MONOCLONAL ANTIBODIES TO UVEAL MELANOMA

By

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Thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy

Faculty of Medicine

October 1987

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To my family

INDEX

SUMMARY

This thesis concerns the immunology of uveal melanoma. It is introduced by a critical review of the literature on this neoplasm, and on tumour immunology with special reference to melanoma. Two main conclusions could be drawn from this review. Firstly, further progress in the immunology of uveal melanoma is dependent on a better understanding of the antigenicity of this tumour and on the availability of suitable antigens for testing the anti-tumour immune response. Secondly, the best chance of achieving these objectives, at present, is by the application of monoclonal antibody technology. Accordingly, the main aims of this study were to determine the feasibility and scope of producing monoclonal antibodies to uveal melanoma.

Monoclonal antibodies were prepared using the rat hybridoma system. Tissue specimens from over 80 uveal melanomas were used to immunise rats and for the necessary immuno-assays. Hybridomas were screened by ELISA.

Eleven monoclonal antibodies were produced. Most of these were of the IgM isotype and all reacted with intracellular antigens. The most specific was mAb 4A3. Nevertheless, immunohistochemistry on frozen and fixed sections and immunofluorescence microscopy using cell suspensions showed that this antibody reacted with several types of normal and malignant cells.

Western blotting demonstrated that the 4A3 antigen had a molecular weight of 55-62 kD. The 4A3 antigen was detected in the subretinal fluid of five patients with uveal melanoma with the use of this technique, and in one of two patients with rhegmatogenous retinal detachment.

ELISA reactivity of the rat monoclonal antibodies with uveal melanoma cells was inhibited by serum from 10 out of 12 patients with uveal melanoma and from two out of eight healthy individuals.

B Lymphocytes of patients with uveal melanoma were EBV transformed *in vitro* and tested by ELISA for reactivity with autologous tumour. The results were similar to those obtained with lymphocytes from healthy individuals. This suggested that such techniques were inadequate for analysing humoral immunity to melanoma and unsuitable for the production of human monoclonal antibodies to this tumour.

In conclusion, specific monoclonal antibodies to uveal melanoma could be very useful, but such antibodies are unlikely to be produced using conventional methods. Better results will probably be achieved if uncultured tissue from a single tumour were used for all immunisation and assay procedures required for one fusion, and if more efficient techniques for selecting hybridomas were available.

ACKNOWLEDGEMENTS

I should like to express my sincere gratitude to the following:

Professor Wallace S. Foulds, who encouraged me to undertake this project, for his teaching and guidance and for providing me with specimens of tumour and subretinal fluid.

Dr Ailsa M. Campbell for providing me with all the necessary laboratory facilities in the Department of Biochemistry, for supervising this project and for her constructive criticism and encouragement.

Mrs Brenda J. McGuire for her technical support.

Professor William R. Lee for providing me with fresh tissue from numerous tumour specimens and for his support, encouragement and advice throughout this study.

Professor R.M.S. Smellie and Professor M. D. Houslay for the opportunity to undertake research in the Department of Biochemistry.

Professor Rona M. MacKie for offering me the facilities of the Department of Dermatology, for providing me with tumour specimens, and for her helpful advice and support.

Mrs Celia A. Cannon for testing culture media and for her useful advice.

Mrs Dorothy A. Aitken for performing electron microscopy.

Mr James A. Stewart, Dr Marlyn L. Turbitt and Mr James A.W. Ralston for technical assistance with the immunohistochemical studies.

Mr Alan Hughes and colleagues, Medical Illustration Department of the University of Glasgow, for preparing the illustrations.

Miss Marion A. McCormack, Dr Ronald Strang, Dr Donald Allan, Mrs Shirley M. Mitchell, Dr William Cushley, Dr John R. Kusel, Mrs Sophia S. Cameron, Dr Souravi Ghosh, and Dr Delyth M. Reid for their assistance.

The Scottish Hospital Endowments Research Trust for providing support for this project.

The Scottish Home and Health Department for my employment.

Finally, I am grateful to all the patients and colleagues who donated blood samples.

CONTENTS

ABBREVIATIONS

The abbreviations used are those recommended by the Biochemical Journal in 'Instructions to Authors', 1981, with the following exceptions:

A	Aminopterin
Ab	Antibody
AEC	3-Aminoethylcarbazole
AET	2-Aminoethylisothiouronium bromide hydrobromide
BSA	Bovine serum albumin
CsA	Cyclosporin A
DAB	Diaminobenzidine
DMSO	Dimethylsulphoxide
EBV	Epstein-Barr virus
ELISA	Enzyme linked immunoabsorbent assay
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
HAT	Hypoxanthine aminopterin thymidine
HT	Hypoxanthine thymidine
Ig	Immunoglobulin
IgM, IgG	Immunoglobulin classes; M & G
mAb	Monoclonal antibody
OPD	Orthophenylenediamine
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PMSF	Phenylmethyl sulphonyl fluoride
P/S	Penicillin/streptomycin
RPMI	Rosewell Park Memorial Institute (medium)
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SRBCs	Sheep red blood cells

LIST OF TABLES

Chapter 1

INTRODUCTION

1.1 Uveal melanoma

1.1.1 Introduction

Uveal melanoma is a malignant neoplasm which originates in the pigmented tissues of the eye. The current management of this tumour is not ideal for a number of reasons. Firstly, it can sometimes be very difficult to diagnose clinically. Secondly, eradication of the primary tumour usually requires mutilating surgery. Thirdly, following enucleation, about 50% of cases in most of the published series develop metastatic disease, which is invariably fatal. Finally, prediction of metastatic disease is inaccurate in the individual case.

Thousands of articles have been published on uveal melanoma. The aspects of the literature which are relevant to the present study are summarised in this section. A historical review of the early literature on this subject has been written by Duke Elder and Perkins (1966).

1.1.2 Origin

Intraocular melanomas occur in the uvea, which is a highly vascular and pigmented tissue supporting the refractive and sensory structures of the eye (Fig. 1.1). The uvea is divided anatomically into the iris, ciliary body and choroid. The iris (Fig. 1.2) regulates the

Figure 1.1 The eye. Sagittal section demonstrating choroid (a), ciliary body (b), iris (c), lens (d), retina (e),optic nerve (f), sclera (g), and cornea (h). A choroidal melanoma (i) is situated inferior to the optic nerve and is associated with a localised retinal detachment. (Haematoxylin-eosin, x 5.5).

Figure 1.2 The iris. Light micrograph demonstrating the pigment epithelium (a), and stroma (b) (Haematoxylin-eosin, x 37).

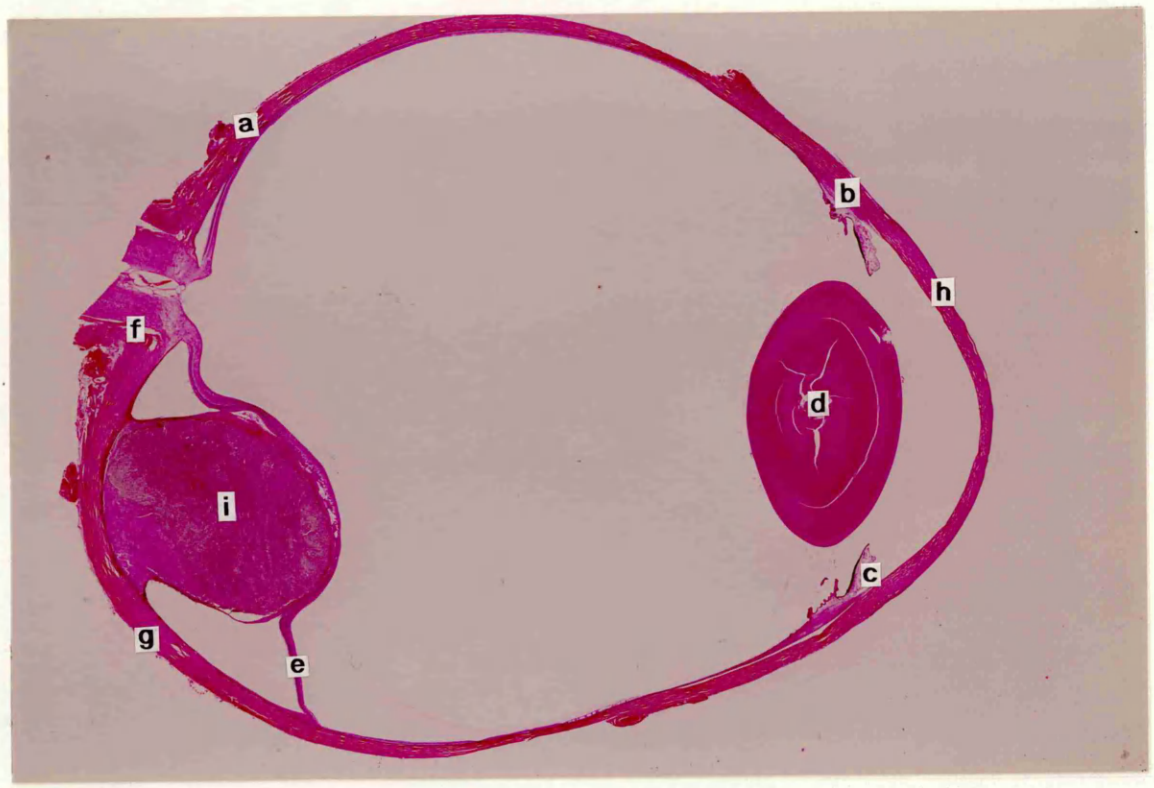


Fig. 1.1

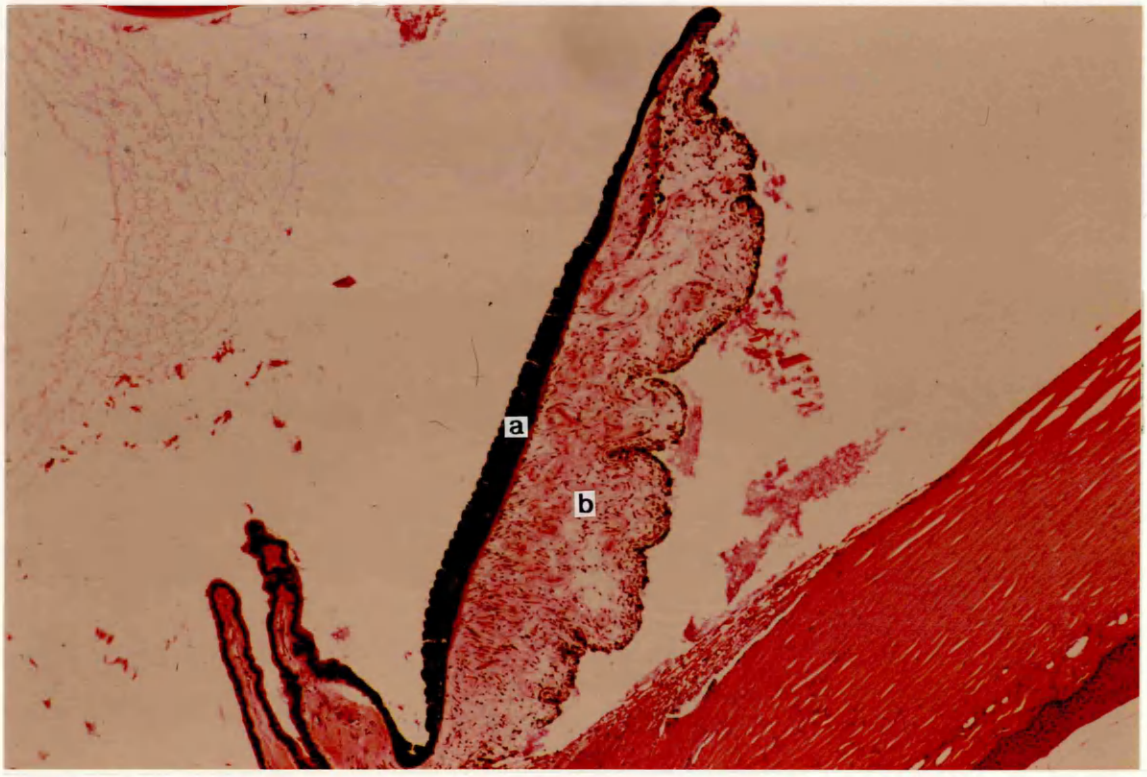


Fig. 1.2

amount of light reaching the retina. The ciliary body (Fig. 1.3) alters the refractive power of the crystalline lens and produces aqueous humour. The choroid (Fig. 1.4) provides the outer retina with its metabolic requirements.

Melanomas are thought to develop from melanocytes, which are of neural crest origin (Rawles 1947). It is therefore to be expected that these tumours are antigenically similar to non-ocular melanomas and to neural tumours.

1.1.3 Epidemiology

The incidence of uveal melanoma is approximately six per million per year in caucasians (Jensen 1963) and much lower in the pigmented races (Apple, Garland & Boutros 1983). An increasing prevalence has been reported (Swerdlow 1983). This may be due to the longer life expectancy (Teikari & Raivio 1985). The sex ratio is approximately equal and the mean age of presentation is 53 years (Benjamin et al 1948).

1.1.4 Pathogenesis

It is estimated that most uveal melanomas are derived from naevi (Yanoff & Zimmerman 1967). These are benign melanocytic tumours which occur in 2-6% of the population (Ganley & Comstock 1973). Uveal melanoma has been associated with melanocytosis (Ferry 1983; Velasquez & Jones 1983), with cutaneous melanoma (reviewed by Albert et al 1985) and with non-ocular, non-melanomatous, malignant neoplasms (reviewed by de Wolff-Rouendaal 1985). In addition, familial occurrence of ocular melanomas has

Figure 1.3 The ciliary body. Light micrograph demonstrating ciliary epithelium (a), ciliary muscle (b), fragments of zonule (c), root of iris (d), and sclera (e) (Haematoxylin-eosin, x 37).

Figure 1.4 The choroid and adjacent structures. Light micrograph demonstrating retina (a), retinal pigment epithelium (b), choroid (c), which contains many pigmented melanocytes, and sclera (d) (Haematoxylin-eosin, x 230).

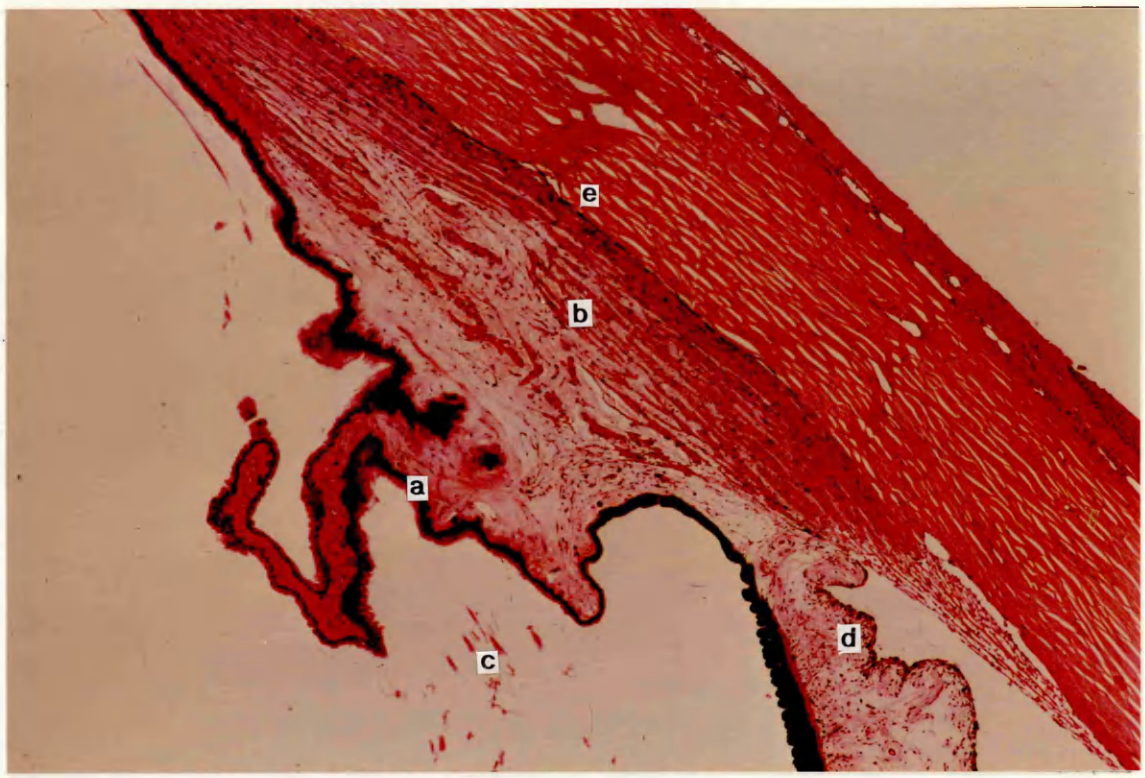


Fig. 1.3

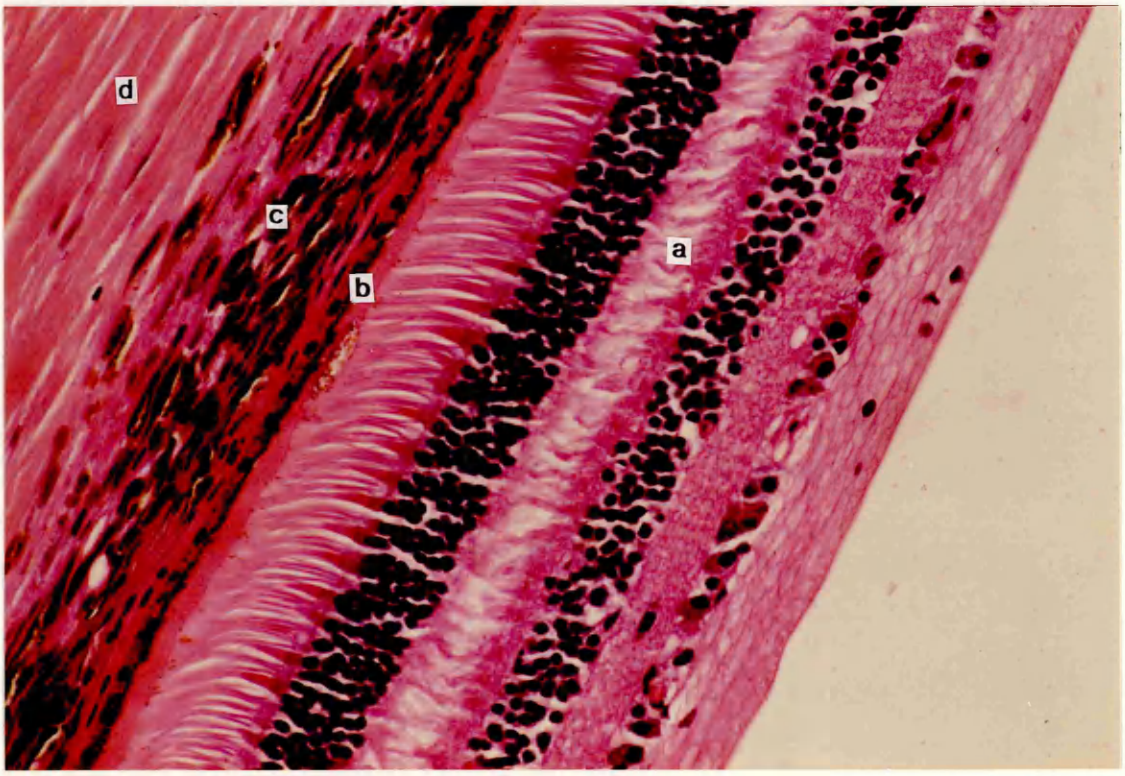


Fig. 1.4

been reported (reviewed by Green et al 1978; Lynch, Anderson & Krush 1968). A cluster of cases in a chemical factory suggests that exposure to environmental carcinogens may cause this disease (Albert et al 1980). A viral aetiology has been postulated (Albert 1979), although the evidence for this is of doubtful validity.

1.1.5 Histology

On light microscopy, uveal melanomas consist of spindle cells, which are narrow and fusiform, and/or epithelioid cells, which are large and pleomorphic (Fig. 1.5). The cell type of uveal melanomas is a useful indicator of the prognosis for survival following enucleation, the presence of epithelioid cells being associated with a poorer outlook (Callender et al 1942). Analysis of the antigenic phenotype of uveal melanoma cells, by immunohistochemistry or flow-cytometry, has not yet been achieved but could improve prognostication. Aggregates of lymphocytic cells are seen in about 5-12% of all uveal melanomas (Lang, Davidorf & Baba 1977). Necrosis is evident in a similar proportion of such tumours. The significance of these features is not entirely understood.

1.1.6 Diagnosis

The presenting clinical features of a uveal melanoma (reviewed by Shields 1983; Foulds 1985) are determined by its location within the eye and by its effects on adjacent ocular structures (Figs. 1.6, 1.7, 1.8). Most patients with intraocular melanoma initially

Figure 1.5 Histological appearances of uveal melanoma. (i) Spindle melanoma cells (Haematoxylin-eosin, x 230). (ii) Epithelioid melanoma cells (Haematoxylin-eosin, x 230).

