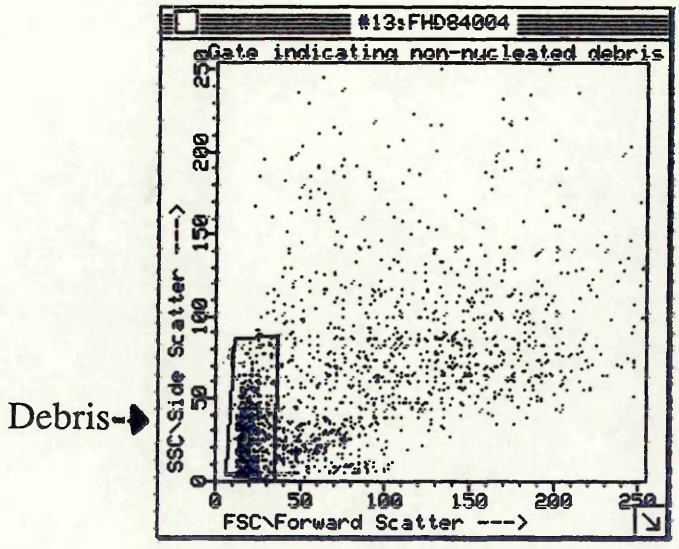


Figure 2.5: FSC versus SSC plot for the identification of non-nucleated debris. This material is contained within the box shown and is excluded form any analysis peformed.

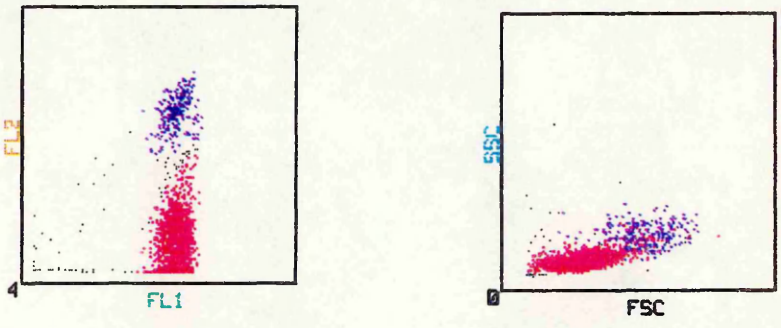


Figure 2.6: Becton Dickinson "Paint- a- Gate" software used to identify lymphocytes (red) and monocytes (blue) on FL1/FL2 (using the "Leucogate" antibody: anti -leucocyte FITC (FL1) and anti- Leu M3 PE (FL2)). These cells then show a distinctive pattern on the FSC and SSC plot.

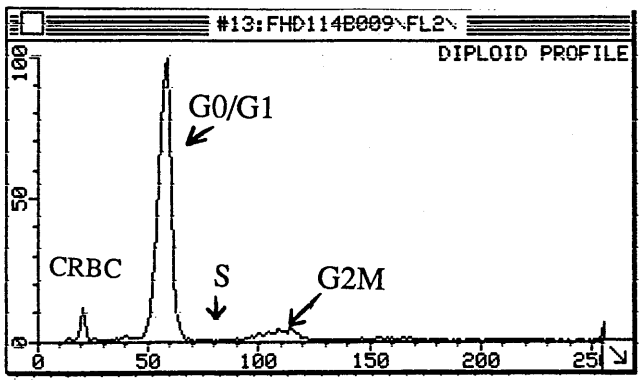


Figure 2.7: DNA showing diploid characteristics. Peaks indicate the G0/G1 (Gap 0 and 1) and G2M (Gap 2 and Mitosis) phases of the cell cycle. The position of chick red blood cell (CRBC) DNA is also identified.

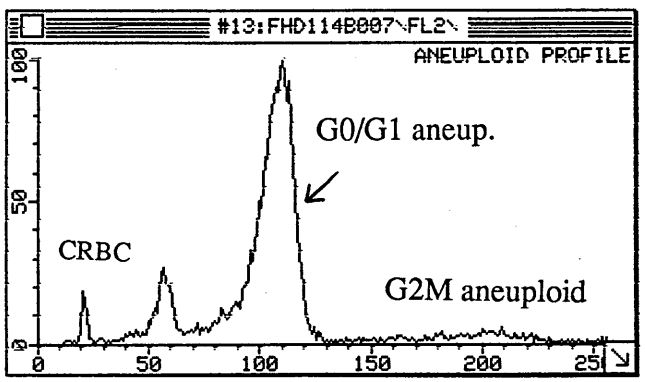


Figure 2.8: DNA showing aneuploid characteristics. Peaks indicate the position of G0/G1 and G2M of the aneuploid peaks.

2.2.6 4-Hour ^{51} Cr release cytotoxicity assay

Effector cells were prepared, washed in RPMI 1640 medium supplemented with 10 % FCS, resuspended in appropriate concentrations (effector : target ratios of 20:1 to 1:1) and added in 100µl aliquots to microtitre wells(serially diluted across the plate in triplicate wells). Target cells were incubated in 0.7 ml PBS with 200µCi Na_2 ^{51}Cr O_4 for 90 minutes at 37°C in a water bath. After this time the cells were washed 3 times with complete medium. Cells were counted and the cell count adjusted to the appropriate number ($1x10^5$ per well). Aliquots of 100µl were added to the wells containing the effector cells. For each target, additional wells were also prepared, containing 100µl of target cell suspension with 100µl of complete medium, and 100µl of target cells with 100µl of 3N HCl, corresponding to spontaneous and maximal release respectively. The culture plate was then centrifuged at 150g for 5 minutes and incubated at 4 hours at 37°C, 5 % CO_2 and 95% O_2. After this incubation period the plate was centrifuged at 150g for 5 minutes, and 100µl of supernatant was carefully removed, without disturbing the cells, and added to gamma counter tubes . The tubes were then counted on an LKB Wallac 1275 Minigamma counter on isotope selection mode fro ^{51}Cr. Results were analysed by calculating the % lysis of the target cells. The following formula was applied:

$$\% \text{ lysis} = \frac{\text{experimental cpm - spontaneous cpm}}{\text{maximum cpm - spontaneous cpm}} \times 100$$

CHAPTER 3

FLOW CYTOMETRIC ANALYSIS OF TUMOUR INFILTRATING AND PERIPHERAL BLOOD LYMPHOCYTES OF UVEAL MELANOMA PATIENTS

3 FLOW CYTOMETRIC ANALYSIS OF TUMOUR INFILTRATING LYMPHOCYTES AND PERIPHERAL BLOOD LYMPHOCYTES OF UVEAL MELANOMA PATIENTS

3.1 Phenotypic analysis of tumour infiltrating lymphocytes

3.1.1 Aims of the Study

Approximately 5-12% of uveal melanomas contain TILs. This section examines the differences between these TILs and the parallel PBLs from such individuals. The lymphocytes were analysed with respect to the relative proportions of lymphocyte subsets and the markers of activation expressed on their cell surface.

3.1.1.1 Activation markers

Activation markers used in this study included 2 well known antigens on the surface of T cells that are reported to be upregulated when the cell is stimulated by antigen or mitogen.

(1) HLA DR

HLA DR has been demonstrated as a T cell activation marker which is absent on resting T cells (Reinherz *et al.*, 1979) but is present on T cells stimulated by antigen, in mixed lymphocyte cultures (Pawelec *et al.*, 1982). This antigen is thought to be associated with antigen presentation (Section 1.4.4.2).

(2) IL-2 Receptor (Tac, CD25)

On antigen stimulated T cells the expression of the low affinity β chain (p55) is 5 to 10 fold greater than that of the intermediate affinity chain. IL-2 binding to high affinity receptors decreases their expression by 50%, but increases the

expression of the β chain 10 to 20 fold (Smith, 1989a) (Section 1.4.5). This increase in the β chain makes their presence a good guide to the increase in IL-2 receptors after activation, antigen stimulation or the presence of IL-2.

3.1.2 Results

Phenotypic analysis of TILs and appropriate PBLs from 5 uveal melanoma patients, was performed by applying 2 colour flow cytometric analysis. TILs and PBLs were characterised according to their expression of CD3, CD19, CD4 and CD8 on the cell surface. The $CD4^+$ and $CD8^+$ T cells were then analysed for their expression of the activation markers (i) HLADR and (ii) IL-2 receptor (Tac or CD25). The antibodies were murine antibodies to human cell surface markers and directly conjugated to either PE or FITC. (Table 3.1).

Antibody	CD	Conjugate	Reactivity
Anti- leu 2(a)	CD8	PE	Suppressor/ Cytotoxic
Anti- leu 3(a)	CD4	PE	Helper T cell
Anti- leu 4	CD3	FITC	T lymphocytes
Anti- leu 12	CD19	PE	B lymphocytes
HLA DR	NA	FITC	Class II MHC antigen
IL-2R	CD25	FITC	Interleukin 2 receptor

Table 3.1: Antibodies used to detect lymphocyte populations and activation markers (CD nomenclature for HLA DR not available (NA)

Spilled cells from 27 tumour samples were prepared (Section 2.2.1.2), and then incubated with the relevant antibodies for 20 minutes, at 4°C and washed ready for FACscan analysis (Section 2.2.2.1). Similarly, PBLs from each patient that contained sufficient TILs, were isolated from whole blood, using a ficoll- density gradient (Section 2.2.1.4), and stained as before. Lymphocytes were identified in 5 out of the 27 tumour samples by the use of the Leucogate antibody (Becton Dickinson) (Figure 3.1) which is a prepared antibody mixture containing a FITC conjugated anti- leucocyte marker and a PE conjugated monocyte/ macrophage marker (anti- leu 3M). This antibody identified an extensive infiltrate in 4 of the tumours. The 5th did contain analysable lymphocytes but to a much lesser extent. This 5th tumour sample was included to investigate the possibility that this lymphocytic infiltrate was perhaps a reflection of hemorrhage in the tumour. This antibody preparation allows lymphocytes, and this situation TILs, to be identified according to their position on a FL1 versus FL2 plot (Figure 3.1). It also allows an approximate gate to be placed on the FSC versus SSC scatter plot so that TILs could be collected within the tumour cell suspension. Live cells were also identified by using PI, detected on the FL3 fluorescence channel (Section 2.2.4.1). Simultest reagent (Becton Dickinson) kits for the identification of T and B cells and also for the identification of $CD4^+$ and $CD8^+$ cells were used.

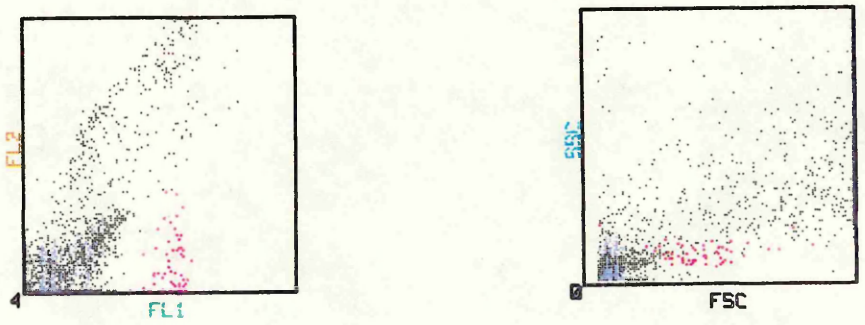


Figure 3.1: Identification of TILs (red) on the FL1/FL2 plot and the corresponding FFC/SSC plot. These cells are stained with the "Leucogate" antibody and analysed using the "Paint- a- Gate" software (Becton Dickinson).

Figure 3.2 indicates data obtained from flow cytometric analysis of (a) TIL and (b) PBL samples showing the classification of the cells into the individual lymphocyte subpopulations. Quadrants were set according to the fluorescence profile of cells incubated with an irrelevant antibody (FITC-IgG_1 and PE- IgG_{2a}), so as to allow approximately 99% of the cells to be within the lower left (LL) quadrant. When the cells were incubated with the appropriate PE and FITC labelled antibody, any spread of fluorescence along the FL1 channel to the right of the vertical marker indicated a positive result. Statistical analysis identified the percentages of each lymphocyte sub-population and expressed it as a percentage of the cells within the lymphocyte gate. The actual percentage of each T lymphocyte sub-population expressing the particular marker to be calculated.

$$\% \text{ Positive} = \frac{\% \text{ in UR}}{\% \text{ in UL} + \% \text{ in UR}} \times 100$$

In Figure 3.3, the data was obtained in a manner similar to that demonstrated in Figure 3.2. All 5 tumour samples are presented with the corresponding data from the PBL of four of the patients. Table 3.2 and 3.3 indicates the actual data obtained for the PBLs and TILs respectively. For all patients it can be seen that T cells predominate in the TILs (Figure 3.3(a)) with insignificant levels of B cells. The $CD8^+$ T cells predominate in the TILs whereas $CD4^+$ T cells generally predominate in the PBL samples (Figure 3.3(b)) When considering the activation markers Figure 3.3(c) indicates that the TILs express substantially more HLA DR than the PBLs even in patient 5. Table 3.3 indicates the mean values for the PBL and TIL samples. It shows the increased expression of the HLA DR on the TILs compared to the PBLs samples.

Figure 3.2(a): Data from the analysis of TILs of uveal melanomas. TILs were identified using the previously mentioned "Leucogate" antibody and then the $CD4^+$ and $CD8^+$ T cells were identified and analysed for the expression of HLA DR and CD25.

(a)

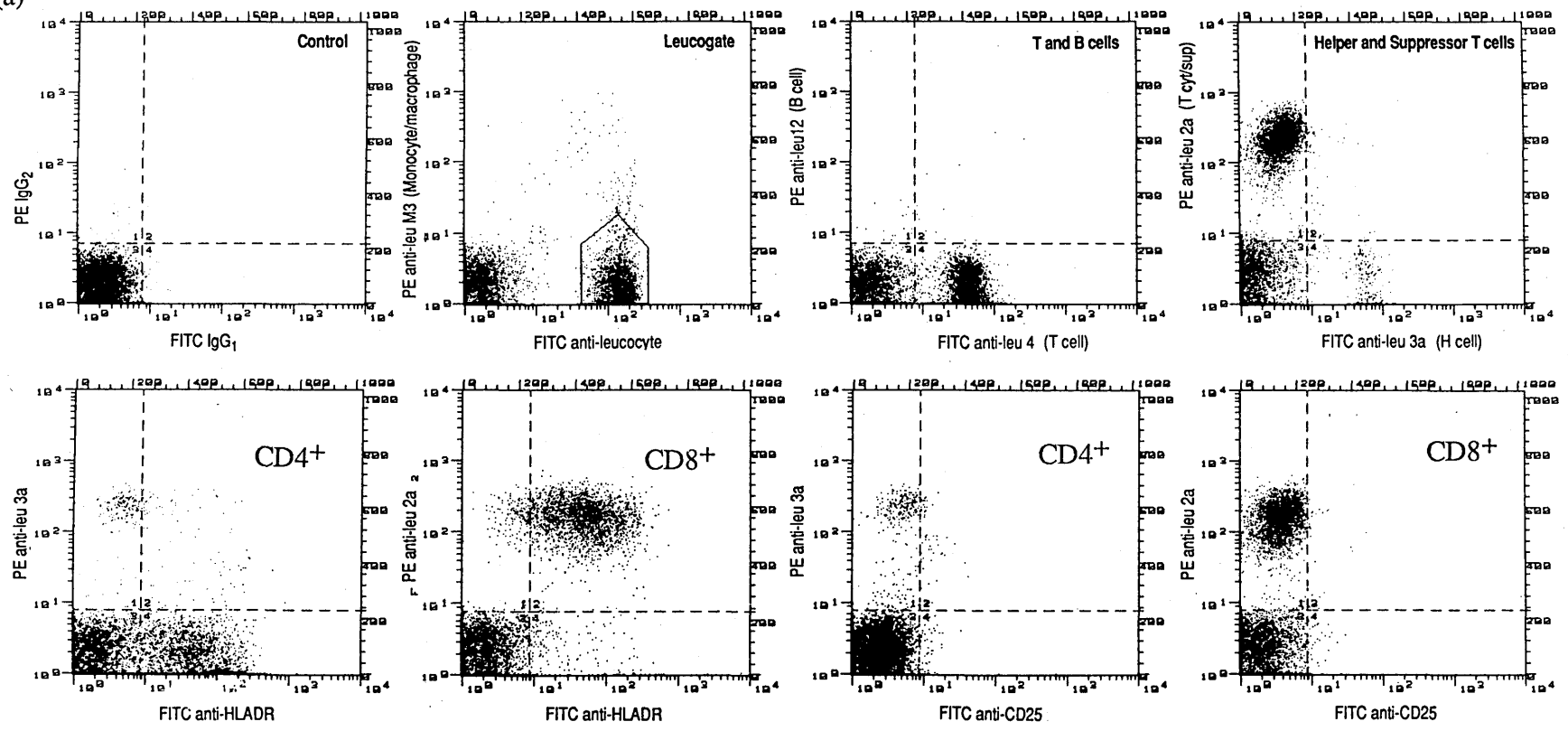
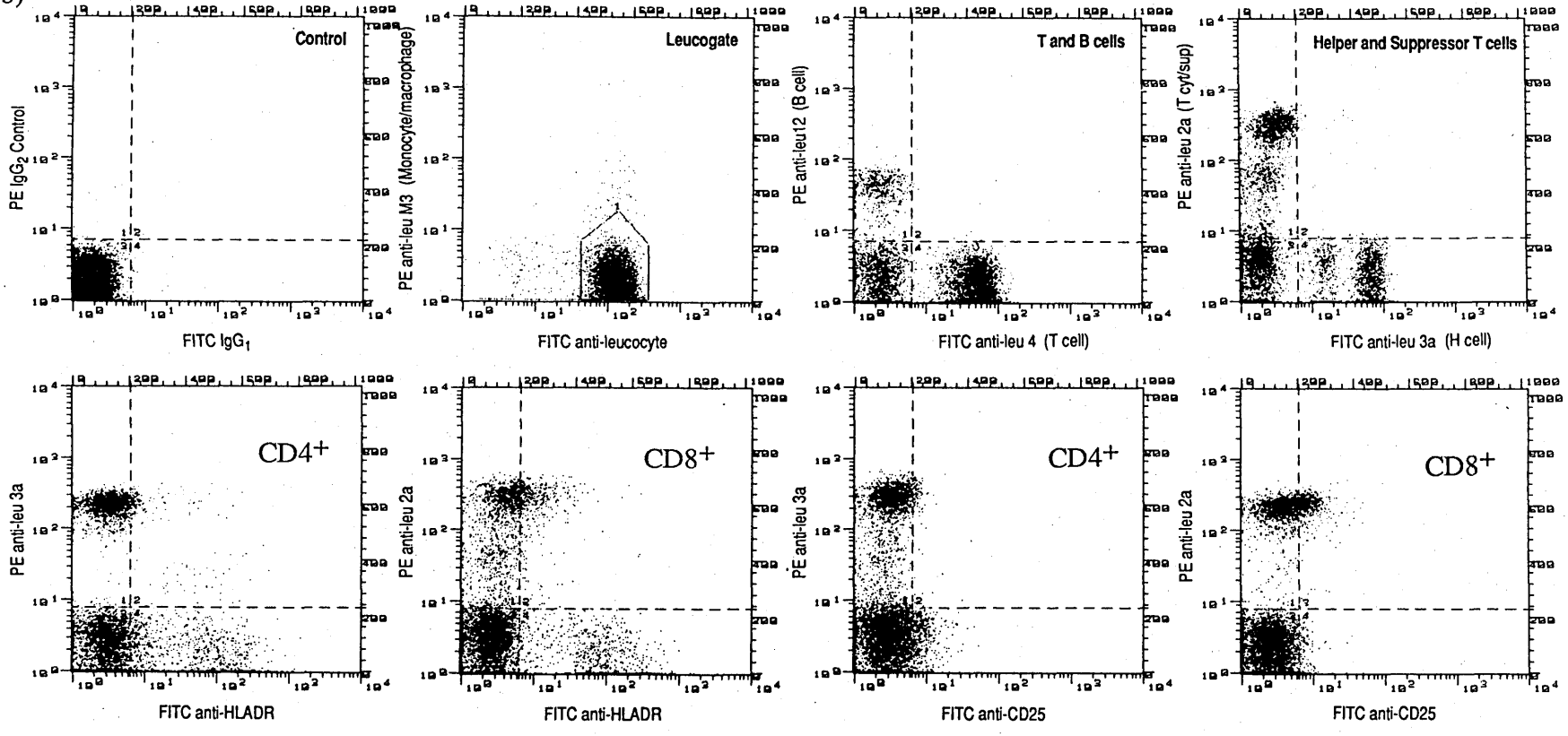

Control
PE IgG2
FITC IgG1
Leucogate
PE anti-leu M3 (Monocyte/macrophage)
FITC anti-leucocyte
T and B cells
PE anti-leu12 (B cell)
FITC anti-leu 4 (T cell)
Helper and Suppressor T cells
PE anti-leu 2a (T cyt/sup)
FITC anti-leu 3a (H cell)
CD4+
PE anti-leu 3a
FITC anti-HLADR
CD8+
PE anti-leu 2a
FITC anti-HLADR
CD4+
PE anti-leu 3a
FITC anti-CD25
CD8+
PE anti-leu 2a
FITC anti-CD25

Figure 3.2(b): Data obtained form the analysis of PBLs from uveal melanoma patients. PBLs were identified using the previously mentioned "Leucogate" antibody and then the $CD4^+$ and $CD8^+$ T cells were identified and analysed for the expression of HLA DR and CD25.

(b)


Control
PE IgG2 Control
FITC IgG1
Leucogate
PE anti-leu M3 (Monocyte/macrophage)
FITC anti-leucocyte
T and B cells
PE anti-leu12 (B cell)
FITC anti-leu 4 (T cell)
Helper and Suppressor T cells
PE anti-leu 2a (T cyt/sup)
FITC anti-leu 3a (H cell)
CD4+
PE anti-leu 3a
FITC anti-HLADR
CD8+
PE anti-leu 2a
FITC anti-HLADR
CD4+
PE anti-leu 3a
FITC anti-CD25
CD8+
PE anti-leu 2a
FITC anti-CD25

					HLA DR$^+$		CD25$^+$	
Patient	T cell	B cell	CD4$^+$	CD8$^+$	CD4$^+$	CD8$^+$	CD4$^+$	CD8$^+$
1	56.6	13.4	31.6	29.6	7.6	20.7	18.6	2.7
2	81.7	10	42.5	35.9	22.6	36.3	32.3	5.4
3	NA	NA	NA	NA	NA	NA	NA	NA
4	50.1	10.5	30.3	29.4	10.1	16.0	28.3	4.2
5	59.7	11.4	33.9	34.8	14.4	43.6	7.6	3.3

Table 3.2: Percentage of PBLs positive for the indicated phenotype.

					HLA DR$^+$		CD25$^+$	
Patient	T cell	B cell	CD4$^+$	CD8$^+$	CD4$^+$	CD8$^+$	CD4$^+$	CD8$^+$
1	54.1	0.3	5.4	43.9	37.1	93.6	16.5	3.5
2	50.3	0.3	11.6	31.9	64.0	73.0	11	3.3
3	85.6	2.4	18.4	73.9	83.0	55.0	26.0	3.4
4	73.1	1.2	8.2	77.3	80.0	88.2	30.0	8.1
5	6.7	0.0	3.0	5.0	87.8	55.5	7.9	4.0

Table 3.3: Percentage of TILs positive for the indicated phenotype

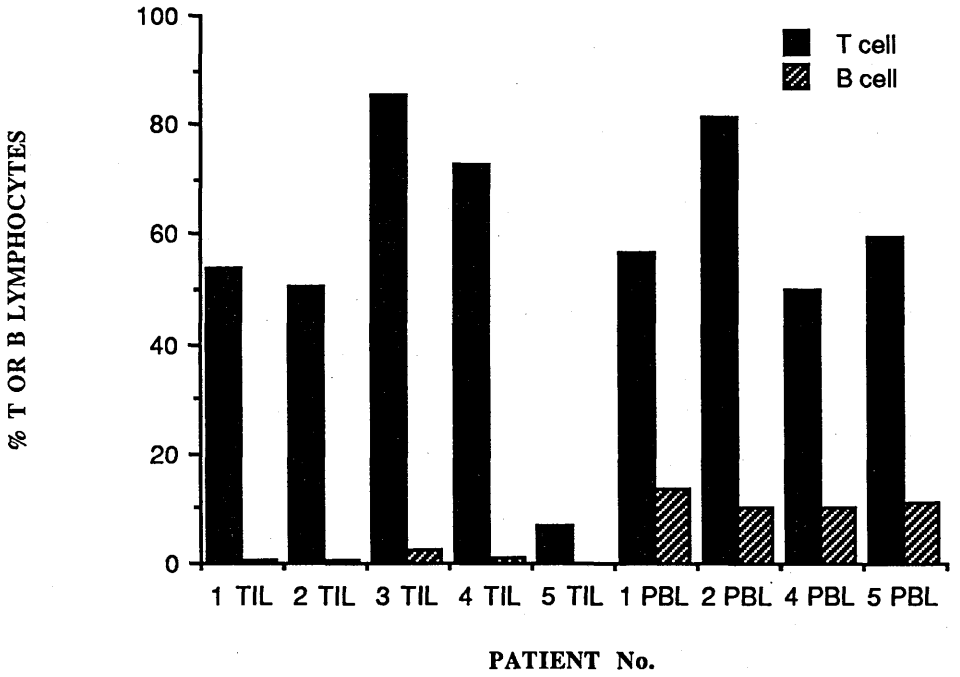
The $CD4^+$ are consistently more positive for the CD25 marker than the $CD8^+$ T cells (Figure 3.3 (d) and Table 3.4) not only in the TIL but also in the PBL samples available.

		PBL	TIL
T cell	Marker	mean	mean
CD4+	HLA DR+	13.7	70.3
CD8+	HLA DR+	29.1	73.0
CD4+	CD25+	21.6	18.2
CD8+	CD25+	3.9	4.4

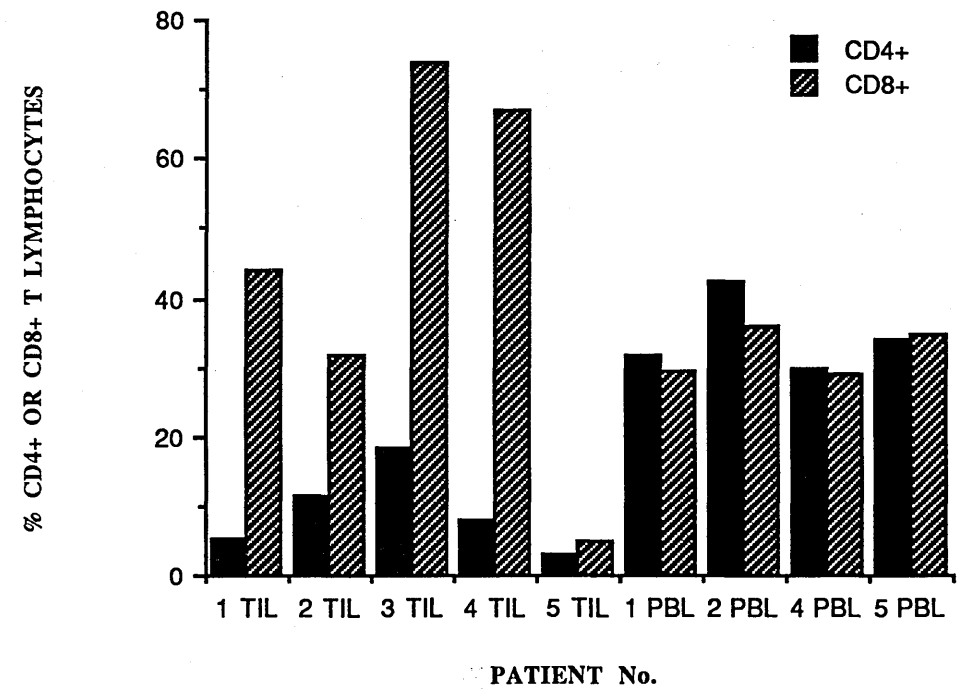
Table 3.4: Mean data for the expression of HLA DR and CD25 on the T cell subsets.

Figure 3.3: Histograms indicating the percentage of (a) T and B cell populations, (b) $CD4^+$ and $CD8^+$ T cells populations, (c) HLA DR^+ $CD4^+/CD8^+$ populations and (d) $CD25^+$ $CD4^+/CD8^+$ populations, in the TILs and PBLs of uveal melanoma patients.

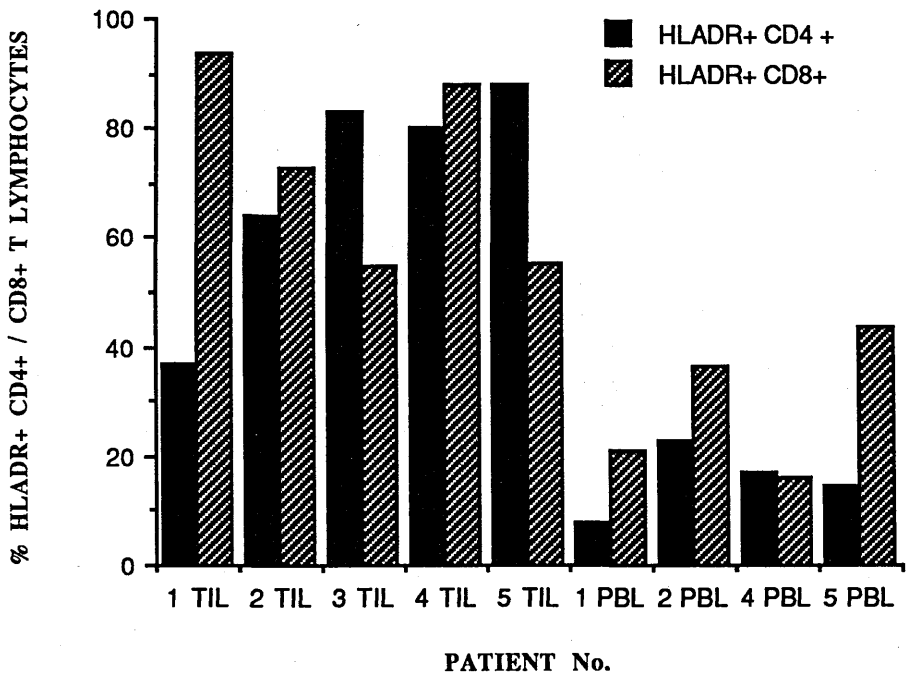
(a)


100
80
60
40
20
0
% T OR B LYMPHOCYTES
T cell
B cell
1 TIL
2 TIL
3 TIL
4 TIL
5 TIL
1 PBL
2 PBL
4 PBL
5 PBL
PATIENT No.

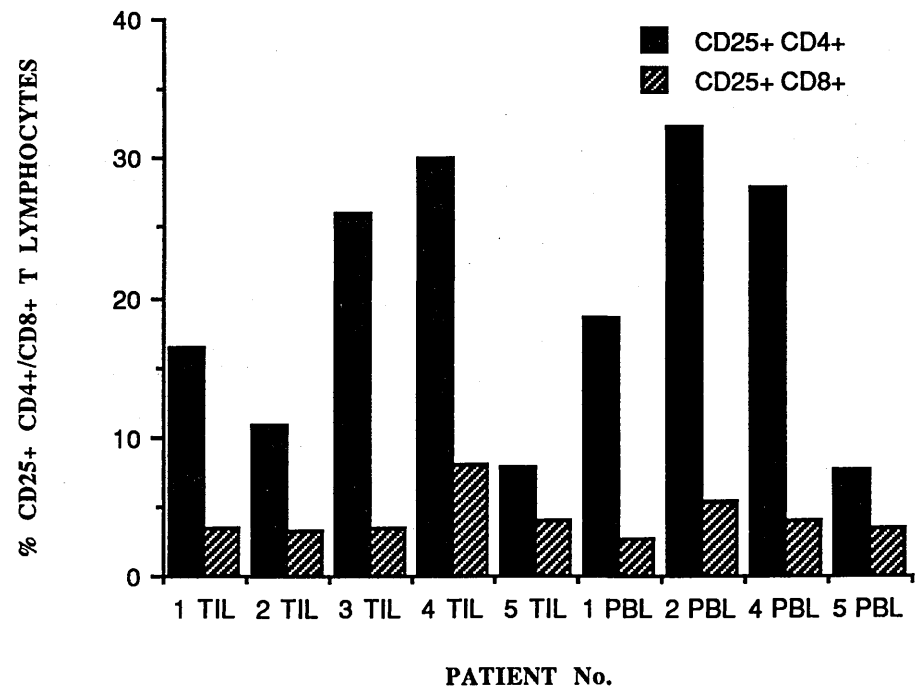
(b)


80
60
40
20
0
% CD4+ OR CD8+ T LYMPHOCYTES
CD4+
CD8+
1 TIL
2 TIL
3 TIL
4 TIL
5 TIL
1 PBL
2 PBL
4 PBL
5 PBL
PATIENT No.

(c)


% HLADR+ CD4+ / CD8+ T LYMPHOCYTES
100
80
60
40
20
0
HLADR+ CD4 +
HLADR+ CD8+
1 TIL
2 TIL
3 TIL
4 TIL
5 TIL
1 PBL
2 PBL
4 PBL
5 PBL
PATIENT No.

(d)


% CD25+ CD4+/CD8+ T LYMPHOCYTES
40
30
20
10
0
CD25+ CD4+
CD25+ CD8+
1 TIL
2 TIL
3 TIL
4 TIL
5 TIL
1 PBL
2 PBL
4 PBL
5 PBL
PATIENT No.

3.1.3 Discussion

In this particular study dominant populations of T cells as opposed to B cells were identified within the tumour infiltrate. This is in agreement with other workers (Whitford *et al.*, 1990; Itoh *et al.*, 1985; Ralfkiiaer *et al.*, 1987; Muul *et al.*, 1987; Belldegrun *et al.*, Topalian *et al* .,1987; Kurnick *et al.*, 1886) who have performed similar studies in other neoplasms. Within the T cells identified, we also found the predominance of $CD8^+$ T cells and this is also reported by other workers (Topalian *et al.*, 1987; Kurnick *et al.*, 1986; Giorno, 1983; Naukkarinen and Syrjanen, 1983; Bilik *et al.*, 1989), although some studies do disagree with these results, finding the predominance of $CD4^+$ T cells (Muul *et al* .,1987; Belldegrun *et al.*, 1986; Kradin *et al.*, 1987; Whitwell *et al.*, 1984; Gottlinger *et al.*, 1985). These conflicting results may be due to the methods used on the cell populations . Most groups study tissue sections stained with enzyme or fluorochrome conjugated antibodies and thus the question of intra -sample variation occurs. This is important in view of tumour heterogeneity (Heppner, 1984; Edwards *et al.*, 1985), the effect of which is reduced by analysing larger amounts of tumour.

The activation of $CD4^+$ T cells marks the beginning of an effective immune response, and the successful completion of antigen presentation by the antigen presenting cell (Section 1.4.4). As mentioned, during this activation process, the surface of the T cell acquires the HLA DR antigens and receptors for IL-2.

The presence of high levels of HLA DR on the surface of the $CD8^+$ TILs may be indicative of the cells presenting some foreign antigen. It is possible that after recognition of the tumour antigen combined to the Class I MHC complex and destruction of the tumour cell, the $CD8^+$ cytotoxic cells present part of the antigenic structure of the foreign cell within the groove of their HLA DR complex to signal to other cells of the immune system. The $CD4^+$ T cells also seem to express high levels of the HLA DR and this again

could be indicative of the antigen presenting capabilities of these T cells to other T cells to augment the immune response. Several groups have found HLA DR to be expressed by an increased number of T cell that infiltrate breast tumours (Rotstein *et al.*, 1988; Ben- Ezra and Sherbari, 1987) ovarian tumours (Heo *et al.*, 1988) and liver tumours (Takagi *et al.*, 1989).There is great variation in results with those studies involving long preparation times or fixing of cells. Whiteside *et al* (1986) performed a study on breast carcinomas, whereby the tumours were collagenase digested, and then exposed to a long incubation period prior to the staining of the lymphocytes that were consequently released. It was found that there were few lymphocytes bearing any activation markers and it is likely that these were cleaved during the enzymatic digestion and incubation period.

Very little difference is evident between the expression of CD25 on the surface of the T cells between the TILs and the PBLs, but there is still significantly more CD25 on the $CD4^+$ T cells than the $CD8^+$ T cells. This receptor only increases on cells after they have been stimulated by antigen and so its presence again signifies that these cells may have possibly recognised some tumour antigen alongside the Class I MHC complex. This marker is also a marker of cell division, and since it seems to be expressed on high percentages of the $CD4^+$ cells, then these cells have an increased turnover and possible expansion within the tumour. The $CD8^+$ T cells show little evidence of division thus this may be due to the existence of a terminal differentiation point into functional cytotoxic cells.

These finding may be relevant when considering TIL immunotherapy (Section 1.5.7). The rationale for this treatment is that the cytotoxic T cells have already been primed with antigen within the tumour mass, and thus will greatly increase in numbers within the IL-2 culture. Consequently, upon returning the cells to the patient, they will destroy metastatic deposits (Rosenberg *et al.*, 1986). Measurements of IL-2 receptor on the surface of the

T cell subsets in melanomas have been performed by other workers (Lotze *et al.*, 1987; Balch *et al.*, 1990) and they also found that the $CD4^+$ cells seemed to express higher levels of this activation marker. Although some groups who phenotyped the cells after IL-2 culture, found $CD8^+$ T cells to predominate (Kurnick *et al.*, 1986; Topalian *et al.*, 1988), others found that the greatest expansion to be in the $CD4^+$ T cell population, with the $CD8^+$ T cells dying away after 2 or 3 weeks. This indicated that the cells being returned to the patient were actually cells expressing the CD4 phenotype (Belldegrun *et al.*, 1989; Muul *et al.*, 1987; Heo *et al.*,1988; Kradin *et al.*, 1987; Skornick *et al.*, 1990). It is evident that this treatment has been partially successful in malignant melanoma (Rosenberg *et al.*, 1990), it thus seems likely that these results have actually been mediated via the CD4 cells . How these responses have been mediated must be clarified to allow further development of this therapeutic approach.

Little work has been performed on uveal melanoma involving culturing of the TILs in IL-2, perhaps due to the low levels of lymphocytic infiltrate within the tumours in general. The problem arises when isolating the lymphocytes from the tumour cell suspensions, due to the predominance of tumour cells. Consequently these TILs cannot be readily phenotyped, after IL-2 stimulation, to determine if it is indeed the $CD4^+$ population that will predominate.

3.2 Vβ TCR REGION ANALYSIS OF TUMOUR INFILTRATING LYMPHOCYTES

3.2.1 Aims of the study

It was decided to investigate the origin of the TILs. Among many other possibilites these could have expanded and located in the tumour site or, alternatively, have been attracted to the tumour by selective expression of adherence molecules (Section 4.1) by the tumour cells. In attempt to investigate this question of clonality it was decided to study the Vβ TCR region expression on TILs of uveal melanoma and also breast carcinoma another type of cancer readily available to studying. This was performed to assess whether the TILs were clonal or polyclonal, in their Vβ TCR expression, and if so, which Vβs were preferentially expressed. If a restricted usage of a particular Vβ TCR region in the TILs is present then this may be indicative of a specific antigen or superantigen that is being targeted in these malignant neoplasms. It also gives information as to whether TILs can expand into a clonal population at the site of the tumour.

3.2.2 Results

Vβ TCR region expression on TILs and associated PBLs, from 4 breast carcinomas (patient 1-4) and 6 uveal melanoma tumour samples (patients 5-10) was performed by simultaneous two colour flow cytometric analysis. $CD3^+$ lymphocytes were identified using a PE labelled CD3 antibody (detected on FL2) and the expression of the Vβ TCR region associated with these cells was also identified by using a panel of murine FITC labelled monoclonal antibodies (detected on FL1), specific for a selected number of sub-families of human TCR Vβ regions (Table 3.5).

Name	Specificity
Vβ5(a)	Vβ5 subfamily
Vβ5(b)	Vβ5 subfamily
Vβ5(c)	Vβ5.1 (not a subset of 5(a))
Vβ8(a)	Vβ8 subfamily
Vβ12(a)	Vβ12 subfamily
Vβ6(a)	Vβ6 subfamily (allotype Vβ6.7 epitope)

Table 3.5: Subfamilies of the human TCR Vβ regions

Spilled cells from both the breast carcinoma and uveal melanoma tumour samples were prepared (Section 2.2.1.2), and then incubated with the relevant antibodies for 20 minutes, at 4°C and washed ready for FACScan analysis (Section 2.2.2.1). Similarly PBLs from each patient were isolated from whole blood, using a ficoll- density gradient (Section 2.2.1.4) and stained as before (Section 2.2.2.1). Once the cell suspensions were prepared, the relevant information was collected by the flow cytometer. Each sample was collected with a live /dead discrimination gate present (Section 2.2.4.1) and in the case of the TILs an approximate lymphocyte scatter gate was also present. This lymphocyte gate was placed according to the position of isolated PBLs on a FSC versus SSC plot (Section 2.2.4.2).

Figures 3.4 and 3.5 represent the data obtained from the relevant analysis of the $CD3^+$ $V\beta^+$ populations for the uveal melanoma and the breast carcinoma respectively. Quadrants were placed so as to maintain the $CD3^-$ $V\beta^-$ population in the lower left quadrant (LL), the $CD3^+$ $V\beta^-$ population in the upper left quadrant (UL) and the $CD3^+$ $V\beta^+$ population in the upper right quadrant (UR). Statistics were then derived by calculating the population of

the $CD3^+$ $V\beta^+$ cells as a percentage of the total CD3 population. This information constitutes analysis using the gating method 1 (R1).

$$\% \text{ positive (R1)} = \frac{\% \text{ in UR}}{\% \text{ in UL} + \% \text{ in UR}} \times 100$$

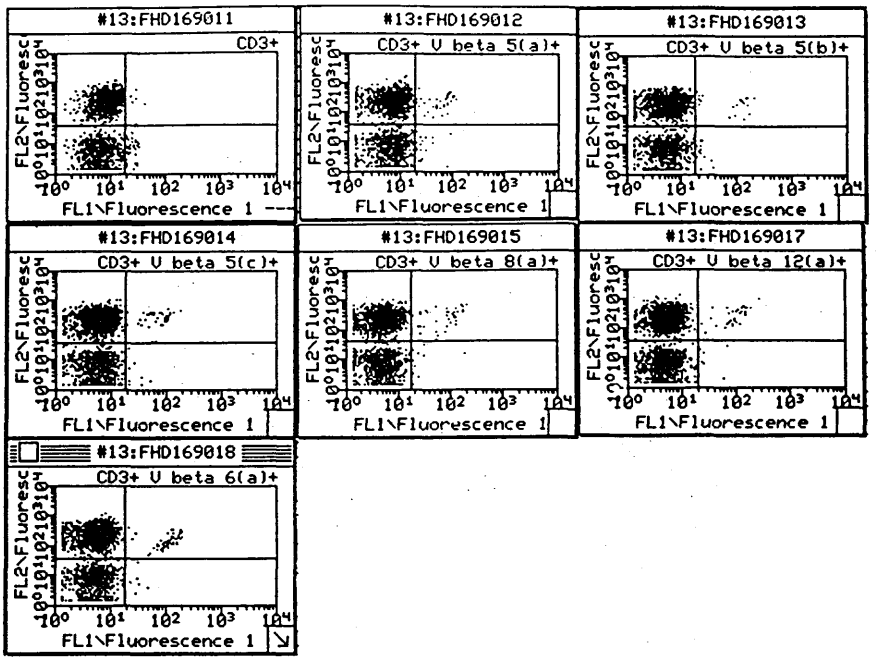
Methods of analysis were complicated by the fact that the antibodies appeared to bind in a non specific manner to some of the TIL samples, especially the uveal melanoma samples. This was indicated by a high fluorescence obtained at the 3rd or 4th log position of the FL1 scale and also leaking through of non-lymphoid cells on the FL2 scale (Figure 3.6). To overcome this the samples were analysed using a different gate setting that would remove any detected non -specific binding. The PBL cell preparations seemed to identify relatively uniform patterns of expression of the Vβ TCR regions, and thus a second gating method (R2) was put into operation. This gating system involved placing a "gate" round the PBLs considered to be positive for the specific Vβ expression and thus maintained to analyse the TIL samples (Figure 3.7 and 3.8). The percentage positive was that determined by calculating the percentage within this gate and stating it as a percentage of the whole $CD3^+$ population.

$$\% \text{ positive (R2)} = \frac{\% \text{ in "gate"}}{\% \text{ in UL} + \% \text{ in UR}} \times 100$$

To identify the TILs in the analysis the $CD3^+$ population was identified and using the "Paint-a-Gate" software (Becton Dickinson), the corresponding population of cells in the FSC versus SSc plot is identified (Figure 3.9).

Figure 3.4: Flow cytometric analysis of (a) PBLs and (b) TILs of uveal melanoma patients in terms of their Vβ TCR expression. Quadrants represent the gating 1 method of analysis (R1).

(a)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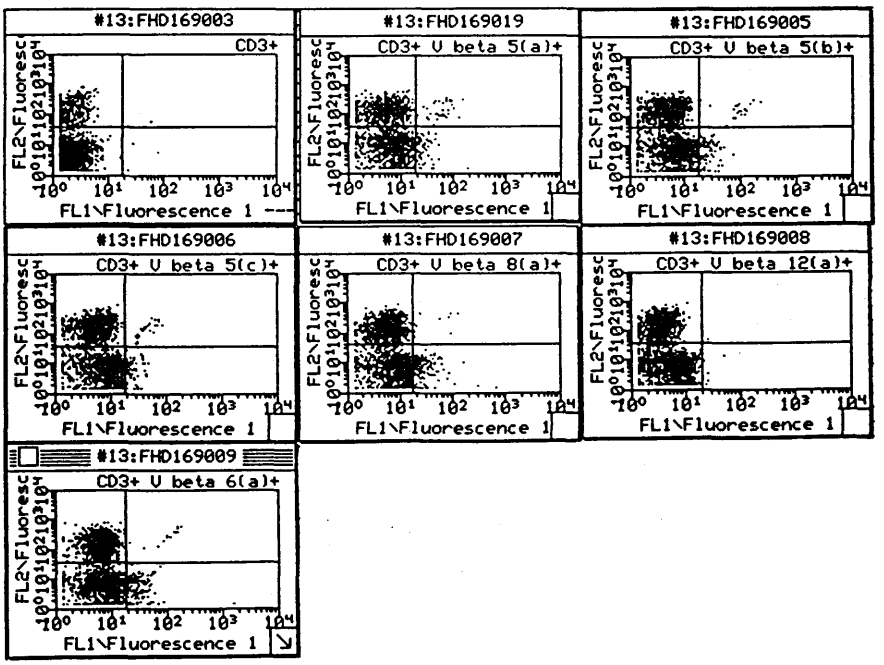
CD3+
#13:FHD169012
CD3+ V beta 5(a)+
#13:FHD169013
CD3+ V beta 5(b)+
#13:FHD169014
CD3+ V beta 5(c)+
#13:FHD169015
CD3+ V beta 8(a)+
#13:FHD169017
CD3+ V beta 12(a)+
#13:FHD169018
CD3+ V beta 6(a)+
FL2\Fluorescence 1
FL1\Fluorescence 1

(b)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CD3+ V beta 5(a)+
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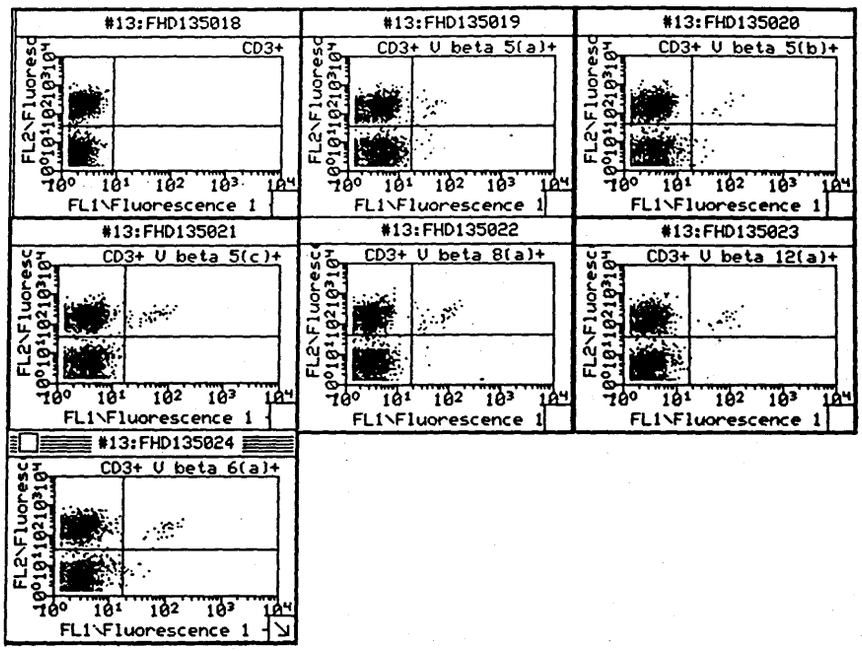
CD3+ V beta 5(c)+
#13:FHD169007
CD3+ V beta 8(a)+
#13:FHD169008
CD3+ V beta 12(a)+
#13:FHD169009
CD3+ V beta 6(a)+
FL2\Fluorescence 1
FL1\Fluorescence 1

Figure 3.5: Flow cytometric analysis of (a) PBLs and (b) TILs of breast carcinoma patients in terms of their Vβ TCR expression. Quadrants represent the gating 1 method of analysis (R1).

(a)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CD3+
#13:FHD135019
CD3+ V beta 5(a)+
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#13:FHD135022
CD3+ V beta 8(a)+
#13:FHD135023
CD3+ V beta 12(a)+
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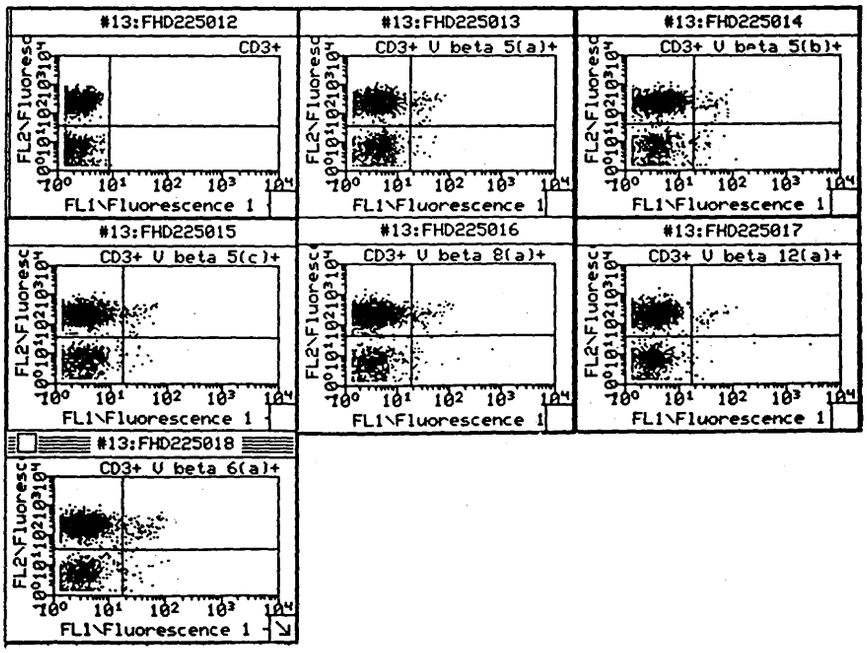
CD3+ V beta 6(a)+
FL2\Fluorescence 1
FL1\Fluorescence 1

(b)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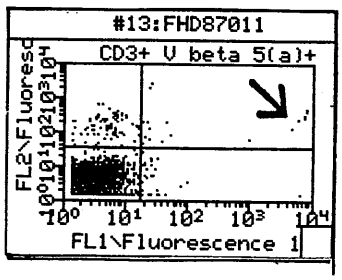
CD3+ V beta 5(c)+
#13:FHD225016
CD3+ V beta 8(a)+
#13:FHD225017
CD3+ V beta 12(a)+
#13:FHD225018
CD3+ V beta 6(a)+
FL2\Fluorescence 1
FL1\Fluorescence 1

(a)



(b)

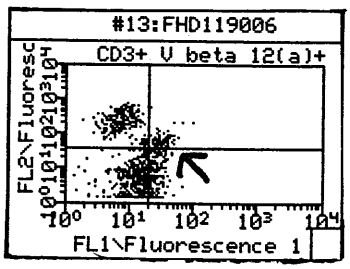
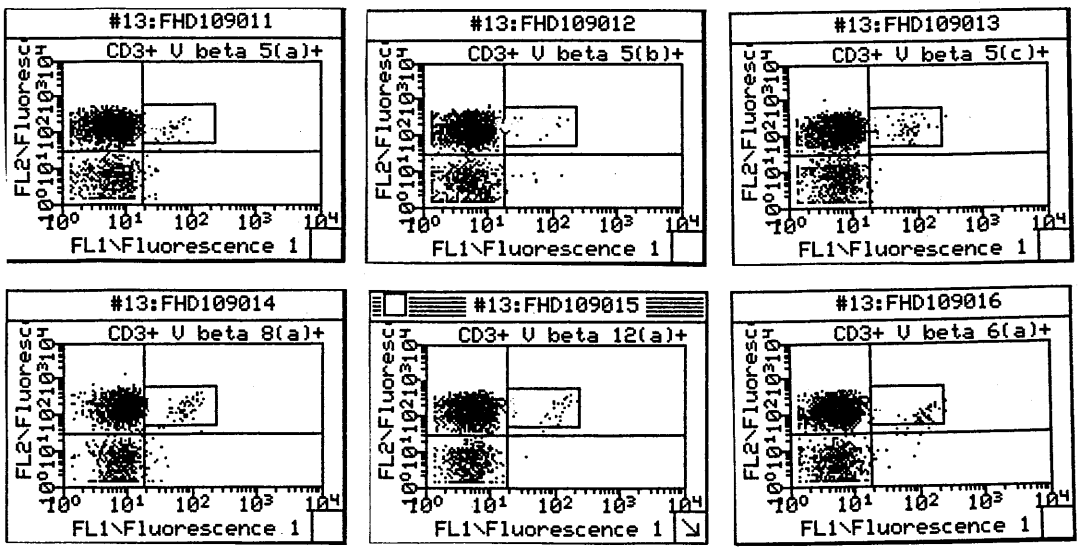


Figure 3.6: Non- specific staining of Vβ TCR region antibodies. (a) indicates high fluorescence in the 3rd and 4th log position. (b) Indicates leaking of non- lymphoid cells on the FL2 scale.

Figure 3.7: Gating method 2 (R2) used to analyse (a) PBLs and (b) TILs of uveal melanoma patients for the analysis of Vβ TCR region expression.

(a)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CD3+ V beta 5(a)+
#13:FHD109012
CD3+ V beta 5(b)+
#13:FHD109013
CD3+ V beta 5(c)+
#13:FHD109014
CD3+ V beta 8(a)+
#13:FHD109015
CD3+ V beta 12(a)+
#13:FHD109016
CD3+ V beta 6(a)+
FL2\Fluorescence 2
FL1\Fluorescence 1

(b)

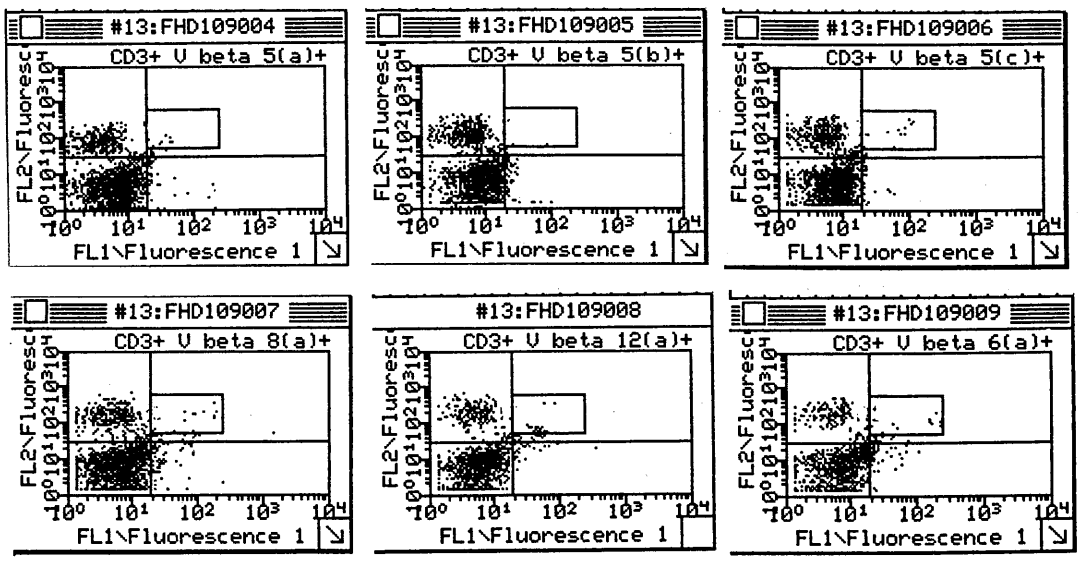
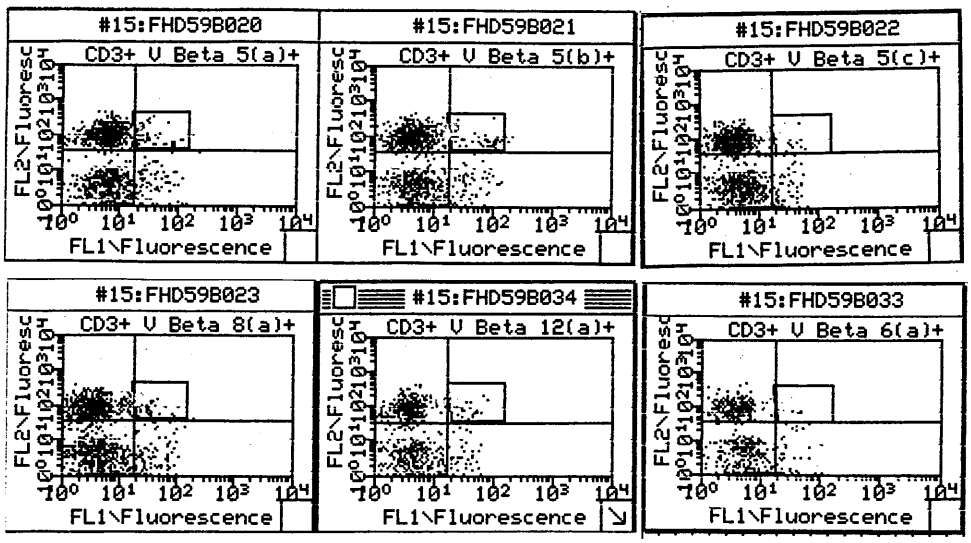
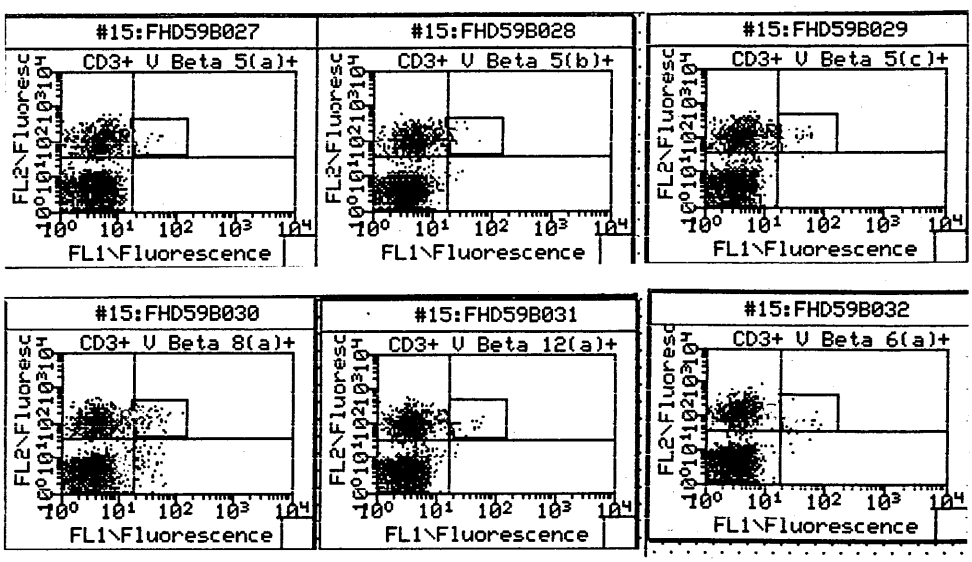

#13:FHD109004
CD3+ V beta 5(a)+
#13:FHD109005
CD3+ V beta 5(b)+
#13:FHD109006
CD3+ V beta 5(c)+
#13:FHD109007
CD3+ V beta 8(a)+
#13:FHD109008
CD3+ V beta 12(a)+
#13:FHD109009
CD3+ V beta 6(a)+
FL2\Fluorescence 2
FL1\Fluorescence 1

Figure 3.8: Gating method 2 (R2) used to analyse (a) PBLs and (b) TILs of breast carcinoma patients for the analysis of Vβ TCR region expression.

(a)


#15:FHD59B020
CD3+ V Beta 5(a)+
#15:FHD59B021
CD3+ V Beta 5(b)+
#15:FHD59B022
CD3+ V Beta 5(c)+
#15:FHD59B023
CD3+ V Beta 8(a)+
#15:FHD59B034
CD3+ V Beta 12(a)+
#15:FHD59B033
CD3+ V Beta 6(a)+
FL2\Fluorescence
FL1\Fluorescence

(b)


#15:FHD59B027
CD3+ V Beta 5(a)+
#15:FHD59B028
CD3+ V Beta 5(b)+
#15:FHD59B029
CD3+ V Beta 5(c)+
#15:FHD59B030
CD3+ V Beta 8(a)+
#15:FHD59B031
CD3+ V Beta 12(a)+
#15:FHD59B032
CD3+ V Beta 6(a)+
FL2\Fluorescence
FL1\Fluorescence

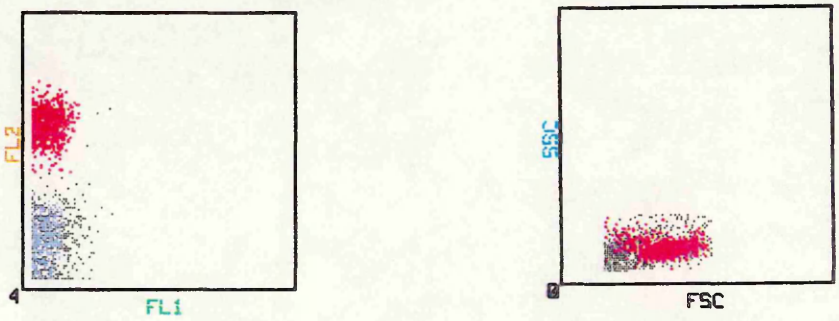


Figure 3.9: Identification of $CD3^+$ TILs (red) on the FL1/ FL2 plot and the corresponding position of these cells on the FSC/ SSC plot. These cells were identified using the "Paint- a- Gate" software (Becton Dickinson).

Tables 3.6 and 3.7 express the $CD3^+$ $V\beta^+$ populations for the PBLs and the TILs respectively, and compares the percentages obtained using the different gating methods described earlier. Tables 3.8 expresses the mean percentages of the specific Vβ for the PBL and the TILs. For the PBLs the analysis using gating method 1 (R1) always gave a slightly higher value but the deviation was not great, the largest difference in mean being a value of 0.3% for Vβ5(a). The mean percentage for the TILs indicates the importance of setting the correct gate, since the percentages vary considerably. It was decided to include the gating method 2 (R2) for analysis for these particular samples since it seemed to be more representative of the actual Vβ positive population and thus removed any possibility of non- specific binding of the antibodies.

Patient	Gate	Vβ5(a)	Vβ5(b)	Vβ5(c)	Vβ8(a)	Vβ12(a)	Vβ6(a)
1	R1	3.2	1.8	2.9	4.5	1.8	3.8
	R2	2.7	1.6	3.0	4.2	1.6	3.5
2	R1	3.0	1.4	5.7	5.2	1.1	2.6
	R2	2.9	1.3	5.7	5.2	1.1	2.3
3	R1	2.9	0.4	6.5	5.2	3.1	6.9
	R2	2.9	0.4	5.6	4.7	3.1	5.1
4	R1	2.8	3.9	3.4	7.9	2.1	3.2
	R2	2.8	3.9	3.4	7.9	2.1	3.2
5	R1	3.2	1.7	4.3	3.6	2.1	0.92
	R2	2.8	1.5	4.0	3.4	2.0	0.7
6	R1	15.0	12.5	10.1	7.1	4.6	5.3
	R2	14.2	12.1	9.8	6.8	4.1	5.0
7	R1	2.8	0.8	3.5	4.3	1.4	4.2
	R2	2.7	0.8	3.5	4.2	1.3	3.7
8	R1	4.2	2.0	6.6	5.5	3.0	5.1
	R2	3.9	1.7	6.6	5.9	3.3	4.1
9	R1	4.6	1.8	3.9	3.4	2.2	4.5
	R2	4.6	1.8	3.9	3.4	2.2	4.5
10	R1	6.3	2.6	3.8	1.6	2.7	7.4
	R2	6.3	2.3	3.8	1.3	2.5	7.4

Table 3.6: Data corresponding to $CD3^+$ $V\beta^+$ PBLs obtained with gating method 1 (R1) and 2 (R2).

Patient	Gate	Vβ5(a)	Vβ5(b)	Vβ5(c)	Vβ8(a)	Vβ12(a)	Vβ6(a)
1	R1	3.0	4.0	4.4	6.3	4.1	7.1
	R2	2.9	3.4	4.3	6.0	3.7	6.5
2	R1	7.3	6.4	3.3	6.6	2.8	8.4
	R2	7.7	6.4	3.3	6.6	1.9	6.0
3	R1	1.5	0.9	3.3	5.5	3.3	6.1
	R2	1.5	0.9	3.1	5.0	3.3	4.8
4	R1	8.1	8.1	2.8	7.3	7.3	3.7
	R2	7.2	7.4	2.5	6.2	6.9	3.0
5	R1	29.1	14.6	17.1	17.7	15.1	18.7
	R2	8.5	7.1	9.6	8.9	5.7	6.4
6	R1	9.3	3.8	3.5	1.6	2.8	9.9
	R2	7.8	1.3	1.2	1.6	0	4.4
7	R1	5.4	2.6	4.1	11.4	18.2	16.5
	R2	2.7	1.4	3.0	6.5	10.9	7.8
8	R1	19.1	25.7	8.6	31.9	33.6	19.1
	R2	2.6	5.5	2.7	10.1	6.6	2.9
9	R1	2.6	3.0	2.8	0.2	2.9	5.4
	R2	2.2	2.7	2.5	0.1	2.9	5.4
10	R1	32.2	18.5	27.1	23.9	20.0	33.3
	R2	8.0	4.2	9.8	9.8	3.4	4.0

Table 3.7: Data corresponding to $CD3^+$ $V\beta^+$ TILs obtained using gating methods 1 (R1) and 2 (R2).

	PBL		TIL	
Specific Vβ	R1	R2	R1	R2
Vβ5(a)	4.8	4.5	11.7	5.1
Vβ5(b)	2.8	2.7	8.7	4.0
Vβ5(c)	5.0	4.9	7.7	4.2
Vβ8(a)	4.8	4.7	11.2	6.0
Vβ12(a)	2.4	2.3	11.0	4.5
Vβ6(a)	4.3	4.1	12.8	5.1

Table 3.8: Mean data corresponding to $CD3^+$ $V\beta^+$ PBLs and TILs using gating method 1 (R1) and 2 (R2).

Figure 3.10 represents the comparison between the individual Vβ TCR region expression on the PBL and TIL samples for the 10 tumour samples. There is much variation between the different Vβs, but generally Vβ expression is higher on the TILs than on the PBLs with the exception of patient 6 who seems to express more of Vβ 5(a) on the PBLs than on the TILs. Indeed it would seem this occurs for all the Vβs for this particular patient. There does not seem to be one particular Vβ that is present on any one tumour sample to the exclusion of the other tumour samples.