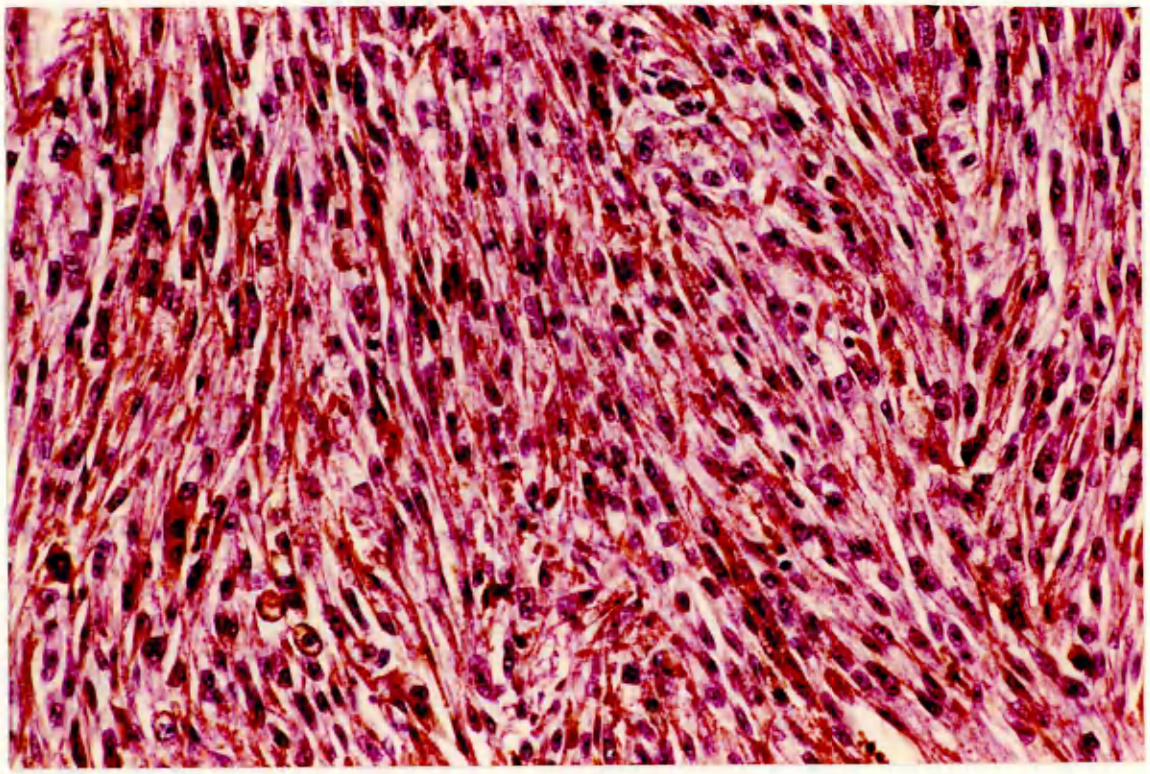


Fig. 1.5 (i)

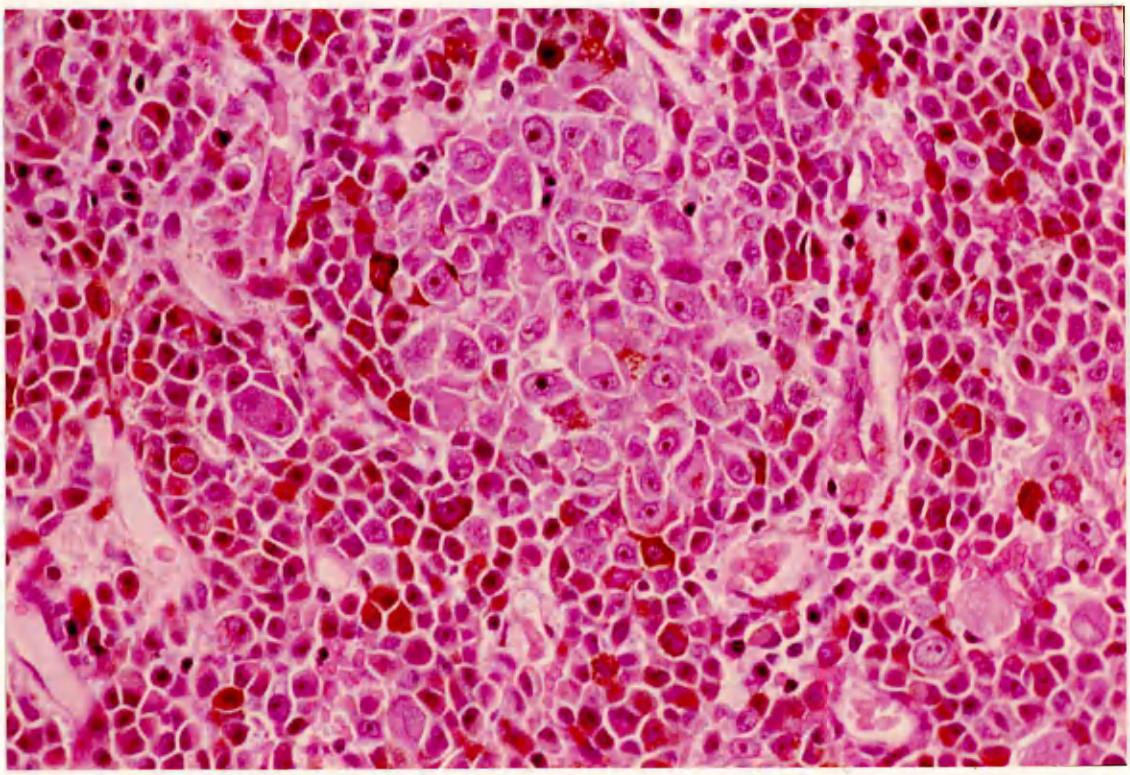
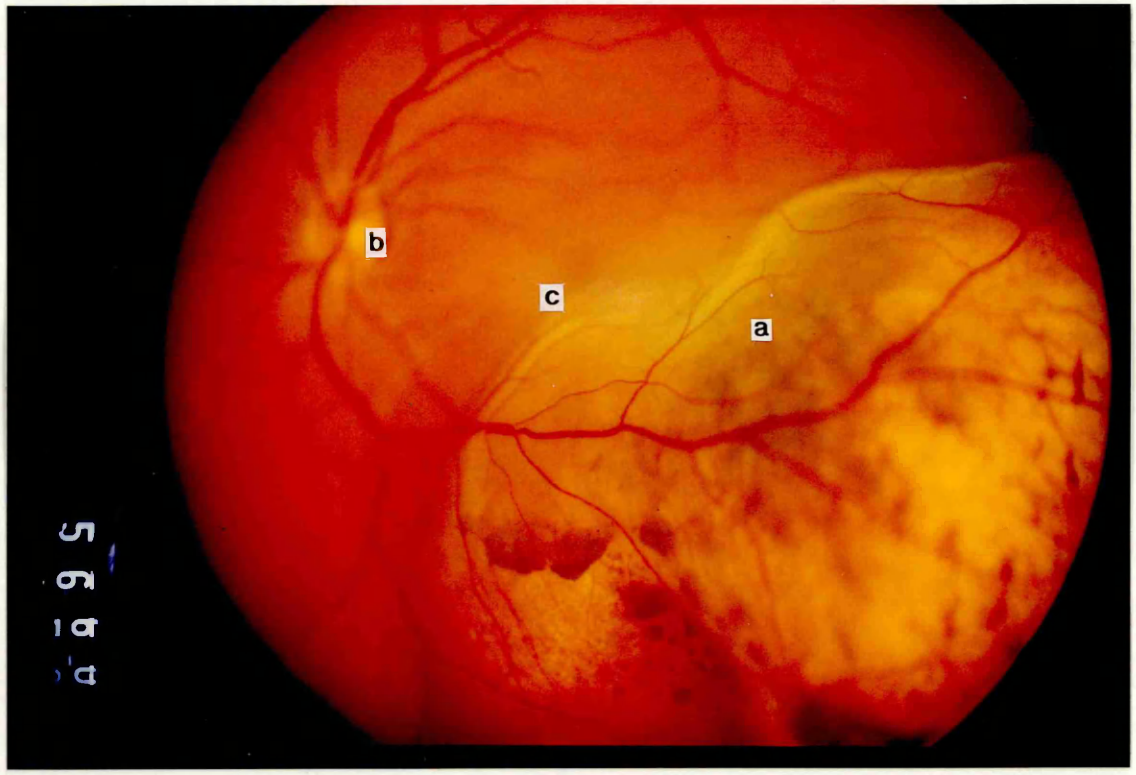


Fig. 1.5 (ii)

Figure 1.6 Choroidal melanoma. Fundus photograph of left eye showing a large amelanotic choroidal melanoma (a) which is situated inferotemporal to the optic disc (b) and involving the fovea (c). The retina, which is transparent, is draped over the tumour and still intact.

Figure 1.7 Ciliary body melanoma. Slit-lamp photograph of right eye showing an inferonasal ciliary body melanoma (a) which is situated behind the lens (b). The iris (c) and cornea (d) are normal. Dilated episcleral 'sentinel' vessels (e) are also visible in the overlying sclera.



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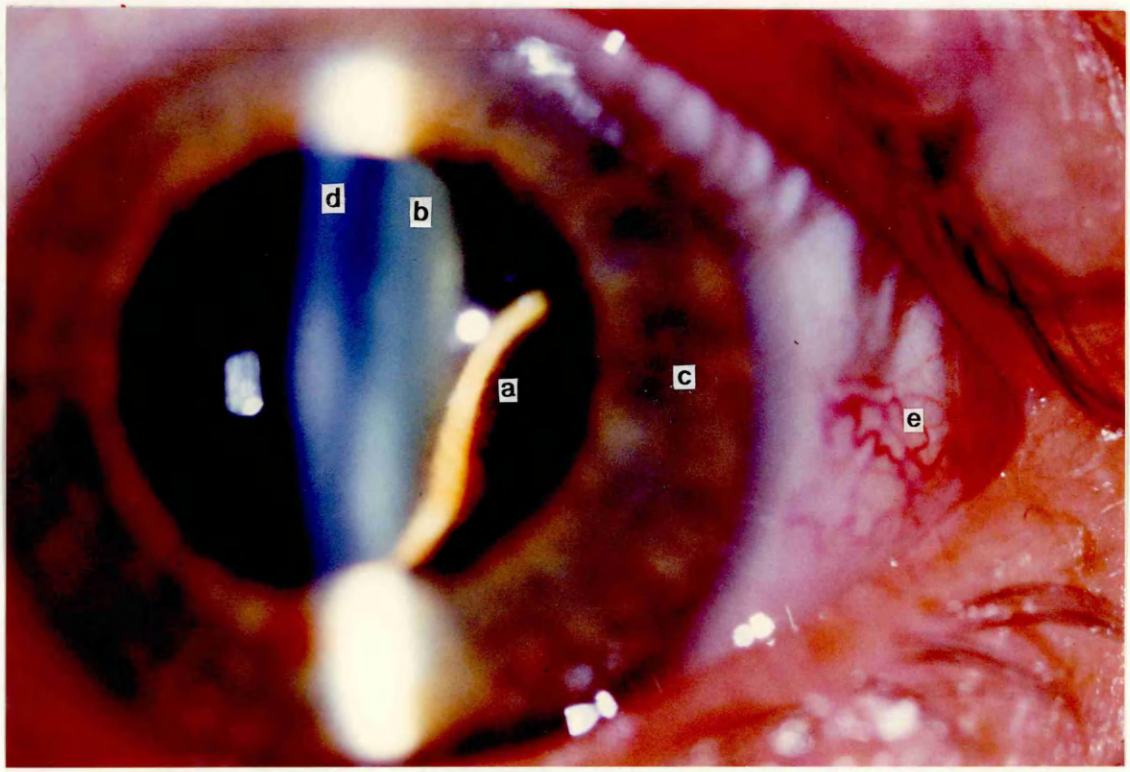


Fig. 1.7

Figure 1.8 Iris melanoma. Anterior segment photograph of right eye showing an inferonasal iris melanoma (a) which is almost completely occluding the pupil (b). The overlying cornea (c) is clear and transparent as seen by the light reflection.

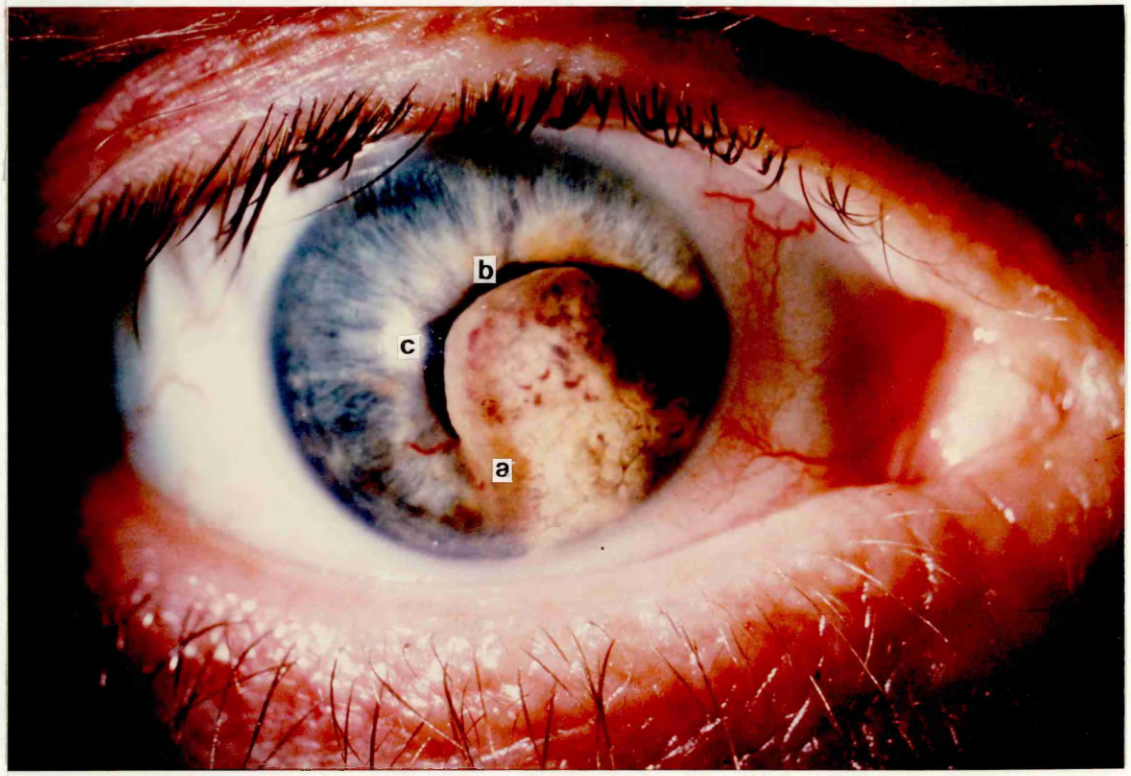


Fig. 1.8

complain of painless deterioration of vision. In some patients, the tumour is found either during routine ocular examination or is discovered pathologically following enucleation of a blind painful eye in which the media are opaque (Makley & Teed 1958). Examination by ophthalmoscopy can be supplemented by fluorescein angiography (Norton et al 1964), transillumination, ultrasonography (Coleman et al 1974), computerised tomography (Mafee, Peyman & McKusick 1985), and magnetic resonance imaging (Chambers et al 1987). Further useful information about the tumour can be obtained by measuring its uptake of radioactive tracers, such as P^{32} (reviewed by Packer 1984), and by computerised ultrasonography, although this is still an experimental procedure (Coleman et al 1985). At present the most definitive means of achieving the diagnosis is by histological examination of tissue samples which can be obtained by needle (Augsburger et al 1985), incisional or excisional biopsy (Foulds et al 1985). These investigations, as well as a greater awareness of the numerous diagnostic pitfalls, have improved the diagnostic accuracy from 80% (Ferry 1964) to over 98% in specialised centres (Davidorf et al 1983). There is still a need, however, for less invasive tests which could establish the diagnosis in difficult cases.

1.1.7 Treatment

With the exception of iris tumours, which can be easily excised, the standard form of treatment for uveal melanomas has until recently been enucleation. Questions regarding the possible adverse effects of this mode of

therapy on survival (Section 1.1.8) have led to the use of adjunctive radiotherapy (Char & Phillips 1985; Lommatzsch & Dietrich 1976), and to an increasing number of conservative forms of therapy aimed at eradicating the primary tumour whilst preserving a functional eye (reviewed by Foulds & Damato 1986a). These include photocoagulation (Meyer-Schwickerath & Bornfeld 1983; Foulds & Damato 1986b), photochemotherapy, using haematoporphyrins and other photosensitising agents (Tse et al 1984), radiotherapy (reviewed by Fairchild 1984; Lommatzsch 1986), and surgical resection (Foulds 1978). In specialised centres these conservative forms of therapy, used alone or in combination, are successful in over 80% of cases (Fig. 1.9), and seem to prevent metastatic disease as effectively as enucleation.

1.1.8 Prognosis

The behaviour of untreated primary uveal melanomas is not entirely predictable in an individual case. Small tumours can show rapid growth (Friberg, Fineberg & McQuaig 1983), or extend extraocularly (Duffin et al 1981), and can even cause metastasis without apparent clinical change (McKnight & Christensen 1984). Larger tumours, on the other hand, can remain stationary for long periods of time (Gass 1977). Spontaneous regression has been reported in a few cases (Reese et al 1970; Jensen & Andersen 1974). Although this phenomenon has been attributed to an immune response to the tumour, there is no evidence to support such speculation.

Figure 1.9 Uveal melanoma treated by local resection. (i) Right inferonasal choroidal melanoma (a) in a 42 year old female. (ii) Fundus appearance a few weeks following local surgical resection performed by the author in November 1984. A large surgical coloboma (a) is visible and contains a small resolving subretinal haemorrhage (c). The overlying retina is still intact as seen by the retinal blood vessels (b). Two years post-operatively, the visual acuity in this eye was 6/9, and the patient was still in good health.

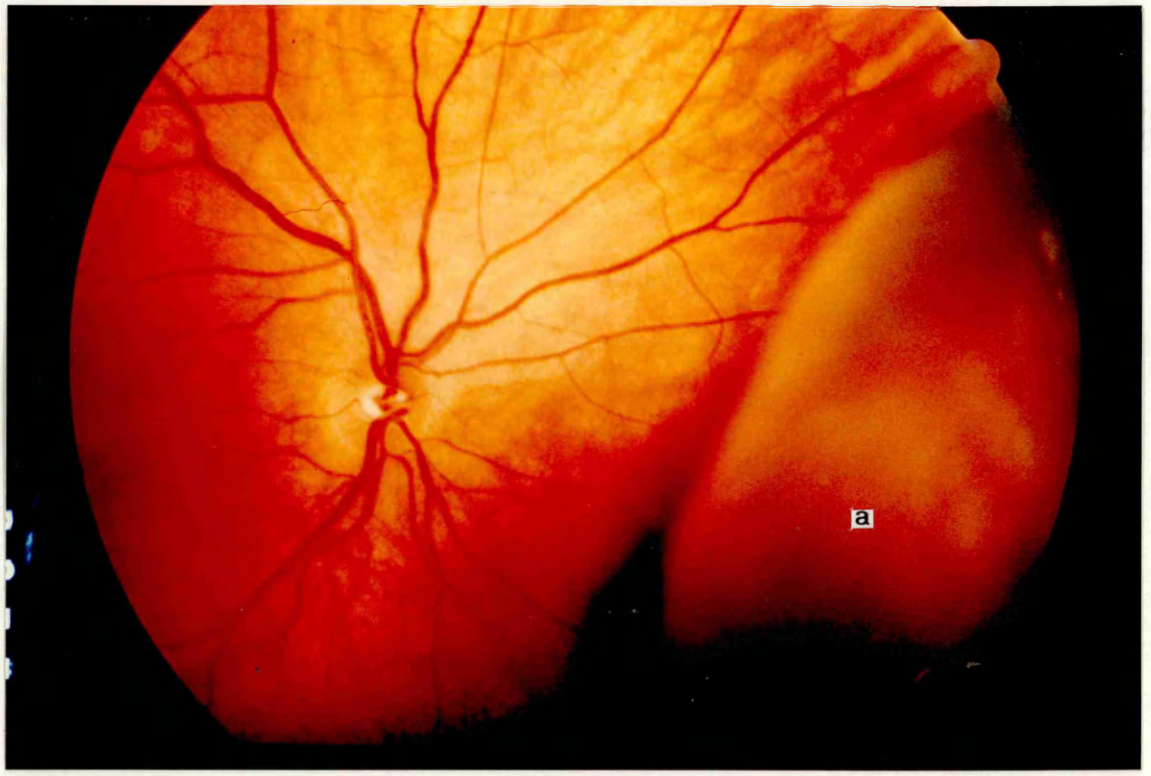


Fig. 1.9 (i)

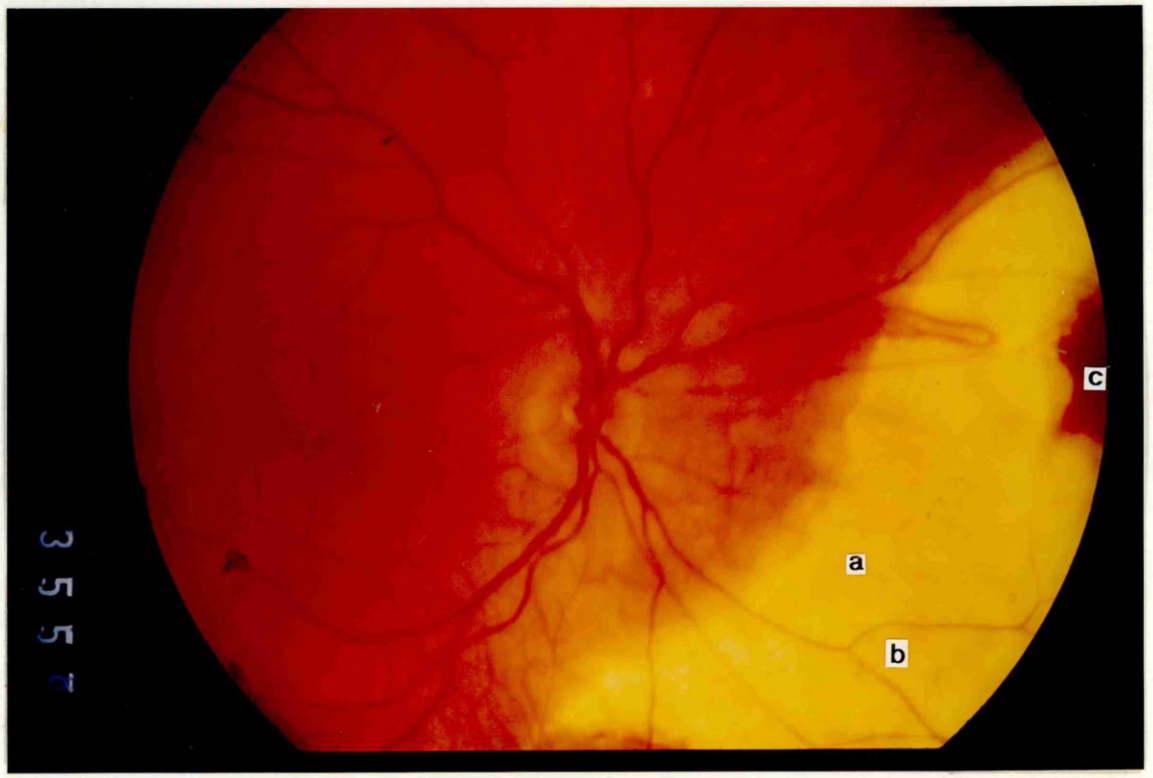


Fig. 1.9 (ii)

Following enucleation, orbital recurrence occurs in 2.5-12% of cases and overt metastatic disease ultimately arises in about 50% of all patients, usually within the first five post-operative years (Jensen 1982). The risk of metastatic disease has been investigated by a number of workers (Davidorf & Lang 1974; Shammas & Blodi 1977; Packard 1980; Seddon et al 1983; Gamel et al 1985; Kidd et al 1986; Pach et al 1986). These studies have shown that poor prognosis is related to tumour size, anterior location, extraocular extension, epithelioid cellularity and nucleolar pleomorphism. A rare subtype of uveal melanoma, consisting of flat diffuse tumours is associated with a particularly high mortality (Font et al 1968). Other factors which have been considered to have an adverse effect on survival include liver disease (Pascal et al 1985), intercurrent illness (Leff et al 1985), pregnancy (reviewed by Apple, Garland & Boutros 1983), and smoking (Keeney, Waddell & Perraut 1982).

The effect of enucleation on the survival of patients with uveal melanoma is a controversial subject. Doubts on the value of this procedure have been expressed by a number of authors (von Hippel 1922; Westerveld-Brandon & Zeeman 1957; Gass 1977). A peak in the mortality has been observed in the second year following enucleation (Benjamin et al 1948) and this has been attributed by some authors to the surgery itself (Zimmerman, McLean & Foster 1978). Other workers, however, have challenged the statistical methods on which such impressions are based (Seigel et al 1979) and consider prompt enucleation to be life-saving (Manschot &

van Peperzeel 1980). This latter hypothesis is supported by reports of a very high mortality from metastatic disease in patients with uveal melanoma who refused to have this operation (Sobanski et al 1965; Raivio 1977). Some workers have suggested that removal of the primary tumour interferes with protective immunological mechanisms (Zimmerman & McLean 1979). Their hypothesis seems to be supported by evidence from animal experiments (Niederkorn 1984) (Section 1.3.3.3).

1.1.9 Metastasis

Metastases from uveal melanoma first tend to declare themselves in the liver (Jensen 1970) and usually cause death within a few months (Char 1978; Rajpal, Moore & Karakousis 1983). At present, treatment is only palliative in nature. There is, therefore, much scope for further research into immunological methods of preventing and treating this disease.

1.2 Tumours

1.2.1 Malignancy

A malignant neoplasm is a tumour consisting of cells which grow in an unco-ordinated manner and which tend to spread beyond their normal confines to other parts of the body to cause metastatic disease and death. The state of cellular autonomy that characterises malignant transformation is usually reached through several steps each reflecting the activation, mutation or loss of different genes (Klein & Klein 1985). The understanding of the genetic basis of neoplastic disease has recently

improved greatly following the discovery of viral oncogenes in viral tumours (reviewed by Bishop 1985) and of homologous DNA sequences in normal cells (Waterfield, Scrace, Whittle et al 1983). There is evidence that aberrant activation of these genes causes excessive production of factors which normally have key roles in growth and differentiation, and that these factors stimulate tumour growth (the autocrine stimulation concept) (Sporn & Todaro 1980). Such autostimulatory factors are known to be produced by melanoma cells (Richmond et al 1985).

1.2.2 Tumour-associated antigens

1.2.2.1 Specificity

When a cell undergoes malignant transformation it expresses molecules which when recognised immunologically, not necessarily by the host of the tumour, are referred to as tumour-associated antigens or markers. To date, with the possible exception of idiotypes on B cell lymphomas (Miller et al 1982), no antigens have been discovered that are completely tumour-specific. Such molecules have always been demonstrated in normal tissues albeit in small amounts. Terms like 'tumour-specific antigens', or 'tumour-unique antigens', are therefore no longer used. A method of classifying tumour-associated antigens has been introduced whereby antigens expressed solely by a single tumour are categorised 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).

1.2.2.2 Nature

Tumour-associated antigens, which can be present in the membrane, cytoplasm or nucleus of the cell, may be carbohydrates, proteins or lipids or combinations of each (reviewed by Reading & Hutchins 1985; Hakomori & Kannagi 1983; Feizi 1985). Such antigens can arise as a result of a variety of biochemical changes. Firstly, new molecules can be synthesized. Secondly, precursor molecules can accumulate due to the blockage of metabolic pathways. Thirdly, previously 'cryptic' molecules can be exposed by organisational changes in the cell membrane (Hakomori & Kannagi 1983).

Most antigens recognised by monoclonal antibodies can be placed into certain categories. (i) Oncofoetal antigens such as OFA-1 in melanomas (Rees, Irie & Morton 1981) are normally expressed only during embryonic development. (ii) Differentiation antigens occur in different sub-populations of cells in adult tissues and reflect distinctive stages of differentiation. Such antigens have been extensively studied in lymphoid tumours (Greaves 1982), and, to a lesser extent, in cutaneous melanoma (Houghton et al 1982). (iii) Lineage-associated determinants reflect the tissue from which the tumour is derived. Melanoma-associated antigens, for example, tend also to be expressed on neural tumours (Section 1.5).

(iv) Class 1 and Class 2 histocompatibility antigens tend to be expressed by malignant tumours, including melanomas (Ruiter et al 1984), even when they are normally absent in the parent tissues. (v) Tumours induced by viruses can express antigens which are specific for the causative virus, such as the expression of Epstein Barr virus antigens in African but not American Burkitt's lymphoma cells (Pagano et al 1973). When the antigens described above cause rejection of experimental neoplasms, they are referred to as tumour-associated transplantation antigens.

The antigenic expression of melanomas has been extensively reviewed (Hellström & Brown 1979; Carey 1982; Burk 1984; Cochran 1985; Houghton, Cordon-Cardo & Eisinger 1986).

1.2.2.3 Heterogeneity

Malignant neoplasms, including melanomas, show much intra- and inter-tumour antigenic heterogeneity (Miller 1982; Natali, Cavaliere et al 1983; Heppner 1984; Bystryn et al 1985; Edwards 1985). Such variable antigenic expression has significant implications for the immunological diagnosis and therapy of tumours. This phenomenon also appears to be of functional significance. Animal studies have shown that antigens expressed by the primary tumour are related to the distribution of metastatic deposits (Shearman, Gallatin & Longenecker 1980; Yogeeswaran, Stein & Sebastian 1978; Poste & Nicolson 1980; Berthier-Vergnes, Portoukalian & Dore 1985). Tumour-associated antigens also appear to

influence the degree of allogeneic lymphocyte stimulation by these cells (De Baetselier et al 1980; Zehngebot et al 1983). Immunohistochemical studies have revealed that antigenic profiles of excised cutaneous melanomas (Suter et al 1983), and other tumours such as breast carcinomas (Wilkinson et al 1984), are related to the risk of subsequent metastatic disease. Such methods of prognostication could usefully be applied to uveal melanomas.

1.3 Tumour immunology

Tumour immunology is the study of the immunological inter-relationships between a neoplasm and its host. It is also concerned with the development and application of immunological tools for use in the clinical situation and in research. The subject has been extensively reviewed (Lachman 1984; Bast 1985; Roitt, Brostoff & Male 1985; Evans 1986).

1.3.1 Immune response to cancer

The immune response to cancer is thought to be a complex interplay of different effector cells whose behaviour is influenced by a wide diversity of substances known as biological response modifiers.

1.3.1.1 Effector cells

The classes of cells which mediate anti-tumour immune responses (reviewed by Rees & Ali 1985) are thought to include lymphocytes, macrophages, polymorphonuclear leucocytes and platelet fragments. T lymphocytes are

responsible for antigen-specific cytotoxicity and require direct contact with the target cells. Natural killer cells can lyse target tumour cells without prior sensitisation (Herberman & Ortaldo 1981; Hanna 1985). Other cells thought to participate in the anti-tumour immune response include natural cytotoxic cells (Djeu et al 1983) and lymphokine-activated killer cells (Grimm et al 1982). Activated macrophages apart from their phagocytic and modulatory functions can kill tumour cells by producing a variety of toxins (Adams, Lewis & Johnson 1983), including tumour necrosis factor (Beutler & Cerami 1986).

1.3.1.2 Biological response modifiers

Biological response modifiers exert anti-tumour effects indirectly through effector cells and include interferons, lymphokines, antibodies, helper and suppressor factors, and differentiation and growth factors. Interferons are a group of proteins and glycoproteins which possess antiviral, immunomodulatory and antiproliferative activities (reviewed by Krown 1986). They can also alter the antigenicity of tumour cells, including melanoma cells (Liao, Kwong et al 1982b; Giacomini et al 1985). Lymphokines are proteins secreted by lymphocytes that regulate immune responses in a variety of ways (reviewed by Gillis et al 1986; Cohen & Cohen 1986). Antibodies may be cytotoxic to target cells through complement action, or they may mark tumour cells for attack by antibody- dependent cellular cytotoxicity. Besides activating different inflammatory cells,

biological response modifiers can also influence the local milieu of a neoplasm by mediating vasoactive effects and by supporting the growth of fibroblasts and capillary endothelial cells.

1.3.1.3 In vivo tumour immunity

Current knowledge regarding the immune response to cancer is necessarily based on experiments conducted *in vitro* or using animal models. The relevance of such studies with respect to the human *in vivo* situation is often uncertain. For example, whereas *in vitro* anti-tumour antibodies result in antigen-specific tumour cell lysis (Section 1.3.1.1), *in vivo* anti-tumour antibodies occasionally seem to have an apparently detrimental effect on the host, apparently blocking the immune response (Sjögren *et al* 1971; Currie & Alexander 1974). The hypothesis that an immune response, analogous to that in graft rejection or infectious disease, is mounted against tumours remains controversial, some workers even suggesting that the immune response may stimulate tumour growth (Fidler 1974; Prehn & Prehn 1987; Grossman & Herberman 1986).

1.3.2 The immunology of melanoma

The immunology of melanoma has been extensively investigated (reviewed by Cochran 1978; Hellström & Brown 1979; Carey 1982; Peter 1983; Burk 1984). This type of tumour is thought to be particularly likely to elicit an immunological response. Spontaneous regression of cutaneous melanomas appears to be frequent in relation to

other tumours (Nathanson 1976). In addition, melanomas have been associated with autoimmune phenomena such as vitiligo, ocular abnormalities (Sober & Haynes 1978; Chang et al 1986) and the disappearance of co-existing naevi (Epstein et al 1973). Most cutaneous melanomas contain lymphoid infiltrates consisting predominantly of T cells (Ralfkiaer et al 1987).

1.3.3 Immunology of uveal melanoma

1.3.3.1 General aspects

Uveal melanomas, like their cutaneous counterpart, are considered to be relatively susceptible to immunological influences because of reports of spontaneous regression (Jensen and Andersen 1974; Reese et al 1970), of the development of vitiligo and halo naevi (Albert et al 1982) and of the delayed appearance of metastatic disease, sometimes decades after enucleation (Newton 1965).

The milieu of uveal melanomas is very different from that of cutaneous melanomas. Firstly, neither the eye nor the orbit are drained by lymphatics. Secondly, studies using animal models have shown that it is much easier to transplant allogeneic tumours by intraocular, than by subcutaneous, inoculation (Niederkorn & Streilein 1983). Such intraocular transplantation can be prevented by prior adoptive transfer of humoral or cellular immunity to the recipient (Niederkorn & Streilein 1984). This suggests that sensitisation is reduced or delayed when tumour-associated antigens are presented intraocularly. Furthermore, the existence of an 'anterior-chamber

associated immune deviation' has been recognised whereby the presence of a growing intraocular tumour specifically suppresses delayed type hypersensitivity to the antigen (Niederkorn & Streilein 1983). Despite these considerations, patients with uveal melanoma do apparently tend to develop humoral and cellular immune responses to their tumour and about 10% of these tumours contain lymphocytic infiltrates although these do not seem to be related to prognosis (Lang, Davidorf & Baba 1977).

1.3.3.2 Immune status of patients with uveal melanoma

According to the immunological surveillance theory, cells which undergo malignant transformation are detected and destroyed by the immune system unless this is deficient (Burnet 1967; discussed by Klein 1980). Patients with uveal melanoma, however, do not usually show any definite evidence of immunological insufficiency (Priluck et al 1979; Felberg & Shields 1984) or any striking HLA or ABO antigen associations (Völker-Dieben et al 1983). Although patients with this tumour tend to show significant increases in the numbers of lymphocytes in the peripheral blood (Flynn et al 1986), such changes may be the result rather than the cause of the malignancy.

1.3.3.3 Immunological prevention of metastatic disease

Different workers, using animal models, have independently produced evidence suggesting that 'splenic T-cell-dependent immunity' (Niederkorn & Streilein 1983, Niederkorn 1987), and NK activity (Yokoyama et al 1986) prevent metastatic disease from intraocular tumours. It

has even been suggested that the primary tumour plays a beneficial role in such defensive processes (Gershon, Carter & Kondo 1967). Such a concept, known as 'concomitant immunity,' has recently been supported by a series of experiments using animal models of intraocular malignancy (Niederkorn, Shadduck & Albert 1982; Niederkorn & Streilein 1983). Other animal studies, however, have yielded results which are not in keeping with this hypothesis because enucleation of tumour containing eyes improved survival (Burns, Fraunfelder & Klass 1962). Different animal models, therefore, seem to produce different results.

In patients with uveal melanoma, cell-mediated immunity becomes depressed in advance of clinically recognised metastatic disease (Priluck et al 1979). This could be the result of subclinical metastases rather than the cause. There is some evidence that tumour cells can stimulate suppressor T cells (Schatten et al 1984) or release antigens which act as blocking factors (Currie & Alexander 1974; Hellström & Hellström 1974).

1.3.3.4 Humoral immunity to uveal melanoma

Initial attempts at detecting humoral immunity to uveal melanomas were not very successful (Howard & Spalter 1966; Nairn et al 1972). In 1971, Rahi demonstrated serum antibodies which were reactive against autologous tumours (Rahi 1971). These findings were later supported by Wong & Oskvig (1974) who, using immunofluorescence techniques, demonstrated that the serum of most patients contained IgG and IgM reactive against

autologous and allogeneic melanoma cells. Many patients with uveitis or primary biliary cirrhosis, however, showed anti-cytoplasmic antibodies and over a third of 1,083 control subjects seemed to show serum antibodies reacting against antigens present on the melanoma cell membrane. Further studies using immunofluorescence and subsequently immunoperoxidase techniques have achieved essentially similar results (Brownstein, Sheikh & Lewis 1976; Brownstein, Sheikh & Lewis 1977; Federman, Lewis & Clark 1974; Federman et al 1974; Brownstein, Phillips & Lewis 1978; Felberg et al 1979). Brownstein, Sheikh & Lewis (1977) have suggested that serum antibodies to melanoma cell membrane antigens are only present in patients with small tumours. Only 12 patients with uveal melanoma, however, were included in this study. Federman and associates (1974), implied that the degree of humoral immunity might be related to prognosis. This impression, however, was only based on their finding that in a group of 15 uveal melanoma patients the individual with the largest tumour and, therefore, with the highest statistical chance of metastatic disease apparently showed the poorest antibody response.

The level of tumour-associated antibodies in the serum of patients with uveal melanoma has been reported to increase following treatment by photocoagulation (Federman et al 1979). Such a rise in antibody titres was also seen following radiotherapy but not following enucleation (Felberg 1977).

In 1979, Malaty, Rahi and Garner (1979) demonstrated that the incidence of tumour-associated antibodies in patients and control subjects correlated well with the incidence of antibodies to smooth muscle. This result prompted Felberg, Donoso and Federman (1980) to repeat their previous measurements of tumour-associated antibodies in the serum of patients with uveal melanoma and in control subjects, this time performing absorption of the sera with actin and thrombosthenin prior to the ELISA. The result was that a partial reduction in the number of false positive results was achieved. Such findings, similar to those of Whitehead (1973) in his studies of humoral immunity to cutaneous melanoma, were a further indication of the problems caused by the use of non-specific antigens and antibodies. They demonstrate that more refined reagents are required for studies of humoral immunity to uveal melanomas. The preparation of such reagents has become possible due to the development of hybridoma technology for the production of monoclonal antibodies (Köhler & Milstein 1975).

A number of workers have recently suggested that further insights into the humoral immune response to tumours might also be gained by immortalising peripheral lymphocytes by infection with the Epstein Barr virus (Cote et al 1983; Houghton et al 1983; Campbell et al 1986). Such a technique had not been applied to uveal melanoma immunology prior to the present study.

1.3.3.5 Cellular immunity to uveal melanoma

After initially discouraging results (Hart, Reznikov & Hughes 1968; Nairn et al 1972), cellular immunity to uveal melanomas was demonstrated by a variety of methods including skin testing (Char et al 1974), the leucocyte migration inhibition assay (Char et al 1975; 1977), the macrophage migration inhibition assay (Manor et al 1978), the lymphocyte transformation test (Noor Sunba et al 1980), the leucocyte adherence inhibition assay (Cernak & Kalafut 1983; Diekhues 1986), a one-stage capillary leucocyte migration assay and a two-stage lymphokine generation assay using normal human leucocytes as indicator cells (Cochran et al 1985). Like the humoral immune response, cellular immunity has been shown to decline with the development of metastatic disease (Unsgaard & O'Toole 1975; Char 1977; Felberg & Shields 1984).

1.3.4 Clinical applications of tumour immunology

1.3.4.1 Immunodiagnosis of cancer

Many immunological assays have been investigated in the hope of improving the diagnosis of cancer and for monitoring the tumour burden in treated patients (reviewed by Sulitzeanu 1985; Shuster et al 1980). The types of immunological tests which have found a place in clinical practice are (i) the detection and measurement of tumour products in the serum or urine, (ii) radio-immunolocalisation and (iii) immunohistochemistry. Immuno-assays of human chorionic gonadotrophin, carcino-embryonic antigen, alpha-foetoprotein and

antigen CA 125 (Klug et al 1984) are routinely used for monitoring the response to treatment of tumours such as colonic, hepatic and ovarian carcinomas as well as teratocarcinomas and choriocarcinomas (reviewed by Bagshawe 1980; Klavins 1983). The measurement of melanoma-associated antigens in the serum also appears to show similar potential (Ross et al 1984; Morgan, Crane & Rossen 1984). Preliminary studies, using highly-absorbed polyclonal antisera or monoclonal antibodies labelled with appropriate radioactive isotopes, appear to have scope for the localisation of tumours (reviewed by Rankin & McVie 1983; Zalcberg 1985; Murray et al 1987). The immunohistochemical detection of recognized antigens, not necessarily tumour-associated (Warner 1985), has established itself as a useful method for diagnosing undifferentiated tumours (Gatter et al 1985) as well as for the detection of small numbers of tumour cells in different tissues and body fluids (Ghosh, Mason & Spriggs 1983).

1.3.4.2 Immunodiagnosis of uveal melanoma

Studies of humoral immunity to uveal melanoma have tended to suffer from lack of sensitivity and specificity. One of the more recent and extensive investigations was performed by Felberg, Donoso & Federman (1980) who also absorbed the serum samples with actin and thrombosthenin before performing ELISA for the detection of melanoma-associated antibodies. Positive results were obtained in 72% of 73 patients with uveal melanoma and 13% of 106 control subjects. These authors suggested that

the efficiency of such tests of humoral immunity could be improved by using more specific antigens, obtained with the aid of monoclonal antibodies. Other studies suggest that most of the auto-antibodies detected in patients with malignancy seem to be directed against normal tissue antigens (Whitehead 1973; Old 1981; Ruffatti et al 1983; Thomas et al 1983) and, conversely, antibodies against tumour-associated antigens can be detected in a significant proportion of apparently healthy individuals (Muna, Marcus & Smart 1969; Nairn et al 1972; Houghton et al 1980; Avrameas, Guilbert & Dighiero 1981). If such findings do indeed represent a genuine biological phenomenon, and not merely laboratory artefact, the scope for developing diagnostic tests based on humoral immunity to tumours may be limited.

By means of the cutaneous delayed hypersensitivity response to a soluble melanoma antigen, Char and associates demonstrated that whereas 26 out of 27 patients with ocular melanomas produced positive results, all 12 individuals without this condition produced negative results (1974). Although the results of this investigation were encouraging, the authors considered it unethical to inject tumour extracts into individuals who might not be be suffering from a malignant process. With an in vitro migration inhibition assay, positive results were obtained in 10 out of 11 patients with uveal melanoma and in 3 out of 32 control subjects (Char et al 1975; Char 1977). In a similar study, using a macrophage migration inhibition assay, a positive result was obtained in 7 out of 8 patients with choroidal melanoma and in none of 25

control subjects (Manor et al 1978). Using a modification of the leucocyte adherence inhibition assay, Kalafut, Cernak & Manh Hung (1979) obtained a positive result in 21 out of 22 patients with uveal melanoma and one out of 26 control subjects. Noor Sunba et al (1980) investigated the lymphoproliferative response in 25 patients with uveal melanoma. Only half of the patients showed a strong response to autologous melanoma extracts and the reactivity with pooled allogeneic extracts tended to be weaker. The results of these studies suggest that tests of cellular immunity have an encouraging degree of specificity. One problem has been a shortage of sufficient amounts of standardised antigens. Furthermore, inconsistent results were obtained when extracts from different uveal melanomas were used to test the cellular immunity of the same patient, (Char et al 1975; Manor et al 1978). This problem could, in theory, be solved by extracting the same antigen from different uveal melanomas using monoclonal antibodies.

1.3.4.3 Immunotherapy of cancer

Immunotherapy of cancer, otherwise known as 'biological response modification', has been practised since the nineteenth century (Currie 1972). A number of different strategies exist and these have been extensively reviewed (Mitchell 1985; Bystryn 1985; Oldham & Smalley 1985; Mihich 1986).