Table 3.9 shows the mean values for the individual tumour types of the PBLs and the TILs, respectively, and this information is graphically illustrated in Figure 3.11. Generally that the PBLs from patients with breast carcinoma and uveal melanoma have lower Vβ expression than the TIL from these patients, except for Vβ5(c) which seems to be expressed to a slightly higher extent in the PBLs than the TILs from the corresponding sample. In general, the expression of Vβs in the breast carcinoma PBL samples is lower

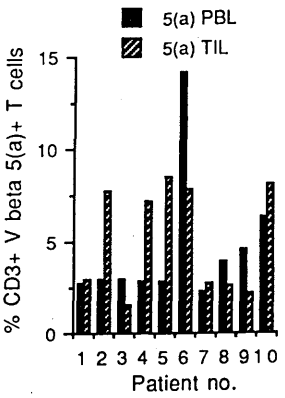
than that of the uveal melanoma patient PBLs, except for, Vβ8(a) where the change is very slight (5.5% and 4.7%, respectively). In uveal melanoma the expression of the TCR Vβ regions shows a general trend of being greater than the breast carcinoma, except for Vβ5(b), where there is a slight reduction.

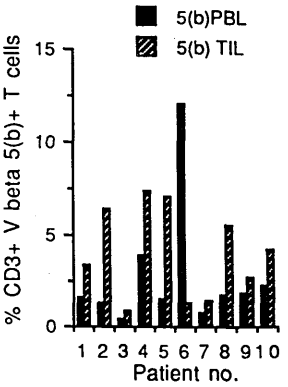
Tumour	Cell type	Vβ5(a)	Vβ5(b)	Vβ5(c)	Vβ8(a)	Vβ12(a)	Vβ6(a)
Breast	PBL	2.8	1.8	4.4	5.5	1.9	3.5
	TIL	4.8	4.5	3.3	5.9	3.9	5.0
Uveal	PBL	6.2	3.3	5.2	4.7	2.5	4.2
	TIL	5.3	3.7	4.8	6.1	4.9	5.1

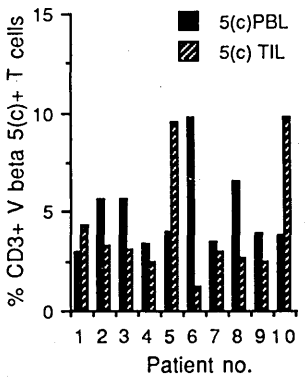
Table 3.9: Comparison of mean data for $CD3^+$ $V\beta^+$ PBLs and TILs for breast carcinoma and uveal melanoma (gating method 2 (R2) used).

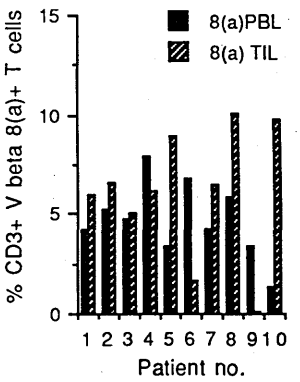
These changes in expression are not large and therefore may not be significant but taken as an entity the change is significant when one considers that only 1-5% of the PBLs are positive for any specific Vβ. This seems reasonable since other workers (Kappler *et al.*, 1989; Karpati *et al.*, 1991) have also shown similar Vβ expression statistics in other Vβ induction systems.

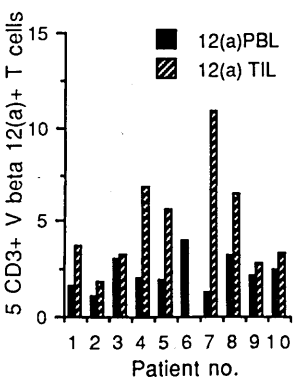
Figure 3.10: Comparison of each of the individual Vβ TCR regions analysed on the 10 tumour samples Data obtained by analysis using gating method 2 (R2).

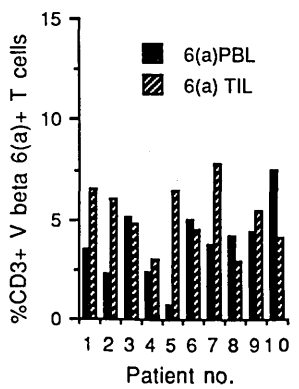

5(a) PBL
5(a) TIL
% CD3+ V beta 5(a)+ T cells
15
10
5
0
1 2 3 4 5 6 7 8 9 10
Patient no.


5(b)PBL
5(b) TIL
% CD3+ V beta 5(b)+ T cells
15
10
5
0
1 2 3 4 5 6 7 8 9 10
Patient no.


5(c)PBL
5(c) TIL
% CD3+ V beta 5(c)+ T cells
15
10
5
0
1 2 3 4 5 6 7 8 9 10
Patient no.


8(a)PBL
8(a) TIL
% CD3+ V beta 8(a)+ T cells
15
10
5
0
1 2 3 4 5 6 7 8 9 10
Patient no.


12(a)PBL
12(a) TIL
5 CD3+ V beta 12(a)+ T cells
15
10
5
0
1 2 3 4 5 6 7 8 9 10
Patient no.


6(a)PBL
6(a) TIL
%CD3+ V beta 6(a)+ T cells
15
10
5
0
1 2 3 4 5 6 7 8 9 10
Patient no.

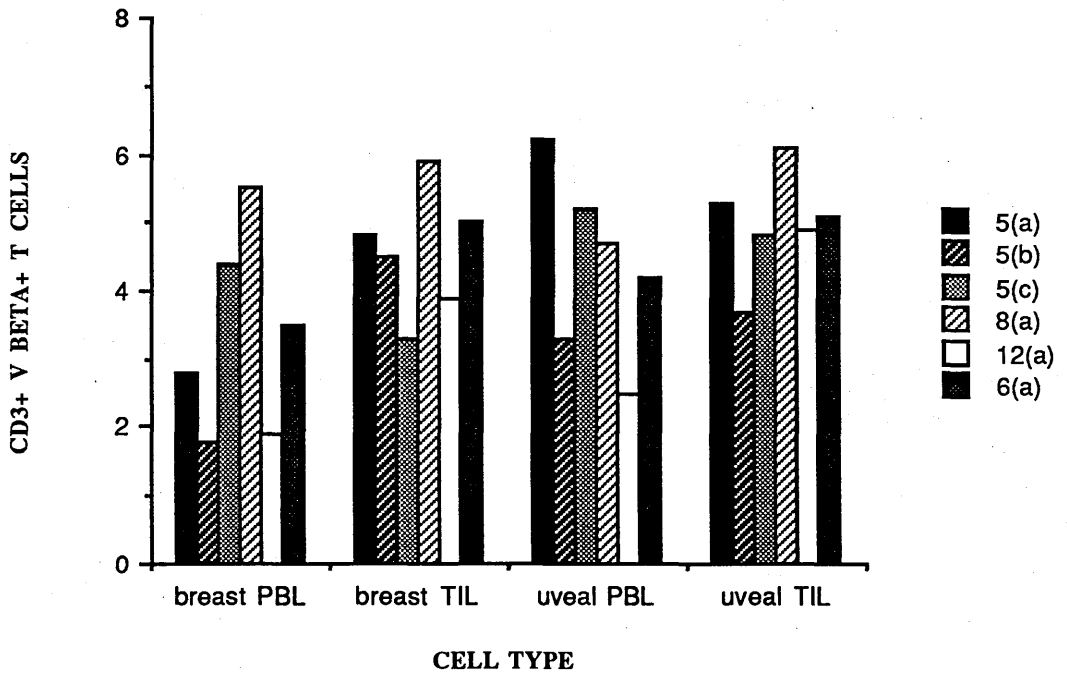


Figure 3.11: Mean data corresponding to the Vβ TCR region expression of breast carcinoma PBLs and TILs, compared to the uveal melanoma PBLs and TILs.

3.2.3 Discussion

Very few studies have been concerned with the expression of Vβ TCR regions in TILs, although much work has been done on the association of Vβ expression with reactivity to exotoxins which are produced by bacteria.(Choi *et al.*, 1989; White *et al.*, 1989; Kappler *et al.*, 1989) and superantigens produced by the recently characterised products of mouse MMTV virus (Choi *et al.*, 1991).However there has been one report on the Vα TCR gene expression in uveal melanoma (Nitta *et al.*, 1990), where it was found that Vα7 was found in 7 of the 8 uveal melanomas studied. This was done by using PCR techniques with Vα primers. Thus it was suggested that TILs may have a restricted TCR repertoire. Vα genes are seldom analysed in diversity studies because they are dominated by the Vβ regions in monoclonal antibody production, and consequently few antibody probes to Vα products on the surface of the cells under study, are available.

The present study was performed on 6 uveal melanoma tumour samples and 4 breast carcinomas by the technique of flow cytometry using fluorescently labelled murine antibodies to the Vβ regions of the human TCR. These antibodies identify minor populations of PBLs (approximately 1-5%).

It can be seen from Figure 3.4 and 3.5 that all the Vβs seem to be expressed on the TILs and PBLs analysed and no one Vβ is preferentially expressed. This indicates that the TILs are polyclonal for the Vβ TCR region measured. In addition it should be noted that Jα genes and Dβ and Jβ genes may add to the diversity of any one expressing specific Vβ or Vα genes. Thus the lack of clonal diversity observed may indicate a difference between the α and β chains of the TCR in an oligoclonal response. Belldegrun *et al* (1989) reported oligoclonality in bulk IL-2 generated cultures of TILs derived from human tumours. However, the same laboratory, studying transplanted chemically- induced murine tumours, in inbred strains of mice, has more

recently reported that freshly excised TILs are polyclonal in their Vβ repertoire and that oligoclonality is only observed after culture in IL-2 (Karpati et al., 1991). However, some restriction in the TCR expression might still exist, although this restriction might or might not be reflected in restricted Vβ usage because TCR-α and TCR-β regions other than Vβ contributed to the determination of antigen specificity. Also the panel of antibodies used included a limited number of the possible Vβ repertoire, thus the question of clonality cannot be completely addressed. In addition, lymphocytes may not only enter the tumour but may also leave the tumour, thus the TILs are only being measured at one specific moment in time. The expression of the Vβ on a particular population of TILs may change during the course of time. Finally, the TIL population may contain only a limited number of tumour- specific T cells and a larger number of "passenger" T cells, making it difficult to detect selective TCR expression in the response to tumours.

There are slight differences between the expression of the Vβ on the TILs compared to the PBLs, the TILs generally expressing more of any given Vβ. This may be due to practical problems in the analysis since activated cells are larger and thus more fluorescent. Differences may also be due to the TILs recognising a particular antigen that is not Vβ specific. For example, as mentioned, the recognition of an epitope on the mucin polypeptide core in the case of pancreatic and breast adenocarcinoma (Jerome *et al.*, 1990). In the case of superantigen involvement, any direct parallel to murine *Mls* model (Dyson *et al.*, 1991; Marrack *et al.*, 1991) would be expected to be observed as an absence of a particular Vβ clone in both PBLs and tumour cells and we do not observe this within the panel studied. Likewise, any equivalent to a bacterial superantigen in the tumour would be expected to be observed as a non- MHC restricted selective accumulation of a particular Vβ clone in all patients bearing the same tumour (Herman *et al.*, 1991; Kappler *et al.*, 1989).

In the data of Nitta *et al* (1990) there is a clear suggestion of superantigen involvement for Vα chains of the TCR. Within the limitations of the available panel of antibodies, there is no evidence of this in the presented data.

It is relatively simple to collect 10,000 events for both the PBLs and the TILs of the breast carcinoma samples, due to the abundance of cells present. Unfortunately, the uveal melanomas do not contain high levels of TILs (Table 3.10) even in the small number of a relatively highly infiltrated tumours. Only 10-12% of the uveal melanoma tumour samples contain lymphocytes and of these approximately 1-6% of the cell suspension obtained will represent lymphocytes. It is therefore impossible to isolate these lymphocytes from the tumour cells and consequently the cell suspension that is incubated with the antibodies will invariably contain mostly tumour or non-lymphoid cells. For example patient samples 6 and 9 contained high levels of lymphocytes whereas patients samples 5,7,8,and 10 only contained 1.1%,1.3% 1.1% and 1.6% lymphocytes, respectively (Table 3.10). The high levels of non specific binding occuring in these patients may be due to the antibodies binding to the tumour cells present within the gate placed round the lymphocytes.

Patient no.	Tumour	%TIL
1	Breast	30
2		29
3		11
4		20
5	Uveal	1.1
6		4.6
7		1.3
8		1.1
9		5.3
10		1.6

Table 3.10: Percentage of cell suspension containing TILs

The breast carcinoma samples were included in the study, since they are readily available and act as a different cell type to compare with the uveal melanoma TILs. Breast carcinomas exhibit TILs in approximately 70% of tumours and the levels of TILs are significantly higher than in uveal melanoma (Whitford *et al.*, 1990) (Table 3.10). Thus it is interesting to see the same pattern occuring in the increased level of expression of the Vβ in the TILs compared to the PBLs.

The conclusion from this study is that TILs may be oligoclonal or polyclonal with the capacity for selective homing. They are not, however, monoclonal. Thus therapeutic strategies for TIL expansion in IL-2 culture must accommodate selective alterations in the recognition profile of the T cells.

3.3 INVESTIGATION INTO THE SYSTEMIC IMMUNE RESPONSE TO UVEAL MELANOMA

3.3.1 Aims

In consequence of the controversy over the role of enucleation in the management of patients with uveal melanoma (Section 1.3), it was decided to determine the immune status of patients prior to and after the operation to remove the tumour. This would (1) identify whether or not the operation had altered the immune status of the patient in general (so far as it is reflected in these parameters) and (2) test the hypothesis of Zimmerman *et al* (1978) that the primary tumour was generating a protective response and thus should be left intact. In the first case, an earlier report (Flynn *et al.*, 1986) had suggested that the tumour had a significant effect on the phenotypic population in the systemic circulation.

Initially, the proposal was to look at the lymphocytes in the systemic circulation prior to the operation and then 1 week, 4 weeks and 6 months after. However this was seldom practical as patients did not always return for check- ups at the expected times and, there were strategic problems in obtaining the samples from the medical staff. Thus the data collected was eventually only 8 patients pre- operatively and 1 week post- operatively. In addition, the control samples were intended to include uveitis patients as there is strong evidence for an immune response (although not necessarily systemic) in this condition. However the only samples available were retinitis pigmentosa (RP) patients who have an eye disease which shows little evidence of any, let alone systemic, autoimmunity. A series of control samples from "normal" individuals were collected but the data was subsequently discovered to be improperly analysed.

The analysis was by the methods described previously (Section 3.1.1)

3.2.2 Results

Blood samples (10ml) were obtained from 8 uveal melanoma patients immediately prior to the operation to remove the tumour, and also 1 week post operatively. Blood was also obtained from 4 RP patients. PBL were isolated using a ficoll- density gradient (Section 2.2.1.4) and cells were then classified into T or B cells or either $CD4^+$ or $CD8^+$ by using directly labelled antibodies to the T cell subsets (Table 3.1). The individual markers were identified by using the previously described FITC labelled murine antibodies to HLA DR and CD25 (Table 3.1). Data was analysed as before (Section 3.1) where the percentage of cells positive for the marker were calculated.

Tables 3.11, 3.12 and 3.13 indicate the percentage of the relevant phenotypes in pre- operative PBLs, post- operative and control patients respectively. The mean data are also indicated on the relevant table and then graphically represented on Figures 3.12 to 3.15. It can be seen from Figure 3.12 that the levels of T cells and B cells do not change between the pre- and post- operative blood samples and indeed the control patients also seem to have similar levels of the different lymphocyte cell types (Table 3.14). This is also true not only for the levels of CD4 and CD8 positive cells but also for the expression of HLA DR and CD25 on the $CD4^+$ and $CD8^+$ T cells. Table 3.14 shows the significance values between the pre- operative, post- operative and control samples and indicated that no significant differences exist.

					HLA DR$^+$		CD25$^+$	
Patient	T	B	CD4$^+$	CD8$^+$	CD4$^+$	CD8$^+$	CD4$^+$	CD8$^+$
1	74.2	10.7	58.5	19.0	7.9	55.5	39.1	49.2
2	69.2	9.8	44.5	23.5	4.6	21.9	47.3	38.0
3	71.1	3.6	30.9	43.7	30.9	78.9	64.3	51.1
4	54.3	30.5	32.7	24.0	14.0	65.0	46.1	41.8
5	63.4	25.3	33.1	30.3	12.5	37.1	22.8	52.0
6	44.2	44.8	20.3	19.9	32.6	62.7	70.6	51.2
7	67.7	6.8	30.9	37.8	24.3	56.8	61.1	46.2
8	47.7	18.5	33.0	25.6	14.4	25.5	49.1	36.9
mean	61.6	18.8	35.5	28.0	17.7	50.4	50.1	45.9
±SD	±11.4	±14.0	±11.3	±8.8	±10.4	±20.2	±15.5	±6.0

Table 3.11: Percentage of pre- operative PBL positive for the specific cell markers.

					HLA DR$^+$		CD25$^+$	
Patient	T	B	CD4$^+$	CD8$^+$	CD4$^+$	CD8$^+$	CD4$^+$	CD8$^+$
1	57.3	13.9	48.3	20.4	12.4	23.1	66.5	66.3
2	37.7	43.7	19.8	28.1	9.8	36.9	40.4	32.1
3	60.1	7.5	35.0	33.0	35.7	49.7	60.1	39.6
4	46.2	4.0	21.4	11.9	25.4	45.0	73.1	32.3
5	67.7	22.5	28.5	35.9	21.3	74.7	57.1	66.3
6	70.6	18.0	32.3	26.7	20.4	44.0	55.4	25.7
7	58.6	10.7	29.1	26.3	17.8	55.3	67.6	38.8
8	67.9	11.0	56.2	21.3	10.2	27.4	37.1	37.6
mean	58.2	16.4	33.9	25.5	19.1	44.5	57.2	42.4
±SD	±11.3	±12.4	±12.6	±7.6	±8.7	±16.3	±12.8	±15.5

Table 3.12: Percentage of Post- operative PBL positive for the specific cell markers.

					HLA DR$^+$		CD25$^+$	
Patient	T	B	CD4$^+$	CD8$^+$	CD4$^+$	CD8$^+$	CD4$^+$	CD8$^+$
1	64.7	10.1	40.5	26.3	20.0	73.4	45.5	43.4
2	53.7	11.8	41.7	15.9	23.8	61.0	59.0	45.7
3	43.5	15.6	27.0	25.7	43.7	61.7	73.7	39.8
4	74.3	14.4	56.0	20.1	34.0	34.7	69.7	48.1
mean	59	12.9	41.3	22	30.3	57.7	61.9	44.2
±SD	±13.5	±2.4	±11.8	±4.9	±10.6	±16.3	±12.6	±3.5

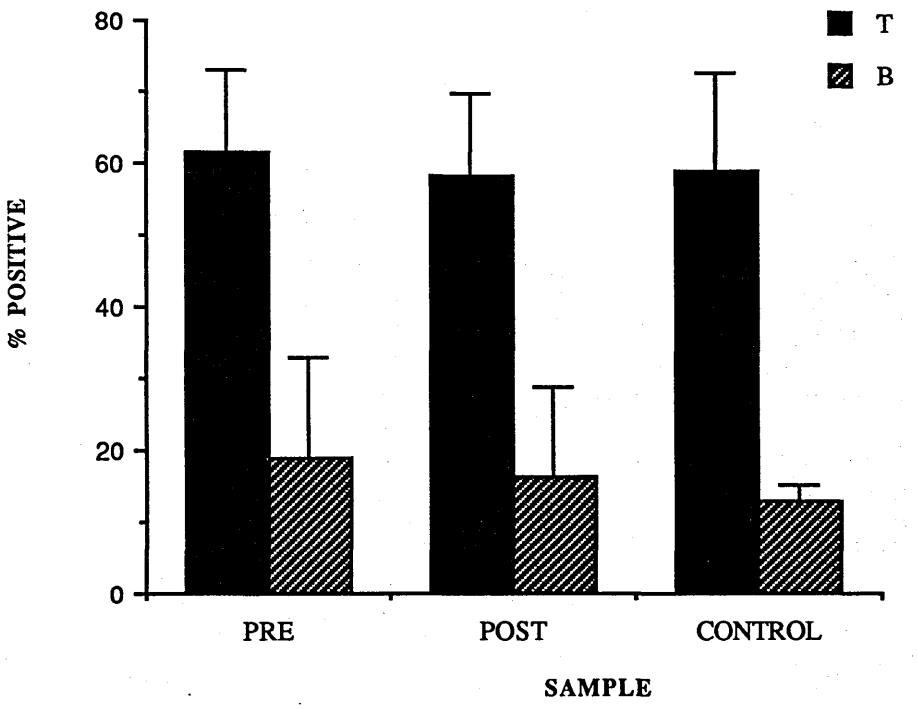
Table 3.13: Percentages of control patient PBL positive for the specific cell markers.

	Correlation		
Marker	Pre/Post	Pre/Control	Post/Control
T cell	$p<0.288$	$p<0.477$	$p<0.500$
B cell	$p<0.440$	$p<0.488$	$p<0.500$
$CD4^+$	$p<0.337$	$p<0.452$	$p<0.443$
$CD8^+$	$p<0.242$	$p<0.454$	$p<0.414$
$CD4^+$HLA DR^+	$p<0.288$	$p<0.386$	$p<0.398$
$CD8^+$HLA DR^+	$p<0.242$	$p<0.466$	$p<0.420$
$CD4^+CD25^+$	$p<0.242$	$p<0.431$	$p<0.443$
$CD8^+CD25^+$	$p<0.288$	$p<0.454$	$p<0.409$

Table 3.14: The significance values for the correlation between the activation markers on the pre- operative and post-operative T cells (using the Wilcoxon Signed Rank correlation) and between the pre-/ post- operative T cells with the control samples (using the Mann-Whitney test). A $p<0.05$ is considered to be significant.

Figure 3.12: Mean data obtained for T and B cell populations in pre- operative, post- operative and control (RP) PBL samples.

Figure 3.13: Mean data obtained for $CD4^+$ and $CD8^+$ T cell populations in pre- operative, post- operative and control (RP) PBL samples.


80
60
40
20
0
% POSITIVE
T
B
PRE
POST
CONTROL
SAMPLE

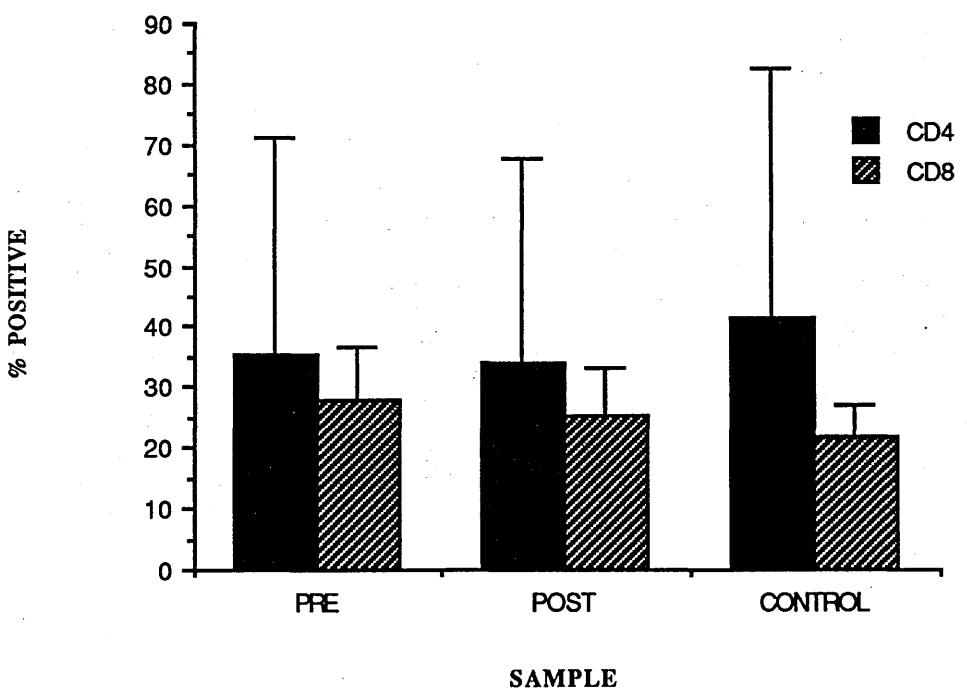
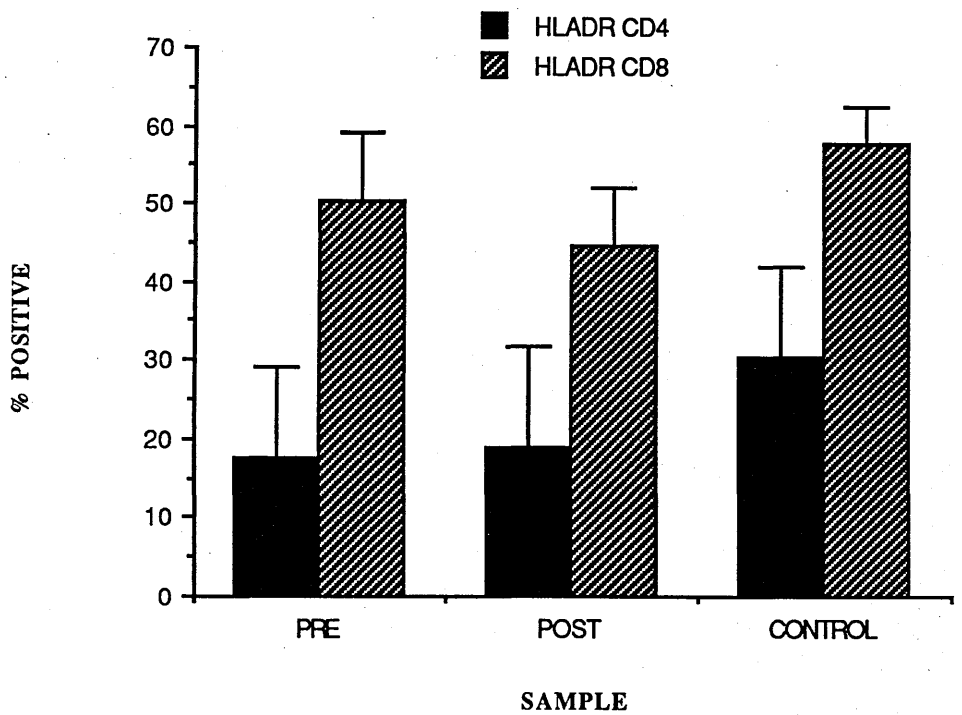
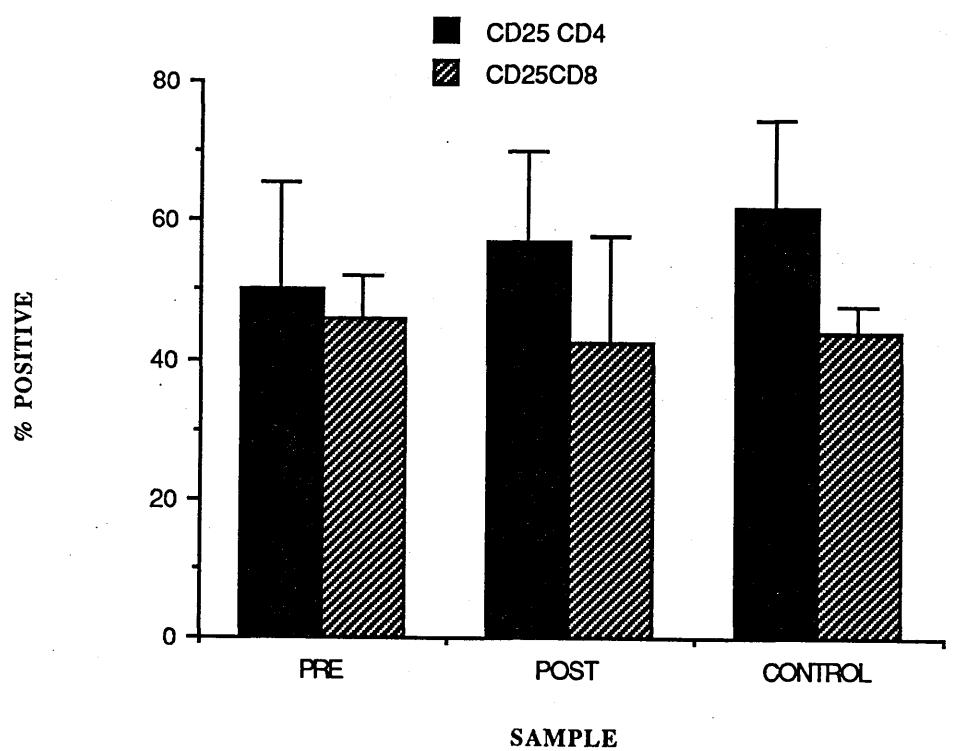

90
80
70
60
50
40
30
20
10
0
% POSITIVE
CD4
CD8
PRE
POST
CONTROL
SAMPLE

Figure 3.14: Mean data obtained for HLA DR^+ $CD4^+/CD8^+$ T cell populations in pre- operative, post- operative and control (RP) PBL samples.

Figure 3.15: Mean data obtained for $CD25^+$ $CD4^+/CD8^+$ T cell populations in pre- operative, post- operative and control (RP) PBL samples.


HLADR CD4
HLADR CD8
70
60
50
40
30
20
10
0
% POSITIVE
PRE
POST
CONTROL
SAMPLE


CD25 CD4
CD25CD8
80
60
40
20
0
% POSITIVE
PRE
POST
CONTROL
SAMPLE

3.3.3 Discussion

During the 1970's much work was carried out into tumour antigen and tumour- host relationships which was reviewed by Baldwin and Price (1976) and also Hollstrome (1976). The latter established that lymphoid cells from tumour- bearing as well as tumour- free donors were cytotoxic *in vitro* for the appropriate target cells, but that tumour- bearing serum specifically inhibited these effects. This phenomenon was rationalised in terms of circulating humoral factors. In addition, the effects of tumour- bearer serum were demonstrated using other *in vitro* procedures to monitor cell mediated immunity to tumour cells including inhibition of leucocyte adherence (Maluish and Halliday, 1974) and lymphocyte stimulation (Vanky *et al.*, 1974). Such immune responses were identified in patients with colonic cancer (Nind *et al.*, 1975) and also in malignant melanoma where experiments showed that PBLs initially exhibited little cytotoxicity for melanoma cells *in vitro*, but reactivity was detected after repeated washing. Furthermore, the cytotoxicity of washed PBLs could apparently be inhibited again by addition of the patient's serum (Currie and Basham, 1973). At this time the circulating tumour antigens or immune complexes were implicated in this type of inhibitory response, since serum activity decreased in animals rendered tumour- free by local resection of their tumour grafts (Robins and Baldwin, 1974; Baldwin *et al.*, 1973). There have been reports that uveal melanoma are susceptible to dissemination into the venous circulation by manipulation of the tumour (Zimmerman *et al.*, 1978; Zimmerman and McLean, 1979) although others are in disagreement with this idea (Manschot *et al.*, 1979; Seigal *et al.*, 1979). Zimmerman *et al* (1978) concluded that enucleation of a melanoma- containing eye led to a significant increase in the, metastasis of the tumour, by removal of a systemic response elicited by the primary tumour.

A more recent study by Flynn *et al* (1986) indicated an increase in the level of Tc, Th and the level of B cells in the PBLs of patients with uveal

melanoma compared to control patients, it was decided to determine whether PBLs from patients with uveal melanoma displayed any alteration in the cell activation status when the tumour was removed. There is wide patient variability (Table 3.11 to 3.13) as is common with clinical studies, and consequently mean values were used for comparisons. Control patients with a condition RP, a term used to describe a heterogeneous group of progressive retinal dystrophies affecting pigment epithelium function (Reviewed by Forrester *et al.*, 1987) were used in this study to act as another eye condition, although non- neoplastic, in case disease induced changes in the eye itself may elicit an immune response.

It was found that there was little difference between the levels of T cells and B cells between the PBL samples taken at different time intervals and indeed between the control patients (Figure 3.12). The levels of cytotoxic T cells within the PBL did not seem to change significantly when the tumour was removed, and indeed the activation status of the T cells did not seem to be impaired or accentuated (Figure 3.14 and 3.15). Previous reports indicated the inhibition of the activation of T cells (Vanky *et al.*, 1974). It must be noted that the blood samples were only 1 week post- operative and perhaps a greater time lapse may have given a better indication if the tumour was inducing some immunosuppressive activity upon the PBLs. It must also be noted that as previously mentioned (Section 1.1.4) the eye is classically referred to as an immunologically privileged site, being isolated from cells of the immune system. Thus perhaps no systemic immune response would be expected. There also appears to be little difference between the sample T cells and the control cells which is in disagreement with the findings of Flynn *et al* (1986) who did however, include a greater number of patients and unlike our study, included normal control PBL samples.

In retrospect, the view of the immune response in the 1970's was oversimplified and is now greatly altered by knowledge of the nature of T cell recognition and function (Section 1.4.1) and also of the largely self nature of oncogene products (Section 1.6.1). In addition experimental methods were of limited accuracy in comparison to those now available. It is likely, in outbred patients with many and variable recent and past immunological stimuli, that analysis of simple markers such as those used here will be so dominated by other environmental stimuli as to make any anti- tumour response undetectable.

CHAPTER 4

CELL SURFACE ANALYSIS OF ADHESION MOLECULES ON TUMOUR CELLS AND TILS

4 CELL SURFACE ANALYSIS OF ADHESION MOLECULES ON TUMOUR CELLS AND TILS

4.1 Background and Aims

Section 3.2 shows that there is no evidence of clonal expansion of TILs due to site post recognition. An alternative hypothesis is that they migrate to and accumulate at the tumour site in response to molecules expressed on the tumour cells. Adhesion molecules are reported to be involved in the metastasis of primary tumour cells (Section 1.5.5). The surface markers measured in this study, on 20 uveal melanoma tumour samples, included HLA DR and ICAM-1 (CD54) which have been shown to have expression patterns correlating with the probability of metastasis in cutaneous melanoma. In addition, two further markers were studied: LFA-1 (CD11a), the relevant ligand for ICAM-1, particularly on the TIL samples, and also Class I MHC antigen. The TILs were also studied for the expression of these molecules to investigate the possibility that there may be differences between the $CD4^+$ and $CD8^+$ T cell populations within the tumour. The expression of these molecules and also of the MHC antigens were investigated for possible correlations with differing tumour cell types.