Non-specific systemic immunotherapy aims to boost the complete spectrum of the patient's immune system in a non-specific manner in the hope of boosting anti-tumour

activity (reviewed by Hersh 1985; Borden & Hawkins 1986). Many different biological response modifiers have been investigated for this purpose. (1) The attenuated live mycobacterium, Bacillus Calmette Guerin (BCG), has received most attention following observations that intralesional inoculation of cutaneous melanoma usually causes regression of the injected tumour and, occasionally, also of distant deposits (Morton et al 1970). Extensive prospective randomised studies, however, have shown that despite its local therapeutic value BCG does not have any beneficial systemic effects (discussed by Borden & Hawkins 1986). (2) Levamisole is an antihelminthic drug which has been found to have immunomodulatory properties, enhancing T cell and macrophage function in vitro. Clinical trials, however, have shown that it is ineffective in the treatment of melanoma when used as an adjunct to surgery (Spitler et al 1982) or chemotherapy (Costanzi et al 1984): it also has many serious side effects (Parkinson et al 1977). (3) Polyadenylic-polyuridylic acid (poly A:poly U), a double stranded complex of synthetic polynucleotides, stimulates antibody production and cell mediated immune responses. Trials with this biological response modifier have shown increased survival in patients with breast cancer (Lacour et al 1984). (4) The recent production of interferons by recombinant DNA technology has made it possible for clinical trials to be undertaken (reviewed by Borden & Hawkins 1986). These have produced encouraging results in the treatment of melanoma (Legha 1986). In one study, however, the observed anti-tumour activity of

interferon could not be related to immunological changes resulting from this treatment so that its presumed immunological mode of action was questioned (Neefe, Phillips & Treat 1986).

Adoptive immunotherapy aims to restore deficient immunological functions to effective levels of activity. For example, autologous lymphokine-activated killer (LAK) cells cultured *in vitro* have been re-infused into patients in combination with recombinant interleukin-2 (Rosenberg 1985). This approach, although cumbersome, has caused at least partial tumour regression in patients with a variety of advanced malignancies, including melanoma. Other workers have also used adoptive immunotherapy in the treatment of melanoma with encouraging results (Slankard-Chahinian *et al* 1984; Balsari *et al* 1986). Serum-blocking of cell mediated immunity seen in patients with advanced melanoma has apparently been 'unblocked' by the transfusion of serum taken from patients with spontaneously regressed melanoma (Hellström *et al* 1971) or from normal black individuals (Hellström *et al* 1973). Prospective, randomised, double-blind studies, however, did not confirm the efficacy of such serotherapy (Wright *et al* 1978). Perfusion of plasma over immobilised Protein A has been investigated, the rationale being to remove blocking factors such as immune complexes from the serum (Bertram 1985). Cyclophosphamide appears to antagonise the known detrimental effects of suppressor T cells and is sometimes used as an adjunct to other forms of therapy (reviewed by Mastrangelo, Berd & Maguire 1986).

Specific active immunotherapy is aimed at boosting the anti-tumour immune response with the use of autologous or allogeneic tumour cells which have been inactivated by irradiation (Ikonopisov *et al* 1970), or treated with neuraminidase in order to enhance their immunogenicity (Rios & Simmons 1974). Although enhanced tumour immunity has often been achieved, evidence of beneficial clinical effects has been limited.

Passive immunotherapy consists of infusing antibodies into patients in the hope of supplementing the humoral response. This approach has become more efficient since the development of hybridoma technology for the production of monoclonal antibodies *in vitro* (discussed by Houghton & Scheinberg 1986). Anti-tumour monoclonal antibodies can theoretically destroy target cells by activating complement (Houghton *et al* 1985) or mediating antibody dependent cell mediated immunity (Adams *et al* 1984). There is also a possibility that infused antibodies may act as 'surrogate antigens' thereby boosting the host's immune system (Koprowski, *et al* 1984; Herlyn, Ross & Koprowski 1986). If the autocrine hypothesis (Section 1.2.1) is proved correct it may be possible to alter tumour behaviour by blocking the relevant hormone receptors using monoclonal antibodies. This hypothesis has been supported by the results of experiments performed both *in vitro* and *in vivo* in animal models (Cuttitta *et al* 1985).

Monoclonal antibodies are also under investigation as selective vectors for a variety of chemical toxins, photodynamic substances (Mew et al 1983), and radio-isotopes (discussed by Baldwin & Byres 1986; Frankel et al 1986; Embleton 1986; Cobb & Humm 1986). There are many potential obstacles to the successful application of anti-tumour monoclonal antibodies in vivo. The ligand may dissociate from the antibody (discussed by Zalcberg 1985) or interfere with its activity (Matzku et al 1985). The antibody may react with shed tumour-associated antigens circulating in the serum (discussed by Zalcberg 1985), or with anti-antibodies (discussed by Chatenoud 1986; Cobb & Humm 1986). The antibody may be cleared from the circulation by reticulo-endothelial cells in the liver or by the kidney (discussed by Zalcberg 1985). Access of the antibody to the tumour may be limited by lack of vascularity of the tumour or by the inability of the antibody to pass through the vascular endothelium. Even if the monoclonal antibody does reach the interstitial space of the tumour it may fail to attach to sufficient numbers of tumour cells because of antigenic heterogeneity or modulation, or both (Poste 1986). Finally, antibodies attaching to the cell membrane may not be internalised (Matzku et al 1986). A number of pharmacokinetic studies suggest that these problems can, to some extent, be overcome by various means. Firstly, separation of a radioisotope from the carrier could be reduced by selecting a more suitable ligand (Rosenblum et al 1985). Secondly, antibody fragments could be used to improve access to the tumour, to reduce clearance by the

reticuloendothelial system (Zalcberg 1985; Larson et al 1983; Ballou et al 1985) and to prevent antigenic modulation (Cobbold & Waldmann 1984). Thirdly, prior, or simultaneous, administration of non-specific antibodies could reduce uptake by reticuloendothelial cells (Rosenblum et al 1985; Zalcberg 1985). Fourthly, cocktails of anti-tumour monoclonal antibodies could compensate for low antigen density (Capone, Papsidero & Ming Chu 1984) or antigenic heterogeneity (Krizan et al 1985). Finally, the immune response to the monoclonal antibodies could be circumvented by the sequential use of different antibodies to the same tumour (discussed by Chatenoud 1986) or avoided by the use of human or chimaeric rodent-human monoclonal antibodies (discussed by Cobb & Humm 1986).

Despite the frequent reporting of encouraging results and anecdotes of dramatic responses (Miller et al 1982; Treleaven et al 1984; Houghton et al 1985), immunotherapy has not yet found a place in routine clinical practice. The recent advances in tumour immunology have led to a less naive attitude to the subject and to the realisation that effective immunotherapy is not a short-term prospect but a long-term goal.

1.3.4.4 Immunotherapy of uveal melanoma

A few trials of non-specific immunotherapy of uveal melanomas have been initiated (Frenkel et al 1977; Shields 1983; Char 1977; Davidorf 1978) but, to date, published results have not been forthcoming.

1.4 Hybridoma technology

1.4.1 Limitations of conventional antisera

Until recently lymphoid cells could only be maintained *in vitro* for short periods. The serological analysis of tumour-associated antigens, therefore, required the use of antisera taken from immunised animals or patients with cancer. The use of such sera was associated with a number of problems. Firstly, despite performing absorption with appropriate antigens, the antibodies, while specific for a single determinant, were nevertheless heterogeneous. Secondly, the same combination of specific antibodies was impossible to reproduce. Thirdly, only small amounts of antibody could be produced by these methods.

1.4.2 Hybridoma production

Monoclonal antibodies of predefined specificity can be produced in unlimited amounts from immortalised B lymphocytes (Köhler & Milstein 1975). These cells are obtained by fusing antibody-producing lymphocytes with myeloma cells (ie, B lymphocytes which have undergone malignant transformation). The technique was originally developed to study factors regulating immunoglobulin gene expression (Köhler & Milstein 1975).

Following a fusion procedure, the hybridomas are separated from the unfused myeloma cells with the use of metabolic inhibitors (Littlefield 1964). In normal cells, synthesis of purines and pyrimidines from amino acid and carbohydrate precursors takes place via two metabolic pathways, (i) the main pathway and (ii) the 'salvage'

pathway. If the main pathway is blocked by aminopterin, a folic antagonist, nucleotide synthesis can continue via a 'salvage' pathway, which involves two enzymes, hypoxanthine-guanine-phosphoribosyl transferase and thymidine kinase. The myeloma cells used for the generation of somatic cell hybrids are deficient in these two enzymes and are unable to grow in the presence of aminopterin unless they have fused with the normal lymphocytes. Since unfused normal lymphocytes only live for short periods in culture, the only cells that can persist indefinitely in culture medium containing hypoxanthine, aminopterin and thymidine (HAT) are the hybrid cells. The hybridomas producing the desired antibodies are then identified by means of an appropriate assay and subcloned repeatedly until cellular homogeneity has been attained.

Most antibodies have been produced from rodent hybridomas. Recently the production of appropriate human lymphoblastoid cell lines has allowed human hybridoma technology to be developed (reviewed by Olsson 1985). Sufficient numbers of lymphocytes for fusion can be obtained by taking a small sample of peripheral blood and propagating the lymphocytes in vitro following transformation with the Epstein-Barr virus (Steinitz 1979; Roder, Cole & Kozbor 1986).

1.4.3 Monoclonal antibodies

Monoclonal antibodies have a number of advantages over conventional antisera. Firstly, they can be raised against antigens without the need for prior identification

and purification of these antigens. Secondly, unlimited amounts of pure and standardised antibodies can be produced. The monoclonal nature of such antibodies does not guarantee specificity and can sometimes cause problems. For example, unlike polyclonal antisera, many monoclonal antibodies do not react with fixed tissues due to changes in the antigenic structure.

1.5 Monoclonal antibodies to melanoma

Since 1978, when the first monoclonal antibodies to melanoma-associated antigens were reported (Koprowski et al 1978), the total number of such reagents has grown dramatically. The results achieved to date using such antibodies illustrate the possibilities and limitations of the application of hybridoma technology to oncology. They are discussed in some detail in the present section because they relevant to this work.

1.5.1 Production

The usual strategy has been to immunise mice with cells from cultured cell lines derived from primary or metastatic cutaneous melanoma and to screen the resulting hybridomas against cultured cells from a variety of tumours. Most workers have used immunoprecipitation of internally-labelled cultured melanoma cells for the investigation of antigens detected by these antibodies. Some workers have departed from this protocol, such as Harper and associates (1984) who immunised mice with membrane-enriched fractions instead of whole cells, and Atkinson and associates (1985) who screened hybrids by

immunohistochemistry using fixed tissue. Recently, monoclonal antibodies have also been produced from human cell lines. These are discussed later in this section.

1.5.2 Specificity

Nearly all of the monoclonal antibodies to melanoma have reacted with other types of cell (Table 1.1). Although some antibodies were originally reported to be specific, often on the basis of subjective assessment, subsequent evaluation by independent workers has shown such claims to be incorrect (Brown et al 1981; Clemente 1985; Real et al 1985; Godal et al 1986). A recent report stating that a new monoclonal antibody is highly specific for melanoma in fixed tissues (Gown et al 1986), although encouraging, needsto be confirmed by other workers.

Non-specific monoclonal antibodies have tended to be directed against histocompatibility antigens (Mitchell et al 1980), foetal tissue antigens (Liao, Clarke, Khosravi et al 1982a), proliferation-dependent antigens (Real et al 1985), as well as foetal calf serum constituents (Yamaguchi et al 1987). Many of the monoclonal antibodies have cross-reacted with neural tumours (Koprowski et al 1978; Carrel et al 1980; Dippold et al 1980; Liao, Clarke et al 1981), and other types of malignancy (Woodbury et al 1980; Saxton et al 1982). Monoclonal antibody 6.1, for example, cross-reacted with antigens on renal and breast carcinomas although these antigens differed in molecular weight from those on melanomas (Loop et al 1981). The p97 antigen, recognised by hybridoma 4.1, was initially thought to be expressed

Table 1.1 Reactivities of monoclonal antibodies generated against cutaneous melanoma. (1) Representative monoclonal antibodies reported by different groups of workers (MAbs). Human monoclonal antibodies are indicated by an asterisk. (2) Proportion of cultured or uncultured melanomas (Mel) recognised by the monoclonal antibodies. (3) Cross-reactivity of monoclonal antibodies with non-melanomatous carcinomas (Ca), sarcomas (Sa) and normal tissues. The apparent specificity of each monoclonal antibody varies according to the assay used and from laboratory to laboratories. (4) References to articles in which monoclonal antibodies were first reported or in which information regarding their reactivities was published.

1	2	3	4
MAb	Mel	Other	References
Wistar Institute, Pennsylvania, USA			
Nu-4B	All	Astrocytoma	Koprowski et al 1978
		Emb. fibroblasts	Mitchell et al 1980
		Dysplastic naevi	Thompson et al 1982
19-19		Naevi	
		Astrocytomas	
		Emb.fibroblasts	
		Collagenous tiss.	
		Keratinocytes	
ME491	58/70	Some carcinomas	Atkinson et al 1984
		Naevi	Atkinson et al 1985
ME492	33/35	Macrophages,naevi	
		Mucous glands	
		Hematogenous cells	
		Prostatic Ca	
		Colon Ca	
Fred Hutchinson Cancer Research Center, Seattle			
3.1	Imm. melanoma line only		Yeh et al 1979
4.2	25/29	Normal lung	Yeh et al 1982
		Normal kidney	Nudelman et al 1982
	3/17	Other tumours	
4.1	90%	55% other tumours	Woodbury et al 1980
		Foetal colon	Brown et al 1981b
5.1	50%	50% carcinomas	Loop et al 1981
		Adult brain	
		Foetal tissues	
6.1	50%	80% renal Ca.	
		Normal cell lines	
Departments of Otolaryngology and Pathology, University of Washington, Seattle			
HMB-45	97%	Basal cell Ca	Gown et al 1986
		Hair follicles,	
		Naevi	
		Spitz tumours	
		0/168 tumours	
The Memorial Sloan-Kettering Cancer Center, New York			
I12	11/16	Astrocytomas	Dippold et al 1980
		Renal/bladder Ca	
		Renal epithelium	
N9	13/16	Astrocytoma	Albino et al 1983
		Renal/bladder Ca	
		Colon Ca	
		Renal epithelium	
		Foetal brain	
R24	all	Astrocytomas	Pukel et al 1982
(Mel-1)		Melanocytes	
		Emb. brain & lung	
		Cultured T cells	
		Histiocytoma	Clemente 1985
		Fibroblasts	
		Breast Ca	
		Naevi	
M19	10/16	Astrocytomas	
		Renal & ovarian Ca	
TA99	6/12	Pigmented cells	Thomson et al 1985
Ma4*	5/23	Other tumours	Houghton et al 1983
Ri37*	2/2	Bladder Ca,	Cote et al 1983
		Lung Ca	
		Mononuclear cells	

Table 1.1 Contd.

MAb	Mel	Other	References
Department of Surgery, Duke University Medical Center, Durham, North Carolina, USA			
D6.1	13/13	Breast Ca Ovarian Ca Colon Ca Lung Ca	Johnston et al 1985
Scripps Clinic and Research Foundation, California			
165.28T	All	Astrocytoma	Imai et al 1981
473.54S	All	Lymphoid cells (wk)	
376.74T	All	Astrocytoma Colon Ca Breast Ca Prostate Ca	
345.134S	5/5	Other tumours Lymphoid cells	Wilson et al 1982
376.96S	4/5	Naevi, skin Ca Breast Ca	
225.28S	5/5	Astrocytoma 54% Skin Ca Schwannoma Hair follicle Foetal skin Foetal kidney	Kageshita et al 1985
465.12S		Colon Ca Breast Ca Bladder Ca	Wilson et al 1982
		Colon, rectum	Giacomini et al 1984
		Most normal tiss.	Natali et al 1982
9.2.27	6/6	'Nil'	Morgan, Galloway & Reisfeld 1981
		Sarcomas	Godal et al 1986
155.8	7/8	Nil	Harper, Bumol & Reisfeld 1984
Oncology Division, University of California, Los Angeles			
L55*	over50%	Foetal brain Carcinoma/sarcoma Fibroblasts	Irie, Sze & Saxton 1982
L72*	over50%	Neural tumours	Cahan et al 1982
Division of Surgical Oncology, John Wayne Clinic, UCLA			
705F6	14/14	Other tumours Foetal brain Foetal fibroblasts Neural tumours Foetal cell lines	Saxton et al 1982
436G10	10/14	Other tumours	
Cancer Center, Wadley Institute of Molecular Medicine, Dallas			
WI-MN-1	16/17	Epidermoid Ca Amnion cells Leukaemia	Khan et al 1983
University of Southern California School of Medicine, LA			
2-139-1*		Colon Ca Liver Ca Lung Ca Pancreatic Ca Prostatic Ca Ducts	Imam et al 1985

Table 1.1 Contd.

1	2	3	4
MAb	Mel	Other	References
General and oncologic surgery, City of Hope Medical Centre, Duarte, California			
F11	7/8	5/5 Ca lines	Chee et al 1982
Department of Dermatology and Syphilology, Detroit,			
FKH1	79-100%	Naevi, lung Ca, Phaeochromocytoma Mast cells,	Fukaya et al 1986
Department of Experimental Dermatology, Munster, Germany			
M-2-2-4	92%	Sebaceous glands Breast Ca Naevi	Suter et al 1983
M-2-7-6	30%	Cutaneous nerves Brain tissue Leiomyoma	
M-2-10-15	30%	Cutaneous nerves Brain tissue Naevi	
A-10-33	3/13	Capillaries Neuroblastoma Teratoma Lung Ca	
A-1-43	4/13	Epidermal cells Sweat ducts Cutaneous nerves Lung Ca Naevi Basal cell Ca Squamous cell Ca Leiomyoma	
Ludwig Institute for Cancer Research, Switzerland			
Mel-5	9/10	Glioblastoma	Carrel et al 1980
Mel-14	10/10	Glioblastoma	
Mel-7	5/10	Nil	
Me3-NE4	6/10	Glioblastoma	
Me4-H3	7/10	Nil	
Me4-H4	9/10	Nil	
Me5-D5	9/10	Nil	
Me4-F8	9/10	'Nil'	Carrel et al 1980
		Astrocytoma	Herlyn et al 1982
		Adenocarcinoma	Johnson & Riethmuller
		Fibroblasts	1982
		Lymphoid cells	Harper et al 1982
Ontario Cancer Foundation			
7.51	10/10	Neuroblastoma Retinoblastoma Glioma	Liao et al 1981
7.60	9/10	Neuroblastoma Retinoblastoma Glioma	
7.39	7/10	Nil	
140.240	All	Foetal tissues	Liao et al 1982a
		Sweat glands, Naevi	Natali et al 1986

Table 1.1 Contd.

MAb	Mel	Other	References
The Netherlands Cancer Institute, Amsterdam			
C-1	4/5	Nonspecific	Hageman et al 1982
C-2	4/5	Variable	
NKI/C-3	97/99	Naevi	Vennegoor et al 1985
		Carcinoids	
		Medullary Ca	
		Neuroendocrine Ca	Mackie, Campbell &
		Prostatic Ca	Turbitt 1984
		Ovarian Ca	
		Breast Ca	
		Lung Ca	
PAL-M1		Naevi	Ruiter et al 1985
		Other tumours	
		Epithelial cells	
		Lymphoid cells	
PAL-M2		Other tumours	
		Epithelial cells	
		Lymphoid cells	
AMF-7	25/25	Vasc. End. cells	De Vries et al 1986
		Naevi,	
AMF-6	30/30	Perineurium, naevi	
Institut für Immunologie, Munich			
21.43	6/12	Nil	Johnson et al 1981
15.95	8/12	5/5 Ca lines	
		Foetal fibroblasts	
		Adrenal gland	
		Teratoma	
15.75	8/12	4/5 Ca lines	
		Fibroblasts	
16.63	10/12	2/5 Ca lines	
		Lymphocytes	
Department of Dermatology, Sapporo Medical College, Japan			
HMSA-1	16/26	Naevi	Akutsu & Jimbow 1986
		Astrocytomas	
		Pinealoma	
University of Calgary, Canada			
LS62	All	Fibroblasts	Sikora et al 1987
		50% other tumours	
		Naevi	
		Vasc. end.	
		Mast cells	
		Glandular ep.	

Table 1.1

only by melanomas (Woodbury et al 1980). Although this impression was later found to be incorrect levels of this antigen were 50 times higher in melanomas than in normal human tissues (Brown, Woodbury et al 1981). This demonstrates that the results of quantitative analyses of the number of antigen molecules per cell are more meaningful than descriptions of antigens as specific or non-specific. The apparent specificity of an antigen depends greatly on the assay that is used (Pukel et al 1982). For example, an 88K protein antigen detected by antibodies C-2,6 and 7 (Hageman et al 1982), was apparently non-specific when tested against a variety of cell lines using a radiolabelled probe but showed a high degree of specificity on immunohistochemistry. Furthermore, the results of assays performed with cell lines do not necessarily apply to uncultured cells of the same tumour type (Johnson et al 1981). For these reasons detailed investigations of the 'specificity' of a monoclonal antibody are ultimately determined by its intended purpose.

1.5.3 Antigens identified by different antibodies

A significant proportion of monoclonal antibodies, often produced in different laboratories, have been found to react against the same antigen (Yeh et al 1979; Brown, Nishiyama et al 1981; Dippold et al 1980; Thurin et al 1985; Liao et al 1987). An example of such an antigen is an intracellular glycoprotein which is related to melanin pigment (Tai et al 1983; Thomson et al 1985).

1.5.4 Reactivity with animal melanomas

Monoclonal antibodies raised against human melanomas have cross-reacted with melanomas in animal models (Natali, Aguzzi *et al* 1983) and *vice versa* (Vollmers & Birchmeier 1983; Hirabayashi *et al* 1985). Syngeneic monoclonal antibodies to B16 melanoma when infused into animals were shown to alter the metastatic behaviour of this tumour (Herd 1987).

1.5.5 Applications

1.5.5.1 Characterisation of antigens

Some of the antigens have undergone extensive characterisation. Antigen p97, recognised by mAb 4.1 (Woodbury *et al* 1980) appears to be a monomeric sialoglycoprotein containing a stable 40kD fragment (Brown, Nishiyama *et al* 1981) which is structurally and functionally related to transferrin (Brown *et al* 1982). Its gene, like the genes of transferrin and the transferrin receptor, is expressed on chromosome 3 which commonly shows rearrangements in malignant tumours (Plowman *et al* 1983). The amino acid sequence of the p97 antigen has recently been elucidated by determining the nucleotide sequence of cloned and purified p97 mRNA (Rose *et al* 1986)

Monoclonal antibody 140.240 (Liao, Clarke *et al* 1982a) recognises a non-sialylated 83K glycoprotein on a 77K polypeptide backbone, which is converted to gp87 by stepwise modification of the high mannose oligosaccharides to the more complex sialylated sugars (Khosravi, Dent & Liao 1985).

Monoclonal antibody, 4.2, (Yeh et al 1982) recognises a ganglioside that is similar to brain GD3 ganglioside except that its ceramide is characterised by a predominance of longer chain fatty acids (Nudelman et al 1982). This antibody also binds to a structural analogue, disialylparagloboside, which has a different biosynthetic pathway (Brodin et al 1985).

Three antigens identified by Atkinson and associates (1985) were identical or in close proximity, and these showed heterogeneity in charge and molecular weight as a result of non-uniform processing of a single protein core whose amino acid sequence was partially elucidated (Ross et al 1985).

Two human monoclonal antibodies produced against oncofoetal antigen (Irie, Sze & Saxton 1982) recognised two molecules which differed from each other by only one sialic acid residue (Cahan et al 1982; Irie, Tai & Morton 1985).

1.5.5.2 Classification of melanomas

By means of a panel of 34 monoclonal antibodies, some new and others previously described, Houghton and colleagues (1982) defined differentiation antigens expressed on melanomas, newborn melanocytes or adult melanocytes and suggested that these could be used to classify melanomas in an analogous fashion to the classification of lymphocytic tumours. Holzmann and associates, using a different panel of monoclonal antibodies, have also succeeded in correlating antigenic expression with the degree of tumour progression (1987).

1.5.5.3 Investigation of the functional significance of antigens

Further insight into the nature and distribution of antigens recognised by monoclonal antibodies has been gained from immunohistochemical studies. The prognosis of cutaneous melanomas has been correlated with the expression of certain well-defined antigens, despite the fact that these antigens were not entirely specific for melanoma (Brüggen et al 1984).

1.5.5.4 Detection of serum antigens

Some workers have detected circulating melanoma-associated antigens in the serum which have shown a correlation with the extent of the malignancy (Ross et al 1984). Other melanoma-associated antigens have also been found in the serum of healthy contols, albeit in smaller concentrations than patients with melanoma (Giacomini et al 1984).

1.5.5.5 Diagnostic immunohistochemistry

Monoclonal antibodies are already proving useful in clinical practice (Mackie et al 1984; Gatter et al 1985). Many are not reactive with formalin fixed tissues but there have been a few exceptions (Dippold, Knuth & Meyer Zum Büschenfelde 1984, Atkinson et al 1985; Vennegoor et al 1985; Akutsu & Jimbow 1986; Gown et al 1986).

1.5.5.6 Targeting of monoclonal antibodies

Targeting of radiolabelled antibodies has been demonstrated in animal models (Wilson et al 1982) and in human patients (Natali, Aguzzi et al 1983; Murray et al 1987). Antibody fragments were found to be better than whole immunoglobulins for this purpose (Larson et al 1983; Buraggi et al 1985). Experiments using athymic nude mice have shown accumulation of monoclonal antibodies in human melanoma implants (Wilson et al 1982; Beaumier 1985), and tumour regression (Cheresh et al 1985). An animal containing an implanted human tumour is not an ideal model, however, because monoclonal antibodies recognising human rather than melanoma-associated antigens would still accumulate within the tumour. Monoclonal antibodies have been infused into patients with widespread metastatic disease. Of 12 such patients treated with a monoclonal antibody, three showed a major anti-tumour response characterised clinically by pain and inflammation at the tumour sites and histologically by tumour necrosis, mast cell and lymphocytic infiltration, complement deposition and demonstrable mouse immunoglobulin (Houghton et al 1985). In this context it is remarkable that this antibody specifically blocked autologous T-cell cytotoxicity for melanoma cells (Dippold, Knuth & Meyer Zum Büschenfelde 1984). Using murine monoclonal anti-melanoma antibodies attached to ricin A chain toxin, Spitler and associates (1987) claimed to obtain an encouraging tumour response in a few of their patients, with side effects which did not cause serious distress to the patient.

1.5.5.7 Tumour destruction

A number of *in vitro* studies have demonstrated that some monoclonal antibodies can induce antibody-dependent cell mediated cytotoxicity (Herlyn *et al* 1979; Hellström, Brown & Hellström 1982; Cheresh *et al* 1984), sometimes only after puromycin treatment (Wilson *et al* 1982). Some antibodies activated complement (Wilson *et al* 1982), and others induced tumour cell lysis by both mechanisms (Yeh *et al* 1979; Cheresh *et al* 1984). In one experiment, antibody binding to tumour cells was apparently greatly reduced by recombinant immune interferon (Giacomini *et al* 1985).

1.5.6 Human monoclonal antibodies

The production of human monoclonal antibodies to melanoma-associated antigens has only been achieved relatively recently, due to the technical difficulties involved. Of 771 immunoglobulin-secreting cultures, six reacted with cell surface and 27 with intracellular antigens (Houghton *et al* 1983). Another group of workers has also reported similarly disappointing results (Yamaguchi *et al* 1987).

Some of the antibodies have reacted against previously recognised antigens such as OFA-1 (Irie, Sze & Saxton 1982), whereas others have recognised new antigens (Cote *et al* 1986).

Two antibodies detect an antigen in fixed tissues which is present in all melanomas tested, including uveal melanomas, but not naevi. It is interesting that the same antigen was also reported to be present in sweat glands (Imam et al 1985).

Intralesional injection of a human IgM monoclonal antibody, Hu-mAb L72, into cutaneous melanomas has been followed by regression of these tumours and in one patient there was also regression of an untreated tumour satellite (Irie & Morton 1986).

1.5.7 Reactivity with uveal melanomas

A few monoclonal antibodies generated against cutaneous melanoma cells lines have been tested for reactivity with uveal melanoma. Of these, some have reacted only with the cutaneous form of tumour (Mitchell et al 1980; Atkinson et al 1985), whereas others have reacted with both types of melanoma (Herlyn et al 1980; Folberg et al 1985; Imam et al 1985; van der Pol et al 1987; Bomanji, Garner et al 1987). These have been used in immunohistochemical studies to demonstrate hepatic metastases (Donoso, Folberg, Naids et al 1985) and antigenic variation in ocular tumours (Donoso et al 1986; van der Pol et al 1987). In addition, van der Pol and associates have shown that the expression of a tumour-associated antigen was diminished by prior radiotherapy (1987). Recently, radioimmunoscintigraphy of ocular melanoma has been achieved with ^{99m}Tc labelled cutaneous melanoma antibody fragments (Bomanji, Hungerford et al 1987).

When the present study was initiated, monoclonal antibodies had not been raised against uveal melanomas, probably because uveal melanoma cell lines were not available. In 1985 Donoso and associates immunised mice with cultured cutaneous melanoma cells and viable uveal melanoma cells and, following fusion, selected hybridomas by immunohistochemistry according to their reactivity with formalin fixed uveal melanoma tissue. One antibody reacted strongly with over 90% of all uveal melanomas tested, weakly with normal choroidal melanocytes, and focally with retinal pigment epithelial cells. This antibody also reacted with cutaneous melanomas and naevi as well as salivary and bronchial glands, and pulmonary and colonic carcinomas (Donoso, Folberg, Edelberg et al 1985). In 1986, the monoclonal antibodies produced in the present study were reported (Damato et al 1986). These were produced entirely with the use of uncultured uveal melanoma cells.

1.6 Objectives of this research

The aims of the present study are:

1. To determine whether it is possible to generate rodent and human monoclonal antibodies to relevant antigens expressed by human uveal melanomas.

2. To establish whether such techniques will yield monoclonal antibodies that are likely to have useful clinical or research applications.

3. To evaluate the Epstein-Barr virus lymphocyte transformation technique, firstly, as a means of analysing the humoral immune response to uveal melanoma in patients with this tumour and, secondly, as a method for the production of human monoclonal antibodies to this tumour.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

The materials used during the course of this study are listed below with the names of the manufacturers, who are based in Britain unless otherwise stated.

2.1.1 Cell Culture materials

RPMI-1640 and Aminopterin (Methotrexate) were supplied by Flow Laboratories, Irvine.

Foetal calf serum and Fungizone (Amphotericin B) were obtained from Gibco Bio-Cult Ltd., Paisley.

Penicillin and Streptomycin were obtained from Glaxo Pharmaceuticals Ltd., London.

Hypoxanthine and ouabain-octahydrate were obtained from Sigma London Chemical Co Ltd., Poole.

Thymidine and phenylmethylsulphonyl fluoride (PMSF) were obtained from Boehringer-Mannheim, Sussex.

Cyclosporin A was obtained from Sandoz International, Switzerland.

2.1.2 Disposable materials

Tissue Culture Flasks, manufactured by Nunc, were supplied by Gibco Bio-Cult Ltd., Paisley.

Tissue Culture Plates were supplied by Costar Northumbria Biologicals Ltd, Northumbria.

MicroELISA Plates were supplied by Dynatech Laboratories Ltd., Billinghurst.

Sterile universal containers were obtained from Sterilin Ltd., Teddington.

Lab-Tek Tissue culture 8-well Chamber/Slides were obtained from Miles Laboratories Ltd., Slough.

Multispot microscopy slides were obtained from C.A. Hendley (Essex) Ltd., Oakwood.

Glass microscopic slides and coverslips were obtained from Chance Propper Ltd., Warley.

Millipore filters were obtained from Millipore (U.K.) Ltd., London.

Eppendorf tubes,(1.5 ml capacity), manufactured by Eppendorf, Gerateban, Netheler and Hinz, were supplied by Anderman and Co., East Molesey.

Dialysis tubing was obtained from Visking Tubing, Scientific Instruments Centre Ltd., London.

Sterile plastic syringes were obtained from Becton Dickinson, Dublin.

Sterile needles were obtained from Gillette Surgical, Middlesex.

2.1.3 Cell lines

The rat myeloma line Y3.Ag.1.2.3 (Galfré et al 1979) was obtained from C. Milstein, M.R.C., Cambridge.

The KR-4 human plasmacytoma cell line (Kozbor, Lagarde & Roder 1982) was obtained from Dr John Roder, Department of Microbiology and Immunology, Queen's University, Kingston, Ontario, Canada.

The EBV secreting marmoset cell line, B95-8 (Miller & Lipman 1973) was obtained from Dr D. Crawford, Hammersmith Hospital, London.

The HeLa cell line was obtained from Flow Laboratories, Irvine.

The B008 human melanoma cell line (Creasey et al 1979) was obtained from Dr T.E. Wheldon, Glasgow Institute of Radiotherapeutics and Oncology, Belvedere Hospital, Glasgow.

2.1.4 Animals

DA rats were obtained from OLAC Ltd., Blackthorn, Bicester and maintained as breeding stock in the University of Glasgow West Medical Buildings Colony.

2.1.5 Chemicals

Hypoxanthine, Staphylococcus protein A (Cowan strain), Agarose, Poly-L-lysine, Glutaraldehyde, Polyoxyethylene sorbitan monolaurate (Tween 20), Periodic acid, Phenylmethyl sulphonyl fluoride (PMSF), Tris, sodium barbitone, 2-aminoethylisothiouronium bromide hydrobromide (AET), ortho-phenylenediamine (OPD), 6-thioguanine (2-amino 6-mercaptopurine), diaminobenzidine (DAB), 3-aminoethyl carbazole (AEC) and Sodium azide were obtained from Sigma Chem. Co. Ltd., Poole.

Sodium chloride, ammonia, ammonium chloride, ammonium sulphate, calcium chloride, glycine, xylol, Hydromount, formdimethylamide, ortho-phosphoric acid and citric acid were all of analytical grade and were supplied by B.D.H Chemicals Ltd., Poole.

Polyethylene glycol (PEG) 4000 and 1500 were obtained from Merck, Darmstadt, West-Germany.

Hydrogen peroxide was obtained from Fisons, Loughborough.

Dimethylsulphoxide (DMSO) was obtained from Koch-Light Laboratories Ltd., Colnbrook.

Sulphuric acid, sodium di-hydrogen phosphate and di-sodium hydrogen phosphate were obtained from Riedel-De Haen AG, Seelze-Hannover.

Hydrochloric acid, formaldehyde, acetone, glycerol and sodium citrate were obtained from May and Baker Ltd., Dagenham.

2.1.6 Electrophoretic Materials

Sodium dodecyl-sulphate, acrylamide, N,N,N'N'tetra-methylene diamine (Temed), butan-2-ol, N,N'-methylene bis acrylamide and ammonium persulphate were obtained from B.D.H. Chemicals Ltd., Poole.

2-Mercaptoethanol was obtained from Koch-Light Laboratories Ltd., Colnbrook.

Membrane Filters, 0.45 μm, were supplied by Schleicher and Schull, East Molesley.

Low molecular weight marker proteins were supplied by Pharmacia (GB) Ltd, Hounslow.

2.1.7 Stains

Trypan Blue, Hoechst (Bisbenzimide), Coomassie Blue (R), Coomassie Blue (G) and Naphthol Blue Black were obtained from Sigma Chem. Co. Ltd. Poole.

Silver nitrate was obtained from Johnson Matthey Chemicals, U.K.

2.1.8 Radiochemicals

^{125}I-NaI, specific activity 2.96 Bq/mg, was supplied by the Western Infirmary, University of Glasgow.

2.1.9 Photographic Materials

X-Ray films (Kodak X-Omat S), SX-80 developer and FX-40 X-ray liquid fixer were supplied by Kodak Ltd., Hemel Hampstead.

100 and 400 ASA Photographic films (Kodak) were purchased from a photographic retailer, Glasgow.

2.1.10 Serological Reagents

Rabbit anti-rat IgG (H+L), Rabbit anti-rat IgG (H+L) conjugated to horseradish peroxidase and Rabbit anti-rat IgG (H+L) conjugated to fluorescein were supplied by Miles Laboratories Ltd., Slough.

Normal goat serum and normal rabbit serum were obtained from the Scottish Antibody Production Unit, Wishaw, Scotland.

2.1.11 Enzymes

Neuraminidase was obtained from Sigma Chemical Co. Ltd., Poole.

Trypsin and chymotrypsin were obtained from Worthington Biochemical Corporation, Freehold, New Jersey, USA.

2.1.12 Miscellaneous

Bovine serum albumin was supplied by Sigma Chemical Co. Ltd., Poole.

Iodo-gen (1,3,4,6-tetrachloro-3a, 6a-diphenyl glycouril) was obtained from Pierce Chemical Co. Rockford, Illinois, U.S.A.

Sepharose 6B, Columns PD-10, Ficoll-Paque and Percoll were obtained from Pharmacia (GB) Ltd., Hounslow.

Sheep red blood cells were obtained from Gibco Bio-Cult Ltd., Paisley.

OCT-Compound was obtained from Raymond Lamb Ltd., London.

Multichannel micro-pipettes were obtained from Flow Laboratories, Irvine.

Micropipettes were manufactured by Gilson France S.A. and supplied by Anachem Ltd., Luton.

All other reagents were of analytical grade or of the highest available purity.

2.2 Standard Solutions and Buffers

2.2.1 Fusion and Cell Culture Media

2.2.1.1 L-Glutamine stock solution

A 100 mM stock solution of L-Glutamine in distilled water was prepared and stored in 1 ml aliquots at 4°C after sterilisation by Millipore filtration.

2.2.1.2 RPMI-1640 medium

This medium was supplied in liquid or powdered form. Shortly before use, the liquid form was supplemented with 1% L-Glutamine stock solution (v/v) to achieve a final concentration of 2 mM. The powdered form was reconstituted with distilled water in the Cell Culture Unit of the University of Glasgow Biochemistry Department as instructed by the manufacturers. The medium was sterilised by Millipore filtration and stored at 4°C. This medium is referred to elsewhere in this text as RPMI.

2.2.1.3 Foetal calf serum (FCS)

Foetal calf serum was purchased in bulk after samples from different batches were tested by growth efficiency tests. These were performed by Mrs C. Cannon in the Biochemistry Department of the University of Glasgow: hybridomas were grown and subcloned in different samples of FCS and the numbers of hybrids arising in these samples were counted and compared. This serum was inactivated at 56°C for 30 minutes and stored at -20°C. Repeated freezing and thawing was avoided. After opening a container, any residual serum was stored at 4°C until use.

2.2.1.4 Antibiotics and antifungal agents

A solution containing $1x10^7$ I.U. Sodium benzyl penicillin and 10 g streptomycin in 1 l of distilled water (P/S) was obtained in a constituted form and stored at -20°C until use.

Fungizone, 2.5% (w/v) in distilled water, was obtained in a constituted form and was stored in 1 ml aliquots at -20°C until use.

2.2.1.5 Complete Medium

Complete medium was made up by the addition of 2% P/S stock solution (v/v), 1% fungizone (v/v) and either 10% or 20% FCS (v/v) to RPMI, for the culture of myeloma cells and hybridomas respectively. These solutions are referred to elsewhere in this text as 10% and 20% complete medium respectively. In the latter stages of this project the addition of antibiotics and antifungal agents to the two types of complete medium was discontinued.

2.2.1.6 Hypoxanthine/Thymidine stock solution (HT)

Hundred-fold concentrated HT stock solution was prepared which consisted of $3x10^{-4}$M glycine, $1x10^{-2}$M hypoxanthine and $1.6x10^{-3}$M thymidine in distilled water. The solution was then sterilised by Millipore filtration, aliquoted and stored in darkness at -20°C.

2.2.1.7 HT-complete medium

HT-complete medium was made up as described by Littlefield (1964) by diluting 1.0 ml of the sterilised stock solution with 100 ml of complete medium (Section 2.2.1.5).

2.2.1.8 Aminopterin stock solution

A 0.1 mM-Aminopterin stock solution in distilled water was prepared and the pH adjusted to 7.5. The solution was then sterilised by Millipore filtration and stored at -20°C in 0.4 ml aliquots.

2.2.1.9 HAT-complete medium

A 0.4 ml of stock aminopterin solution was added to 100 ml of HT medium to give a final concentration of 4×10^{-7}M.

2.2.1.10 Ouabain stock solution

A 10 mM-Ouabain solution in RPMI was prepared. The mixture was sterilised by Millipore filtration and stored in 5 ml aliquots at 4°C protected from light.

2.2.1.11 Thioguanine stock solution

A Thioguanine solution, 10 mg/ml, in 0.1 N NaOH was prepared. The mixture was sterilised by Millipore filtration and stored at 4°C.

2.2.1.12 Ouabain/Thioguanine-complete medium

Ouabain solution, 5% (v/v), and thioguanine solution, 0.3% (v/v), were added to 10% complete medium.

2.2.1.13 Polyethylene glycol solution

Polyethylene glycol solution, 50% (w/v) in RPMI, was prepared by autoclaving 2 g PEG at 15 lb/sq. in. for 15 minutes and adding 2 ml sterile RPMI before the PEG had cooled and solidified.

2.2.2 Saline buffers

2.2.2.1 Phosphate buffered saline

A x10 solution of 1.4 mM NaCl, 9.5 mM Na_2HPO_4 and 12.8 mM NaH_2HPO_4 in distilled water was prepared and adjusted to pH 7.4. This was diluted, 1 in 10, in distilled water, as required, immediately before use.

2.2.2.2 PBS-Tween

Tween 20, 0.05% (v/v), in PBS.

2.3 Methods

2.3.1 Animal Handling

2.3.1.1 Maintenance

A maximum of two animals of the same sex were housed in R.M.2 cages and fed on Labsure Animal Diet, CRM Nuts. Water was provided ad libitum from drop bottles. The ear of one animal in each cage was clipped for identification purposes.

2.3.1.2 Anaesthesia

Five to ten millilitres of ether was poured into a glass jar containing dry cotton wool and covered by a mattress of clean tissue paper. A single animal was placed in this jar which was then loosely closed. When the animal no longer responded to tactile stimuli it was transferred from the jar to the working surface. If further anaesthesia was required, an open universal container containing ether-soaked cotton wool was applied close to the animal's muzzle.

2.3.1.3 Intraperitoneal injection

An inoculum of not more than 1 ml in volume was injected into the peritoneal cavity through the lower abdominal wall, using a 21-G sterile disposable needle.

2.3.1.4 Subcutaneous injection

An inoculum less than 1 ml in volume was injected subcutaneously beneath the dorsal skin, using a 21-G sterile disposable needle.

2.3.1.5 Intravenous injection

The animal was anaesthetised in ether (Section 2.3.1.2). Hot cotton wool soaks were applied to the tail to induce vasodilatation and the intravenous injection was given using a 25-G sterile disposable needle.

2.3.1.6 Test bleeding

The animal was anaesthetised in ether (Section 2.3.1.2). The terminal 3 mm length of the tail was amputated using a number 11 Bard Parker scalpel blade and 0.25-1.0 ml of blood was collected in an Eppendorf tube. Further bleeding was arrested by squeezing the tail until the animal had recovered from the anaesthetic. During animal sacrifice, blood was collected by trans-diaphragmatic cardiac puncture performed with a 21-G sterile disposable needle while the heart was still beating.

The serum was separated by centrifugation of the blood specimen at 300xg for five minutes at room

temperature. Whereas this procedure was adequate for rat serum, mouse blood was more prone to haemolysis. With these animals the specimen was first incubated for 30 minutes at room temperature to allow the clot to form and then overnight at 4°C to allow the clot to retract. If the serum was to be stored by freezing it was first heat-inactivated at 56°C for 30 minutes. An anticoagulant was not used.

2.3.1.7 Euthanasia

The animals were killed by cervical dislocation performed under anaesthesia.

2.3.1.8 Removal of spleen

The anaesthetised animal was placed supine on the working surface and its abdomen cleaned with 70% alcohol. Longitudinal midline incisions were made, first through skin and abdominal muscle and then, after further rinsing of the instruments in alcohol and flaming over a bunsen burner, through the peritoneum. The spleen was removed, avoiding damage to adjacent organs, and placed in a sterile dry universal container for immediate transportation to a sterile hood.

2.3.2 Antigens

2.3.2.1 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 (Foulds 1978) or by enucleation. The tumour tissue was first

transported dry or in saline to the Pathology laboratory. Triangular blocks (3-6 mm) were excised from the apical part of tumours greater than ten millimeters in diameter 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 where further processing was undertaken within 45 minutes of their excision.