4.2 Results

4.2.1 Tumour cell surface antigen analysis

Primary cell suspensions were prepared (Section 2.2.1.2) and stained for FACScan analysis using the secondary antibody method (Section 2.2.2.1), except in the case of HLA DR expression where the antibody was already directly conjugated to FITC. The relevant unconjugated antibodies used in this particular study included W6/32 (anti- Class I), 6.5B5 (anti-ICAM-1, CD54), TS1/22 (anti- LFA-1, CD11a) (Table 4.1).

Antibody	Isotype	Reactivity	Cell Source
W6/32	IgG_2	HLA A/B/C	ECAC (Barnstable *et al*, 1978)
6.5B5	IgG1	CD54	(Wellicome *et al*, 1990)
TS1/22	IgG1	CD11a	ATCC

Table 4.1: Antibodies used in this study

The antibodies 6.5B5 and TS1/22 were supplied as cell supernatants , containing 0.05% sodium azide, and 10µl of each was added to the cell suspension (50µl). W6/32 was purified in the laboratory to a concentration of 20µg/ml and diluted 1:5 with 0.1% sodium azide. Again 10µl of this antibody was added to a 50µl cell suspension. After the primary incubation the cells were washed as usual and then stained with the FITC conjugated secondary antibody (sheep anti mouse IgG whole antibody (Section 2.1)). Once the unbound antibody had been washed from the cell suspension, PI was added to allow a live/ dead discrimination gate to be applied upon collection (Section 2.2.4.1).

Two gates were placed on the final collection of the tumour cells. These included the live/ dead discrimination gate, as described previously (Section 2.2.4.1), and also a scatter gate (Figure 4.1) to remove the possibility of any lymphocytes present influencing the results obtained for the tumour cells. Prior to the analysis the cells were analysed for the presence of any infiltrate using the previously defined Leucogate antibody (Section 3.1.2) (Figure 3.1). Once the position of the lymphocytes was determined on the

scatter plot, an exclusion gate for the lymphocytes was applied (Figure 4.1). Figure 4.2 gives an illustration of the histograms obtained on analysis of the cell surface expression of each individual antigen on a representative tumour sample.

Table 4.2 shows the percentages of the cells within the tumour cell suspensions expressing each of the individual surface markers and also the percentage of the suspension that contained lymphocytes. There is much patient variability for all of the antigens studied. This can be seen from the mean data and appropriate standard deviations presented. The percentages of cells bearing Class I and Class II (HLA DR) antigens vary considerably between patients and Figure 4.3 shows that Class II (HLA DR) gain does not correlate with Class I loss. There is a trend toward a high percentage of ICAM-1 expressing cells and a negative correlation between percent ICAM-1 positive cells and percent Class II(HLA DR) +ve cells ($p< 0.012$) (Table 4.3 and Figure 4.4). The levels of TILs present in the tumour suspensions does not correlate with Class I or ICAM-1(Figures 4.5 and 4.6). The statistics for this data are shown in Table 4.3. There is a correlation between the levels of TILs present and the expression of Class II present ($p<0.001$) (Figure 4.7)

Pathological reports for the tumours were analysed to investigate possible correlations between the expression of these markers and the cell type. The cell types are pure epithelioid cells (EP, n=2), pure spindle cells (SP, n=9) and mixed spindle and epithelioid (EP/SP, n=9) cells (Section 1.3.3). Figures 4.8 to 4.10 show the levels of the individual cell surface antigens and the appropriate cell type and Table 4.4 indicates the mean values. Class I is expressed on a higher percentage of the mixed cell population with a mean value of 34.5% and both epithelioid cell samples have a lower percentage of cells expressing this MHC antigen (mean value of 16.3%). HLA DR is expressed on a slightly higher percent of the pure spindle cells than the mixed cell population with mean values of 8.9% and 7.6%

respectively. Again the two pure epithelioid tumours have a lower percentage of cells bearing this MHC antigen. ICAM-1 is present on similar percentages of all the 3 cell types.

Patient No.	% TILs	Class I	HLA DR	ICAM-1
1	2.2	13.1	2.3	82.3
2	0.8	3.1	8.0	65.5
3	0.6	22.0	6.3	89.5
4	4.2	34.4	26.3	55.4
5	0.4	22.1	1.2	83.4
6	7.3	19.5	9.1	67.4
7	3.8	33.3	16.0	61.5
8	3.2	79.8	7.4	94.9
9	6.1	8.1	9.6	84.0
10	2.4	4.9	5.1	88.3
11	0.1	12.8	0	95.3
12	0.6	31.3	1.5	93.9
13	6.0	7.7	3.0	91.1
14	0.3	13.4	0.7	92.9
15	1.1	30.7	4.0	91.1
16	4.6	18.2	10.3	54.7
17	0.2	9.2	0.8	46.1
18	1.1	23.5	27.9	57.6
19	5.3	88.6	11.6	84.5
20	1.6	59.9	8.5	74.9
mean ± SD	2.7± 2.2	26.7± 23.6	7.9± 7.8	77.7± 5.9

Table 4.2: Percentages of tumour cells expressing antigens thought to be involved in metastasis.

Variable 1	Variable 2	Significance	Correlation Equ
ICAM-1	HLA DR	$p<0.012$	$y=87.0-1.16x$
% TILs	Class I	$p<0.67$	$y=80.1-0.89x$
% TILs	Class II	$p<0.001$	$y=5.16+1.08x$
% TILs	ICAM-1	$p<0.316$	$y=80.1-0.89x$

Table 4.3: Statistical analysis on the tumour cell surface marker expression data represented in Table 4.2. Significance values were calculated using the Spearman Rank correlation model. It is considered that a p value of less than 0.05 ($p<0.05$) indicates a significant correlation.

Antigen	EP	SP	EP/SP
Class I	16.3	22.3	34.5
HLA DR	5.7	8.9	7.6
ICAM-1	74.9	71.5	84.6

Table 4.4: Mean values of the cell surface antigen expression on the different cell types (epithelioid (EP), spindle (SP) and mixed epithelioid/ spindle (EP/SP)) of uveal melanoma.

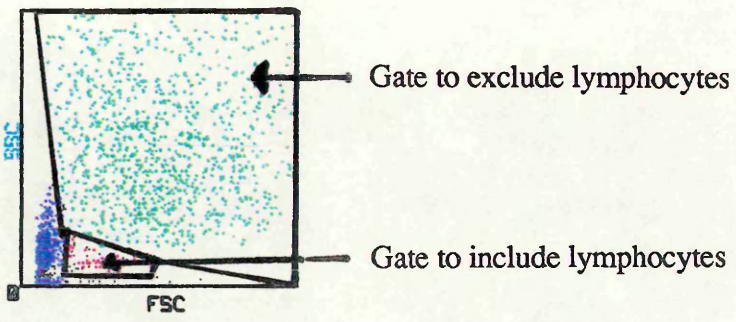


Figure 4.1: Scatter plot indicating gates applied when analysing the cell surface expression of the relevant adhesion molecules on the uveal melanoma tumour cells (excluding TILs) and TILs

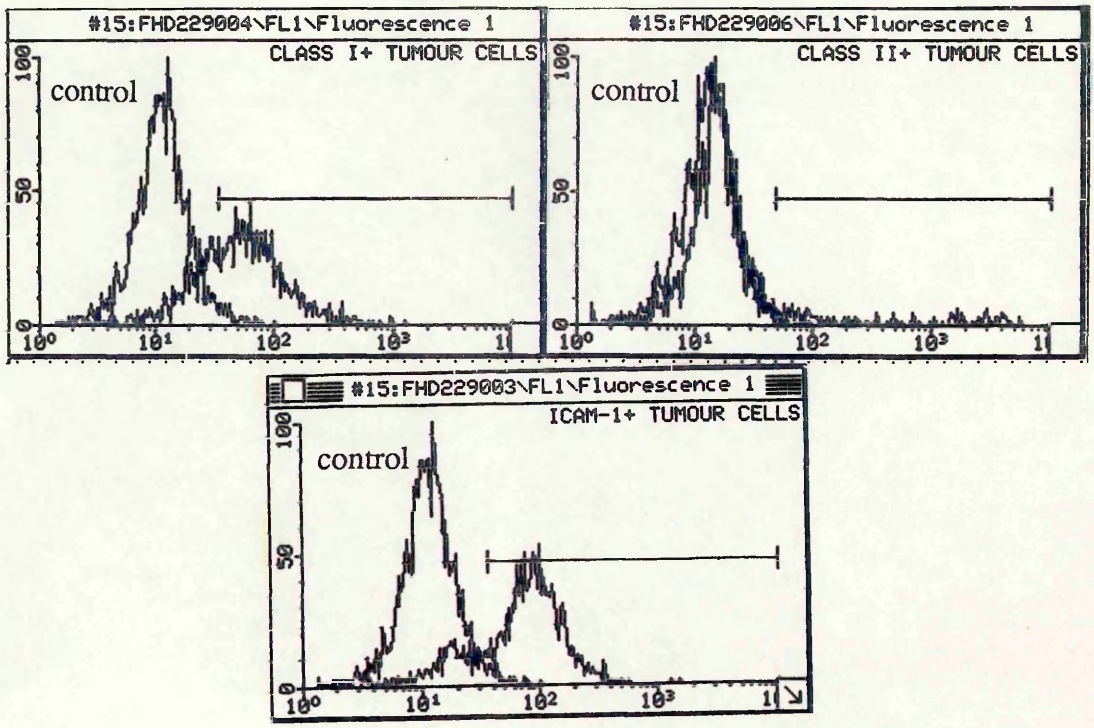
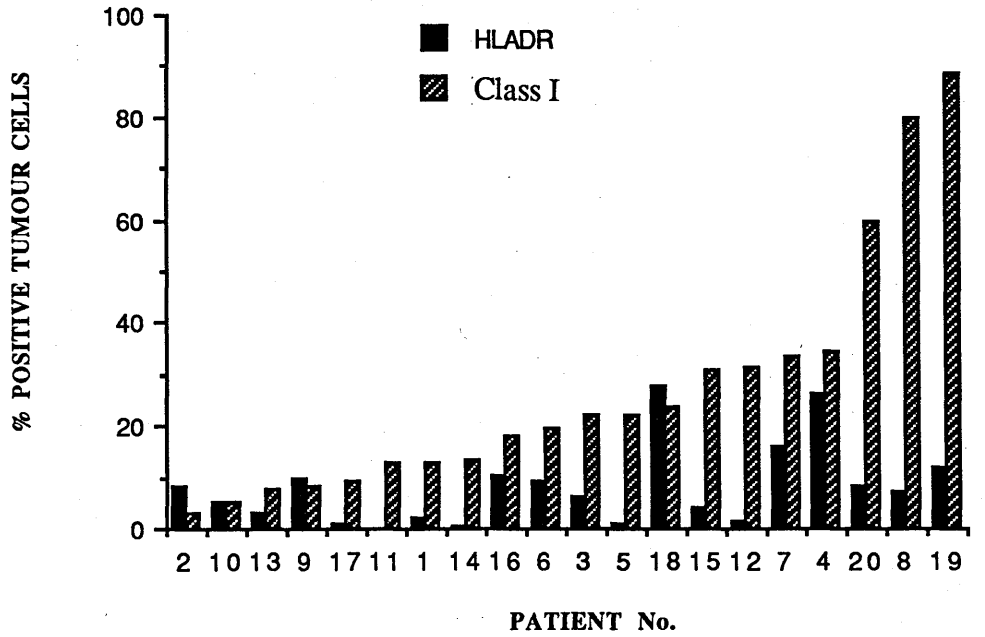


Figure 4.2: Histograms obtained when analysing the expression of individual antigens on the surface of the uveal melanoma tumour cells.

Figure 4.3: Graph indicating the correlation of Class I MHC expression with the level of HLA DR expression.on the uveal melanoma tumour cells.

Figure 4.4: Graph indicating the correlation of HLA DR with ICAM-1 being expressed on the uveal melanoma cells.


HLADR
Class I
% POSITIVE TUMOUR CELLS
100
80
60
40
20
0
2 10 13 9 17 11 1 14 16 6 3 5 18 15 12 7 4 20 8 19
PATIENT No.

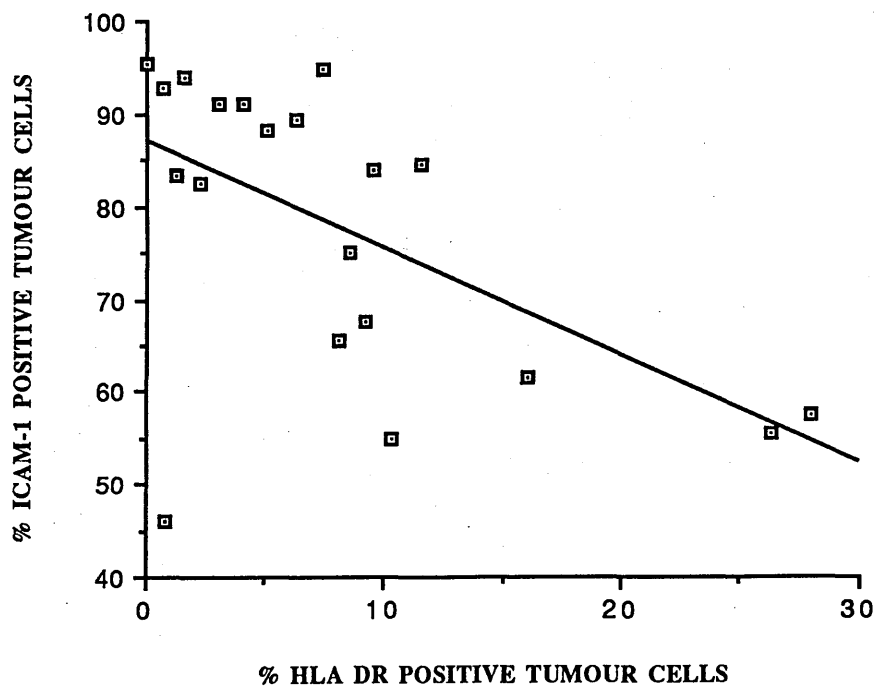
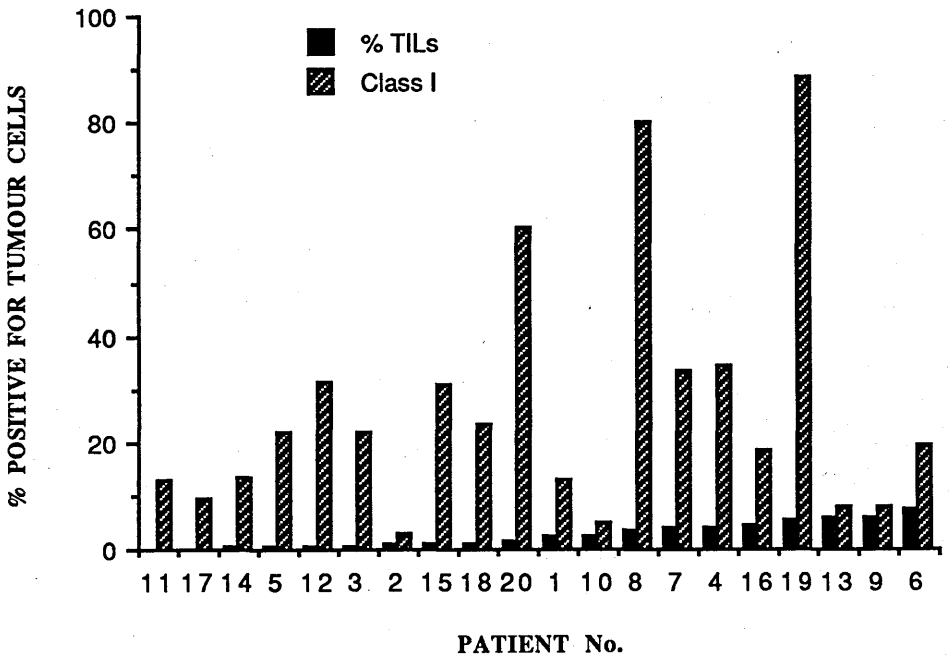
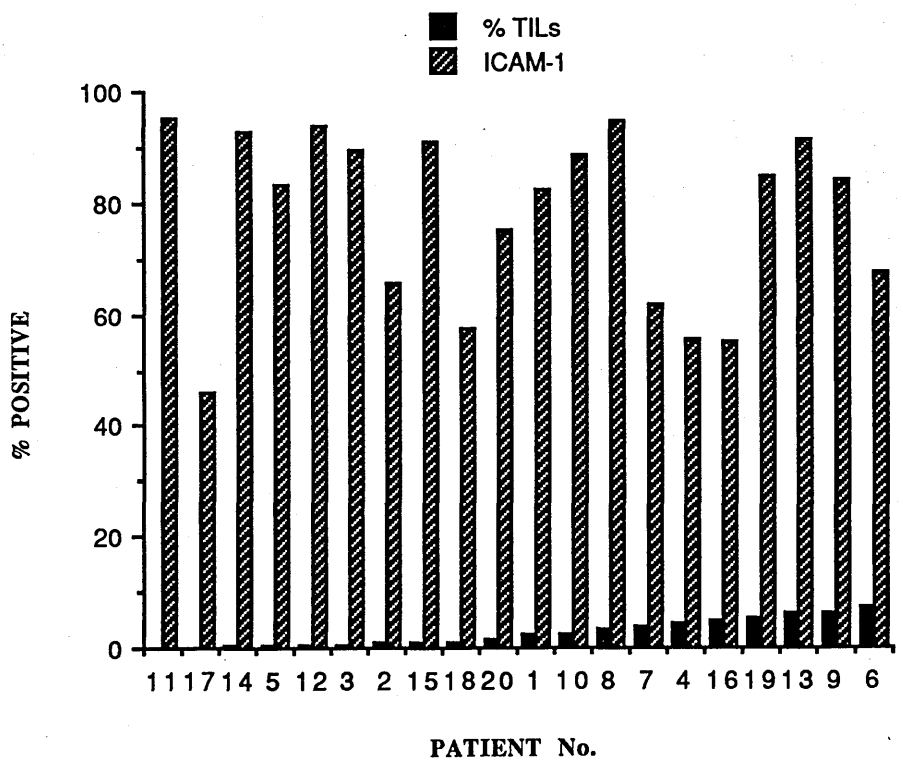

% ICAM-1 POSITIVE TUMOUR CELLS
100
90
80
70
60
50
40
0
10
20
30
% HLA DR POSITIVE TUMOUR CELLS

Figure 4.5: Graph indicating the correlation of Class I MHC being expressed on the tumour cells with the level of TILs present within the tumour cell suspension.

Figure 4.6: Graph indicating the correlation of ICAM-1 being expressed on the tumour cells with the levels of TILS present within the tumour cell suspension.


% TILs
Class I
% POSITIVE FOR TUMOUR CELLS
100
80
60
40
20
0
11 17 14 5 12 3 2 15 18 20 1 10 8 7 4 16 19 13 9 6
PATIENT No.


% TILs
ICAM-1
% POSITIVE
100
80
60
40
20
0
11 17 14 5 12 3 2 15 18 20 1 10 8 7 4 16 19 13 9 6
PATIENT No.

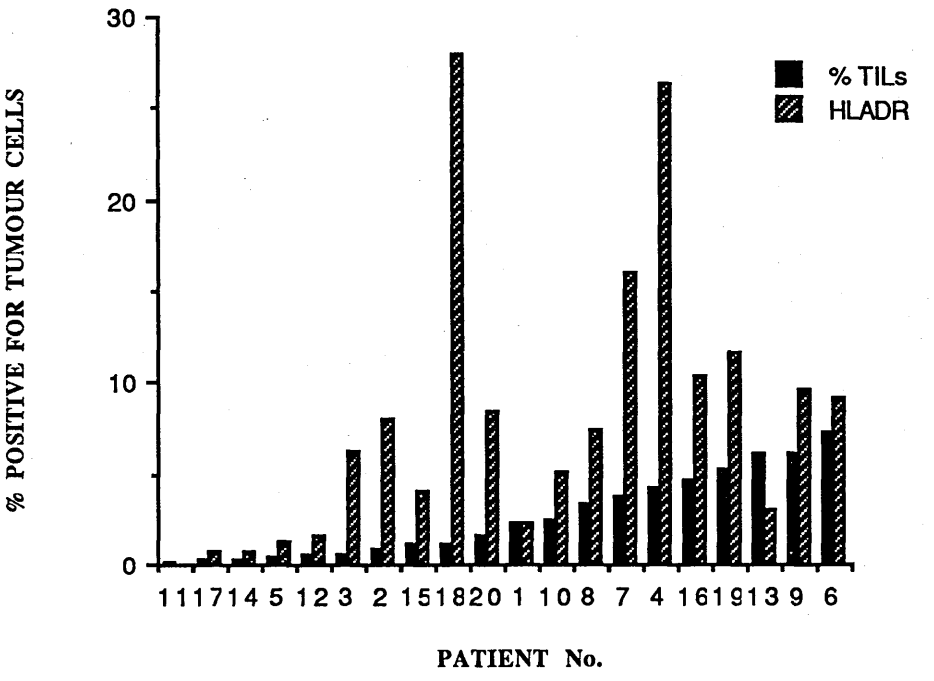
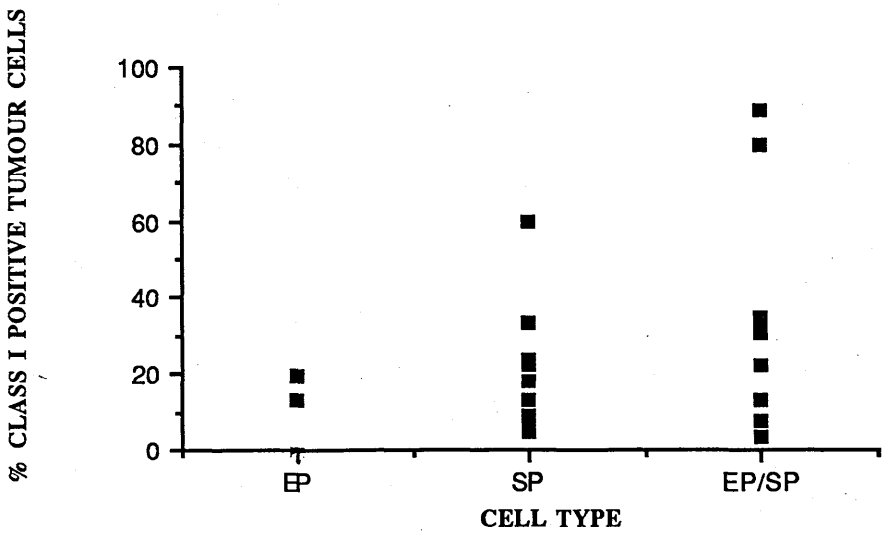


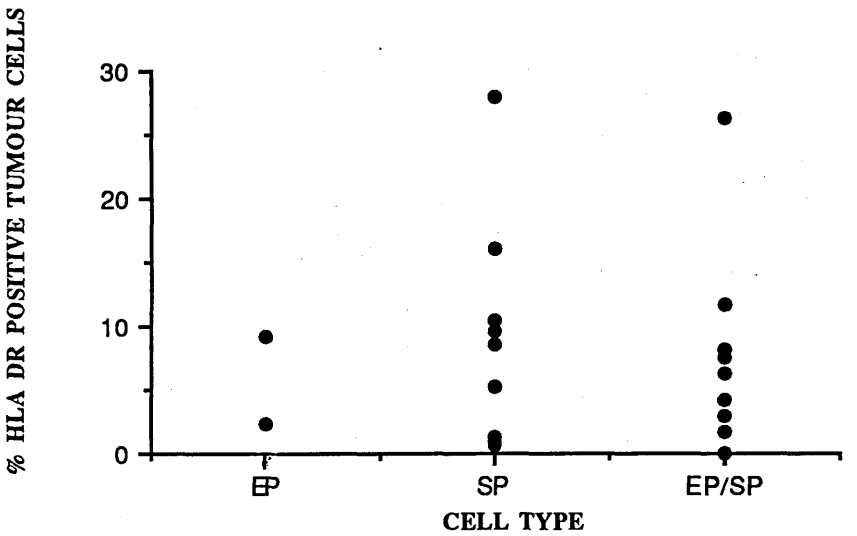
Figure 4.7: Graph indicating the correlation of HLA DR (Class II MHC) being expressed on the tumour cells with the level of TILs present within the tumour cell suspension.

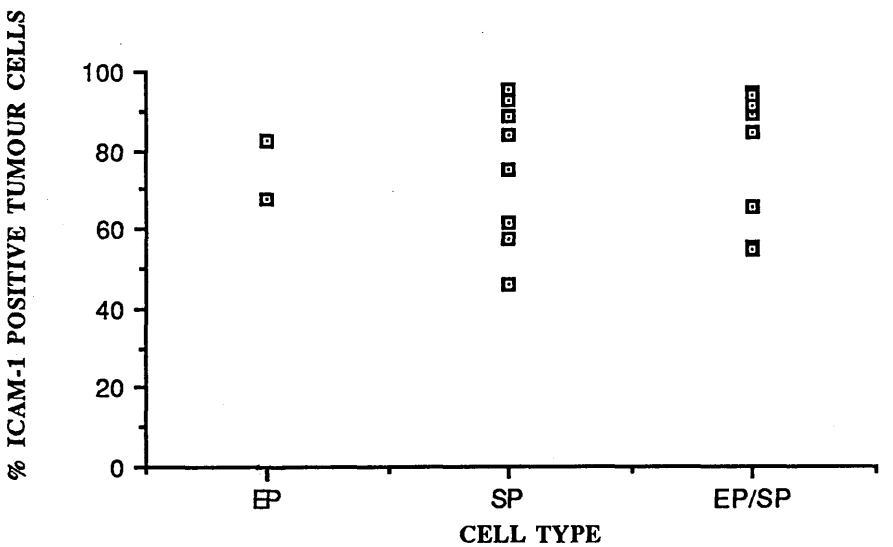
Figure 4.8: Class I expressed on the epithelioid (E), spindle (SP) and the mixed epithelioid/spindle (EP/SP) tumour cell populations.

Figure 4.9: HLA DR (Class II MHC) expressed on the epithelioid (E), spindle (SP) and the mixed epithelioid/spindle (EP/SP) tumour cell populations.

Figure 4.10: ICAM-1 expressed on the epithelioid (E),spindle (SP) and the mixed epithelioid/spindle (EP/SP) tumour cell populations.


% CLASS I POSITIVE TUMOUR CELLS
100
80
60
40
20
0
EP
SP
EP/SP
CELL TYPE


% HLA DR POSITIVE TUMOUR CELLS
30
20
10
0
EP
SP
EP/SP
CELL TYPE


% ICAM-1 POSITIVE TUMOUR CELLS
100
80
60
40
20
0
EP
SP
EP/SP
CELL TYPE

4.2.2 Cell surface antigen analysis of TIL subpopulations

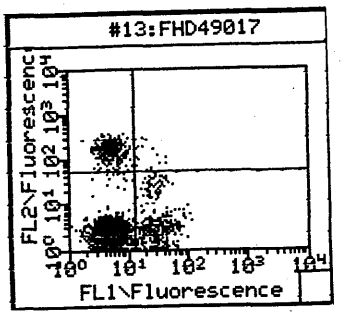
Five uveal melanoma tumour samples that contained sufficient levels of TILs were used in this study to measure the percentage expressing cell surface markers relevant to adhesion (Section 4.2.1) i.e. Class I, HLA DR, ICAM-1 and LFA-1. These molecules were also measured on 4 of the 5 corresponding patient PBL samples. The cells were stained as described before (Section 4.2.1) . Before the PE conjugated antibody could be added to the cell, any free binding sites of the FITC labelled secondary antibody were blocked with 10% mouse serum. Three of the TIL samples were those obtained from the previous study (Section 4.2.1), namely samples 16, 19 and 20, and an additional 2 TIL samples not included in the previous study. For the purpose of this study these samples will be referred to as TIL and PBL samples 1, 2, 3, 4, and 5. Figures 4.11 and 4.12 illustrate FACScan analysis diagrams obtained for (a) PBLs and (b) TILs. Percentages were calculated as in Section 3.1.2.

Table 4.5 shows the percentage positive cells measured in the TIL and PBL samples for each marker. It was decided not to continue measure the levels of LFA-1 on the lymphocytes since preliminary data indicated that it was present on >97% of the cells. This provided more cells to measure the other perhaps more variable cell surface markers.

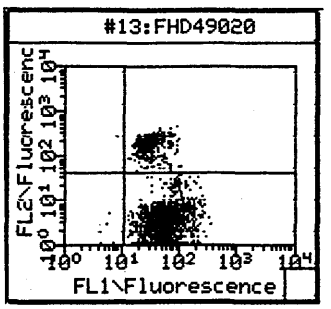
Figure 4.11: Illustrative FACSan diagrams obtained for the analysis of $CD4^+$ (A) and $CD8^+$(B) PBL subsets. Each diagram indicates the staining for (a) $CD4^+$(A)/ $CD8^+$(B) PBL in the UL quadrant, (b) Class I MHC^+ staining PBL subsets in the UR quadrant, (c) HLA DR^+ staining PBL subsets in the UR quadrant and (d) ICAM-1^+ staining PBL subsets in the UR quadrant. In each case the percentage non-specific staining of the $CD4^+/CD8^+$ T cells present in the UR quadrant of panel (a) was subtracted from the positive staining for the other antigens.

A

(a)


#13:FHD49017
FL2\Fluorescenc
FL1\Fluorescence

(b)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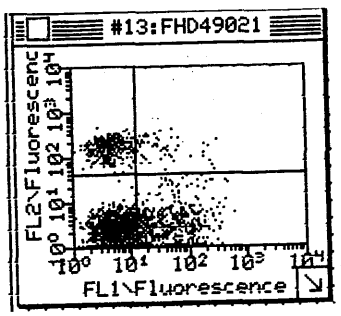
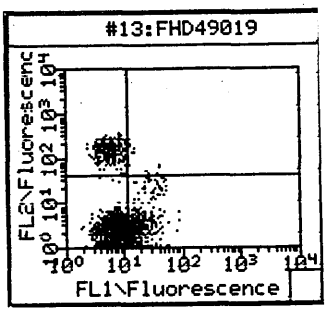
FL2\Fluorescenc
FL1\Fluorescence

(c)

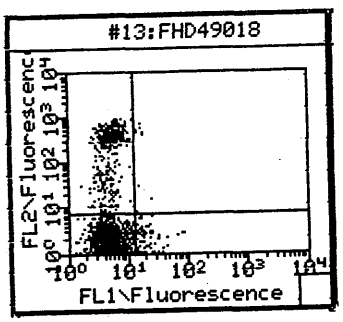

#13:FHD49021
FL2\Fluorescenc
FL1\Fluorescence

(d)


#13:FHD49019
FL2\Fluorescenc
FL1\Fluorescence

B

(a)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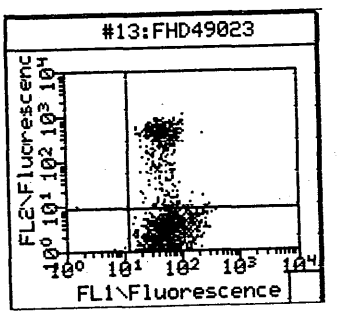
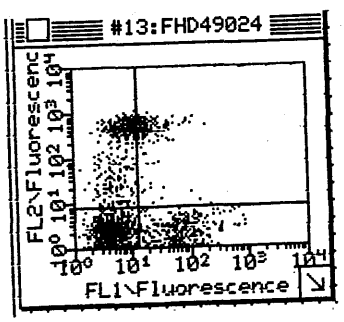
FL2\Fluorescenc
FL1\Fluorescence

(b)


#13:FHD49023
FL2\Fluorescenc
FL1\Fluorescence

(c)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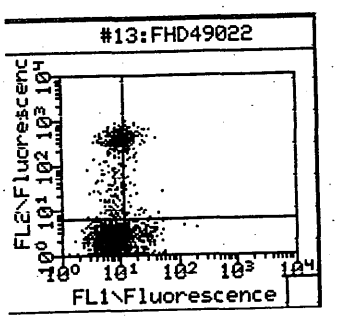
FL2\Fluorescenc
FL1\Fluorescence

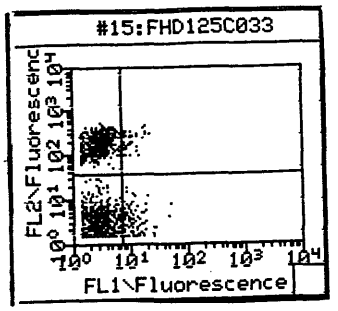
(d)


#13:FHD49022
FL2\Fluorescenc
FL1\Fluorescence

PFigure 4.12: Illustrative FACSan diagrams obtained for the analysis of $CD4^+$ (A) and $CD8^+$(B) TIL subsets. Each diagram indicates the staining for (a) $CD4^+$(A)/ $CD8^+$(B) TILs in the UL quadrant, (b) Class I MHC^+ staining TIL subsets in the UR quadrant, (c)HLA DR^+ staining TIL subsets in the UR quadrant and (d) ICAM-1^+ staining TIL subsets in the UR quadrant. In each case the percentage non-specific staining of the $CD4^+/CD8^+$ T cells present in the UR quadrant of panel (a) was subtracted from the positive staining for the other antigens.