Melanoma tissue intended for animal immunisation or immuno-electrophoretic procedures was stored in dry freezing vials in a gas-phase liquid nitrogen freezer. Tissue intended for immunohistochemistry on frozen sections was stored in OCT-Compound embedding medium in freezing vials in liquid nitrogen. Melanoma tissue and cell suspensions intended for the preparation of ELISA plates were stored in PBS at 4°C usually for two or three hours only and never for periods exceeding 24 hours from the time of the tumour excision. Storage of the ELISA plates is described in Section 2.3.5.1.

2.3.2.2 Collection and storage of subretinal fluid

During local resection of choroidal melanomas, incision of the choroid was often followed by a sudden escape of subretinal fluid which pooled in the operative field. This was immediately collected into a 2 ml disposable syringe using a lacrimal cannula. Contamination with blood was minimised by cleaning the operative field immediately prior to the anticipated release of subretinal fluid.

When subretinal fluid was obtained from an eye enucleated for melanoma the eye was immediately placed on a Tudor Thomas stand to which it was secured by means of a 4/0 black silk suture passed through the optic nerve. The location of the retinal detachment was determined by indirect ophthalmoscopy and the subretinal fluid aspirated through the overlying sclera into a disposable syringe using a 25-G needle.

During surgery for rhegmatogenous retinal detachment, if it was necessary to drain subretinal fluid by means of a scleral and choroidal incision, this fluid was collected as it accumulated in the operative field using a disposable 2 ml syringe fitted with a lacrimal cannula.

Subretinal fluid was stored at -20°C until it was required, either in the syringe in which it was collected or in an eppendorf tube.

2.3.3 Cell membrane preparation

Cell membranes were prepared as described by Standring and Williams in 1978.

Reagents

i. 0.15 M NaCl

ii. 100 mM PBS pH 7.4

Stock solution A: 200 mM Na_2HPO_4

in distilled water.

Stock solution B: 200 mM NaH_2HPO_4.

100 mM-PBS was prepared by adding 40.5 ml solution A

to 9.5 ml solution B and making up to 100 ml with distilled water. The pH of the solution was adjusted to 7.4.

iii. EDTA, 1 mM in 10 mM PBS. pH 7.4

iv. 0.5 M Sucrose in 10 mM PBS, pH 7.4

Procedure

A suspension of $2\text{-}6\text{x}10^8$ cells was prepared in 10 ml saline (Section 2.3.4.4) and centrifuged at 300xg for five minutes at room temperature. The pellet was then washed by re-suspending it in 10 ml saline and centrifuging at 300xg for five minutes at room temperature. The cells were re-suspended in 2-5 ml PBS and homogenised using a mechanical homogeniser. A volume 2.5 ml of 0.5 M sucrose/phosphate buffer was added to the cell suspension which was then centrifuged at 300xg for five minutes. The supernatant was carefully removed with a pipette and loose material lying on top of the pellet was transferred to an SW 65 rotar tube for centrifugation at $1\text{x}10^5$ g for one hour at 4°C. After carefully removing the supernatant with a pipette, the resulting pellet was resuspended in 0.5 ml PBS. The protein concentration of this membrane suspension was estimated by the Bradford assay (Section 2.9).

2.3.4 Cells

2.3.4.1 Culture

Unfused rat or mouse myeloma cells were grown in 10% complete medium (Section 2.2.1.5) in a 5% CO_2/air mixture at 37°C. Hybridoma cells were grown in 20%

complete medium under similar conditions. The cell suspension was inspected every one to three days using an inverted microscope and fresh culture medium was added in ratios of 1:1 to 3:1 to maintain a concentration of 10^5 to 10^6 cells/ml. The size and type of cell culture flask used depended on the volume of the cell suspension required.

2.3.4.2 Cryopreservation

Cells were stored in liquid nitrogen as described by Galfré and Milstein (1981).

The number of cells available for storage was determined by measuring their concentration in an 0.5 ml sample (Section 2.3.4.3). The cell suspension was centrifuged (500xg for five minutes at room temperature) and the pellet was re-suspended in a 10% solution of DMSO in 90% FCS to achieve a final concentration of $2\text{-}5\text{x}10^6$ cells/ml. This cell suspension was transferred in 1.5-2.0 ml aliquots to freezing vials which were placed in a -70°C freezer overnight prior to storage in a gas-phase 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 a 15 ml volume of RPMI. This was centrifuged at 500xg for five minutes at room temperature and the pellet of washed cells was re-suspended in 3 ml of 10% or 20% complete medium, depending on the cell type (Section 2.3.4.1), in a 15 ml cell culture flask. This flask was

incubated upright at 37°C in a humidified CO_2 incubator until cell growth required the addition of further complete medium.

In the initial stages of this research suspensions of uveal melanoma cells (Section 2.3.4.4) were frozen as described above except that the freezing solution consisted of 0.25 M sucrose, 1.5 mM $MgCl_2$, and 10% glycerol (v/v) in 10 mM Hepes. Although this procedure was later discontinued in favour of the use of fresh cells (for reasons discussed in Section 3.3), material stored in this way was used for the preparation of antigens for SDS-PAGE and immunoblotting (Section 2.11).

2.3.4.3 Cell viability and cell count

Reagents

i. Trypan Blue Dye Solution: One part of 1% (w/v) trypan blue dye solution was mixed with four parts of 1% (w/v) NaCl. Immediately before use this solution was diluted with PBS to produce a 0.1% solution.

ii. Ammonium chloride solution: A 0.9% solution (w/v) of ammonium chloride in distilled water was stored at -20°C in 300 µl aliquots.

Procedure

The trypan blue exclusion test was sometimes used to determine cell viability (discussed by Adams 1980). An aliquot of the cell suspension was mixed with an equal volume of the dye solution and the respective numbers of live and dead cells were counted with a Neubauer

Haemocytometer. The mean of two measurements was taken as the final result. If the presence of red blood cells interfered with the count these cells were lysed by incubating a 50 µl aliquot of the cell suspension with 300 µl ammonium chloride solution for five minutes at room temperature.

2.3.4.4 Preparation of melanoma cell suspension

Using the plunger of a disposable 10 ml syringe, the tumour tissue was passed through a stainless steel wire mesh into a 6 cm petri dish containing RPMI. A sample of the cell suspension was counted with a Neubauer Haemocytometer so that the required cell concentration could be achieved by the addition of an appropriate amount of RPMI. Before this dilution was made the integrity of the cells was determined by examining a parallel sample by phase contrast microscopy.

2.3.4.5 Preparation of spleen lymphocyte suspension

The animal spleen was placed in a small volume of RPMI in a 6 cm petri dish and any fat or fibrous tissue was removed. The tissues were teased apart into minute fragments using two sterile disposable 21-G needles. Remaining cell clumps were disaggregated by aspirating the suspension successively two or three times through 21-G and 25-G needles. The cells were transferred to a universal container and washed twice by centrifugation at 500xg for five minutes at room temperature. The rat and

mouse spleens were assumed to contain $2x10^8$ and $1x10^8$ cells respectively. If the size of the spleen seemed abnormal, a 50 µl aliquot of the cell suspension was counted (Section 2.3.4.3).

2.3.4.6 Preparation of human lymphocyte suspension

B and T lymphocytes were extracted from peripheral human blood as described by Kaplan and Clark (1974).

Reagents

i. 2-Aminoethylisothiouronium bromide hydrobromide (AET) solution: 102 mg AET dissolved in 9 ml distilled water, adjusted to pH 9.0, and filter sterilised

ii. AET-sheep red blood cells: 20 ml 50% sheep RBC's in Alsever's solution were centrifuged at 300xg for five minutes at room temperature. A 1 ml volume of packed cells was transferred to a fresh universal container and washed twice by centrifugation in 20 ml RPMI at 300xg for five minutes at room temperature. The pellet was resuspended in 4 ml AET solution and incubated at 37°C for 20 minutes. After washing by two further centrifugations in 20 ml volumes of RPMI (300xg for five minutes at room temperature) the cells were resuspended in 9 ml RPMI. Immediately before use this solution was diluted 1:5 in RPMI to obtain a 2% solution.

Procedure

Ten to 20 ml of blood was removed from the antecubital vein and transferred without delay to the laboratory in a lithium/heparin container. The sample of

whole blood was added to an equal volume of 2% FCS in RPMI and this mixture was layered over an equal volume of Ficoll-Paque. Centrifugation (300xg for 15 minutes at room temperature) sedimented the white cells onto the fluid interface so that they could be collected with a pipette. These cells were then washed by centrifugation in 10-15 ml RPMI at 200xg for ten minutes at room temperature, resuspended in 5 ml RPMI and counted. If T and/or B lymphocytes were required separately, the T lymphocytes were extracted from the B cells and monocytes by E rosetting with AET-treated sheep red blood cells.

The lymphocytes were suspended in RPMI at a concentration of $1x10^7$ cells/ml. Equal volumes of 2% AET-treated sheep red blood cells in RPMI and FCS were then added, in this sequence, to the lymphocyte suspension. The mixture was centrifuged at 200xg for ten minutes at room temperature and incubated in an ice bath for 90 minutes. The pellet was then re-suspended in its supernatant by gently rocking the test tube and a small sample was examined under the microscope to ensure that rosettes had formed. The whole sample was layered onto an equal volume of 70% v/v Percoll in RPMI and the mixture centrifuged at 500xg for 15 minutes at room temperature. The B cells were harvested from the fluid interface. If T cells were required they were collected from the pellet at the bottom of the tube after removal of the remainder of the Percoll: a few drops of distilled water were added to the pellet for five seconds in order to lyse the red blood cells and then the container was immediately filled

with RPMI. The B and T cells respectively in RPMI were washed by centrifugation at 500xg for five minutes at room temperature, suspended in 5 ml RPMI and counted. They were then adjusted to a suitable concentration in RPMI or 20% complete medium depending on their intended use (Sections 2.3.5.1 and 2.5.2).

2.3.5 Enzyme-Linked Immunosorbent Assay (ELISA)

This assay, developed by Engvall and Perlmann in 1971, was performed essentially as described by Heusser, Stocker & Gisler (1981).

2.3.5.1 Preparation of 'wet' ELISA plates

Reagents

i) 0.01% (w/v) poly-L-lysine in PBS.

ii) 0.25% (v/v) glutaraldehyde in PBS

iii) PBS with and without Tween 20, 0.05%, v/v.

iv) Storage medium: 5% BSA (w/v), 0.01% sodium azide (w/v), 0.75% glycine (w/v) and 1% heat-inactivated normal goat serum (v/v) in PBS.

v) Blocking solution: 3% BSA and 1% heat-inactivated normal goat serum in PBS

Procedure

Flat-bottomed, 96-well microtitre plates were rinsed briefly in warm distilled water and incubated with poly-L-lysine solution (100 µl/well) for one hour. The plates were then washed with PBS and 10^4 fresh primary uveal melanoma cells or control cells (Sections 2.3.4.4 and 2.3.4.6) in 100 µl PBS were dispensed to all wells except for the wells in row 1 of the plate which were left

empty in order to calibrate the spectrophotometer. The plates were then centrifuged at 500xg for five minutes at room temperature and treated with 100 µl of glutaraldehyde solution for three minutes. After washing with PBS, 300 µl of storage solution were added to each well. The plates were wrapped in cling-film and stored at 4°C until use.

2.3.5.2 Preparation of 'dry' ELISA plates

This method was performed as described by Effros et al 1985.

Flat-bottomed, 96 well microtitre plates were rinsed briefly in warm distilled water. $1x10^4$ cells in 100 µl PBS were dispensed to all wells except for the wells in row 1 of the plate which were left empty in order to calibrate the spectrophotometer. The plates were incubated in air at 37°C until they had dried completely, then wrapped in cling-film, and stored at 4°C until use.

2.3.5.3 Routine screening ELISA

Reagents

i. Diluting solution: 0.5% BSA (w/v) in PBS-Tween 20.

ii. Second antibody: rabbit anti-rat IgG (H+L) conjugated to horseradish peroxidase diluted 1/1000 in diluting solution.

iii. McIlvaine's Citrate Phosphate Buffer Stock solution

A: Citric acid (0.1 M)

Stock solution B: $Na_2HPO_4.2H_2O$ (0.2 M) Working diluent: 17.9 ml of citric acid and 32.1 ml of Na_2HPO_4 were mixed and the volume made up to 100 ml to give a solution of pH 6.0.

iv. 0.1% (v/v) Hydrogen peroxide in distilled water

vi) Substrate: 0.04% O-phenylene diamine (w/v) in 0.05 M sodium citrate 0.15 M sodium phosphate buffer pH 6 containing 0.01% H_2O_2 (v/v).

Procedure

In early experiments, shortly before the assay was to be performed, storage medium was discarded and replaced with a blocking solution for 4-12 hours at 4°C. Latterly, this replacement with blocking medium was discontinued because it was no longer considered necessary. After washing the plates with PBS-Tween, the wells were incubated with 100 µl volumes of hybridoma supernatant or doubling dilutions of serum in diluting solution overnight at 4°C. After further washing, 100 µl aliquots of horseradish peroxidase-labelled second antibody were added to all relevant wells for 30 minutes at room temperature. The wells were washed with PBS-Tween and incubated in darkness for 30 minutes with 100 µl volumes of freshly prepared substrate mixture. The reaction was arrested by the addition of 50 µl of 4 N H_2SO_4 to each well and the light absorbance was monitored at 492 nm on a Titretek Multiskan spectrophotometer.

Control specimens were used routinely, subject to availability. Depending on the type of fluid tested, positive controls consisted of immune rat sera or known anti-melanoma monoclonal antibodies, and negative controls consisted of pre-immune rat sera or tissue culture medium. A positive result was judged to be greater than two Standard Deviations above the mean of the negative control values or, if such a calculation was not appropriate, more than 120% of the greatest control value.

2.3.5.4 Competition ELISA

The procedure was performed as described in Section 2.3.5.3 except that prior to the addition of the monoclonal antibody the wells were incubated with serial doubling dilutions of patient or control human serum in dilution buffer for 24 hours at 4°C and washed with PBS-Tween.

2.3.6 Immunohistochemistry

The immunohistochemistry was performed in a conventional manner (Sternberger 1979).

Reagents

i. Wash buffer: 2% FCS in RPMI

ii. Blocking solution: 1% normal goat serum (v/v) in PBS

iii. Diluting medium: 10% FCS in RPMI

iv. Monoclonal antibody: neat concentrated antibody, 1/2, 1/4 and 1/8 dilutions in diluting medium

v. Second antibody: unfrozen horseradish peroxidase

linked rabbit anti-rat IgG (H&L) antibody, diluted 1:50 in diluting medium

vi. Diamino benzidine (DAB) substrate: 100 mg DAB dissolved in 500 ml of 0.066% stock H_2O_2 (v/v) in PBS, prepared in a fume cupboard immediately before use and kept away from bright light

vii. Veronal acetate buffer, pH 5.0

Stock solution A: prepared by dissolving 1.94 g sodium acetate trihydrate and 2.94 g sodium barbitone in 100 ml distilled water to produce a 0.142 M solution

Stock solution B: prepared by adding 0.85 ml 0.1 M HCl to 100 ml distilled water The incubating solution was prepared by mixing 9 ml stock solution A to stock solution B.

viii. 3-amino-9-ethyl carbazole (AEC) substrate: 0.5 ml of 0.4% stock AEC added to 9.5 ml 0.05 M acetate buffer, pH 5.0, containing 10 µl 30% H_2O_2 and filtered

ix. Histomount or Hydromount for DAB or AEC slides respectively

2.3.6.1 Preparation of frozen sections

The tissue was collected and stored as described in Section 2.3.2.1. On removal from storage, it was embedded in 7.5% gelatin in 0.15 M NaCl and snap frozen in isopentane cooled in liquid nitrogen. Cryostat sections were sectioned in a conventional cryostat at 5-10 µm and picked up on gelatin-prepared glass slides. If these were to be used within 24 hours of preparation they were stored in a dry atmosphere, that is, in a

desiccator or covered by tissue paper and aluminium foil. If the sections were to be used at a later date they were prefixed in 100% acetone at -20°C for five seconds and dried in air before being stored at -20°C in sealed containers. Immediately before use the sections were fixed in 100% acetone at room temperature for three minutes and then dried in air at room temperature for 30 seconds.

2.3.6.2. Preparation of paraffin sections

Fresh tissue was collected as described in Section 2.3.2.1, fixed in buffered formalin and embedded in paraffin. Blocks were sectioned at 4-5 μm with a conventional microtome and mounted on freshly polished slides which had been degreased in 99% alcohol for at least 24 hours or which had been coated with 0.1% poly-L-lysine. The sections were dried by incubation in air at 37°C overnight and then at 56-60°C for 15 minutes. Paraffin sections were de-waxed by immersion in xylene for five minutes followed by immersion in two changes of absolute ethanol for four minutes. They were then stored in distilled water until use.

2.3.6.3 Staining of sections

After a brief rinse with wash buffer, sections were blocked with normal goat serum for 30 minutes. Excess blocking solution was shaken off the slides which were then incubated with 50 μl rat monoclonal antibody dilutions in a moist chamber for one to two hours at room temperature. The sections were given a further rinse

with wash buffer and incubated with second antibody overnight at room temperature in a moist chamber. After a brief rinse in wash buffer or acetate buffer, depending on whether the substrate was DAB or AEC respectively, the sections were incubated in the substrate solution for five to ten minutes. The slides were rinsed in water for a few seconds and counterstained lightly with haematoxylin. Finally the slides were mounted with glass coverslips using the appropriate mounting medium.

2.3.7 Immunofluorescence Assay

Reagents

i) 0.01% (w/v) poly-L-lysine in PBS

ii) Blocking solution: 3% BSA and 1% heat-inactivated normal goat serum in PBS

iii) Diluting solution: 0.5% BSA (w/v) in PBS-Tween 20, RPMI or PBS

iv) Second antibody: rabbit anti-rat IgG (H+L) conjugated to FITC diluted 1/20 in diluting solution

v) Mounting fluid: $NaHCO_3$, 0.0715 g, and Na_2CO_3, 0.016 g, dissolved in 10 ml distilled water mixed with 90 ml glycerol. pH 8-9

vi) Hoechst stain (10 μg/ml) in diluting solution

Procedure

2.3.7.1 Multispot slides

Gelman Hawksley multispot microscope slides were washed with methanol and air dried. They were then incubated with a solution of poly-L-lysine for 40 minutes at room temperature, washed and dried. Ten microlitre aliquots of the cell suspension at a concentration of 10^5 cells/ml in PBS were applied to each spot and the slides were placed in a humidified chamber at room temperature. After 30 minutes, any surplus fluid containing unattached cells was removed with a pipette and 10 µl aliquots of blocking solution were dispensed onto each spot. The slides were placed in the humidified chamber for 30 minutes and washed. They were then immersed in absolute alcohol for two minutes, dried in air and stored at -70°C until use.

After rinsing the slides with PBS-Tween, 10 µl aliquots of dilutions of serum or monoclonal antibody in PBS were added to all spots and the slides were incubated in a humidified chamber at room temperature for 30 minutes. The slides were then rinsed with PBS and incubated with 10 µl/spot aliquots of a 1:100 dilution of FITC-conjugated rabbit anti-rat IgG (H+L) antibody in diluting solution for 30 minutes in a humidified chamber at room temperature. After a final wash with PBS the slides were covered with glass coverslips mounted in buffered glycerol and examined with a Leitz Orthoplan fluorescent microscope. Negative controls consisted of

unimmunised rat serum or cell culture medium. Positive controls consisted of anti-melanoma monoclonal antibody, when this was available, or pre-immune serum.

2.3.7.2 Tissue culture chamber/slides

Cells were cultured in 8-well tissue culture chamber/slides until they had almost formed a confluent monolayer. When the assay was performed using live cells, the supernatant was removed and replaced by filter-sterilised monoclonal antibody in RPMI. After overnight incubation at 37°C, the wells were washed with RPMI and incubated with FITC labelled rabbit anti-rat IgG (H&L) for one hour at 37°C. When the assay was performed using fixed cells, the slides were detached from the culture chamber and immersed in acetone or absolute alcohol for two minutes. The overnight incubation with monoclonal antibody in PBS was performed at 4°C. After a further wash, the cells were incubated with Hoechst stain for 20 minutes at 37°C. The slides were given a final wash, mounted and examined alternately by fluorescence microscopy and phase contrast microscopy. Positive and negative controls consisted of immunised rat serum and 20% complete medium respectively.

2.4 Production of rat-rat hybrid myelomas

2.4.1 Animal immunisation

2.4.1.1 Inoculation

A 1-2 ml cell suspension containing $3\text{-}10\times10^6$ cells was prepared (Section 2.3.4.4) from uveal melanoma tissue which was either freshly excised or which had been stored

in liquid nitrogen (Section 2.3.2.1). This suspension was drawn up into an appropriate syringe and injected subcutaneously, intraperitoneally or intravenously (Section 2.3.1).

2.4.1.2 Immunisation schedule

Animals were inoculated intraperitoneally with 2-5 x 10^6 uveal melanoma cells from one patient and boosted, about 4-8 weeks later, with intraperitoneal injections of similar numbers of cells taken either from the same patient or from different individuals depending on the type of antigen that was being sought. The periods between injections were determined by the availability of fresh tissue, when this was used, or by the timing of fusions in relation to other aspects of this research.

The strength of the animals' immune response was determined four days after the latest boost by ELISA (Section 2.3.5.1) on blood samples taken from the tail vein (Section 2.3.1.6). When an adequate immune response was demonstrated, (ie, a titre of 1/1000 or greater), a further boosting dose was given, intravenously if possible, and fusion was performed three to four days later (Section 2.4.3).

2.4.2 Preparation for fusion

2.4.2.1 Preparation of myeloma cell suspension

The myeloma cells to be used for the fusion were grown in 10% complete medium in a spinner culture flask equilibrated with 5% CO_2 and maintained in a 37°C water

bath equipped with a magnetic stirrer. On the day of the intended fusion, the trypan blue exclusion test was performed on a sample of the myeloma cells to ensure that more than 95% were viable (Section 2.3.4.3) and to determine the concentration of the cell suspension. An appropriate volume of myeloma cells was removed from the spinner culture flask and washed twice by centrifugation in RPMI (500xg for five minutes at 24°C).