A

(a)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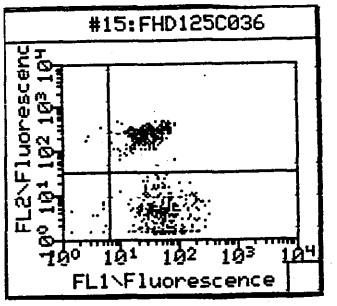
FL2\Fluorescenc
FL1\Fluorescence

(b)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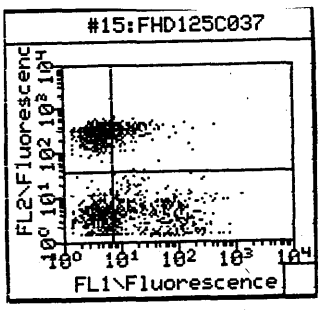
FL1\Fluorescence

(c)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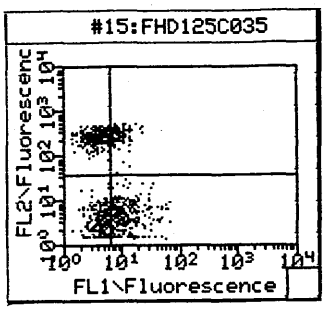
FL1\Fluorescence

(d)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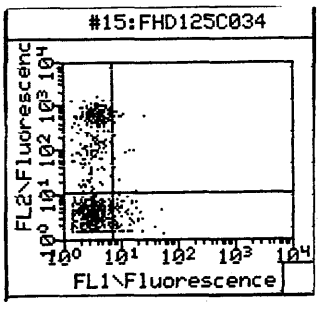
FL2\Fluorescenc
FL1\Fluorescence

B

(a)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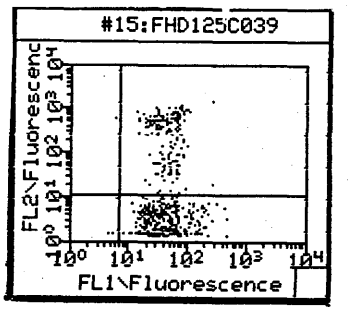
FL2\Fluorescenc
FL1\Fluorescence

(b)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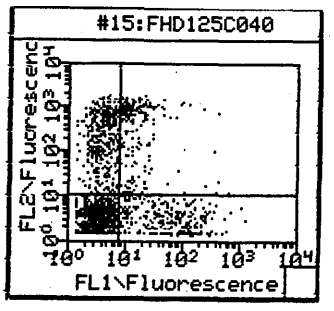
FL2\Fluorescenc
FL1\Fluorescence

(c)


#15:FHD125C040
FL2\Fluorescenc
FL1\Fluorescence

(d)

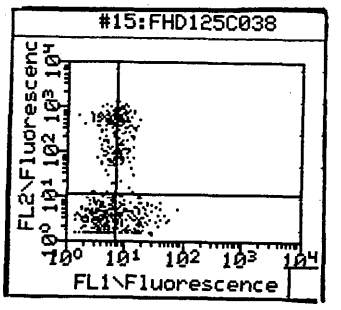

#15:FHD125C038
FL2\Fluorescenc
FL1\Fluorescence

Table 4.6 and Figure 4.13 indicate the mean percentages of cells positive for the measured markers. The Class I MHC antigen is present on a high percent of the each of the lymphocyte subpopulations in the TILs and the PBLs (Figure 4.14) (although it should be noted that the average data may be influenced by the low percentage of $CD8^+$ Class I^+ PBL on patient 2 (24.7%)). It can be seen that HLA DR is present to greater extent on the TILs than the PBL samples (Figure 4.15). This is similar to the previous study (Section 3.2), with a higher percentage of $CD8^+$HLA DR^+ cells than the $CD4^+$ HLA DR^+ cells. In both the PBL and TIL samples ICAM-1 is present on a higher percentage of $CD8^+$ T cells than $CD4^+$ T cells (Figure 4.16).

As a consequence of the observation of Whitford *et al* (1991) of a strong correlation between HLA DR expression on $CD8^+$ TILs and both Class I and Class II MHC antigens on breast carcinoma cells, it was relevant to inspect the more limited uveal melanoma data for any such trends. For 3 of the TIL samples, data for the matching tumour cell sample was available (Section 4.2.1). Although there is a very small sample number there does appear to be a trend towards correlation between HLA DR bearing $CD4^+$ cells (Figure 4.17) and also between HLA DR expressing tumour cells and HLA DR bearing $CD8^+$ TILs (Figure 4.18). Figures 4.19 and 4.20 indicate that a similar correlation does not exist between HLA DR expression on the tumour cells and HLA DR bearing CD4+ TILs ($p<0.667$), or the expression of Class I on the tumour cells with HLA DR bearing CD8+ TILs ($p<0.667$). However, with only three patients such an analysis obviously has limited validity.

	CD4±			CD8±		
Sample no	Class I	HLA DR	ICAM-1	Class I	HLA DR	ICAM-1
1 TIL	96.6	67.2	29.0	92.5	74.0	47.8
2 PBL	90.9	16.7	24.7	24.7	28.2	38.3
2 TIL	94.5	53.8	36.8	92.0	73.6	72.4
3 PBL	98.6	13.5	27.9	97.0	97.0	67.1
3 TIL	95.2	42.3	70.0	96.3	61.5	82.4
4 PBL	98.5	1.8	14.0	98.9	39.5	32.9
4 TIL	91.2	55.5	68.9	98.4	81.5	63.0
5 PBL	94.0	9.9	5.0	97.9	28.4	28.2
5 TIL	81.4	22.0	19.8	94.0	70.7	48.2

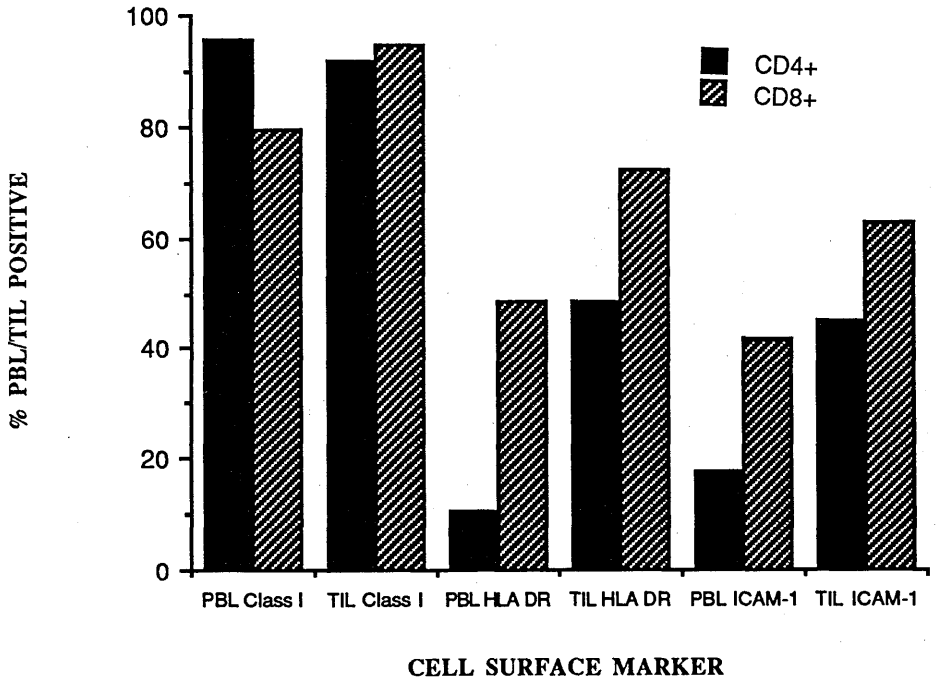
Table 4.5: Data obtained from the analysis of cell surface expression of Class I, HLA DR and ICAM-1 by uveal melanoma containing TIL and relevant PBL samples.

	PBL		TIL	
Antigen	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$
Class I^+	95.5± 3.7	79.6± 36.5	91.7± 6.1	94.6± 2.6
HLA DR^+	10.4± 6.4	48.2± 32.8	48.1± 17	72.2± 7.2
ICAM-1^+	17.9± 10.4	41.6± 17.4	44.9± 23.2	62.7± 15.1

Table 4.6: Mean data obtained from the expression of Class I, HLA DR and ICAM-1 on the surface of $CD4^+$ and $CD8^+$ PBL and TIL samples.

Figure 4.13: Mean percentage of the expression of each of the individual cell surface markers on the PBLs and TILs.

Figure 4.14: Expression of MHC Class I on the $CD4^+$ and $CD8^+$ PBL and TIL subsets.


% PBL/TIL POSITIVE
100
80
60
40
20
0
CD4+
CD8+
PBL Class I
TIL Class I
PBL HLA DR
TIL HLA DR
PBL ICAM-1
TIL ICAM-1
CELL SURFACE MARKER

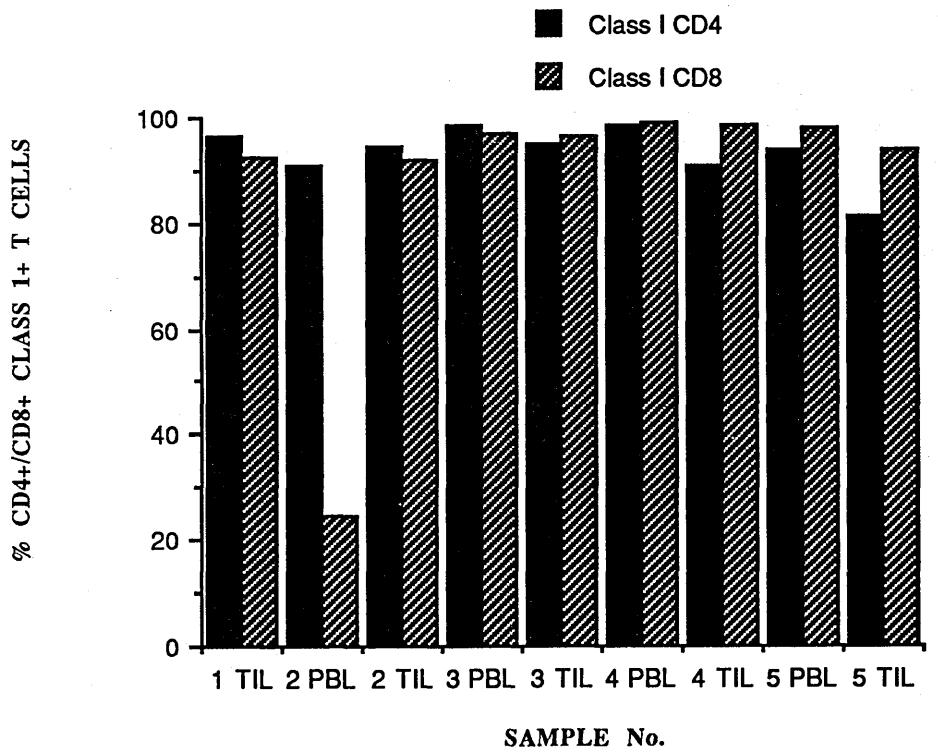
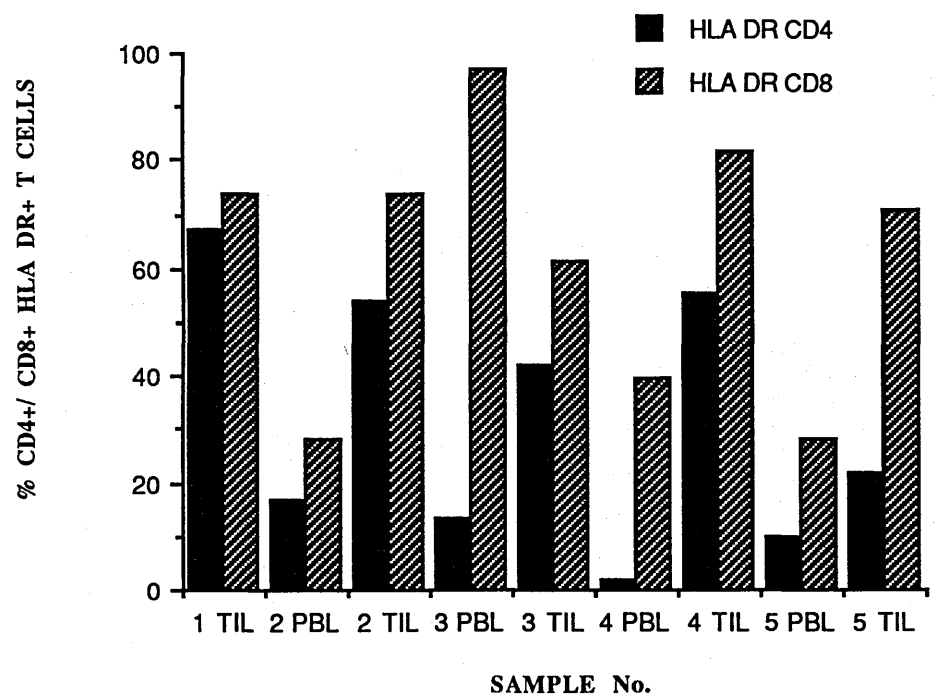

% CD4+/CD8+ CLASS 1+ T CELLS
Class I CD4
Class I CD8
100
80
60
40
20
0
1 TIL
2 PBL
2 TIL
3 PBL
3 TIL
4 PBL
4 TIL
5 PBL
5 TIL
SAMPLE No.

Figure 4.15: Expression of HLA DR (MHC Class II) on the $CD4^+$ and $CD8^+$ PBL and TIL subsets.

Figure 4.16: Expression of ICAM-1 on the $CD4^+$ and $CD8^+$ PBL and TIL subsets.


HLA DR CD4
HLA DR CD8
% CD4+/ CD8+ HLA DR+ T CELLS
100
80
60
40
20
0
1 TIL 2 PBL 2 TIL 3 PBL 3 TIL 4 PBL 4 TIL 5 PBL 5 TIL
SAMPLE No.

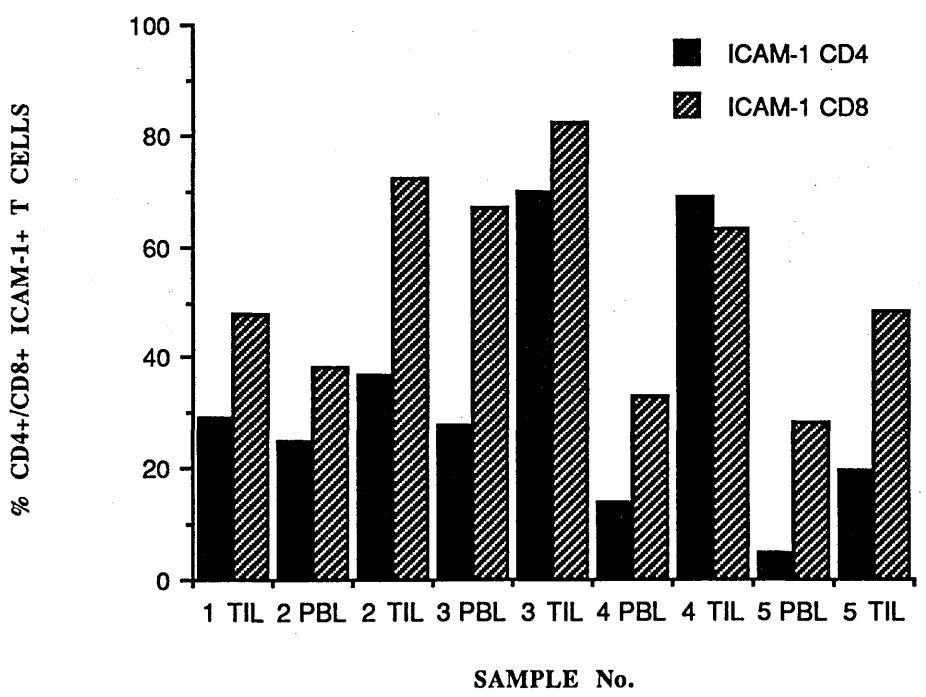
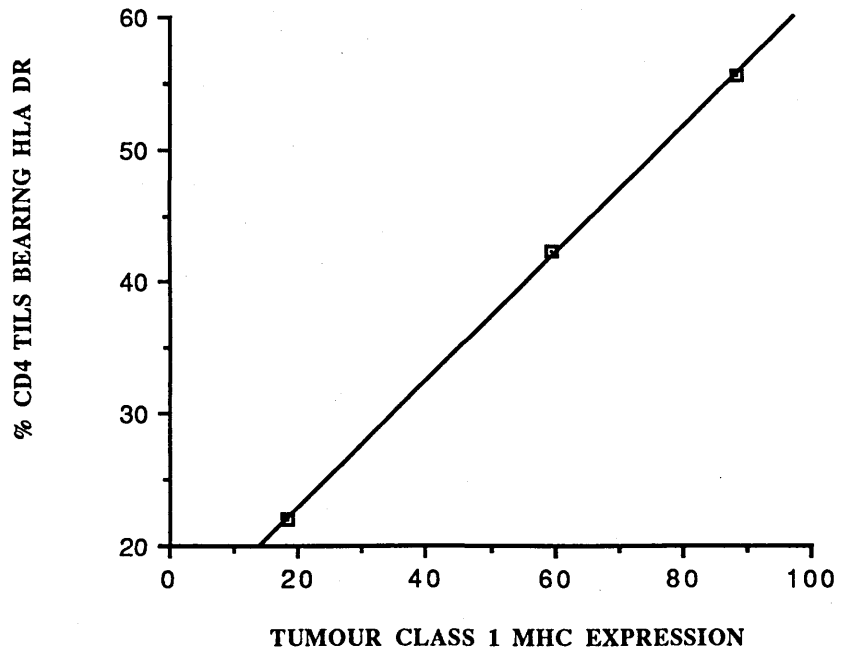

ICAM-1 CD4
ICAM-1 CD8
% CD4+/CD8+ ICAM-1+ T CELLS
100
80
60
40
20
0
1 TIL 2 PBL 2 TIL 3 PBL 3 TIL 4 PBL 4 TIL 5 PBL 5 TIL
SAMPLE No.

Figure 4.17: Correlation of the expression of $CD4^+$ TILs bearing HLA DR with Class I MHC^+ tumour cells.

Figure 4.18: Correlation of the expression of $CD8^+$TILs bearing HLA DR with HLA DR^+ tumour cells.


% CD4 TILS BEARING HLA DR
60
50
40
30
20
0
20
40
60
80
100
TUMOUR CLASS 1 MHC EXPRESSION

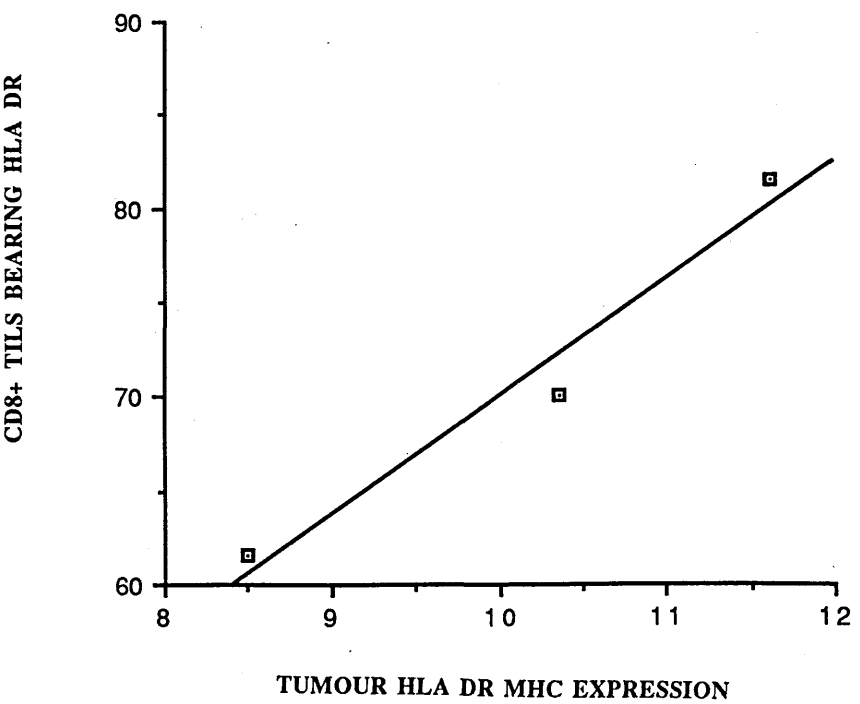
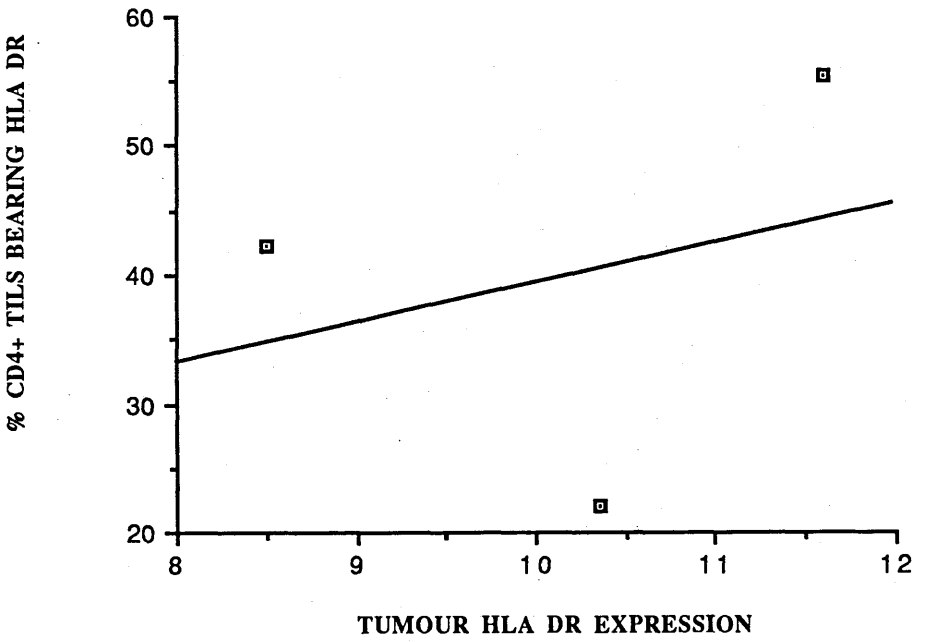
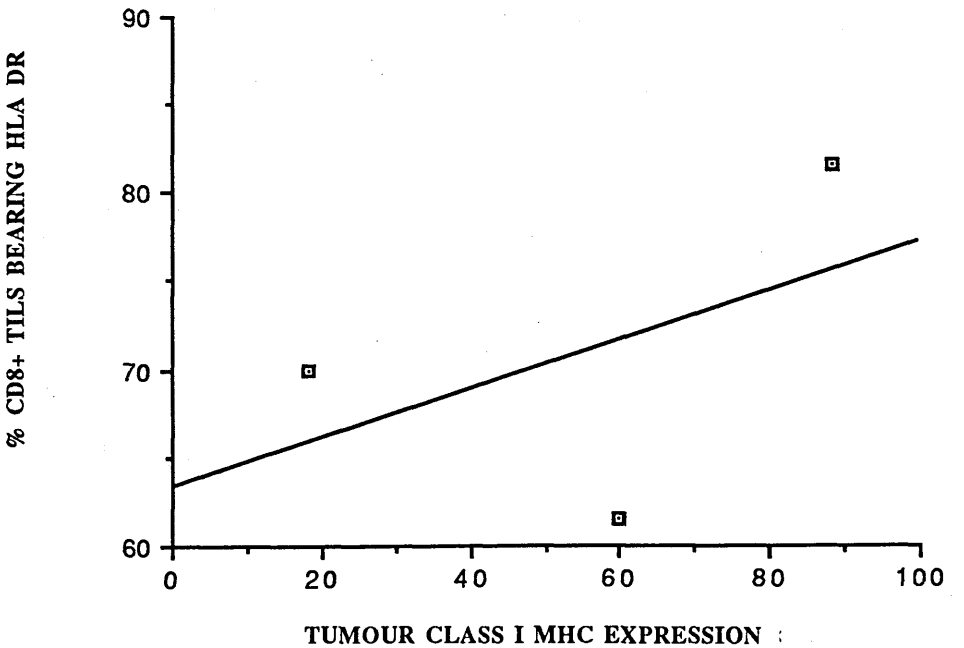

CD8+ TILS BEARING HLA DR
90
80
70
60
8
9
10
11
12
TUMOUR HLA DR MHC EXPRESSION

Figure 4.19: Correlation of the expression of $CD4^+$ TILs bearing HLA DR with HLA DR^+ tumour cells.

Figure 4.20: Correlation of the expression of $CD8^+$ TILs bearing HLA DR with Class I^+ tumour cells.


% CD4+ TILS BEARING HLA DR
60
50
40
30
20
8
9
10
11
12
TUMOUR HLA DR EXPRESSION


% CD8+ TILS BEARING HLA DR
90
80
70
60
0
20
40
60
80
100
TUMOUR CLASS I MHC EXPRESSION

4.2.3 Involvement of ICAM-1 and LFA-1 in cell killing

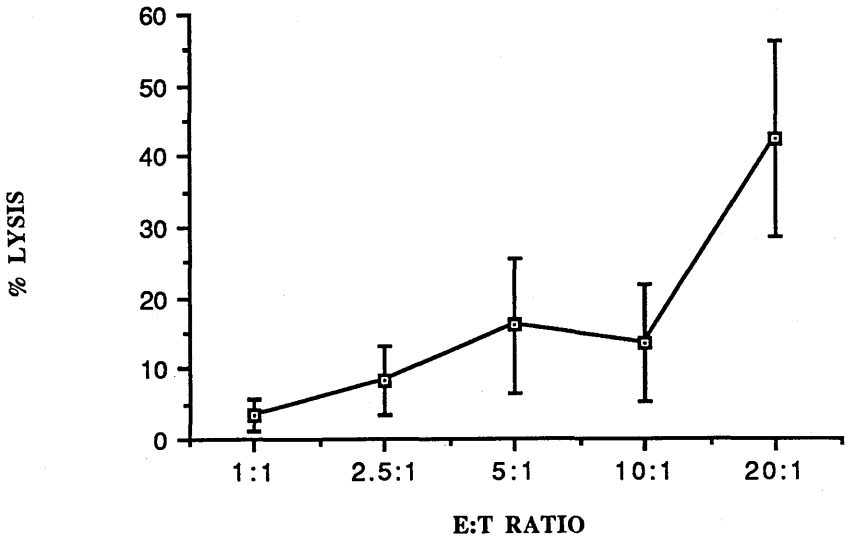
4-hour chromium release cytotoxicity assays (Section 2.2.6) were performed, initially, to investigate the possibility that ICAM-1: LFA-1 interactions, between uveal melanoma cells and the tumour infiltrating lymphocytes, may be involved in the killing of the autologous tumour cells (Anachini *et al*, 1987)). For this study to be performed it was essential that the primary tumour cells could incorporate the ^{51}Cr to a sufficient extent to allow a difference to be detected in the release of the isotope due to the selective inhibition of cytotoxicity by the presence of the adhesion molecules.

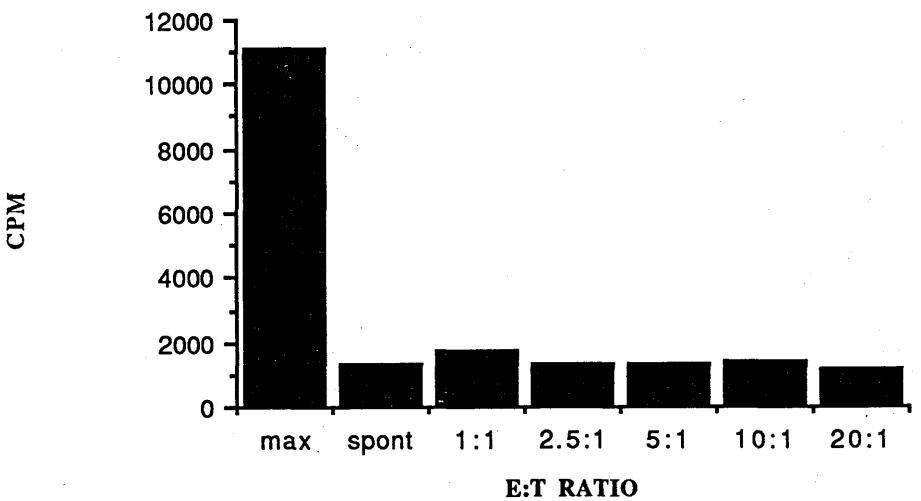
Initially the assay was performed using the natural killer sensitive erythroleukemia cell line K562 (targets) (Lozzio and Lozzio, 1975) and normal PBLs (effector). Figure 4.21 shows the % lysis by each effector : target ratio tested. The mean maximum incorporation was 7700cpm and the mean spontaneous incorporation was 800cpm. It can be seen that increased killing occurs with increased E:T ratio, and thus the assay system functioned as expected. A cutaneous melanoma target cell line, B8, was then used but there was no obvious killing by the PBLs even although the cells were incorporating the ^{51}Cr to reasonable levels (Figure 4.22). This could have been due to the lack of MHC compatibility between the targets and the effectors. When fresh primary tumour cells were used it was observed that little of the ^{51}Cr was being incorporated (Figure 4.23) and this was shown by simply incubating the cells with the isotope and than measuring the spontaneous release over the time period and also the maximum release due to treatment with 3N HCl. The levels of incorporation of the ^{51}Cr (400cpm) were similar to the spontaneous release levels of the K562 control cell line. However, the cell were incorporating some , albeit low, levels of the isotope since the incorporation was above the background of the gamma counter,

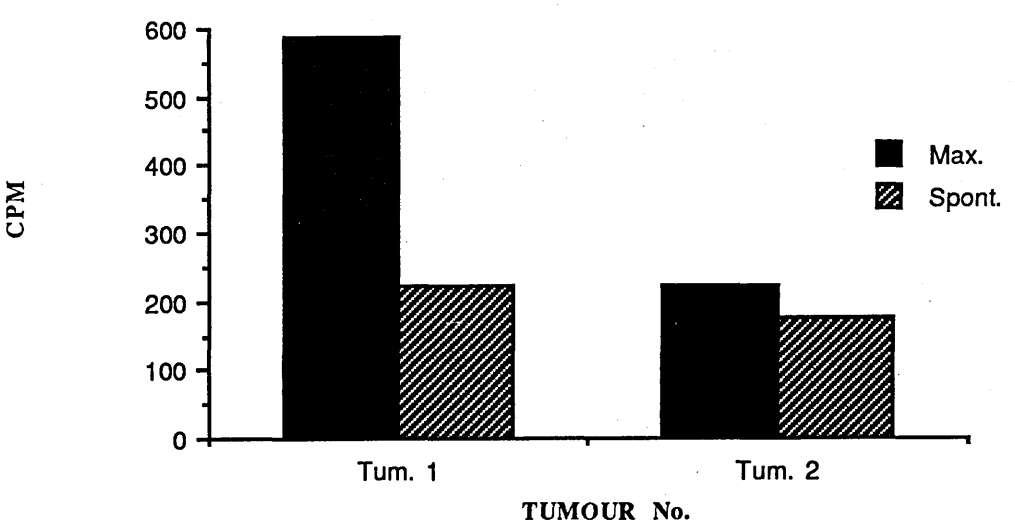
Figure 4.21: Percent lysis obtained using different effector (E):target (T) ratios indicated. The target cells in these experiments were an erytheroleukemic cell line (K562) and the effectors were normal PBLs. Standard deviations are a result of triplicate experiments (n=3).

Figure 4.22: Incorporation of ^{51}Cr in the human cutaneous cell line (B008) and subsequent release due to killing at varying E:T ratios by normal human PBLs.

Figure 4.23: Maximum and spontaneous release of ^{51}Cr by different primary uveal melanoma cell populations.


% LYSIS
60
50
40
30
20
10
0
1:1
2.5:1
5:1
10:1
20:1
E:T RATIO


CPM
12000
10000
8000
6000
4000
2000
0
max
spont
1:1
2.5:1
5:1
10:1
20:1
E:T RATIO


CPM
600
500
400
300
200
100
0
Max.
Spont.
Tum. 1
Tum. 2
TUMOUR No.

4.3 Discussion

4.3.1 Tumour cell surface antigen analysis

The development of the fully metastatic cell is a complex process which can be divided into several different stages often denoted as initiation, promotion and progression (Farber and Cameron, 1980; Weinstein, 1988; Yuspa and Priorier, 1988). Progression to a more malignant phenotype occurs as the tumour cells acquire new characteristics which may represent normal functions in other cell lineages or stages of differentiation (Nicholson, 1988) . These new characteristics may also be involved in the adhesive properties of the metastasising cell to the target tissue. Much work has been performed on cutaneous melanoma to study these melanoma- specific antigens (Holzmann *et al.*, 1987; Wilson *et al.*, 1981) and their relevance in metastasis. It is thought that many of the melanoma- associated antigens which have been examined indicate that these molecules are associated with cell activation and proliferation rather than with malignant transformation (Herlyn and Koprowski, 1988; Johnston *et al*, 1989).

This particular study involved analysing the phenotypic expression of defined cell surface markers on 20 uveal melanoma tumours, including Class I and II MHC (HLA DR) antigens, and the adhesion molecule ICAM-1. It can be seen from Table 4.2 that much patient variation exists for the expression of these markers.

There appears to be no correlation between the loss of Class I and the gain of Class II antigen. HLA DR expression is generally low (7.9%) although there are some tumour cells that express much higher percentages of positive cells. The HLA D region products have a restricted tissue distribution, and are expressed primarily by B lymphocytes and monocytes, and present in higher levels on activated lymphocytes and other lymphokine activated cells. In melanomas it has been reported that HLA DR antigens are frequently expressed on malignant cells which are derived from Class II

negative cells (Carrel *et al.*, 1986). The expression of HLA DR molecules by primary melanomas has been suggested to be a feature of advanced malignant melanomas independent of tumour thickness (Brocker *et al.*, 1985). A prognostic significance of HLA DR expression raises the possibility that the immune system is playing an active role in the control of tumour growth and progression in melanoma. The measure of metastasis of the tumours in this study will of course involve a long term follow- up study of the patients and their survival.

Approximately 70% of primary cutaneous melanomas greater than 1mm in thickness express the glycoprotein defined by monoclonal antibody P.3.58 (ICAM-1) (Holzmann *et al.*, 1985, 1987). *In vitro* ,the expression of P3.58 can be modulated both on lymphocytes and melanoma cells by lymphokines such as IFN and TNF- α (Holzmann *et al.*, 1987). Our studies indicate the presence of ICAM-1 on a high percentage of all cells (77.7%). The flow cytometric data presented here show a negative correlation with the expression of HLA DR ($p<0.012$) (Figure 4.4) and this may indicate the phenotypic changes that take place during the development of the tumour. The TILs in this system seem to lack the potential to potentiate the expression of ICAM-1 (Figure 4.7) but to perhaps increase the level of HLA DR expression (Figure 4.6 and Table 4.3).Brocker *et al* (1988) have observed a positive correlation between the leukocytes containing IFN-γ and the expression of HLA DR and specific melanoma associated antigens, such as gp 89, on the tumour cells and suggested changes in the phenotype of the tumour cells were in part being determined by the infiltrate. In this study there does not seem to be any positive correlation between the expression of Class I or ICAM-1 on the tumour cells with the levels of TILs within the tumour suspension (Figures 4.5 and 4.6), although a correlation ($p<0.001$) between the levels of TILs and the expression of HLA DR is evident (Figure 4.7 and Table 4.3) as mentioned previously. This could be due to the antigen

presenting capabilities of the tumour cells or, as in the study by Brocker *et al* (1988), the HLA DR could be induced by certain factors being released by the lymphocytes present.

An attempt was made to correlate the presence of the cell surface antigens with the particular cell type of the tumour (Figure 4.8 to 4.10). The purely epithelioid cells seemed to express less Class I (5.7%), HLA DR (16.3%) and LFA-1(1.3%) than the other cell types but with only 2 epithelioid tumours in the study group, no conclusions can be drawn. There is little difference in the expression of ICAM-1 between the different cell groups and thus it is not, at this stage, a suitable marker for prognosis or the cell type, even although it is thought that epithelial cells are thought to have a worse prognosis (Section 1.3.3).

4.3.2. Cell Surface Antigen Analysis of TIL Subpopulations

The various cell types within the immune system communicate with each other via membrane contact and soluble factors. Cytotoxic T cells have received much attention since it was originally shown that effector- target contact is essential for lysis (Zagury *et al.*, 1975; Martz 1975). The peptide in the groove serves to allow recognition, and effector function requires additional contact between CD8 and the non-polymorphic regions of MHC Class I(or CD4 and non-polymorphic regions of Class II) (Section 1.4.2). Finally , additional adhesion molecules are also necessary for effector function. Studies have been performed that show that activated lymphocytes can attach to cells within the same species in a process termed "natural attachment" (Galili *et al.*, 1978). It has been suggested that this contact represents the first step in the immunological recognition and that it is the prerequisite for subsequent detection of fine structural deviations on the target cell membranes by the lymphocytes (Klein, 1980).

As shown earlier lymphocytes infiltrate, albeit to small extents, into uveal melanomas, and it is mainly the $CD8^+$ T cells that constitute this infiltrate. When the expression of LFA-1 was studied on these lymphocytes it was found that >97% expressed this marker. Section 4.3.1 shows that high percentages of the tumour cells express the ligand for this particular adhesion molecule, ICAM-1. Attachment of the lymphocytes to the target cell thus may be via this ligand- receptor complex. ICAM-1 has been identified as the ligand of the LFA-1 molecule, by attachment of the LFA-1 positive cells to the ICAM-1 in artificial membranes, to ICAM-1- coated surfaces, and to human malignant and non-malignant cell lines on which ICAM-1 expression has been induced by cytokines (Makgoba *et al.*, 1988; Rotlein *et al.*, 1988). Vanky *et al* (1990) found that human tumours that were lysed by autologous lymphocytes all expressed ICAM-1 and MHC Class I antigens. Figure 4.13 and 4.16 shows that there is a higher level of ICAM-1 expression on the TIL samples, which may be due to the activation state of the lymphocytes, and also that the $CD8^+$ T cells preferentially express this molecule not only in the TIL but also the PBL samples. ICAM -1 can be detected on approximately 40 % of the resting T cell population (Buckle and Hogg, 1990). It has been suggested that these ICAM-1^+ T cells have the phenotypic markers of memory cells $CD45^-$, which can be distinguished functionally from naive T cells, $CD45^+$ by their ability to respond to previously experienced antigen. These cells appear to be in a state of marginal activation in that they also have been reported to express low levels of CD25 (Buckle and Hogg, 1990). Wang *et al* (1990) have suggested that the requirement of ICAM-1 on cytotoxic T cells, as well as on the targets, indicates that the contact between the two molecules can be bi- directional. It has also been shown that on activated lymphocytes the avidity of the LFA-1 molecule is transiently elevated (Van Kooyk *et al* 1989; Dustin and Springer *et al* 1989). This prompt and short- lived change in the avidity of LFA-1, together with the activation- dependant expression of

ICAM-1, is thought to provide the dynamics of regulation of cell interactions, i.e. attachment and detachment to and from the targets, allowing recirculation of the effectors. Within this study, the high levels of ICAM-1 found on TILs compared to PBLs may be indicative of the functionally active lymphocytes, and these cell may interact via their high avidity LFA-1 with the ICAM-1 on the uveal melanomas cell targets and deliver the "lethal hit". Alternatively they could interact through their ICAM-1, with the LFA-1 of the resting lymphocytes and deliver activation signals. Indeed Wang *et al* (1990) found that incubation of lymphocytes with an anti- ICAM-1 antibody did not affect the lytic interaction with target cells, but did abrogate the activation of the resting lymphocytes.

Thus contact between the effector and target cells may be a result of the infiltration of lymphocytes into the tumour, and the consequent dissemination or lytic activity due to the lymphocytes. There also may be the possibility of contacts between the effector cell populations which may be required for the cytotoxic function.

As was also shown in Section 3.1 the TILs have much higher levels of HLA DR compared to the PBLs and as is expected Class I MHC antigen is present on very high levels of the lymphocytes.

Correlations between the expression of both MHC antigens on the tumour cells and HLA DR present on the $CD4^+$ and $CD8^+$ T cells were also investigated in three cases. The positive correlation found in breast cancer by Whitford *et al* (1991) may represent the ability of the tumour cells to present antigens such as oncogene derived peptides. In this particular study there was found to be a strong correlation between the expression of Class I on the tumour cells and HLA DR on the $CD4^+$, and HL ADR expression on the tumour cells with $CD8^+$ T cells expressing HLA DR (Figure 4.17 and 4.18). Perhaps the uveal melanoma cells are also capable of presenting antigen to the

T cells, but obviously a greater sample number is required to make any definite conclusions.

4.3.3 Cytotoxicity Assays

Cytotoxicity assays were performed due to the report of Wang *et al* (1990) that events occurring in ^{51}Cr cytotoxicity assays required ICAM-1 expression on the effector cells. The study was intended to determine the lytic activity of the TILs compared to the resting PBLs. The effects of incubation of the effector or targets with the relevant adhesion molecule antibodies might have indicated the importance of these molecules in the lytic activity or activation abilities of the lymphocytes.

The study was not pursued because of the problems associated with the uptake of the ^{51}Cr by the tumour cells. Primary uveal melanoma cells will adhere to the plastic once in an *in vitro* situation but will not proliferate and consequently will not incorporate the ^{51}Cr to an adequate level. Thus comparative studies involving the inhibition of the release of the isotope due to incubation of either the lymphocytes or the tumour cells with the antibodies to the relevant adhesion molecules cannot be performed.

CHAPTER 5

DNA ANALYSIS OF UVEAL MELANOMA TUMOUR CELLS

5. DNA ANALYSIS OF UVEAL MELANOMA TUMOUR CELLS

5.1. Methods of Cell Cycle Analysis

There are several states that proliferating cells can assume besides mitosis. After completing mitosis (M), the daughter cells enter the Gap1 (G) phase and they spend a period of time in this phase depending upon the tissue type. The cell then enters the DNA synthetic (S) phase where the genetic material of the cell is doubled. Before the cells undergo division again there is a second Gap phase (G2) (Figure 1.13).

There are defined genes that are crucial for yeast cells to pass specific points of the cell cycle. These crucial genes are collectively known as cell-division cycle (cdc) genes and there are indications that other eukaryotic cells may use similar mechanisms. One of these genes, cdc 2 (Nurse, 1981) is particularly intriguing because its correct activity seems crucial for entry into mitosis. It is also required earlier in the cell cycle at the onset of S phase (Mendenhall *et al.*, 1987). Control of the mitotic mechanisms is present universally in all eukaryotic cells, and involves the cdc 2 gene product which is a protein kinase p34 ^{cdc2} and it is activated at both the mitotic and the meiotic M- phase (Reviewed by Nurse, 1990). Activation requires changes in the phosphorylation state of the kinase and its interaction with cyclins, which are proteins that vary in level during the cell cycle and are degraded as the cells exit the M phase.

There are various methods for the study of the various aspects of the cell cycle and much work has been done to correlate the variables that reflect tumour proliferation with metastatic potential, tumour recurrence and overall mortality.

Discussed below are six methods for cell cycle analysis, with the advantages and disadvantages of using such methods.

5.1.1.Mitotic Count

This is defined as the number of mitoses per designated high-power field (HPFs). The mitotic count has long been employed by histopathologists as a diagnostic and prognostic indicator, but mitotic count as a marker of tumour proliferation activity remains controversial, due to inter- observer variation in mitosis counting. Wide variation between observers in mitosis counting have been reported by Silverberg (1976), who expressed concern about the use of the mitotic count as the sole indicator of malignancy.

5.1.2 Thymidine Labelling Index (TLI)

This is a measure of the percentage of tumour cells in the DNA synthetic (S) phase of the cell cycle. TLI is measured *in vitro* by incubating freshly excised tissue in a medium containing tritiated thymidine, which is incorporated into DNA. Autoradiographs are then prepared, and the labelled cells counted and compared with the non- labelled population. This method was first reported in 1961 on a study on breast tissue (Johnson and Bond, 1961). Since then attention has focused on the role of TLI as a prognostic indicator in human cancer. Increased TLI levels have also been observed in colonic mucosa of patients with familial polyopsis coli and familial colonic cancer leading to the suggestion that TLI may be useful as a marker for hereditary disposition to colonic cancer (Maasken and Deschner, 1977).

The use of TLI as an indicator of tumour proliferation has several limitations. TLI documents the number of cells in the S phase, but does not measure the duration of the S phase. Thus a tumour could have a slow rate of cell proliferation but a high TLI. The method also relies upon a sample from one part of the tumour assuming that that no heterogeneous growth patterns exist. In

addition, human inter-observer variation, reproducibility error and small sample size may lead to misleading results.

5.1.3 Bromodeoxyuridine

The S phase cell population can also be measured using BdUR (Section 1.6.2.4). BdUR is a pyrimidine analogue of thymidine which is readily incorporated into cells during the DNA synthetic phase. Recently, various monoclonal antibodies to BdUR have been developed (Gratzner 1982; Morstyne *et al* .,1983; Roberts *et al* .,1985). Tumours which have been exposed to BdUR, *in vivo* or *in vitro*, are stained with the antibody using an immunoperoxidase technique after DNA denaturation. The stained cells are counted using microscopy and expressed as a percentage of 500 or 1000 tumour cells to yield the labelling index. BdUR has obvious advantages over thymidine labelling, since it is sometimes employed as a therapeutic agent and, in consequence, *in vivo* kinetic studies can be conducted without introducing additional procedures or exposing the patient to radioactivity.

5.1.4 Nucleolar Organiser Regions (NORs)

These are loops of DNA which code for ribosomal RNA (Alberts *et al* .,1983). NORs are so called because of their ability to associate into nucleoli. They are located on acrocentric chromosomes, of which man has five pairs located on chromosomes 13,14,15,21and 22 (Lewin 1980). Each acrocentric chromosome bears two NORs: thus, a normal diploid cell should contain 20 NORs. NORs can be demonstrated using *in situ* hybridisation to localised ribosomal genes (Warburton and Henderson 1979). NORs are associated with certain proteins, including RNA polymerase 1. These proteins are known as AgNORs and are thought to play an important role in RNA

transcription. In 1975, it was discovered that NORs could be demonstrated using a simple silver staining method (Goodpasture and Bloom, 1975), and the method could be applied to paraffin- embedded tissue sections (Ploton *et al* .,1986). AgNORs are visualised as black dots within the nucleolus and the mean AgNOR count for 50 or 100 cells is usually calculated. It has been shown that there is a significant difference in AgNOR count between benign and malignant melanocytic (Crocker and Skilbeck, 1987) and breast (Smith and Crocker, 1988) lesions and between low grade and high grade lymphomas (Crocker and Nar, 1987).

Discrepancies have been shown with this technique. Chromosomes fuse during interphase, so that only a single silver positive structure is seen in resting cells, but disperse during metaphase when individual chromosomes and consequently individual AgNORs can be identified (Jan- Mahammed *et al.*, 1989). Thus, the AgNOR count may depend on the stage of the cell cycle. It has been suggested the AgNOR count is not a true count but rather a measure of the degree of dispersal of NOR associated proteins (Underwood and Giri, 1988). Another problem arises in the affinity of the nucleolus itself for silver (Tandler, 1954). This may lead to dificulties in precise counting and consequently to inter-observer variation and reproducibility error. Many of the current disputes about AgNORs, if the inter-observer variation and recognition differences are ignored, could well be failure to obtain properly randomised samples and to minimise sample error related to tumour heterogeneity.

5.1.5 Ki67

Ki67 is a mouse monoclonal antibody which was produced in 1983 (Gerdes *et al.*, 1983). This antibody is reported to recognise a nuclear antigen which is present only in proliferating cells. It appears to be expressed only at certain stages of the cell cycle (Gerdes *et al* .,1984; Barsch and Gerdes, 1987).

The antibody is applied to sections obtained from frozen tissue using conventional immunocytochemistry. The number of cells staining with Ki67 is said to represent the number of cells cycling at any one time (i.e the growth fraction). Results are expressed as the number of positive cells per total number of tumour cells counted. Various workers have reported a good correlation between Ki67 positivity and cell proliferation data obtained using other techniques. (Schrape *et al.*,1987; Sasaki *et al.*, 1988).

As a marker of cell proliferation activitiy, Ki67 positivity may provide an indication of the number of cells cycling at any one time. It appears to begin expression in mid-G1; G0 and early G1 cells are negative. Expression then increases through the cycle , and G2 and M cells are very heavily positive. Drawbacks include the need for fresh tissue as opposed to paraffin embedded tissue sections, and the possibility of observer error. Moreover, sections must be stained immediately after cutting with short acetone fixation.

5.1.6 Flow Cytometry

The final and perhaps the most important method of studying tumour cell cycle is the flow cytometer. Flow cytometry is an automated technique which quantifies cellular DNA content and analyses cell cycle distribution (Quirke and Dyson, 1986; Coon *et al.*,1987). A sample of tissue is prepared and the cells are stained using DNA specific- fluorochrome dyes. The suspension flows through a light beam past stationary fluorescence detectors, at a rate of about 100 cells per second. The light scatter by the cells is registered by the detectors and converted into electronic signals which are analysed by the computer (Section 2.2.5). The computer produces DNA histograms, and the available software can then analyse the results and derive information on the cell cycle (Figure 2.7). Ploidy status is concerned with expressing the DNA content as a DNA index value. This is the

ratio of the G0/G1 cells of the population being studied to that of an internal or external standard diploid cell population. Diploid cells have a DNA index of 1.00. Cells with an index of 2.00 are tetraploid. Cells that contain abnormal quantities of DNA are referred to as being aneuploid and thus have a DNA index less than or greater than 1.The DNA histogram also can determine the S phase content and the proliferation activity (S+ G2/M phases) of the sample although in a mixed population of diploid and aneuploid cells the profile is difficult to analyse with accuracy.

5.2 Aims of the study

The aims of this study were to analyse the cell cycle distribution of the DNA of the uveal melanoma tumour samples by flow cytometry. Correlations between the cell type, level of cellular infiltrate and other pathological features were investigated so as to obtain a better understanding of these tumours at a genomic level.

5.3 Results

Ploidy analysis of 32 uveal melanoma tumours, and the appropriate patient PBL, was performed using the method of Vindeløv *et al* (1983) (Section 2.2.2.2). From this study it was found that only one of the tumours contained abnormal (aneuploid) DNA (Figure 1.5(a)), and the DNA index for this tumour was calculated to be 1.8. This tumour was of epithelial origin, contained no infiltrate and had a mitotic count of 1 per 40 high power field.

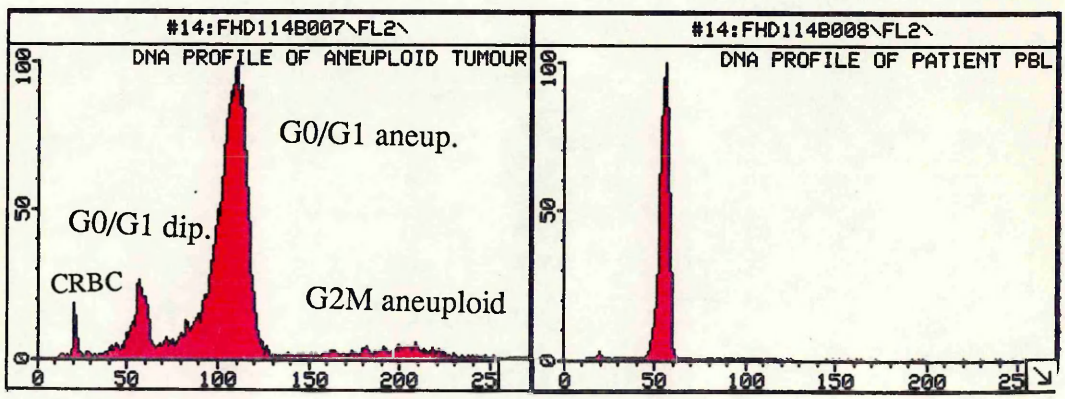
From the remaining 31 diploid tumours (Figure 5.1(b)), 18 were of pure spindle cell type (58%), 10 were mixed spindle and epithelioid cell type (32.2%) and the remaining 3 were purely epithelioid cells (9.6%). The cells in each part of the cell cycle (G1, S, and G2M) were calculated using the Becton Dickinson Software Sum of Broadened Rectangles (Section 2.2.5.2). This model was used since it was preferable to use one method of analysis throughout and under certain circumstances the model would not allow the data to fit the Polynomial model, perhaps due to the presence of debris or due to the presence of too small an S phase. Since this model was used, a reference control was necessary to establish the position of the G0/G1 peak. Chick red blood cells (CRBC) were used since they contain approximately 35% of the DNA content of normal human cells. The proliferation index for each tumour was also determined. This flow cytometric data was then correlated with other pathological features such as the cell type, mitotic count, and the presence or absence of tumour infiltrating lymphocytes.(Table 5.1). It was found that, of the tumours sampled for the study, the mean percentage of cells that were found to be in the G0/G1 phase was 87.3 ± 6.5 with a minimum of 67% and a maximum of 95%. The range of cells in this S phase was from 3% to 27% with a mean S phase presentation of $7.9\% \pm 5.6\%$. Similarly, $4.7\% \pm 2.9\%$ of the cells were in the G2M phase of the cycle with values ranging from 1% to 14%. The summation of the G2,M and S phases

gives the proliferation index, and the range of this index was between 5 and 32 with a mean of 13.0± 7.0.

Patient	G1	S	G2M	G2MS	C.T	Op.	MI	LI
1	89	7	4	11	B	LR	4	X
2	89	7	4	11	B	LR	0	X
3	93	3	4	7	B	LR	1	X
4	76	10	14	24	EP/B	EN	3	X
5	82	14	4	18	EP/B	LR/EN	22	++
6	89	4	7	11	B	LR	7	X
7	87	5	9	14	B	LR	6	X
8	93	4	3	7	B	LR	0	X
9	78	16	6	24	B	LR	0	X
10	92	5	3	8	EP/B	LR	3	+
11	93	4	3	7	AB	LR	2	X
12	85	10	5	15	EP	LR/EN	5	X
13	95	3	2	5	AB	EN	0	X
14	93	4	3	7	EP	LR/EN	5	+
15	85	6	9	15	EP/B	EN	1	X
16	89	9	2	11	B	LR	10	X
17	91	5	4	9	B	LR	2	X
18	86	11	3	14	EP/SP	EN	20	+
19	92	5	3	8	B	LR	1	++
20	92	5	3	8	B	LR	5	X
21	77	20	3	23	B	LR	2	X
22	94	3	3	6	EP/B	EN	4	X
23	92	5	3	8	B	LR	5	X
24	90	6	4	10	B	LR	3	X
25	67	27	5	32	EP/B	EN	5	+
26	78	17	5	22	EP	LR	24	++
27	91	9	1	10	EP/B	LR	5	++
28	82	6	12	18	EP/B	EN	8	++
29	85	7	8	15	AB	EN	0	+
30	93	4	2	6	AB	EN	0	X
31	90	5	6	11	EP/B	LR	2	+

Table 5.1: Patient data concerning percentage of tumour cells in each stage of the cell cycle (obtained by FACScan analysis) and also pathological features. Cell types (CT) include spindle cells (AB), pure epithelioid (EP) and mixed spindle/ epithelioid (EP/B). Operations (Op.) performed were either local resection (LR) and/ or enucleation (EN). Mitotic index (MI) stated per 40 high power fields and lymphocytic infiltration (LI) consisted of no lymphocytes (X), low levels (+) or high levels (++)

(a)



(b)

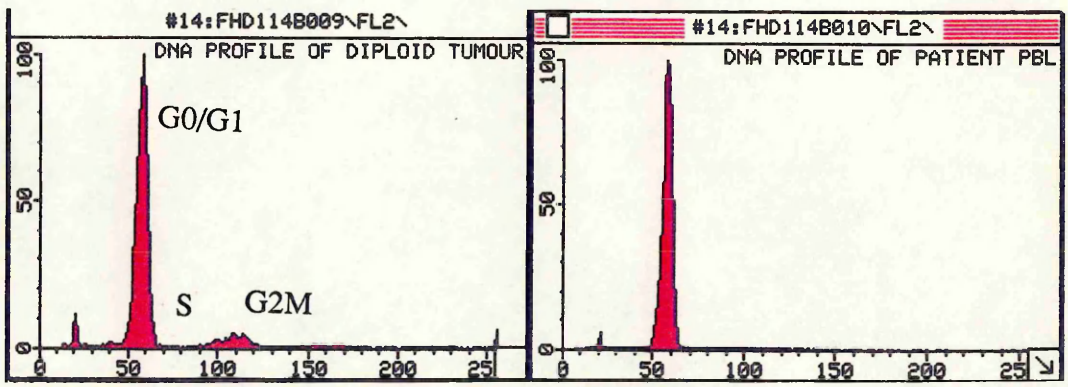


Figure 5.1: DNA histogram of (a) abnormal DNA (aneuploid) containing tumour. G0/G1 of diploid peak and G0/G1 of aneuploid peak are represented. Corresponding patient's diploid PBLs are shown. (b) Diploid tumour and PBL DNA.

When the cells were classified into cell type and correlated with the proliferation index it was found that pure spindle cells exhibited an index of 10.9± 5.3, pure epithelioid cells and the mixed cell type had an index of 14 ±7.5 and 15.6±7.8 respectively (Figure 5.2). A similar correlation was performed with the mitotic index (MI) and the cell type. Pure spindle cells appeared to have a mean MI of 2.66 with a wide range of 0 to 10. Similarly, the epithelial and mixed cells had mean MI of 11.3 and 7.3 with ranges of 5 to 24 and 1 to 22, respectively (Fig 5.3)

When the mitotic index and its relationship with the presence or absence of tumour infiltrating lymphocytes defined by the pathology reports were compared, it was found that tumours containing no TILs had a mitotic index of between 0 and 10, whereas tumours with low lymphocytic infiltration had a range of 0 to 20 and the highly infiltrated group exhibited levels of 1 to 24 (Figure 5.4).

When the flow cytometry data for the determination of the S phase and the pathology data for the mitotic count were compared, it was found that the tumour cells with mitotic counts less than 10 also had low levels of cells in the S phase of the cell cycle- less than 10%. There were also 3 tumours with mitotic indices of 20, 22 and 24 with SPF of 11, 14, and 17, respectively. However, there were 3 tumours with high S phase fractions cells but exceptionally low mitotic counts (Figure 5.5)

As might be expected there is a good correlation with the percentage of cells in S phase and the proliferation index of each individual tumour (Figure 5.6), since, by definition, one of the variables of the proliferation index is the percentage of cells in the S phase.

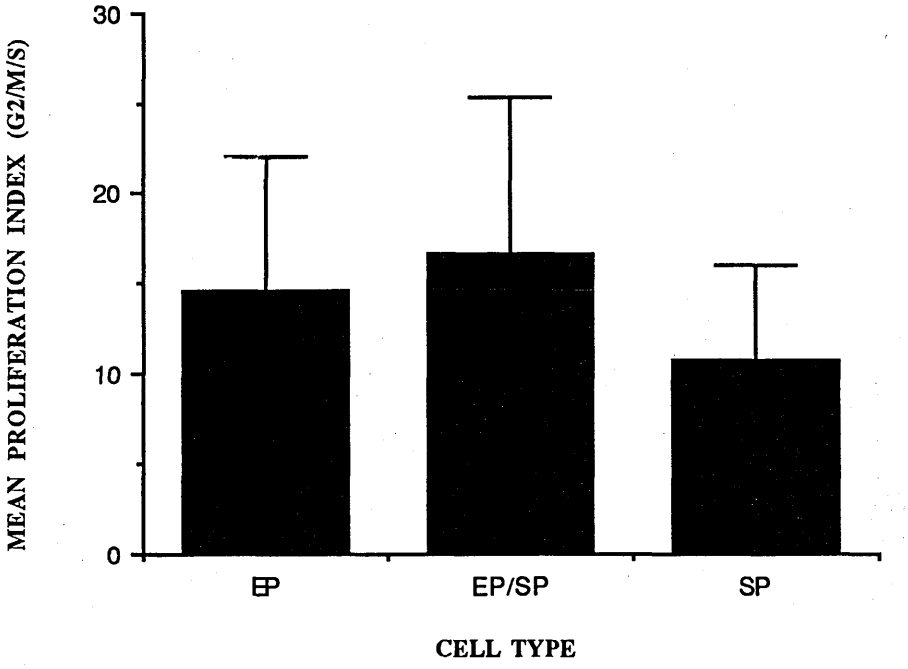
Several attempts were made to perform flow cytometric analysis of fixed paraffin embedded samples sections of archival tissues. It would obviously be of value to be able to carry out a long term study of pathological material and

correlate this with the treatment and survival of the patient, which would already be known by this time.

This was attempted by deparaffinising the tissue in xylene and rehydrating the sections in a sequence of decreasing concentrations of ethanol and finally washing the cells in distilled water (Section 2.2.2.3). The cells were then pepsin digested using the method of Hedley (1983), and then stained as before (Vindeløv, 1983) for cell cycle analysis of the tumour nucleii. After several attempts at this rehydration and digestion protocol, it was decided to terminate this study since the material being obtained at the end of the experiment appeared to be composed largely of debris which could not be analysed using the DNA software package. Since one of the benefits of flow cytometry is the ability to perform analysis of fresh tumour samples it seemed appropriate to concentrate the study to such material in the hope that it might form part of a long term follow- up study in the future.

Figure 5.2: Correlation of proliferation index with individual cell type populations; epithelioid (EP), spindle (SP) and mixed epithelioid/ spindle (EP/SP).

Figure 5.3: Correlation of mitotic index with individual cell type populations; epithelioid (EP), spindle (SP) and mixed epithelioid/ spindle (EP/SP).


MEAN PROLIFERATION INDEX (G2/M/S)
30
20
10
0
EP
EP/SP
SP
CELL TYPE

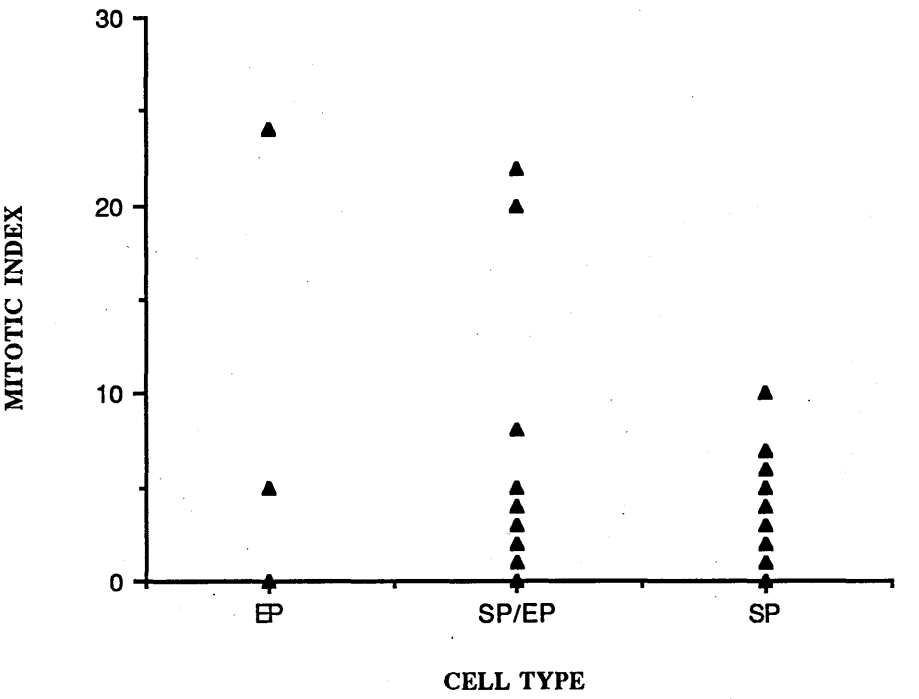
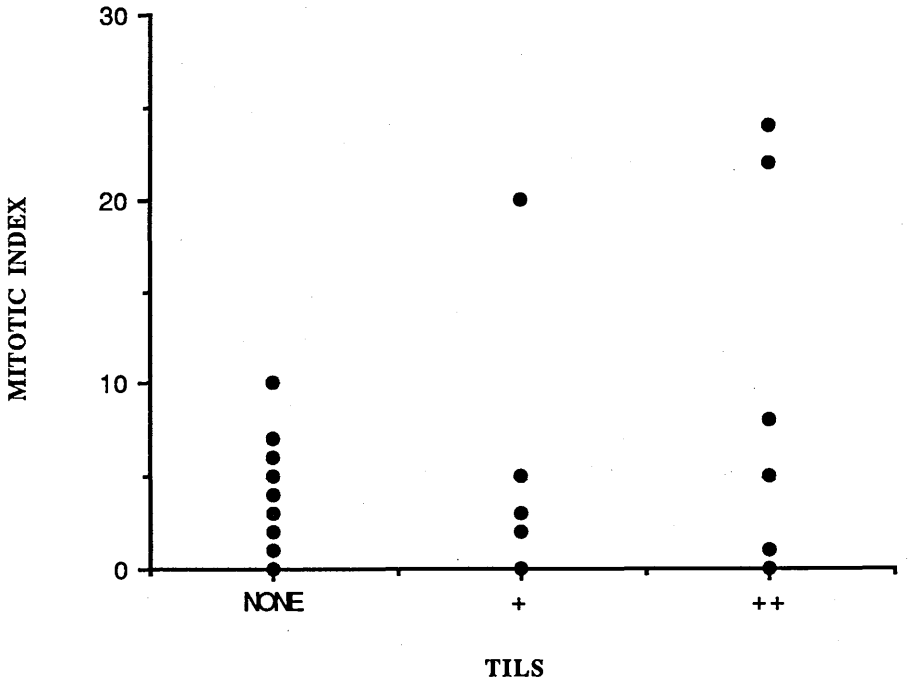
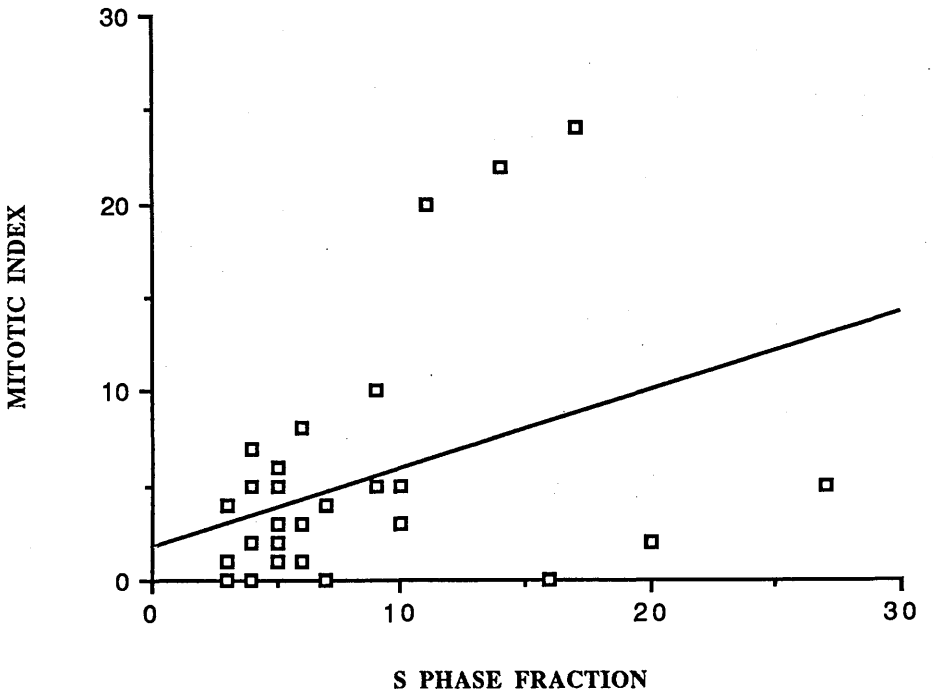

MITOTIC INDEX
30
20
10
0
EP
SP/EP
SP
CELL TYPE

Figure 5.4: Correlation of mitotic index with levels of lymphocytic infiltration (none, + (minimal) and ++ (moderate/ extensive)).

Figure 5.5: Mitotic index correlation with S phase fraction.