2.4.2.2 Preparation of spleen cell suspension

The immunised rat was killed (Section 2.3.1.7) and a suspension of spleen cells was prepared (Section 2.3.4.5). The cells were counted and washed twice by centrifugation in RPMI (500xg for five minutes at 24°C).

2.4.2.3 Preparation of cell-culture plates

For some fusions the plates were coated with a spleen cell feeder layer. A spleen cell suspension was prepared (Section 2.3.4.5) in 20% complete medium at a concentration of 10^5 cells/ml and 1 ml aliquots of this suspension were dispensed into all wells of four 24-well cell culture plates. If feeder cells were not used the wells were filled with 1 ml aliquots of 20% HAT-complete medium.

2.4.2.4 Preparation of polyethylene glycol (PEG) soln.

Polyethylene glycol, 50% (w/v) in RPMI, was prepared as described in Section 2.2.1.13. In some fusions 300 µl DMSO was added to this mixture to prepare a 15% solution (Norwood 1976).

2.4.3 Fusion protocol

Fusion between spleen and myeloma cells was performed according to Köhler & Milstein (1975) with minor modifications (Campbell 1984).

Aliquots of spleen and myeloma cells, in 0.5 ml aliquots, containing $1x10^6$ and $0.5 x10^6$ cells respectively were put aside for use as controls. The remaining spleen and myeloma cells in RPMI were mixed at a ratio of 2:1 and centrifuged at 500xg for five minutes at 24°C. The supernatant was discarded and the pellet was dispersed by gentle tapping of the container. Two millilitres of the 50% PEG was added to the pellet over a period of 30 seconds during gentle shaking of the suspension (Gefter, Margulies & Scharff 1977). The pellet was then re-suspended for 30 seconds and incubated at room temperature (Fazekas de St. Groth & Scheidegger 1980) for another 30 seconds. Five millilitres of RPMI were then added to the suspension over a period of 90 seconds and a further 5 ml of RPMI over a few seconds. The mixture was incubated at room temperature for two to three minutes and centrifuged at 500xg for five minutes.

The cells were then gently re-suspended in a 10 ml volume of 20% complete medium. This cell suspension was then dispensed in 50 µl aliquots into all wells of the cell-culture plates. Similar aliquots of unfused myeloma cells and splenic lymphocytes were transferred to one well of each plate to serve as controls. The plates were incubated as described previously (Section 2.3.4.1).

2.4.4 Culture of hybridomas

One week after the fusion, and again when the first hybrids appeared, 0.5 ml of the supernatant was gently removed from each well, without disturbing the cells, and replaced with the same volume of fresh 20% HAT-complete medium (Section 2.2.1.9). Latterly, rat spleen feeder cells (Section 2.3.4.5) were also included in this medium. The addition of aminopterin to the culture medium was discontinued when all the control Y3 cells had died and hypoxanthine and thymidine were discontinued a week or two later.

When hybridomas showed confluent growth the supernatants were tested for the presence of anti-melanoma antibodies by ELISA (Section 2.3.5). Aliquots of 100 µl were removed from the supernatant for this purpose and these were replaced by 100 µl volumes of 20% complete medium. Positive hybridomas were expanded and subcloned.

2.4.5 Subcloning of hybridomas

A spleen feeder cell suspension was prepared (Section 2.3.4.5) in 20% complete medium at a concentration of 10^5 cells/ml and 100 µl aliquots were dispensed into all wells of an appropriate number of 96-well cell culture plates. One to three plates were usually prepared for each subcloning procedure.

The hybrid cells were suspended in a 20 ml volume of 20% complete medium at a concentration of 10 cells/ml. Two serial doubling dilutions were performed to obtain three 10 ml cell suspensions with concentrations of 10, 5

and 2.5 cells/ml respectively. The cell suspensions were dispensed in 100 µl aliquots to all wells of the three plates so that these contained 1.0, 0.5 and 0.25 cells/well respectively. The plates were incubated as described previously (Section 2.3.4.1).

When subclones showed confluent growth their supernatants were tested for antibody secretion by ELISA (Section 2.3.5). Positive clones from the lowest dilution of cells were expanded in flasks and subcloned for the second and third time by the same procedure. The hybrid colonies established after the third cycle of limiting dilution of cloning were regarded as monoclonal. These were propagated in spinner culture flasks in serum-free RPMI for concentration of the monoclonal antibody. (Section 2.8).

2.4.6 Protection of hybrids

As soon as possible after each subcloning procedure surplus hybrid cells were stored in liquid nitrogen as a precautionary measure (Section 2.3.4.2). Cells were also maintained in culture until it was demonstrated that a sample of cells could be re-established in culture following storage in liquid nitrogen.

2.5 EBV-transformation of peripheral B lymphocytes

The methods used in this study have been described previously (Steinitz et al 1979).

2.5.1 Preparation of Epstein-Barr Virus Stock

B95-8 Cells were grown at $1x10^6$ cells/ml in 5% complete medium. The cells were then grown at a concentration of $0.2x10^6$ cells/ml in 2% complete medium and incubated at 33°C for two weeks. The cell suspension was then centrifuged at 500xg at room temperature for five minutes. After Millipore sterilisation, the supernatant was dispensed into cryotubes in 1 ml aliquots and stored in liquid nitrogen until required.

2.5.2 B Lymphocyte transformation

B Lymphocytes prepared from a 20 ml human venous blood sample (Section 2.3.4.6) were centrifuged at 500xg for five minutes at room temperature and re-suspended, at a concentration of approximately 10^7 cells/ml in a 1 ml volume of Epstein-Barr virus suspension (Section 2.5.1). After a one hour incubation at 37°C the cells were washed by centrifugation at 500xg for five minutes and re-suspended in 10 ml 20% complete medium. This suspension was dispensed in 100 µl aliquots to all wells of a 96-well plate except for the most peripheral wells. These were filled with RPMI in order to reduce evaporation of fluid from the wells containing the lymphocytes. The cells were expanded by incubation at 37°C and fed with fresh 20% complete medium at weekly intervals. When the B lymphocytes were transformed for

assay purposes, the B cells were not extracted by rosetting with AET-treated sheep red blood cells, as described in Section 2.3.4.6. Instead, cyclosporin A was added to the 20% complete medium at a concentration of 1 µg/ml in order to inhibit T cell activity (Shevach 1985) so that suppression of B cell growth would be prevented (Bird et al 1981).

2.6 Production of human-human hybrid myelomas

The methods used in this study have been described by other workers (Kozbor & Roder 1981; Kozbor, Lagarde & Roder 1982; Kozbor & Roder 1983).

2.6.1 Preparation for fusion

2.6.1.1 Preparation of KR-4 cells

KR-4 Cells were maintained in 10% complete medium with ouabain and thioguanine (Section 2.2.1.12) for one week in each month of culture. This was done in order to destroy cells that had regained thymidine kinase or hypoxanthine phosphoribosyl transferase activity (Section 1.4).

2.6.1.2 Preparation of EBV-transformed lymphocytes

EBV Transformed cells (Section 2.5.2), grown in 20% complete medium in 96-well plates, were tested for the production of anti-melanoma antibodies by ELISA (Section 2.3.5). Positive transformants were expanded, either separately or pooled, until they were sufficiently numerous for fusion.

2.6.1.3 Preparation of feeder cells

A 10 ml rat or mouse spleen cell suspension was prepared (Section 2.3.4.5) in 20% complete medium at a concentration of $2-5x10^6$ cells/ml.

2.6.1.4 Preparation of polyethylene glycol solution

Polyethylene glycol, 50% (w/v) in RPMI, was prepared as described in Section 2.2.1.13.

2.6.2 Fusion protocol

The KR-4 and EBV-transformed cell suspensions, each containing $10x10^6$ cells, were collected and centrifuged at 500xg for five minutes at room temperature. The pellets were each re-suspended in 10 ml volumes of RPMI, washed by a further centrifugation at 500xg for five minutes at room temperature, and re-suspended in 10 ml volumes of RPMI. After taking 0.5 ml samples for use as controls, the two cell suspensions were then mixed together in a 20 ml universal container and centrifuged at 500xg for five minutes at room temperature. The pellet was dispersed by tapping the container and 0.5-0.8 ml of warm PEG solution was added over a one minute period, while stirring gently. Further gentle stirring was performed for a further 90 seconds before adding 10 ml of RPMI, at 37°C slowly, with gentle stirring, over a period of six to ten minutes. The cells were then incubated undisturbed at 37°C for 20 minutes, centrifuged at 500xg for five minutes at room temperature and re-suspended in 20 ml RPMI. After further centrifugation, performed as already described, the cells were re-suspended in the

feeder cell suspension (Section 2.6.1.3) and dispensed in 100 µl aliquots into a 96-well cell culture plate. Aliquots of control cells were also added to this plate which was incubated at 37°C.

2.6.3 Culture of hybridomas

One day after fusion 100 µl volumes of 20% complete medium with 2xHAT (Section of 2.2.1.9) were added to each well. Two days later this was replaced by complete medium with 1xHAT and 5% ouabain (v/v). The ouabain and the HAT were discontinued two and three weeks after the fusion respectively.

Hybridomas were first detected 7-12 days after fusion by which time the parental cells had usually died.

2.6.4 Subcloning of hybridomas

Hybridomas were subcloned as described in Section 2.4.5.

2.6.5 Protection of hybridomas from loss

Loss of hybridomas was prevented as described in Section 2.4.6.

2.7 Ouchterlony Assay for antibody class determination

The determination of the class of each monoclonal antibody produced was performed as described by Ouchterlony & Nilsson (1978).

2.7.1 Precipitation

Solution

Agarose, 1% (w/v), in PBS-azide.

Procedure

Glass microscope slides were coated with 3.5 ml of agar solution which was allowed to cool and solidify. A sexagonal array of wells, each 1.5 mm in diameter, was created by trephining rings in the agar with a cork borer and removing the plugs with a fine pipette. Serial doubling dilutions of rabbit anti-rat mu and gamma heavy chains as well as of the monoclonal antibody under investigation were prepared in PBS-azide and 10 µl aliquots were placed in the peripheral wells in order of concentration. The rat immunoglobulin class-specific antisera were pipetted as neat in the centre wells in 10 µl aliquots. Monoclonal antibodies used as neat were ten-fold concentrations of hybridoma culture supernatant (Section 2.8). The slides were incubated in a moist chamber at 4°C for two to three days to allow the precipitation lines to develop.

2.7.2 Staining of precipitation lines

Reagents

i) Wash solution: 5% sodium citrate (w/v) in distilled water

ii) Staining solution: 0.025% w/v Coomassie brilliant blue R in methanol:water:acetic acid 50:45:5 by volume

iii) Destaining solution: Water:acetic acid:methanol 87:8:5 by volume

Procedure

The slides were immersed in the wash solution for one hour and then in five changes of PBS-azide over 24 hours. They were dried by blotting with filter paper for one hour and incubated in air at room temperature overnight. The slides were next immersed in the staining solution for five minutes and then in the destaining solution until the background was clear. After a final wash with distilled water the slides were allowed to dry.

2.8 Concentration of antibodies

Hybridoma cells were grown in 20% or 5% complete medium, or RPMI, in spinner flasks to saturation and then removed by centrifugation at 500xg for five minutes. The supernatant was transferred to Corex tubes of the appropriate size and cooled to 4°C in an ice bath. The amount of ammonium sulphate necessary to produce a 50% saturated solution of this salt was calculated, using a nomogram (Dixon 1953), and added to the stirred supernatant over a period of ten minutes. The precipitated antibody was pelleted by centrifugation at 500xg for ten minutes at 4°C. This pellet was re-suspended in a volume of PBS which was one tenth of the volume of the hybridoma supernatant. The solution was dialysed against 100 volumes of PBS-azide at 4°C for 48-72 hours to remove traces of ammonium sulphate. The sample was then collected and stored at -20°C.

2.9 Protein estimation by Bradford's method

Reagents

i) Stock solution A. 100 ml Phosphoric acid, 85%(w/v), in distilled water

ii) Stock solution B. 100 mg Coomassie Brilliant Blue G-250 in 50 ml of 95% ethanol

iii) BSA, 1 mg/ml in distilled water

iv) Diluting buffer: 0.1% sodium dodecyl sulphate (SDS)

Procedure

Fresh Bradford's reagent (Bradford 1976) was prepared immediately before use by mixing 10 ml of stock solution A with 5 ml of stock solution B, making up to 100 ml with distilled water, and filtering. A duplicate series of tubes containing 10, 20, 30, 40 and 50 µg BSA/100 µl were set up. Aliquots of Bradford's reagent, 0.3 ml in volume, were dispensed into each tube, mixed thoroughly, and incubated for ten minutes at room temperature. The light absorbance was measured at 595 nm using a spectrophotometer and a standard curve was drawn.

Samples of concentrated hybridoma supernatants were treated in the same way and their protein content was determined using the standard curve.

2.10 Conjugation of markers to monoclonal antibody

2.10.1 Conjugation of peroxidase

Reagents

i. 0.1 M Sodium phosphate buffer, pH 7.0

Stock solution A: 0.1 M NaH_2PO_4 in distilled water

Stock solution B: 0.1 M Na_2HPO_4 in distilled water

Stock solution A was added to 50 ml stock solution B until the pH was 7.0.

ii. 0.02% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.0: 5 µl 25% glutaraldehyde was added to 25 ml 0.1 M sodium phosphate buffer, pH7.0.

iii. Horseradish peroxidase: 5 mg of peroxidase dissolved in 0.5 ml of 0.1 M sodium phosphate

iv Immunoglobulin solution: 10x concentration of hybridoma supernatant (Section 2.8)

Procedure

A 30 cm x 1.5 cm column was assembled according to the manufacturer's instructions, with the end of the inflow tubing immersed in the 0.1 M sodium phosphate buffer, pH 7.0, and with the outflow tube passing through a peristaltic pump on its way to a fraction collector. The column was filled with buffer to which sepharose B gel was then added at room temperature. When the gel had settled it was packed by running through at least two column volumes of buffer at a rate of about 20 ml/hour.

Peroxidase solution, 0.5 ml, was added to 1 ml immunoglobulin solution which was then added to 1 ml glutaraldehyde solution. After stirring the mixture continuously for three hours at room temperature the sample was purified by gel filtration as described in literature provided by the supplier of the gel. The column was run at approximately 0.8 ml/min using PBS as the elution buffer. Two millilitre fractions were collected, transferred in turn to a quartz cuvette and their light absorbance monitored at 280 nm.

The efficiency of this procedure was monitored by ELISA. Two 100 µl aliquots from each fraction were dispensed into different wells of an ELISA plate coated with melanoma cells. The ELISA was performed as described in Section 2.3.5 omitting the addition of the horseradish peroxidase linked second antibody to one well of each pair of wells.

2.10.2 Conjugation of fluorochrome

Reagents

i. 0.25 M, carbonate buffer, pH 9.0

ii. Fluorescein isothiocyanate (FITC)

iii. PD-10, Sephadex G-25 M column

Procedure

Hybridoma supernatant was concentrated as described in Section 2.8 and applied to a Sepharose 6B column as described in Section 2.10.1. The fractions containing the highest concentrations of the monoclonal antibody were identified by ELISA (Section 2.3.5) and pooled. The protein concentration of this solution was estimated by

means of the Bradford Assay (Section 2.9). FITC, 0.05 mg/mg of protein in the monoclonal antibody solution, was added to 0.5 ml carbonate-bicarbonate solution and 2 ml of the monoclonal antibody. After mixing for 150 minutes at room temperature, the solution was applied to a PD-10 column containing Sephadex G-25M, according to the manufacturer's instructions (Pharmacia), using PBS as the equilibration buffer. Fractions of 1 ml were collected and a 50 µl sample from each fraction was tested by ELISA (Section 2.3.5). The fractions containing the highest concentrations of FITC-conjugated monoclonal antibody were pooled and stored at 4°C until use.

2.11 Immunoelectrophoretic procedures

2.11.1 SDS-gel electrophoresis of melanoma antigens

2.11.1.1 Preparation of gels

The gels were prepared and run as described by Laemmli (1970).

Reagents

i. Resolving gel buffer: 1.5 M Tris-HCl pH 8.8

ii. 15% ammonium persulphate (w/v) in water

iii. Acrylamide-bisacrylamide, (30:0.8): 30 g of acrylamide and 0.8 g of bisacrylamide dissolved in 100 ml of water, filtered, and stored in a dark bottle at 4°C

iv. Resolving gel: 150 µl ammonium persulphate, 10 µl Temed, 10 ml resolving buffer, and 11.7 ml acrylamide solution dissolved in 18 ml distilled water

v. Stacking gel buffer: 0.5 M Tris-HCl pH 6.8

vi. Stacking gel: 30 µl ammonium persulphate, 10 µl Temed, 1.0 ml acrylamide solution and 2.5 ml stacking gel buffer in 6.5 ml distilled water

vii. Electrophoresis buffer pH 8.3: 10% SDS in 0.025 M-Tris buffer containing 192 mM glycine

viii. Sample buffer: 10% SDS (w/v), 50% sucrose (w/v), 0.005% bromphenol blue with or without 10%(v/v) 2-mercaptoethanol in 0.125 M Tris-HCl adjusted to pH 6.8

ix. Coomassie Blue Stain: 0.025% (w/v) Coomassie brilliant blue R in a solution of 50% (v/v) methanol 5% (v/v) acetic acid

x. Destaining solution: 7.5% (v/v) acetic acid and 5% (v/v) methanol in distilled water

Preparation of slab gels

The mould for the slab gel, 18 cm x 13 cm x 1.5 mm in size, was assembled as follows: Two glass plates separated by two plastic spacers were fastened together by metal bulldog clips to create a mould made watertight by inserting a lining of rubber tubing.

The resolving gel was poured into the mould and allowed to set. A straight upper edge was obtained by placing a small amount of isobutanol on the gel. After washing away the isobutanol with distilled water the stacking gel was poured into the mould and wells of the

appropriate size and number were created by inserting a suitable plastic comb into the stacking gel until this had set.

2.11.1.2 Preparation of melanoma cells for SDS-PAGE

Fresh or frozen uveal melanoma cells (Section 2.3.2.1) were homogenised using a mechanical homogeniser. The cell fragments were transferred to an Eppendorf tube and spun down in a microcentrifuge at 300xg at room temperature for five minutes. The supernatant was discarded and the pellet was re-suspended in sample buffer at a concentration of 2.5×10^6 cells/40 µl by repeated aspiration through 21-G and 25-G disposable needles. The sample was then immersed in boiling water for 100 seconds. On occasion this final step was deliberately omitted.

2.11.1.3 Preparation of cell membranes for SDS-PAGE

Cell membranes, prepared as described in Section 2.3.3 were suspended in appropriate volumes of sample buffer to achieve a protein concentration of approximately 1 mg/ml.

2.11.1.4 Preparation of cytoplasm/nucleus for SDS-PAGE

Preparations containing cell nuclei, cytoplasm and intracytoplasmic organelles were suspended in appropriate volumes of sample buffer to achieve a protein concentration of approximately 1 mg/ml.

2.11.1.5 Preparation of subretinal fluid for SDS-PAGE

The subretinal fluid was mixed with an equal volume of sample buffer in an eppendorf tube and heated by immersion in boiling water for 100 seconds.

2.11.1.6 Electrophoresis procedure

The mould was fitted to the electrophoresis apparatus and made watertight with petroleum jelly (Vaseline). The system was filled with fresh electrophoresis buffer and, using a micropipette, the wells were loaded with 10-40 µl aliquots of the samples being tested. Electrophoresis was performed at 45 mA, 200 V until the Bromphenol blue in the sample buffer had almost reached the end of the gel.

2.11.1.7 Coomassie blue stain

The gel was stained for protein by incubating first in 3-5 volumes of a Coomassie brilliant blue R solution overnight at room temperature and then in destaining solution until a clear background was obtained. The gel was dried in a commercial gel drier.

2.11.1.8 Silver stain

Silver staining of polyacrylamide gels was performed as described by Tsai and Frash (1982).

Reagents

i) Fixative: 40% ethanol (v/v) and 5% acetic acid (v/v) in distilled water

ii) Oxidising solution: 0.7% periodic acid (w/v) in fixative

iii) Staining solution: Ammonia 2 ml, NAOH 0.1 N 28 ml, 20% silver nitrate 5 ml in 115 ml distilled water

iv) Developing solution: 0.005% citric acid (v/v) and 100 µl of 37% formaldehyde in a litre of water

v) Preservative solution: 3% acetic acid in distilled water

Procedure

The gel was placed in the fixative overnight and then in the oxidising solution for five minutes with shaking. After three 15 minute washes in at least five changes of de-ionised water the gel was placed in freshly prepared staining solution for ten minutes with vigorous shaking. After three further ten minute washes in four or five changes of de-ionised water the gel was placed in developing solution for a few minutes until the background showed the first signs of discolouration and/or until the bands developed the desired intensity. Finally the gel was washed in water and stored in preservative solution until it was dried.

2.11.2 Electroblotting

Reagent

i) Transfer buffer: 0.19 M glycine, 20% (v/v) methanol and 0.02% SDS (w/v) in 25 mM Tris-base, pH 8.6.

Procedure

Proteins were electrophoretically transferred onto nitrocellulose paper according to the method described by Towbin and associates (1979) and modified by Burnette (1981) and Batteiger (1982).

A cartridge was loaded with a sandwich comprising the following layers respectively: plastic grid, Scotch-Brite pad, filter paper, gel, nitrocellulose paper, filter paper, Scotch-Brite pad and plastic grid. Care was taken to avoid air bubbles in the system.

This assembly was immersed vertically in the BIO-RAD Tank filled with transfer buffer so that the nitrocellulose paper was closest to the anode. Electrophoretic transfer was performed at 350-400 mA for 2.5-3 hours.