30
20
10
0
MITOTIC INDEX
NONE
+
++
TILS


30
20
10
0
MITOTIC INDEX
0
10
20
30
S PHASE FRACTION

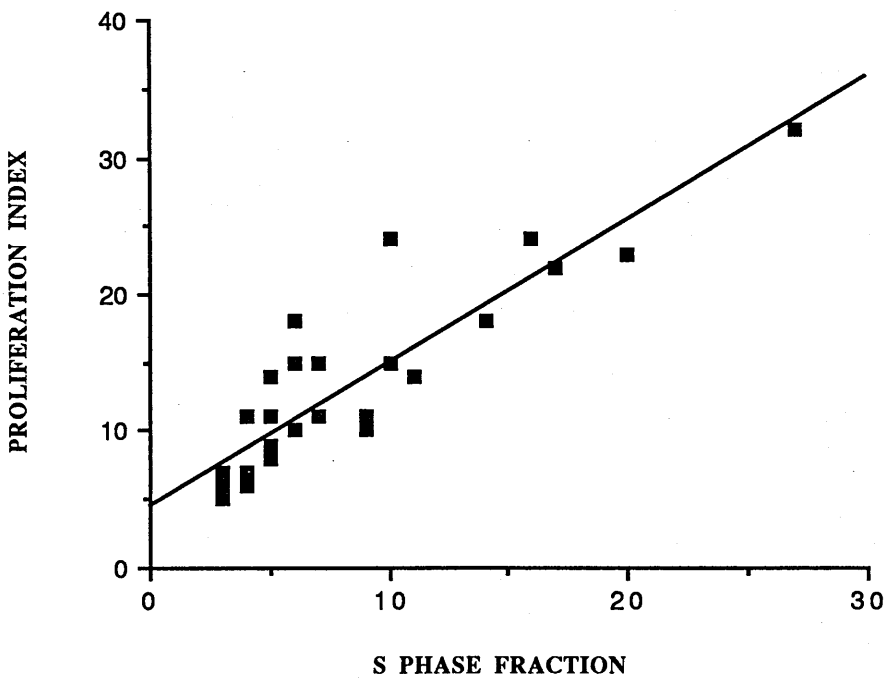


Figure 5.6: Correlation of proliferation index with content of DNA within the S phase fraction.

5.4 Discussion

Several technical points have emerged from this study. The first indicates the benefits of using flow cytometry for DNA analysis since it makes use of modern technology involving lasers and refined analytical computer software. It has the advantage of being entirely objective in data collection, and the subjectivity only occurs when deciding upon the best method of analysis. It also has the ability to collect 5000 to 10000 nuclei in a few minutes. This number of nuclei would not be able to be analysed using frozen sections and applying other immunohistochemical techniques of cell cycle analysis. Unfortunately, FACscan analysis does not give the position of the actively dividing cells within the architecture of the whole tumour. It does however indicate the relative numbers of cells in each phase of the cell cycle of the available tumour piece. Paraffin embedded sections of tissue may be unrepresentative of the whole tumour.

For epithelial tumours and uveal melanomas, reports in the last decade have had a strong bias toward the use of paraffin embedded tissue sections (section 1.6.2.2 and 1.6.2.3) since the survival of the patient is known. The data have not been in concordance with recent findings from fresh tissue, and it appears that fresh samples give a much better set of results to analyse, removing the problems presented by debris that occurs when deparaffinising, and rehydrating tissue samples. Paraffin fixation involves extensive formaldehyde cross- linking, and this makes it difficult to obtain "clean" nuclei not associated with the endoplasmic reticulum containing ribosomal RNA. In the preparation of the nuclei for the DNA analysis, RNAase is added to remove any RNA present, but it may not be completely effective due to the high level of formaldehyde fixation. Storage of paraffin embedded sections, particularly in acid conditions, may break down the phosphodiester bonds in DNA. Paraffin fixed tissue, provided for an attempt at studying DNA analysis of archival tissue. It was found that

analysis on a modern FACS system, and personal communications with other laboratories suggested that this method is recognised as having limited value. In this context, it may be relevant to note that the manufacturers of the FACScan, Becton Dickinson, no longer advise that relevant results can be obtained from paraffin fixed tissue sections.

The data in this section are closer in agreement with Rennie *et al* (1989) than with Shapiro *et al* (1986) and suggest that, when fresh tissue is used, aneuploidy is a comparatively rare event in uveal melanoma. However, the data do not support the suggestion of Rennie *et al* (1989) that epithelial tumours have a higher rate of proliferation than spindle cell tumours. The most likely explanation for this is the small number of epithelial tumours available for study. It does illustrate the lower rate of proliferation of the spindle cells suggesting that perhaps in a mixed spindle, epithelioid tumour it is the epithelioid cells that give the tumour the high proliferation index. Spindle cells seem to have the lowest mitotic index, defined by pathology reports, which would be in concordance with the low proliferation index. It is important to mention that the 3 tumours with the highest mitotic index, all contain epithelioid cells either existing as pure epithelioid tumours or mixed spindle/ epithelioid tumours, and all 3 contain lymphocytes a varying levels. All tumours that do not contain TILs have a low mitotic index. The high mitotic index of the TIL containing tumours is not due to the lymphocytes because they do not exist in high enough quantities to over- shadow the tumour cell nuclei present within the suspension.

Epithelial tumours are known to have a poor prognosis and it seems unlikely that flow cytometric analysis of the cell cycle can have a significant impact on clinical management.

Finally, there is the question as to how the tumour cell cycle is related to tumour aggression and tumour metastasis. Within this question lies the supplementary question as to whether a preponderance of cells in S phase necessarily indicates tumour aggression. Modern molecular biological research has shown that the cell cycle is under a complex series of control mechanisms, many being related to oncogene functions (Section 1.6.1) and that no single index of proliferation, including flow cytometry is likely to give an indication of clinical outcome. Metastasis itself is a complex process involving the ability of the cells to escape from the primary tumour and to establish the secondary growth eslewhere. For this process, the possession of cell surface molecules such as adhesion molecules or of secretory functions such as collagenase, may be more relevant than the cell cycle status of the primary tumour.

CHAPTER 6

DISCUSSION

6 DISCUSSION

6.1 General

The work described in this thesis was initiated to determine if there was any immune response to tumours of the eye, especially uveal melanoma, compared to other tumours that exist outwith this immunologically privileged site of the body (Section 1.1.4). Much work has been performed on cutaneous melanoma, but uveal melanoma is a less studied tumour due to its rarity. A major problem in the research was the availability of tumour samples due to the previously mentioned rarity. The degree of lymphocytic infiltration is also small in comparison to other tumours such as breast and colonic cancer.

6.2 Flow cytometry

Flow cytometry is a method of analysing the cell surface antigen expression of tumour cells with the ability of discriminating live from dead cells. It can quantitate the degree of expression non- subjectively. Other classical methods of phenotyping cells include basic immunohistological techniques that involve staining of fixed tissue sections or cell suspensions with either fluorescent or enzyme linked monoclonal antibodies. This introduces problems because of the nature of the procedures. The cells are fixed and thus perforated, introducing a high degree of non- specific staining of the cells. The quantititive analysis of the cells also becomes very subjective due to the nature of the counting procedures used, and very dependent on the person analysing the results. The individual tissue sections used also introduce an element of inaccuracy, since no two sections will be the same, and if a particular cell population congregates in a particular area of the tumour in preference to another site, then different sections will give different results. Alternatively, flow cytometry can be performed on live cells and any particular cell population can be detected and preferentially analysed

depending on certain physical characteristics that the cells possess. When analysing the tumour it is also important to remember that ideally whole tumours should be analysed and thus removes any possibility preferential screening of individual cell populations, although in this study only apical pieces of the tumour were obtained and thus this problem is not completely removed. Data can also be stored on tapes so that it can be analysed by various methods at a later date.

The accuracy of the machine is also important since it was found that when comparing the levels of TILs from the pathology reports with those found in the tumours determined by FACScan, flow cytometry always revealed an element of infiltration, whereas the pathology reports frequently indicated that there was no infiltrate in the section studied.

6.3 Relevance of Tumour Infiltrating Lymphocytes

Much work has been carried out on the infiltration of human tumours by lymphocytes, and consequently it is an area of great interest with respect to methods of immunotherapy of cancer. The levels of TILs within any tumour type can vary. For example, breast carcinoma has an average of 12% of the tumour containing TILs (Whitford *et al.*, 1990) whereas uveal melanoma can often contain 1-2% TILs. This is an important and relevant finding of this thesis since it illustrates the basic principle that not all tumours have the same immunological features. The levels of TILs may also be indicative of whether the patient will be responsive to therapy involving rhIL-2 (Rosenberg 1986: Topalian *et al.*, 1987; Rosenberg *et al.*, 1990). Most of the work so far has been carried out on malignant cutaneous melanoma which contains sufficient levels of TILs to enable them to be expanded and re- administered to the patient. However uveal melanoma contains such low levels that these TILs will not grow to sufficient quantities.

Another problem exists when analysing the tumours for lymphocytes. This involves the anatomy of the tumour and the location of the test samples obtained. As mentioned previously (Section 6.2) only the apical pieces are obtained, prompting the questions; are these pieces representative of the tumour as a whole? This problem is unavoidable for obvious clinical and pathological reasons.Previous studies involving identification of uveal melanoma TILs have been involved with classical immunohistological or microscopical techniques (De La Cruz *et al.*, 1990) introducing the problems listed above (Section 6.2). Results of phenotyping of TILs represented in this thesis indicate that the TILs are markedly different than the PBLs, not only in the relative proportions of the lymphocytes subsets present, but also in the activation status of the T cells (Section 3.1). Thus the lymphocytes are located within the tumour either because they possess some mechanism contributing to their migration and activation, or the tumour in some way attracts the lymphocytes consequently activating them. In both cases the cells are being selected to infiltrate the tumour for whatever function.

As mentioned (Section 1.5.7), therapeutic strategies have involved culturing of TILs with rhIL-2 and re- administering these to the patient in the hope that the TILs will be more cytotoxic to the tumour cell present. Clonal phenotyping of these cells, in terms of their ability to express specific Vβ regions of the TCR (Section 3.2), has indicated that the TILs, in uveal melanoma, are of polyclonal nature, but also have ability to home into the tumour. Reports of oligoclonality in humans (Belldegrun *et al.*, 1989) and murine (Karparti *et al.*, 1991) IL-2 treated TILs, indicated that expression of these TILs may lead to selective alterations in the recognition profiles of the T cells. Such recognition may be patient specific due to the MHC restriction present within an outbred population.

It would be interesting to elucidate whether the uveal melanoma TILs would be polyclonal or oligoclonal for the Vβ TCR repertoire expression after culturing in IL-2, and also to determine what particular subset of T cell, $CD4^+$ or $CD8^+$, would predominantly be expressed within the culture.

Phenotyping of the PBL samples at different time points (Section 3.3) identified the lack of systemic immune response to uveal melanoma. In the 1970's it was assumed that an immune response was detected due to the identification of a response at the level of serum antibody. It is now apparent that the nature of the response to a small, local and weakly antigenic tumour will not be the same as that detected in bacterial or viral infections.

6.4 Tumour cell surface antigen analysis and relevance in metastasis.

The study of the expression of cell surface antigens (Section 4.1) on the uveal melanomas has revealed some interesting points. The tumours show a high degree of variability in their expression of Class I and Class II (HLA DR) MHC antigen which is in agreement with other studies involving MHC antigen analysis of tumour cell (Whitford *et al.*, 1991). Reports have indicated that HLA DR is preferentially expressed on advanced and metastatic cutaneous melanoma (Johnson *et al.*, 1989). This thesis reports that there is an inverse correlation between the expression of the adhesion molecule, ICAM-1, perhaps involved in the metastasis of tumours, and the HLA DR MHC antigen on the malignant melanoma studied. Perhaps since HLA DR is present on more advanced tumours and the ICAM-1 may not be, then it could be the less aggressive tumours that disseminate and result in metastasis.

The fact that all the tumours expressed a high level of ICAM-1 suggests that this molecule may be involved in the progression of tumours. ICAM-1 is also thought to be absent on normal non- lymphoid tissue and thus

may be regarded, in the case of uveal melanoma, to be a tumour specific antigen.

The TILs, especially the $CD8^+$ T cells, also seem to express ICAM-1 and also its ligand LFA-1. This could indicate three possibilities:

(1) tumour cells adhere to the TILs and it is this attachment that promotes dissemination of the tumour cells into the blood stream, via recirculating lymphocytes, and causes the eventual metastasis of the tumour.

(2) The TILs have the ability to bind to each other via the ICAM-1: LFA-1 interaction, and by some mechanism transmitting the activating signals to the resting lymphocytes.

Finally (3) The binding of LFA-1 on the lymphocytes to the ICAM-1 on the tumours targets, may bring the effector and target cell closer together such that the cytolytic activities of the T cells can come into operation.

The first proposal is of poor prognosis to the patient, whereas the others are of detrimental value to the existing tumour. All three ideas are possible since lymphocytes lacking ICAM-1 expression may still be involved in the dissemination of the tumour cells, allowing the ICAM-1^+ T cells to perform their cytotoxic functions.

6.5 DNA content of uveal melanoma tumour cells and relevance in prognosis.

Tumours are generally associated with an abnormal (aneuploid) DNA content, as in breast carcinoma (Alam *et al.*, 1991) and these tumours are found to be of epithelial origin. Few uveal melanoma tumours are found to be aneuploid which indicates a difference in the characteristics of different neoplasms. Although there is a wealth of ploidy data involved on the analysis of breast and colonic cancer there are few articles on cutaneous melanoma.

This suggests that, like its uveal melanoma counterpart, aneuploidy is not a feature of this type of cancer (Sondergaard *et al.*, 1983; Stenzinger *et al.*, 1984)

The cell cycle parameters also give little information, except that the epithelioid cells, that are already established to give the poorer prognosis in uveal melanoma, may also contribute to the population of cells with the highest turnover rate. This could be indicative of the aggressive nature of the tumour.

6.6 Conclusions

It is important to note, within any clinical study, there will always be great variability in the results obtained between patients. Clinical research only determines trends in results, and thus a greater understanding of more basic science is required to elucidate the underlying problem.

CHAPTER 7

REFERENCES

Abramson, D.H., Ellsworth, R.M., Kitchin, F.D. and Tung, G (1984) Ophthalmology **91**, 1351-1355.

Acha- Orbea, H. and Palmer, E. (1991) Immunology Today **12**, 10, 356.

Acha-Orbea, H.,Mitchell, D.J., Timmerman,L., Wrath, D.C., Tausch, G.S., Waldor, M.K., Zamvil, S.S., McDevitt, H.O. and Steinman, L. (1988). Cell **54**: 263-273.

Alam, S.M., Whitford, P., George, W.D. and Campbell A.M. (1991) European J. Cancer (In press).

Alberts, B., Bray, J., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. (1983) Molecular Biology of the cell. New York and London: Garland: 424-426.

Albino, A.P. (1988). Adv. in pigment cell research **256**: 361-392.

Albino, A.P., Le Strange, R. and Oliff, AI (1984) Transforming ras genes from human melanoma: a manifestation of tumour heterogeneity? Nature **308**, 69-72.

Anichini, A. Fossati,G. and Parmiani, G.. (1987) Immunol. Today **8**, 385-389.

Apple, D.J., Garland, P. and Bautros, G.(1983) In intraocular tumours ed. Lommatzsch, P.K. and Blodi, F.C. Ch 1. 66-74 Berlin, Springer- Verlag.

Armitage, N.C., Robias, R.A., Evans, D.F., Turner, D.R., Baldwin, R.E.and Hardcastle, J.D. (1985) Br J. Surg. **72**, 828-830.

Ashton, N. (1964) Br. J. Ophthalmol. **48**, 650-668.

Auer, G.U., Arrhenius E., Granberg, P.and Fox, C.H. (1980 a) Eur J. Cancer **16**, 273-278.

Auer, G.U., Caspersson, T.O., and Wallgren, A.S. (1980 b) Anal Quant Cytol 1980 b; **2** 161-165.

Augsburger, J.J., Gander, J.R., Amsel, J., Shields, J.A., and Donoso, L.A (1984) Ophthalmology.**91**, 1709-13.

Babbitt, B., Allen, P.M. and Matsueda., G (1985). Nature **317**, 359- 361.

Baker P.E., Gillis S. and Ferm, K.A. (1979) J. Exp. Med. **149**, 273-278.

Baker, P.E., Gillis, S., Ferm, M.M. and Smith, K.A. (1978) J Immunol. **121**, 2168-2173.

Baker, S.J. (1989) Science **244**, 217- 221.

Bakker, M., and Kijlstra. A. (1985). Curr. Eye Research. **4**, 599-604.

Balch, C.M., Murad, T.MN., Soong, S.J., Ingalls, A.L., Halpern, N.B. and Maddox, W.A. (1978) Ann. Surg. **188**, 732-742.

Balch, C.M., Riley, L.B., Bae, Y.J., Salmeron, M.A., Platsoucas, C.D., von Eschenbach, A. and Itoh K. (1990) Archives of Surgery **125**, 200-205.

Baldwin, R.W., Price, M.R. and Robins, R.A. (1973) Int. J. Cancer **11**, 527-535.

Baldwin, R.W.and Price, M.R.(1976) Ann. Review Med. **27**, 151-163.

Baniyash, M., Garcia- Morales, P., Luong, E., Samelson, L.E. and Klausner, R.D. (1988 b). J. Biol. Chem. **263**, 18225.

Baniyash, M.., Garcia- Morales, P., Bonifacino, J.S., Samelson, L.E. and Klausner, R.D. (1988 a) J. Biol. Chem. **263**, 9874.

Barlogie, B., Drewinko, B. and Schumann, J. (1980). Am J. Med., **69**, 195.

Barnd, D.L., Lon, M., Metzgar, R. and Finn, O.J. (1989) Proc. Natl. Acad Sci USA, **86**, 7159-7163.

Barnstable, C.J., Bodmer, W.F., Brown, G., Galfre, G., Milstein, C., Williams, A.F. and Zeigler, A. (1978) Cell **14**, 9-20.

Barsch, H. and Gerdes, J. (1987) Cell Tissue Kinet.**20**, 387-391.

Beer, A.E. and Billingham, R.E. (1976) The Immunobiology of mammalian Reproduction, Prentice- Hall, Englewood Cliffs, New Jersey.

Belldegrun, A., Kasid, A., Uppenkamp, M., Topalian, S.L. and Rosenberg, S.A. (1989) J. Immunology **142**, 4520- 4526.

Belldegrun, A., Linham, W.M. and Robertson, C.N. (1986) Surg. Forum **37**, 671- 673.

Ben- Ezra, J. and Sheibani, K(1987) Cancer **59**, 2037-2041.

Benjamin, B., Cummings, J.N., Goldsmith, A.J.B. and Sorsby, A., (1948). Br. J. of Ophthalmol. **32**, 729-747.

Bernards, R., (1987) Trends Genet. **3**, 298- 301.

Bernengo M.G., Lisa F., Meregalli M., De Matteis A. and Zina G. (1983) Cancer **52**,1841-1848.

Bierer, B.E. and Burakoff, S.J. (1988) FASEB J. **2**, 2584- 2590.

Bilik R., Mor C., Hazaz B. and Moroz C.(1989). Cancer Immunol. Immunotherapy. **28**, 143-147.

Billingham, R.E. and Boswell, T. (1953). Proc. R. Soc. London **141**, 392-406.

Bjorkman, P.J., Sapper, M.A., Samraoui, B., Bennet, W.S., Schrominger, J.L.and Wiley, D.C.(1987) Nature, **329**, 506.

Black R.B., Merrick M.V., Taylor T.V. and Forrest A.P.M.(1975). Br. J. Surgery, **68**,145-146.

Black, C.A. and Horowitz, A.F. (1987) Ann Rev. Cell Biol. **3**, 179- 205.

Bloom, H.J.G., Richardson, W.W. and Field, J.R. (1970) Br. Med. J., **3**,181-188.

Bodmer, W.(1991) Human Immunology **30**, 259- 261.

Bodmer, W.F. (1987) J. Clin Pathol. **27**, 948.

Borges, A.F. and Lineberger, A.S. (1984) Malignant melanoma without metastases in a giant nevus. Am Plast. Surg., **12**, 454.

Borst J., Van de Griend R., Van Oostveen J., Ang S., Melief C., Seidman J., and Bolhuis R. (1987) Nature **325**,683-688.

Borst, J., Van Dongen, J.M., Bolhuis, R.L., Peters, P.J., Hafler, D.A., de Vries, E. and van de Griend, R.J. (1988) J Exp. Med., **167** 1625.

Bos, J.L.(1989). Ras oncogenes on human cancer a review. Cancer **49**, 4682-4689.

Braciale, T.J. and Braciale, V.L. (1991) Immunology Today, **12**, 124- 129.

Braciale, T.J., Sweetser, M.T. and Brown, L.R. (1990) Cold Spring Harbour Symp. Quant. Biol. **54**, 477-485.

Brocker, E.B. Suter, L., Bruggen, J., Ruiter, D.J. Macher, E., Sorg, C. (1985) Int. J. Cancer **36**, 29-35.

Brocker, E.B. Zwalso, G., Holzmann, B., Macher, E. and Sorg, C. (1988) Int. J. Cancer **41**, 562-567.

Brocker, E.B., Suter, L, L. and Sorg, C. (1984) J Invest Dermatol. **82** 244-47.

Brstryn, J.C. (1985). Dermatologic. Clinics, **3**, 327-334

Buckle, A-M. and Hogg, N. (1990) Eur J. Immunol. **20**, 337-341.

Burnet, F.M.(1970) Prog. Exp. Tumour. Res. **13**, 1-27.

Burnet, M. (1964) N. Engl. Med. **20**, 154-158.

Callanan, D., Peeler, J., and Niederkorn, J.Y.(1988) Transplantation **45**, 437-443.

Callender, G.R., Wilder H.C. and Ash, J.E. (1942) Am. J. Ophthalmol. **25**, 962-967.

Carrel, S., Mach, J-P., Ferremi, P., Guiffre, L. and Accolla, R.S.(1986) HLA Class II antigens (Springer, Berlin), 412-428.

Chambers, R.B., Davidorf, F.H., HcAdoo, J.F. and Chakeres, D.W., (1987). Archives of Ophthalmol. **105**, 917-912.

Chambers, R.B., Davidorf, F.H., McAdoo, J.F., and Chakeres, D.W. (1987) Arch. Ophthalmology **105**, 917.

Chandler, J.W., Ray-Keil, L., and Gillette, T.E.(1983). Curr. Exp Res. **2**, 387- 397.

Char, D., Heibron, R.C., Juster, R.P. and Stone, R.D. (1983) Br J. Ophthalmol. **67**, 575-78.

Char, D.H. (1978) Am J of Ophthalmol. **86**, 76-80.

Char,D.H, Huhta, K., and Waldman, F.(1989). Am. J. Ophthalmol. **107**, 65-72.

Chi, H.H., Teng, C.C. and Katzin, H.M. (1965) Ophthalmology **59**, 186-191.

Choi, Y., Kappler, J.W. and Marrack, P. (1991) Nature **350,** 203.

Choi, Y., Kotzin, B., Herron, L., Callahan, J., Marrack, P., and Kappler, J.(1989) Proc. Natl. Acad. Sci.USA. **86**,8941-8946.

Clark, G.M., Dressler, L.G., Owens, M.A., Pounds, G., Oldaker, T. and McGuire, W.L. (1989). New Eng. J. Med. **320**, 627.

Cleasby, G.W. (1958) Arch Ophthalmol. 60, 403-417.

Cole, M.D. (1986) Annu. Rev. Genet. 20: 361-384.

Coleman, D.J., Abrahmson, D.H., Jack, R.L. and Franzen, L.A. (1974) Arch. Ophthalmol. **91**, 344-354.

Coon, J.S. Landlay, A.L. and Weinstein, R.S. (1987) Advances in flow Cytometry for diagnostic pathology. Lab invest.,**57**, 453-479.

Cornelisse, C.J., Van De Velde, C.J.H., Caspers, R.J.C., Moolenaar, A.J. and Hermans, J. (1987). Cytometry **8**, 225.

Cresswell, P., Blum, J.S., Kelner, D.N. and Marks, M.S.(1987) CRC Crit. Rev.Immunol. **7**, 31-53

Crocker, J. and Nar P. (1987) J. Pathol. **151**, 111-118.

Crocker, J. and Skilbeck, N. (1987) J.Clin. Patho., **40,** 885-889.

Currie, G.A., Basham, C. (1972) Br, J, Cancer **31**, 620-629.

Cutler, S.J. and Connelly, R.R. (1969) Cancer **23**, 767-771.

D'Alessandro, G., Zardawi, I., Grace, J. (1987). Pathology **19**, 339-346.

De Fano, C. (1912) Fifth Scientific report on the Imperial Cancer Research Fund, 57.

De La Cruz, P.O., Specht, C.S. and McLean, I.W. (1990) Cancer **65**, 112-115.

Doherty, P.C., Knowles, B., and Wettstein, P.J. (1984) Adv. Cancer Research. **42**, 1-65.

Doll, R. (1980) Cancer (Amsterdam) **45**, 2475- 2485.

Dowle, C.S., Owainti, A., Robbins, A. (1987).Br. J. Surg. **74**, 133.

Duijvestijn, A. and Hamann, A. (1989) Immunol. Today **10**, 23.

Duke, J.R., Dunn, S.N.(1958) Arch Ophthalmol. **59**, 204- 214.

Durie, F.H., Campbell, A.M., Lee, W.R., and Damato, B.E. (1990). Invest. Ophthalmol. and Visual Sci. **31** 2106.

Dustin, M.L. and Springer, T.A. (1988). J. Cell Biol. **107**, 321-331.

Dustin, M.L., and Springer, T.A. (1989) Nature **341**, 619.

Dyson, P.J., Knight, A.M., Fairchild, S., Simpson, E. and Tomonari, K. (1991) Nature **349**, 531-532.

Ediden, M., Aszalos, A., Damjanovich, S., and Waldman, T.A. (1988). J.Immunol. **141**, 1206- 1210.

Edwards, P.A.W.(1985) Br.J. Cancer **51**,149-160.

Eisenmann, R.N. (1989) . In R. Weinberg (Ed), Oncogenes and the molecular origins of cancer 175- 221.

Eskelinen, M.J., Pajarinen, P., Collen, Y., Personen, E., Alhava, E., Kettunen, K., and Nordling, S. (1989). Br. J. Surg. **76**, 830.

Fairchild, R.G. (1984) Seminars in Nuclear medicine **14**, 35-45.

Farber, E. and Cameron, R. (1980) Adv. Cancer Res. **35**, 125- 226.

Fearon, E,R., Cho, K.R., Nigro, J.M.(1990) Science, **247**, 49.

Feichter, G.E., Mueller, A., Kaufman, M.,Haag, D., Barn, I.A., Abel, U., Klinga, K., Kubi, F. (1988). Int. J. Cancer **41**, 823.

Feldman, M., Eisenbach, L.(1988).Cancer surveys **7**, (4), 555-572.

Fidler, I.J. and Kripke, M.L. (1977). Science, **197**, 893.

Figge, J., Webser, T., Smith, T.F. and Paucha, E. (1988). J. Virol. **62**, 1814-1818.

Fisher, B., Bauer, M., Wickerham, L., Redmont, C.K. and Fisher, E.R. (1983) Cancer, **52**,1551-1557.

Flynn, K., Feldberg, N.T., Koegal, A., Hager, R., Shields, J.A., Augsburger J.J. and Donoso L.A. (1986) Am. J. Ophthalmology **101**, 160-163.

Foley, E.J. (1953). Cancer Res **13**, 835-837.

Font, R.L. Spaulding, A.G. and Zimmerman L.E. (1986) Transactions of the American Academy of Ophthalmol. and Otolaryngology **72**, 887-895.

Forrester, J.V., Reid, D.M. and Campbell, A.M.(1987) Seminars in Ophthalmology **2**, 2, 81-88.

Foulds, W.S. (1978) Sund- Afrikaanse Argref vir Oftalmologie, **5**, 161-182.

Foulds, W.S. (1985) The diagnosis and management of tumours of the iris and ciliary body.In Ophthalmic tumours, ed. Oosterhurs, A. pp173-220. Dordrecht: Dr.W. Junk.

Foulds, W.S. and Damato, B.E. (1986) Graefe's Arch. for Clinical and Exp. Ophthalmol. **224**, 26-31.

Friedlander, M.L., Hedley, D.W., Taylor, I.W., Russel, P., Coates, A.S. and Tattersall, M.H.N. (1984) Cancer Res., **44** 397-400.

Friedlander, M.L., Russel, P., Taylor I.W., Hedley, D.W. and Tattersall, M.H.N. (1984) Pathology ., **16**, 301-306.

Friedlander, M.L., Taylor, I.W., Russell, P. and Tattersall, M.N.H. (1984) Br J. Cancer., **49**, 173-179.

Galili, U., Galili, N., Vanky, F., and Klein, E. (1978) Proc. Natl. Acad. Sci. USA **75**, 2396.

Geliebter, J., Melvold, R.W., Zeff, R.A. and Nathenson, S.G. (1986) Proc. Natl Acad. Sci. USA, **83**, 3371.

Gerdes, J., Lemke, H., Barsch, H., Wacker, H.H., Schwab, U. and Stein, H. (1984) J. Immunol. **133** 1710-1715.

Gerdes, J., Schwab, U, Lemke, H., Stein, H. (1983). Int. J. Cancer, **31**,13-20.

Gillis, S., Ferm, M.M., Ou, W. and Smith, K.A.(1978) J. Immunology.**120**, 2027-2032.

Giorno, R. (1983) Archives of Pathology and Laboratory Medicine, **107**, 415-417.

Gold, D.P., Clevers, H., Alarcon, B., Dunlap, S., Novotny, J., Williams, A.F., and Terhorst, C. (1987) Proc. Natl. Acad. Sci U.S.A **84**, 7649.

Goodman, T. and Lefrancois, L. (1988) Nature **333**, 855.

Goodpasture, C, Bloom, S.E. (1975) Chromosoma **53**, 37-50.

Gottlinger, H.G., Reiber, P., Gokel, J.M., Lohe, K.J. and Reithmuller, G. (1985) Int. J. of Cancer **35**, 199-205.

Gratzner, H.G. (1982) Science, **218**, 474-478.

Grimm, E.A., Mazumder, A., Zhang, H.Z. and Rosenberg, S.A. (1985) J. Exp. Med. **155**, 1823-1841.

Guntunz, N. (1985) Cytometry **6**, 597.

Gustafson, H., Tribukait, B., Esposti, P.L. (1982) Urol Res 10, 13-18.

Hammering, U., Ronne, H., Widmark, E., Servenius, B., Dernaro, M., Rask, L. and Peterson, P.A. (1985) EMBO J., **4**, 1431.

Hammerling, G.Y., Klar, D., Pulm, W., Momburg, F., and Moldenhauer, G. (1987). Biochim. Biophys. Acta. **907**, 245-259.

Handley, W.S.(1907) Lancet I, 927-933.

Hann, S.R., King, M.W., Bentley D.L., Anderson, D.W. and Eisenmann, R.N. (1988) Cell **52**, 185-195.

Hansen, M.F., Koufos, A., Gallie, B.L., Phillips, R.A., Fodstad, O., Brogger, A., Gedde-Dahl, T., and Cavenee, W.K. (1985) Proc. Natl. Acad. Sci. USA 82: 6212-6220.

Hart, I.R., Goode, N.T. and Wilson, R.E. (1989) Biochem. Biophys. Actc., **989**, 65.

Head, J.R. and Griffin, W.S.T.(1985) Proc. R. Soc. Lond. (Biol), 224-375.

Head, J.R., and Billingham, R.E. (1985) Perspect. Biol. Med. **29**, 115-131.

Heath, P. (1964) Trans Am. Ophthalmol. Soc. **62**, 51-85.

Hedley, D.W., Friedlander, M.L. and Yaylor, I.W. (1985) Cytometry **6**, 327-333.

Hedley, D.W., Friedlander, M.L., Taylor, I.W., Rurr, C.A. and Musgrove, G.A. (1983) J. Histochem. **31**, 1333-1335.

Hedley, D.W., Rugg,. C.A. and Gelber, R.D. (1987). Cancer Res. **47**, 4729.

Hedrick, S.M., Nielson, E.A., Kavalier, J., Cohen, D.I. and Davis, M.M.(1984).Nature **308**, 153.

Hellstrome, K.E., Hellstrome, I. (1974) Adv. Immunol. **18**, 209-277.

Heo, D.S., Whiteside, T.L., Kanbour, A., and Herberman, R.B. (1988) J.Immunol **140**, 4042-4049.

Heppner, G.H. (1984) Cancer Research **44**, 2259-2265.

Heppner, G.H. (1984) Cancer Research, **44**, 2259-2265.

Herlyn, M and Koprowski, H. (1988) Ann. Rev. Immunol. **6**, 283-308.

Herlyn, M., Clark, W.H., Rodeck, U., Mancianti, M.L., Lambrosic, J. and Koprowski, H. (1987). Lab. Invest. **56**, 461-74.

Herman, A., Kappler, J.W., Marrack, P. and Pullen, A.M. (1991). Ann. Rev. Immunol. **9**, 745- 752.

Hewitt, H.B., Blake, E.R., and Walder, A.S. (1976) Br. J. Cancer **33**, 241-259.

Holland, G. (1967) Klin Monatsbl. Augenherlkd. 150, 359-370.

Holt, S., Croton, R. and Leinster, S.J. (1986) Eur. J. Surg. Oncol. **12,** 53-57.

Holzmann ,B., Johnson ,J.P. Kaudewitz, P., Reithmuller, G. (1985) J. Exp. Med. **161**, 366-377.

Holzmann, B. (1989) Proc. Natl. Acad. Sci. **86**. 641- 644.

Holzmann, B., Brocker, E.B., Lehmann, J.M., Ruiter, D.J., Sorg, C., Reithmuller, G. and Johnson, J.P. (1987) Int. J. Cancer **39**, 466-471.

Holzmann, B., Lehmann, J.M., Ziegler- Hertbrock, H.W.L. (1988). Int. J. Cancer **410**, 542-547.

Horsfall, D.J., Tilley, W.D., Orell, S.R., Marshall, V.R. and Cant, E.L. (1986). Br. J. Cancer **33**, 23.

Houghton, A.N., Cordon- Cardo, C., Eisinger, M. (1986) Int. Rev Exp. Pathol.**28**, 317-29.

Hynes, R. (1987) Cell **48**, 549- 554.

Itoh K., Davidorf, F.H., and Baba, N (1977) Cancer **40**, 2388.

Itohara S., Farr, A.G., Lafaille, J.J., Bonneville, M., Takagaki, Y., Haas, W.and Tonegawa, S.(1990) Nature **343**, 754.

Iversen, O E. and Skaarland, E. (1987) Cancer **60**, 82-87.

Jack, A.S., Boylston, A.W., Carrel, S., and Grogor, I. (1990) American J. Pathology **136**, 17-21.

James, C.D., Carlborn, E., Nordenskjold, M., Collins, V.P. and Cavanee, W.K. (1989) Proc. Natn. Acad. Sci. U.S.A. **86**, 2858- 2862.

Jan- Mahammed, R.M., Armstrong, S.J. Crocker, J., Leyland, M.J. and Hulten, M.A. (1989) J. Pathol. **158**, 3-7.

Jaraquemana, D., Marti, M. and Lang, E.D. (1990) J. Exp Med. **172**, 947-954.

Jensen, O.A. (1963) Acta Ophthalmol. **75 (suppl.)** 173-179

Jensen, O.A. (1982) Acta. Ophthalmol. **60**,161-182..

Jerome, K.R., Barnd, D.L., Bendt, K,M., Boyer, C.M., Taylor-Papadimitriou ,J., McKenzie, I.F.C., Bast, R.C. and Finn, O.J. (1991) Cancer Research **51**, 2908- 2916.

Johnson, H.A., Bond, V.P. (1961) Cancer . **14**, 639-650.

Johnson, J.P. Lehmann, J.M. and Reithmuller, G. (1989) Eur. J. Immunol. **18**, 2097-2100.

Johnson, J.P., Stade B.G., Holzman B., Schwable W. and Riethmuller G. (1989) Proc Natl. Acad. Sci, **86**, 641- 644.

Kallioniemi, O., Blanco, G., Alavaikko, M., Hietanen, T., Matilla, J., Lauslahti, K., Lehtinen, M. and Koivula, T. (1988). Cancer **62**, 2183.

Kappler, J., Kotzin, B., Herron, L., Gelfand, E., Bigler, R., Boylston, A., Carrel, S., Posnett, D., Choi, Y. and Marrack, P. (1989) Science **244**, 811-813.

Karpati, R.M., Banks, S.M., Malissen, B., Rosenberg, S.A., Sheard, M.A., Weber, J.S. and Hodes, R.J. (1991) J. Immunol. **146**, 2043- 2051.

Keeney, A.H., Waddell, W.J. and Perrant T.C. (1982) Trans. Am. Path. Society. **80**, 131-142.

Khoudadoust, A.A.,and Silverstein, A.M. (1966). Surv. Ophthalmol. **11**, 435- 443.

Klein, G. and Klein, E. (1985). Nature, **315**, 190.

Klein, M.H.,Concannon, P., Everett, M., Kim, L.D.H., Hunkapiller, T. and Hood, L. (1987). Proc. Natl. Acad. Sci. USA **84**, 6884-6888.

Koning, C.A. (1987). Nature 330 804.

Koprowski, H., Steplewski, Z., Herlyn, D. and Herlyn, M. (1978).Proc. Natl. Acad. Sci. USA **75**, 3405-9.

Kradin, R.L., Boyle, L.A., Preffer, F.I., Callahan, R.J., Barlai- Kovach, M., Strauss, H.W., Dubinett, S and Kurnick, J.T. (1987) Cancer Immunology, Immunotherapy **24**, 76-85.

Kung, P.C., Goldstein, G., Reinherz, E.I., and Schlossman, S.F. (1979) Science **206**, 347.

Kunzler, K. and Vogelstein, A. (1991) Research news ,1366.

Kurnick, J.T., Kradin, R.L., Blumberg, R., Schneeberger, E.E. and Boyle, L.A.(1986) Clin. Immunol. Immunopathol **38,** 367.

Kute, T.E., Muss, H.B., Hopkins, M., Marshall, R., Case, D. and Kammire, L. (1985). Breast Cancer Res. Treat. **6,** 113.

Kuzial, W.A., Takashima, A., Bonyhadi, M., Bergstresser, P.R., Allison, J.P., Tigelaar, R.E. and Tucker, P.W. (1987) Nature **328,** 263.

Kyprianou, N and Isaacs, J.T. (1990) Cancer Res., **50,** 1449.

Lang, J.P., Davidorf, F.H. and Baba, N. Cancer **40,** 2388-2394, (1977).

Lee, H.W., Brookstein, R. and Hong, F.(1987). Science **235,** 1395-1399.

Leff, S.R., Shields, J.A., Augsburger, J.J. and Donoso, L.A. (1985) Am. J. Ophthalmol. **99,** 209.

Leonard, W.J., Depper, J.M., Uchiyama, T., Smith, K.A., Waldmann, T.A. and Greene, W.C. (1982) Nature, **300,** 267-269.

Levine, A.J., Momand, J., and Finlay, C.A. (1991) Nature **351,** 453-456.

Lewin, B. (1980) Gene Expression. 2. Eukaryotic Chromosomes 2nd Ed. New York: Wiley, 875-878.

Liotta, L.A., Nagaswara Rao, C. and Wewer, U.M.(1986) Ann Rev Biochem. **55**, 1037-1057.

Ljunggen, H-G., Stam, N.J., Ohlen, C (1990) Nature **346**, 476-480.

Lotze, M.T., Custer, M.C., Sharrow, S.O., Rubin, L.A., Nelson, D.L. and Rosenberg, S.A. (1987) Cancer Research **47**, 2188-2195.

Lozzio C.T. and Lozzio B.B. (1975) Blood, **45** 321.

Maasken, A.P., Deschner, E.E. J. (1977) Natl. Cancer Inst. 58, 1221-1224.

Mackay, J., Steel, C.M., Elder, P.A.M. and Evans, H.J. (1988) Lancer, **11**, 1384-1385.

Mafee, M.F., Peymen, G.A. and McKusick, M.A. (1985) Radiology **156**, 403-408.

Makely, T.A. and Tweed, R.W. (1958). Arch. of Ophthal. **60**, 475-478.

Makgoba, M.W., Sanders, M.E., Luce, G.E., Dustin, M.L., Springer, T.A., Clark, E.A., Mannoni, P., and Shaw, S. (1988) Nature **331**, 86.

Makgobo, M.W., Sanders, M.E., Luce, G.E.(1989) Eur. J. Immunol. **18**, 637- 640.

Maluish A., Halliday W.J. (1974) J. Natl. Cancer Inst. **52**, 1415-1420

Manschot, W.A. and Van Peperzol, H.A. (1980) Arch. of Ophthalmol. **98**, 71-77.

Marrack, P., Kushnir, E. and Kappler, J. (1991) Nature **349**, 524- 526.

Martin-Jones, J.D. (1946) Br J. Ophthalmol **11** (suppl.) 1-94.

Martz, E. (1987) Hum. Immunol. **18**, 3-37.

Martz, E., (1975) J Immunol. **115**, 261.

Matoba, A.Y., Peter, J.S., and Niederkorn, J.Y.(1986) Invest Ophthalmol. Visual Sci. **27**, 1244-1254.

Matsui, M., Nakonishi, T., Noguchi, T. and Ferrone, S. (1988) J. Immunol. **141**, 1410- 1417.

Maumenee, A.E.(1951). Am. J. Ophthalmol. **34**, 142-152.

Mayer, D.J., Daar, A.S., Casey, T.A., and Fabre, J.W.(1983).Transplant. Proc. **15**, 126-129.

Maziarz, R.T., Burakoff, S.J., Bluestone, J.A. (1988) J. Immunol. **140**, 4372- 4377.

McCartey, W.C. (1922) Annals of Surg., **76**, 9-12.

McCartey, W.C. and Mahle, A.E. (1921) J. Lab. Clin Med, **6**,473-480.

McGovern, V.J., Shaw, H.M., Milton, G.W., Farage, G.A. (1981) Ackerman AB, ed. Pathology of malignant melanoma: Masson Monographs in Dermatology. New York: Masson Publishing 341-344.

Meecham, W.J. and Char, D.N. (1986) Arch Ophthalmol. **104**, 1626-9.

Mellor, A.L., Weiss, E.H., Ramachandran, K. and Flavell, R.A. (1983) Nature, **306**, 792.

Mendenhall, M.D., Jones, C.A., and Reed, S.I. (1987) Cell, **50**, 927-935.

Meyer- Schwickerath, G. and Bornfeld, N. (1983). In Intraocular tumours ed.Lommatzsch, P.K. and Blodi, F.C. Ch 3 pp269-276. Berlin: Springer-Verlag.

Miceli, M.C. and Finn, O.J. (1989) J. Immunol. **142**,81.

Miller,F.R., (1982) Intratumour immunologic heterogeneity. Cancer Metastasis Reviews,**1** , 319-334

Moebius, U., Manns, M., Hess, G., Meyer zum Buschenfeld, K.H. and Meuer, S.C.(1990) Eur. J. Immunol. **20**,889.

Moller, D.R., Konishu, K., Kirby, M., Balbi, B. and Crystal, R.G.(1988). J. Clin. Invest. **82**: 1183-1191.

Momburg, F., Degener, T., Bacchus, E., Maldenhauer, G., Hammerling, G.J. and Moller, P. (1986) Int. J. Cancer **37**, 179-184.

Moore O.S. and Foote F.W. (1949) Cancer, **2**, 635-642.

Moran, R.E., Black, M.M., Alpert, L. and Straus, M.J. (1984). Cancer **54**, 1586.

Morstyne, G, Hsu, S.M., Kinsella, T., Gratznor, A., Russo, A.,Mitchell, J.B. (1983) J. Clin. Invest. **72**, 1844-1850.

Mule, J.J., Shu, S., Schwarz, S.L. and Rosenberg, S.A. (1984) Science, **225**, 1487

Munro, J.M., Pober, J.S. and Contran, R.S. (1989) Am. J. Pathol. **135**, 121.

Murphy, W.M. Chandler, R.W. and Trafford, R.M. (1986) J Urol. **135**, 694-697.

Muul, L.M., Speiss, P.J., Director, E.P. and Rosenberg, S.A. (1987) J. Immunol. **138**, 989-995.

Natali, P., Bigotti, A., Cavalieri, R., Nicotra, M.R., Tecce, R., Manfredi, D., Chen, Y-X, Nadler, L.M., and Ferrone, S.(1986) Hum. Immunol. **15**, 220-233.

Natali, P.G., Cavaliere, R., Bigotti, A., Nicotra, M.R. Russo, C., Ng, A.K., Giarcomini, P. and Ferrone, S. (1983) J of Immunol., **130**, 1462-1466.

Naukkarinen, A. and Syrjanen, K.J. (1990) J Pathology **160**, 217-222.

Neefjes, J.J., Stollorz, V., Peters, P.J. (1990) Cell **61**, 171- 183.

Newton, J.A., Camplejohn, R.S. and McGibbon, D.H. (1987) Br J. Dermatol., **117**, 169.

Newton, J.A., Camplejohn, R.S. and McGibbon, D.H. (1988). Br J. Cancer **58**, 606-609.

Nicholson, G.L. (1988) Biochim. Biophys. Acta. **948**, 175-224.

Niederkorn, J.Y. (1987) Invest. Ophthalmol. Visual Sci. **28**, 1297- 1403.

Niederkorn, J.Y. and Knisely, Y.L.(1988). Curr Eye Res. **7**, 515-526.

Niederkorn, J.Y. and Meunier, P.C. (1985) Invest. Ophthalmol. Visual Sci.**26**, 877-

Nind, A.P.P., Matthews, N., Pihl, E.A.V., Rolland, J.M. and Nairn, R.C. (1975) Br. J. Cancer **31**, 620-629.

Nitta, T., Oksenberg, J.R., Rao, N.A. and Steinman, L. (1990) Science **249**, 672-674.

Norment, A.M., Salter, R.D., Parham, P., Engelhard, V.H. and Littman, D.R. (1988) Nature **336**, 79-81.

Norton, F.H. (1965) Arch. of Ophthal., **73**. 198-199.

Nuchtern, J.G., Biddison, W.E. and Klausner, R.D. (1990) Nature **343**, 74- 76.

Nurse, P and Bisset, Y. Nature (1981) **292**, 558-560

Nurse, P. (1990) Nature **344,** 503-508.

O'Reilly, S.M., Campeljohn, R.S., Barnes, D.M., Millis, R.R., Allen, D., Rubens, R.D. and Richards, M.A. (1990). Br. J. Cancer **61**, 671.

Oksenberg J.R., Stuart, S., Begovich, A.B., Bell, R., Erlich, H.A., Steinman, L. and Bernard, C.C.A.(1990) Nature **345**, 344

Old, L.J. (1981) Cancer research, **41,** 361-375

Olsz, W., Darynkiewicz, Rosen, P.P. Schwartz, M.K. and Melamed, M.R. (1981) Cancer, 1981; 48: 980-984.

Ortaldo, J.R., Mason, A., Overton, J.R. (1986) J Exp. Med **614**, 1193-1205.

Packard, R.B.S.(1980) Br. J. of Ophthalmol, **64**, 565-578.

Packer, S (1984) Seminar in Nuclear medicine, **14,** 21-34.

Pascal,S.G. Saulenas, A.M., Fournier, G.A., Seddon, J.M., Hatfeld, R.M. and Albert, D.M. (1988) Am. J. of Ophthalmol. **100**, 448-453.

Pawelec, G., Blaurock, M., Schneider, E.M., Shaw, S and Wernet, P. (1982) European J. Immunol. **12**, 967-972.

Ploton, D., Menager, M., Jeanneson, P., Himber, G., Pigeon, F, Adnet, J.J. (1986) Histochem, J. **18**, 5-14.

Posnet, D.M., Schmelkin ,I., Burton, D.A., August, A., McGrath, H. and Mayer, L.F.(1990) J. Clin. Invest., **85**,1770.

Poste, G. and Fidler, I.J. (1980) Nature, **242**, 148.

Prehn, R.T. (1975) J. Natl. Cancer Inst. **55**, 189- 190.

Prehn, R.T. (1977). J. Natl. Cancer Inst. **59**, 1043- 1049.

Prehn, R.T., and Bartlett, G.L. (1987) Int. J. Cancer **39**, 106-110.

Price, J.E., Aukerman, S.L., Ananthaswamy, N. (1989) Cancer Res. **49**, 4274-4281.

Quirke, P., Dyson, J.E.D. (1986) J. Pathology **149**, 79-87.

Raivio, I. (1977) Acto Ophthalmologica. Supplementum, **133**, 1-64.

Ralfkiaer, E., Hans- Jensen, K., Gatter, K.C., Drcewrecker, K.T., and Mason, D.Y. (1987) Virchows Arch **410**, 325

Ranes, B, Zimmerman, L.E. (1985) Arch Ophthalmol. 60, 193-205.

Ratnofsky, S.E., Peterson, A., Greenstein, J.L., and Burakoff, S.J.(1987) J. Exp. Med. **166**, 1747- 1757.

Ratzschke, O., Falk, K., Deres, K. (1990) Nature **348**, 252-254.

Raulet, D.H. (1989) Nature **339** 342

Rawles, M.C., (1947) Physiology and Zoology, **20**, 248-266.

Raynor, A.A., Grimm, E.A., Lotze, M.T. (1985) Cancer, **55**, 1327.

Real, F.X., Houghton, A.N., Albino, A.P., Cordon-Cardo, C., Melamed, M.R., Oettgen, H.F., Old, L.J.(1985) Cancer research **45**: 4401-11.

Reed, W.B., Becker, Sr., S.W. Becker, Jr., S.W. (1965). Arch. Dermatol., **91**, 100.

Reinherz E.L., Kung P.C., Pesando J.M., Ritz J., Goldstein G. and Schlossman S.F. (1979) J. Exp Med. **150**, 1472-1482.

Remvikos, Y., Magdelenat, H., Zajdela, A. (1988) Cancer **61** 1629-1634.

Rennie, I.G., Rees, R.C., Parsons, M.A., Lawry J., and Cotton, D. (1989) Eye , **3**, 611-617.

Rhodes, A.R., Silverman, R.A., Harrist, T.J. and Melski, J.W. (1985) Arch. Dermatol., **121**, 1266.

Ridolfi, R.L., Rosen, P.P., Port, A., Kinne, D. and Mike, V. (1977) Cancer **40**,1365-1385.

Robb, R.J., Greene, W.C. and Rusk, C.M. (1984) J. Exp. Med. **160**, 1126-1146.

Robb, R.J., Munck, A. and Smith, K.A. (1981) J. Exp. Med. **154**, 1455-1474.

Roberts, C.G., DeFazio, A., Tattersall, M.H. (1985) Cytobias. **43**, 313-318

Robins, R.A. and Baldwin, R.W. (1974) Int. J. Cancer **14**,589-597.

Rosen ,P.P., Saigo, P.E. Braun ,D.W., Weathers, E. and Kinne, D.W. (1981) Annals of Surg. **194**, 576-584.

Rosenberg, S.A., Aebersold, P., Corenetta, K., Kasid, A. (1990)New Eng J. Med. **9**, 303, 570- 578.

Rosenberg, S.A., Aebersold, P., Cornetta, K., Kasid, A., Morgan, R.A., Moen, R., Karsen, E.M., Lotze ,M.T., Yang, J.C., Topalian, S.L., Merino, M.J., Culver, K., Miller, A.D., Blease, R.M. and Andersen, W.E. (1990) New England J. Med **323**, 570-578.

Rosenberg, S.A., Lotze, M.T., Yang, J.C., Aebersold, P.M., Lineham, W.M., Seipp, C.A. and White, D.E. (1989) Ann. Surg. **210**, 474-485.

Rosenberg, S.A., Packard, B.S., Aebersold, P.M., Solomon, D., Topalian, S.L. Tog, S.T., Simon, P., Lotze, M.T., Yang, J.C. Seipp C.A., Simpson, C., Carter, C., Bock ,S., Schwartentruber, D., Wei, J.P., and White, D.E. (1988) New England J Med. **319**, 1676-1680.

Rosenberg, S.A., Spiess, P. and Lafreniere, L.(1986) Science, **233**, 1218.

Rotlein, R., Czajkowski, M., O'Neill, M.M., Marlin, S.D., Mainolfi, E. and Merluzzi, E. (1990) Cancer Immunol. Immunother. **30**, 19.

Rotstein, S., Blomgren, H., Petrini, B., Wasserman, J., and von Stedingk, L.V. (1988) Breast Cancer Research and Trearment **12,** 75-79.

Rudd, C.E., Trevillyan, J.M., Dasgupta, J.D., Wong, L.I., and Schlossman, S.F. (1988). Proc Natl. Acad. Sci. USA. **85**, 5190.

Ruoslahti, E (1988) Ann Rev. Biochem. **57**, 375- 413.

Sabe, H., Kondo, S., Shimizo, A., Tagaya, Y. Yodoi, J. (1984) Mol. Biol. **2,** 379-378.

Saito, H., Kranz, D.M., Takagaki, Y., hayday, A.C., Eison, H.N. and Tonegan, S.(1984) Nature, **312**, 36.

Samelson, L.E., Harford, J.B., and Klausner, R.D. (1985). Cell (Cambridge, Mass) **43**, 223.

Samelson, L.E., Patel, M.D., Weissman, A.M., Harford, J.B., and Klausner, R.D. (1986). Cell (Cambridge, Mass) **46**, 1083.

Sasaki, K., Matsamura, K., Tatsuo, T., Shionozaki, F., Takahashi, M. (1988) Cancers **62**, 989-993.

Schmelkin, I. (1989) Gastroenterology **96**, A449. Abstr.

Schrape, S., Jones, D.B., Wright, D.H. (1987) Br J Cancer **55**, 283-286.

Schulze, D.H., Pease, L.R., Geier, S.S., Reyes, A.A., Samiento, L.A., Wallace, R.B. and Nathenson, S.G. (1983) Proc. Natl. Acad. Sci. USA, **80**, 2007.

Schumacher, T.N.M., Heemels, M-T., Neefjes, J.J., Kast, W.M., Melief, C.J.M. and Ploegh, H.L.(1990) Cell **62**, 563-567.

Schweinfest, C.W., Fujiwara, S., Lau, L.F. and Papas, T.S. (1988) Mol. Cell Biol. **8**: 3080- 3087.

Seddon, J.M. Albert, D.M. Lavin, P.T. and Robertson, N. (1983) Arch. of Ophthalmol. **101**, 1984-199.

Seeman, G.H.A., Rein, R.S., Brown, C.S. and Ploegh, H.L. (1986) EMBO J., **5**, 547.

Seigal, D., Myers, M., Ferris, F., Steinhorn, S.C. (1979) Am. J. Ophthalmology **87**,761-765.

Shammos, H.F. and Blodi, F.C. (1977) Arch. of Ophthalmol. **95**, 63-69.

Shapiro, B.E., Feldberg, N.T., Donoso, L.A., Augsberger, J.J., Shields, J.J. and Gamel, J (1986) Cancer biochem. Biophys **8**, 235-8.

Silverberg, S.G. (1976) Hum. Pathol. 451-454.

Silverstein, A.M., and Khoudadoust, A.A. (1973) In "Corneal Graft Failure" (R.Porter and J. Knight, eds), pp 105-125. Elsevier/ North- Holland. Publ. Amsterdam.

Sistrunk, W.E. and McCartey, W.C. (1922) Annals of Surg., **75**,61-69.

Skornick, Y., Topalian, S., and Rosenberg, S.A. (1990) J. Biol. Response Modifiers **9**, 431-438.

Sleckman, B.P., Peterson, A., Jones, W.K., Foran, J.A., Greenstein, J.L., Seed, B., and Burakoff, S.J.(1987). Nature **328**, 351-353.

Smith, K.A. (1983) In Genetics of the Immune Response, eds E. Moller, G. Moller, pp151-185. New York: Plenum.

Smith, K.A. (1989a) Ann. Rev. Cell Biology **5**, 397-425.

Smith, K.A., (1980) In contemporary Topics in Immunobiology, ed N.L. Warner. 11, 139 155 New York Plenum.

Smith, K.A., Gillis, S., Baker P.E.,(1979) In The Molecular Basis of the Immune Cell Function. ed. J.G.Kaplin, pp223-237. Amsterdam: Elsevier/ North Holland.

Smith, M.E.F., Bodmer, J.G., Kelly., A.P., Trowsdale, J., Kirkland, S.C. Bodmer, W.F. (1989 b). Cold Spring Harbour Symp. Quant. Biol. 54, 581.

Smith, M.E.F., Marsh, S.G.E., Bodmer, J.G., Gelsthorpe, K., Bodmer, W.F. (1989 c) Proc. Natl. Acad. Sci. USA. 86, 5557, 1989.

Smith, R., Crocker, J. (1988) Histopathology. **12** 113-125.

Sondergaard, K., Larsen, J.K., Moller, U., Christensen, I.J. and Hou-Jensen, K.(1983) Virchows Arch. (cell pathol.), **42**, 43.

Spandidos, D.A. and Anderson, M.L. (1989). J. Pathol. **157**, 1-10.

Spencer, J., Isaacson, P.G., Diss, T.C. and MacDonald, T.T. (1989) Eu. J. Immunol. **19** 1335.

Springer, T.A., Dustin, M.L., Kishimoto, T.K. and Martin, S.D. (1987) Ann. Rev. Immunol. **5**, 223-252.

Staunton, D.E., Martin, S.D., Stratowa, C., Dustin, M.L. and Springer, T.A.(1988). Cell **52**, 925- 933.

Steeg, P.S. and Liotta, L.A. (1990) Proc. Am. Assoc. Cancer Res., **31**, 504.

Steeg, P.S., Bevilacqua, G., Kopper, L. (1988) J. Natl Cancer Inst., **80**,200.

Stenzinger, W., Suter, L. and Schumann, J. (1984) J. Invest. Dermatol., **5**, 63.

Streilein, J.W., McCulley, J., and Niederkorn, J.Y. (1982). Invest. Ophthalmol. Visual. Sci. **23**, 489-500.

Sweetser, M.T., Morrison, L.A., Braciate, V.L. and Braciale, T.J. (1989) Nature **342**, 180- 182.

Takagi, S., Chen, K., Schwarz, R., Iwatsuki, S., Herberman, R.B. and Whiteside, T.L. (1989) Cancer **63**, 102-111.

Takahashi, T.(1989) Science **246**,491-494.

Tanaki, K., Gorelik, E., Watanabe, M., Hozumi, N., and Jay, G. (1988) Mol Cell Biol. **8**, 1857-1861.

Tandler, C.J. (1954) J. Histochem. Cytochem. **2**, 165-166.

Taramelli, D., Balsari, A., Fossati, G., Auichini, A., Gambacorti- Passerini, C., Kirkwood, J.M., Parmiani, G.(1986) Biochim. Biophys. Acta **865**, 235-51.

Taylor, I.W., Musgrove, E.A., Friedlander, M.L., Foo, M.S., Hedley, D.W. (1983) Eur. J. Cancer Clin. Oncol. **19**, 632-628.

Temponi, M., Romano, G., D'Urso, C.M., Wang, Z., Kekish, U., Ferrone, S. (1989) Seminars Oncol. **15**, 595.

Teodori, L., Capurso. L., Cordelli, E. (1984). Cytometry. **5**, 63.

Teshigawara, K., Wang, H.-M., Kato, K. and Smith K.A. (1987) J. Exp. Med. **165**, 223-238.

Thorud, E., Fossa, S.D. Vaage, S, Kadhus, O., Knudson, O.S., Bormer, O, and Shoaib, C.(1986) Cancer **57**, 808-811.

Tiwari, J.L. and Terasaki, P.I. (1985). HLA and disease association. (New York Springer- Verlag

Toikkanen,S., Joensuu, H., and Klemi, P. (1989). Br. J. Cancer **60**, 693.

Topalian, S.L. and Rosenberg, S.A. (1990) Important Advances in Oncology 1990.Ed. De vita, V.T., Hellman S. and Rosenberg S.A. Lippincot. 19

Topalian, S.L., Muul, L.M., Solomon, D and Rosenberg, S.A. (1987) J. Immunol. Methods **102**, 127-141.

Topalian, S.L., Muul, L.M., Solomon, D., and Rosenberg, S.A. (1987) J. Immunol. Methods **102**, 127.

Topalian, S.L., Soloman, D., Avis, F.P., Chang, A.E., Freerksen, D.L., Linehan, W.M., Lotze, M.T., Robertson, C.N., Seipp, C.A., Simon, P., Simpson, C.G. and Rosenberg, S.A. (1988) J. Clinical Oncology **6**, 839-853.

Townsend, A., Elliott, T., Cerundolo, V., Foster, L., Barber, B. and Tse, A. (1990) Cell **62**, 285-295.

Townsend, A., Ohlen, C., Bastin, J., Ljunggren, H-G., Foster, L. and Karre, K. (1989) Nature, **340**, 443-448.

Townsend, A.R.M., Grotch, F.M. and Davey, J. (1985) Cell **42**, 457-467.

Townsend, A.R.M., Rothbard, J., Gotch, F.M., Bahadur, G., Wraith, D., McMichael, A.J. (1986) Cell **44**, 959-968.

Toyonaga, B and Mak, T.W. (1987) Ann. Rev. Immunol. **5**, 585-620.

Treseler, P.A. and Sanfilippo, F.(1986) Transplantation **41**, 248-252.

Treseler, P.A., Foulks, G.N., and Sanfilippo, F.(1984) Am. J. Ophthalmol. **98**, 763- 772.

Treseler, P.A., Foulks, G.N., and Sanfilippo, F.(1986) Transplantation **41**, 229- 234.

Trevor- Roper, P.D. (1972) In "Corneal Grafting".(T.A.Casey ed.) pp 1-10. Appleton- Century- Crofts. New York.

Tse, D.T. Dutton, J.J. Werngeist, T.A. Hermsen, V.M., and Kerslen, R.C. (1984) Arch. of Ophthalmol.**102**, 833-838.

Tsudo, M., Kozak, R.W., Golman, C.K. and Waldmann.T, T.A. (1986) Proc. Natl. Acad. Sci USA **83**, 9694-9698.

Turbitt, M.L., and Mackie, R.M. (1981) J. Dermatol. **104**, 507-513.

Underwood, J.C.E. (1974) Br. J. Cancer, **30**,538-547.

Underwood, J.C.E., Giri, D.D. (1988) J. Pathol. **155**, 95-96.

Van Bleek, G. and Nathenson, S.G. (1990) Nature **348**, 213-216.

Van den Ingh, H.F., Ruiter, D.J., Griffioen, G., Von Muijen, G.N.P., and Ferrone, S. (1987). Br. J. Cancer. **55**, 125-130.

Van Duinen, S.G., Ruiter, D.J., Broecker, E.B. Van der Velde, E.A., Sorg, C., Welvaart, K. and Ferrone, S. (1988) Cancer Research **48**, 1019-1025.

Van Kooyk Y., van de Wiel-van Kemenade P., Weder P., Kuijpers T.W. and Figdor C.G. (1989) Nature **342**, 811.

Van Schravendijk, M.R., Biddison, M.R., Berger, W.E. and Coligan, J.E. (1985) J. Immunol.,**134,** 410.

Van Vreeswijk, H., Ruiter, D.J., Brocker, E.B., Welvaart, K., and Ferrone, S. (1988) J. Invest. Dermatol. **90**, 755-760.

Vanky A., Stjernward J., Klein G., Steiner L., Lindberg L (1974) J. Natl. Cancer Inst. **51**, 25-32.

Vega, M.A., Ezquerra, A., Aparicio, P., Bragada, R. and Lopez de Castro, J.A.(1985) Proc. Natl. Acad Sci. USA, **82,** 7394.

Veillette, A., Bookman, M.A., Horak, E.M., and Bolen, J.B. (1988). Cell (Cambridge, Mass.)**55,** 301.

Versloog, R., Noordermeer, I.A. and Kruse- Wolters, M. (1988) The EMBO Journal **7**(4): 1023- 1029.

Vindeløv, L.L., Christensen, J., and Nissen, N.I. (1983). Cytometry **3,** 323.

Vogetseder, W., Feichtinger, H. and Schulz, T.F. (1989) Int. J. Cancer **15,** 768-773.

Von Boehmer, H., Teh, H.S., and Kisielow, R.C. (1989) Immunol. Today **10**, 57.

Von Roenn, J.H.., Kheir, S.M., Wolter, J.M. and Coon, J.S. (1986) Cytometry. **5**, 63

Wang P., Vanky F., Li S-L., Patarrroyo M and Klein E. (1990) Cellular Immunol **131**, 366-380.

Wang, H.-M., Smith, K.A. (1987) J. Exp.Med. **166**, 1055-1069.

Wantzin, G.L., Ralfkiaer, E., Arnstorp, C., Czajkowski, M., Martin, S.D., Rothlein, R. (1989) J. Am. Acad. Dermatol. 20, 782.

Warburton, D, Henderson, A.S. Cytogenet. Cell Genet 1979; **24**, 168-175.

Weber, J.S. and Rosenberg, S.A. (1990) J. Natl. Canc. Inst. **82**, 755.

Weinstein, I.B. (1988) Cancer Research **48**, 4135-4143.

Weissman, A.M., Baniyash, M., Hou, D., Samelson, L.E., Burgess, W.H., and Klausner, R.D. (1988 a). Science **239**,1018.

Weissman, A.M., Hou, D., Orloff, D.G., Modi, W.S., Seuanez, H., O'Brien, S.J., and Klausner, R.D. (1988 b) Proc. Natl.Acad. Sci. U.S.A.**85**, 9709.

Weissman, A.M., Samelson, L.E., and Klausner, R.D. (1986). Nature **324**, 480.

Wellicome, S.M., Thornhill, M.H., Pitzalis, C., Thomas, D.S., Lanchbury, J.S., Panayi, S.and Haaskard, D.O. (1990) J. Immunol. **144**, 2558

White, J., Herman, A., Pullen, A.M., Kubo, R., Kappler, J.W. and Marrack, P. (1989) Cell **56**, 27-35.

Whiteside, T.L., Miescher, S., Hurlimann, J., Moretta, L and von Fliedner, V. (1986) International J. Cancer **37**, 803-811.

Whitewell, H.L., Hughes, H.P.A., Moore, M. and Ahmed, A .(1984) British Journal of Cancer **49**, 161-172.

Whitford, P., George, W.D. and Campbell, A.M. (1991) Cancer Letters. In Press.

Whitford, P., Mallon E.A., George W.D. and Campbell A.M. (1990) Br. J. Cancer 62, 971-975.

Whyte, P., Buchkovitch, K.J., Horowitz, J.M., Friend, S.H., Raybuck, M., Weinberg, R.A. and Harlow, E (1988) Nature **334**, 124-129.

William, M.A., Kleinschmidt, J.A., Krohme, G., Franke, W.W. (1982) Exp. Cell Res. **137**: 341-351.

Williams, K.A., and Coster, D.J.(1985). Invest. Ophthalmol. Visual. Sci. **26**,23-30.

Wilson, B.S., Imai, K., Natali, P.G. Ferrone, S (1987) Int J. Cancer **28**, 293-300.

Wraith, D.C., McDevitt, H.O., Steinman, L. and Acha-Orbea, H (1989) Cell **57**, 709-715.

Yanagi, Y., Yoshikai., Legget, k., Clark, S.P., Alexander, I., and Mak`, T.W. (1984) Nature **308**, 145.

Yewdell, J W. and Bennink (1990) J. Cell **62**, 203-206.

Yuspa S.H. and Priorier M.C. (1988) Adv. Cancer Research. **50**. 25-70.

Zagury D., Bernard J., Thierness N., Foldman M. and Berke G. (1975) Eur. J. Immunol. **5**, 818.

Zamvil, S , Nelson, P., Trotter, J., Mitchell, D., Knobler, R., Fritz, R. and Steinman, L. (1985) Nature **317**,355.

Zimmerman L.E. and McLean I.W. (1979) Am. J. Ophthalmology, **88**,741.

Zimmerman, L.C., McLean, I.W., Forrester, W.D. (1978) Br. J. Ophthalmology **62**,420-425

Zimmerman, L.E. and Mclean, I.W. (1979) Am J. of Ophthal. **87**, 741-760.

Zimmerman, L.E., Mclean, I.W., and Foster, W.D. (1978) Br. J. Ophthal. **62**, 420-425.

Zingernagel, R.M. and Doherty, P.C. (1979) Adv. Immunol. **27**, 51-177.