2.11.3 Immunoblotting

Reagents

i. Blocking Buffer: 0.5% v/v Tween-20, NaCl 0.15 M, in 20 mM Tris-HCl buffer, pH 7.2.

ii. ^{125}I-labelled Protein A (2.96 Bq/mg) was prepared by Dr A. M. Campbell in the Department of Biochemistry as follows: The internal surface of a glass vial was coated with iodogen by dissolving 1 mg of this substance in 0.5 ml of chloroform which was then allowed to evaporate. One milligram of protein A was dissolved in 0.5 ml of Tris buffer and transferred to the vial coated with the iodogen. The Na^{125}I was added to the vial and incubated at room temperature for 15 minutes with

intermittent shaking. The mixture was then applied to a 10 ml sephadex-G25M column equilibrated with the appropriate buffer. Fractions of 1 ml were collected and 10 µl of each fraction was counted on a LKB 1275 Minigamma counter. The fractions with the peak ^{125}I incorporation were pooled and stored in convenient volumes.

iii. Amido black stain: 0.1% amido black (v/v), 10% acetic acid (v/v), and 40% methanol (v/v) in distilled water

iv. Destaining solution: 5% acetic acid (v/v), 50% methanol (v/v) in distilled water

Procedure

Following the electrophoretic transfer of proteins from the gel to the nitrocellulose paper (Section 2.11.2), this paper was incubated first in blocking buffer at 4°C overnight, and then in a 1:10 dilution of monoclonal antibody in blocking buffer at room temperature for 90 minutes. After washing in five changes of blocking buffer at room temperature for 30 minutes the paper was incubated with a 1:100 dilution of rabbit anti-rat IgG (H+L) in blocking buffer for 90 minutes at room temperature. Further washing was performed as described above and the paper was incubated with iodinated protein A for 60 minutes at room temperature. The washing was repeated and the paper was blotted dry. Autoradiography was performed at -70°C for 16-72 hours. The exposed film (Kodak X-omat S) was developed and fixed according to the manufacturer's instructions: it was immersed in developer solution for five minutes then, after a one minute rinse

in water, immersed in fixative for one minute and then washed in water for at least 40 minutes. The nitrocellulose paper was incubated with amido black solution for five minutes, washed with water for one to two minutes and destained until a clear background was obtained.

Chapter 3

PRODUCTION OF RAT MONOCLONAL ANTIBODIES TO UVEAL MELANOMA ANTIGENS

3.1 Introduction

The strategy followed in the production of monoclonal antibodies to uveal melanomas is summarised as follows: rats were inoculated with unfixed and uncultured primary human uveal melanoma cells (Section 2.4.1) and when an adequate serum humoral immune response to these cells was demonstrated by ELISA (Section 2.3.5) the splenic lymphocytes of these rats were immortalised by fusion with rat myeloma cells (Section 2.4). Hybridomas secreting antibodies to antigens on primary human uveal melanoma cells were identified by ELISA and subcloned until they were monoclonal. The supernatants of these hybridomas were then concentrated by ammonium sulphate precipitation (Section 2.8) so that the monoclonal antibodies and the antigens with which they reacted could be further investigated.

3.2 Source of uveal melanoma tissue

The cells used for the preparation of ELISA plates and for the rat immunisations were obtained from samples of approximately 80 primary melanomas of the uveal tract.

3.3 Preparation of uveal melanoma cell suspensions

When the melanoma cell suspensions were washed and stored in the same fashion as myeloma cells (Section 2.3.4.5) most cells were severely damaged and as a result, in the early stages of this research, the

suspensions used for the rat inoculations and for the preparation of ELISA plates consisted almost entirely of cell nuclei and cytoplasmic debris (Fig. 3.1 i). Subsequently, when intact cells were required the following precautions were taken: (i) fresh unfrozen tissue was used, and (ii) disaggregation of the cell clumps by repeated aspiration through 21-G and 25-G needles, and washing of the cells by repeated centrifugation were omitted. With these measures, the preservation of the morphological integrity of the cells was usually satisfactory (Fig. 3.1 ii).

3.4 Enzyme-linked immunosorbent Assay

The ELISA was performed as described by Engvall & Perlmann (1971) with the modifications described by Heusser, Stocker & Gisler (1981) for the use of whole cells as antigens (Section 2.3.5.1). Preliminary investigations into the reproducibility of the Multiscan readings indicated that the ELISA results were accurate to the first decimal point.

3.5 Rat immunisation

An adequate humoral immune response in the immunised rats, consisting of an ELISA titre of at least 1/1000, was usually demonstrated after only two intraperitoneal inoculations of $2\text{-}5\times10^6$ cells per inoculation (Fig. 3.2).

Figure 3.1 Disruption of melanoma cells by centrifugation and cryopreservation. Electron micrographs of suspensions of melanoma cells (i) following washing by centrifugation and storage in liquid nitrogen and (ii) freshly prepared with minimal washing. Note disruption of membranes and cytoplasm in (i) and the normal architecture of the melanoma cells in (ii). (Magn. (i) and (ii), x 5000).

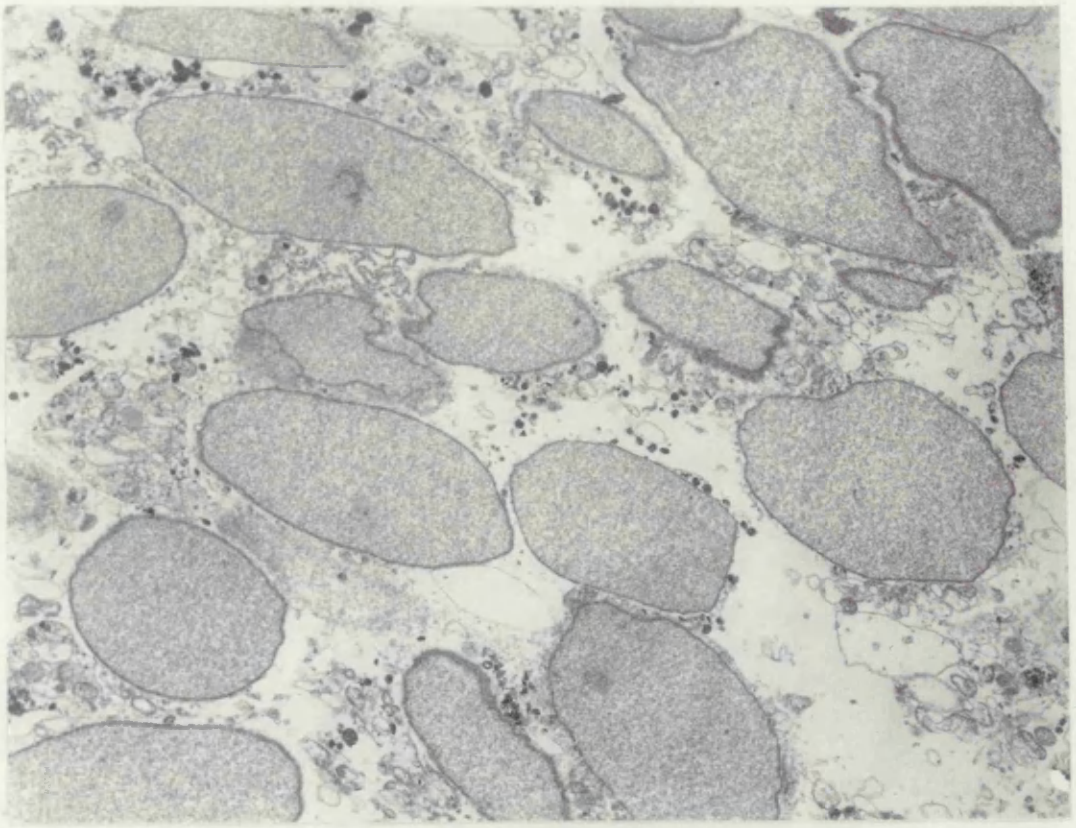


Fig. 3.1 (i)

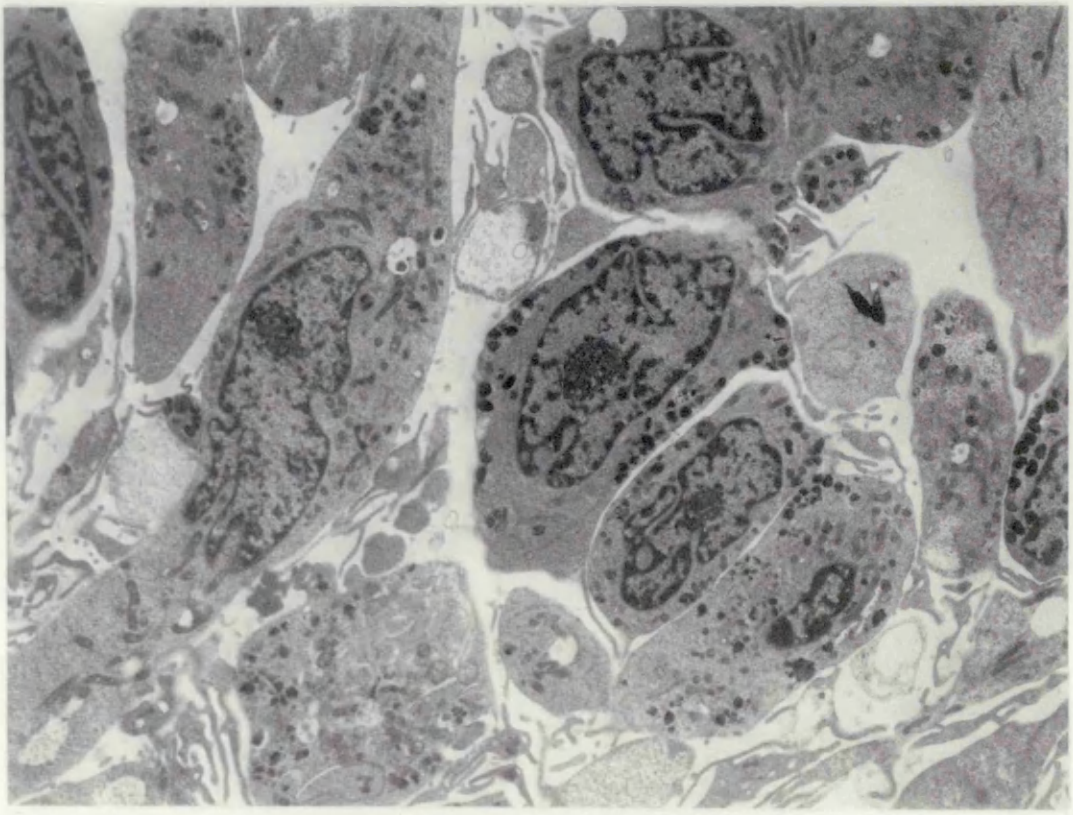


Fig. 3.1 (ii)

Figure 3.2 Rat immune response to melanoma cells. ELISA reactivity with uveal melanoma cells is greater with serum of the two immunised rats than of the unimmunised rat.

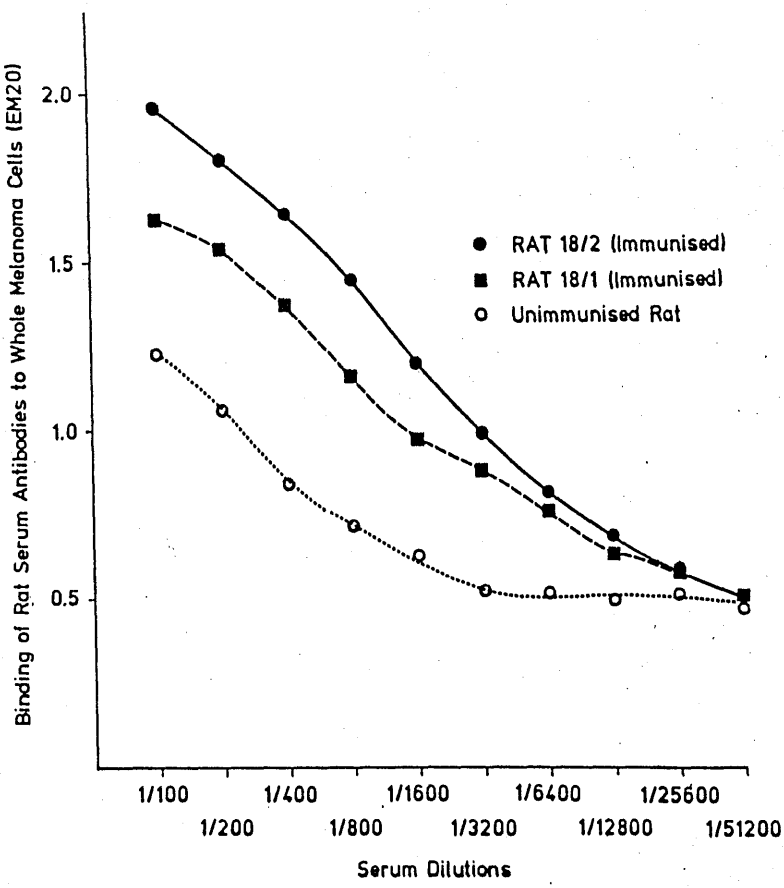

Binding of Rat Serum Antibodies to Whole Melanoma Cells (EM20)
2.0
1.5
1.0
0.5
RAT 18/2 (Immunised)
RAT 18/1 (Immunised)
Unimmunised Rat
1/100
1/200
1/400
1/800
1/1600
1/3200
1/6400
1/12800
1/25600
1/51200
Serum Dilutions

Fig. 3.2

3.6 Fusions

Eight fusions produced numerous hybrids, two produced few hybrids and two were completely sterile. A number of fusions were lost due to bacterial or fungal infection. The fusions which produced monoclonal antibodies were 18/2 and 53/1.

3.6.1 Fusion 18/2

Fusion 18/2 was performed using splenic lymphocytes from a rat which had been successively inoculated with cells from four different melanomas (Table 3.1). Of 37 primary hybrids, 16 reacted significantly with cells from one or more uveal and/or cutaneous melanomas. Six of these were subcloned to monoclonality and the remainder either became negative or were lost in culture. One of the monoclonal antibodies, 1D5, was not studied further because it was subsequently found to be unreactive against all uveal melanomas tested. A preliminary investigation of the sensitivity and specificitiy of the remaining five monoclonal antibodies was performed by ELISA. These were tested simultaneously against a panel of twelve different ocular melanomas, lymphocytes from four healthy volunteers and cells from a breast carcinoma. The results were expressed as a percentage ratio of the mean of two densitometer readings of the monoclonal antibody to four readings from the negative controls (ie, 20% complete medium in place of hybridoma supernatant) and were considered to be positive when they were greater than two standard deviations of the mean of the controls. The antibodies reacted to a different extent with each uveal

Table 3.1 Generation of mAb 4A3.

Event	Date	Tumour	Dose(Cells)
Rat immunisation (IP)	16.5.84	em18	$3x10^6$
Boost (IP)	6.6.84	em19	$3x10^6$
Boost (IP)	11.6.84	em20	$4x10^6$
ELISA	14.6.84	em20&21	
Boost (IP)	15.6.84	em21	$10x10^6$
Fusion 18/2	19.6.84		
ELISA	4.7.84		
ELISA	10.7.84	em19	
4A3 primary hybrid subcloned	24.7.84		
ELISA	17.8.84	em26	
9d (0.25 cells/well) subcloned	18.8.84		
ELISA	5.9.84	em26	
9f (0.25 cells/well) subcloned	13.9.84		
ELISA	24.9.84	em26	
10g (0.5 cells/well) Monoclonal antibody			

Table 3.1

melanoma (Fig. 3.3), whereas the reaction with the control cells showed less variation (Fig. 3.4). In each of the non-melanoma controls the reactivity of the monoclonal antibodies in decreasing order was 1B1, 1C4,4B4,1B4 and 4A3. It was therefore possible to test the relative significance of the binding of the monoclonal antibodies to the uveal melanoma cells by expressing the reactivity of each antibody to each melanoma as a percentage ratio of the mean of the control cells (Fig. 3.5). MAb 4A3 reacted significantly against all of the 12 uveal melanomas; mAb 4B4 against five melanomas; mAb 1B4 against four melanomas; mAb 1B1 against two melanomas and mAb 1C4 against only one melanoma.

3.6.2 Fusion 53/1

Fusion 53/1 was performed using splenic lymphocytes from a rat which had been inoculated three times with cells from the same tumour (ie, em53). Fifty-nine hybrids were produced. Eleven hybrids, which appeared to react the most strongly with melanoma cells on ELISA, were selected for subcloning. Hybridomas were subcloned as soon as possible and residual hybrid cells were then expanded and cryopreserved. When the hybridomas had been subcloned at least once they were cryopreserved in liquid nitrogen until it was possible to evaluate them further.

Five hybridomas produced by fusion 53/1, 3B5,1C2,4B4-2,2D3 and 2B6, were expanded in spinner culture flasks in serum-free medium and their antibodies concentrated by ammonium sulphate precipitation. These antibodies were tested by ELISA against three primary

Figure 3.3 ELISA reactivity of monoclonal antibodies with uveal melanomas (1-12). The results are expressed as a percentage ratio of the mean of two Multiskan readings of the monoclonal antibody to four readings from wells containing 20% complete medium. In each diagram, the line is drawn at the mean +2SD of four control values. Results above this are considered positive.

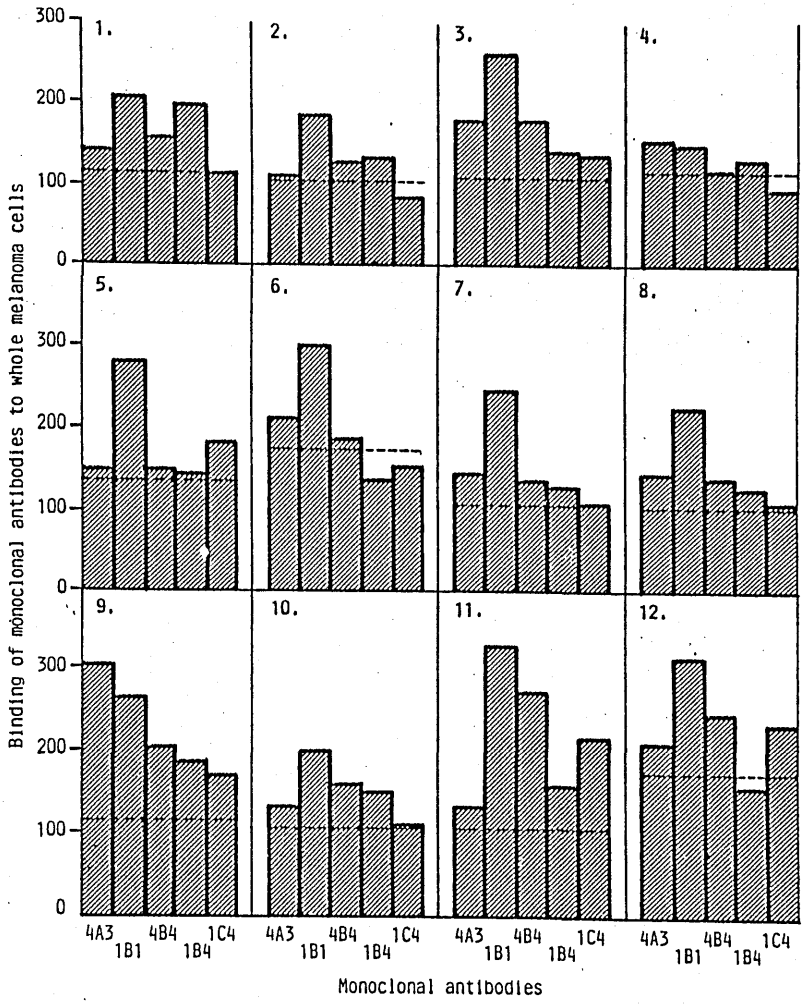

Binding of monoclonal antibodies to whole melanoma cells
300
200
100
0
1.
2.
3.
4.
5.
6.
7.
8.
9.
10.
11.
12.
4A3
1B1
4B4
1B4
1C4
Monoclonal antibodies

Fig. 3.3

Figure 3.4 ELISA reactivity of monoclonal antibodies with control cell preparations. A-E are data from lymphocytes from healthy volunteers and F is from cells of a breast carcinoma. The results are obtained and displayed as in Fig. 3.3.

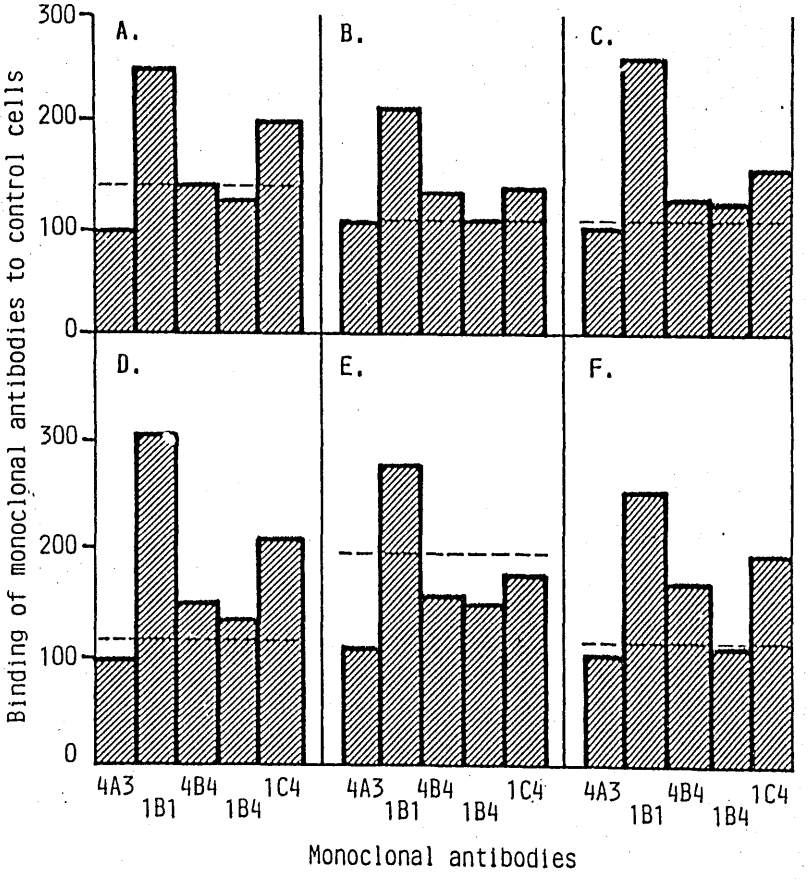

A.
B.
C.
D.
E.
F.
300
200
100
0
Binding of monoclonal antibodies to control cells
4A3
1B1
4B4
1B4
1C4
Monoclonal antibodies

Fig. 3.4

Figure 3.5 ELISA reactivity of monoclonal antibodies with 12 uveal melanomas relative to controls. For each antibody, the results are expressed as a ratio of the results displayed in Fig. 3.3 to the mean of those in Fig. 3.4. In each diagram, the dotted line is drawn at the mean +2 SD of the six control values. Results above this are considered significant.

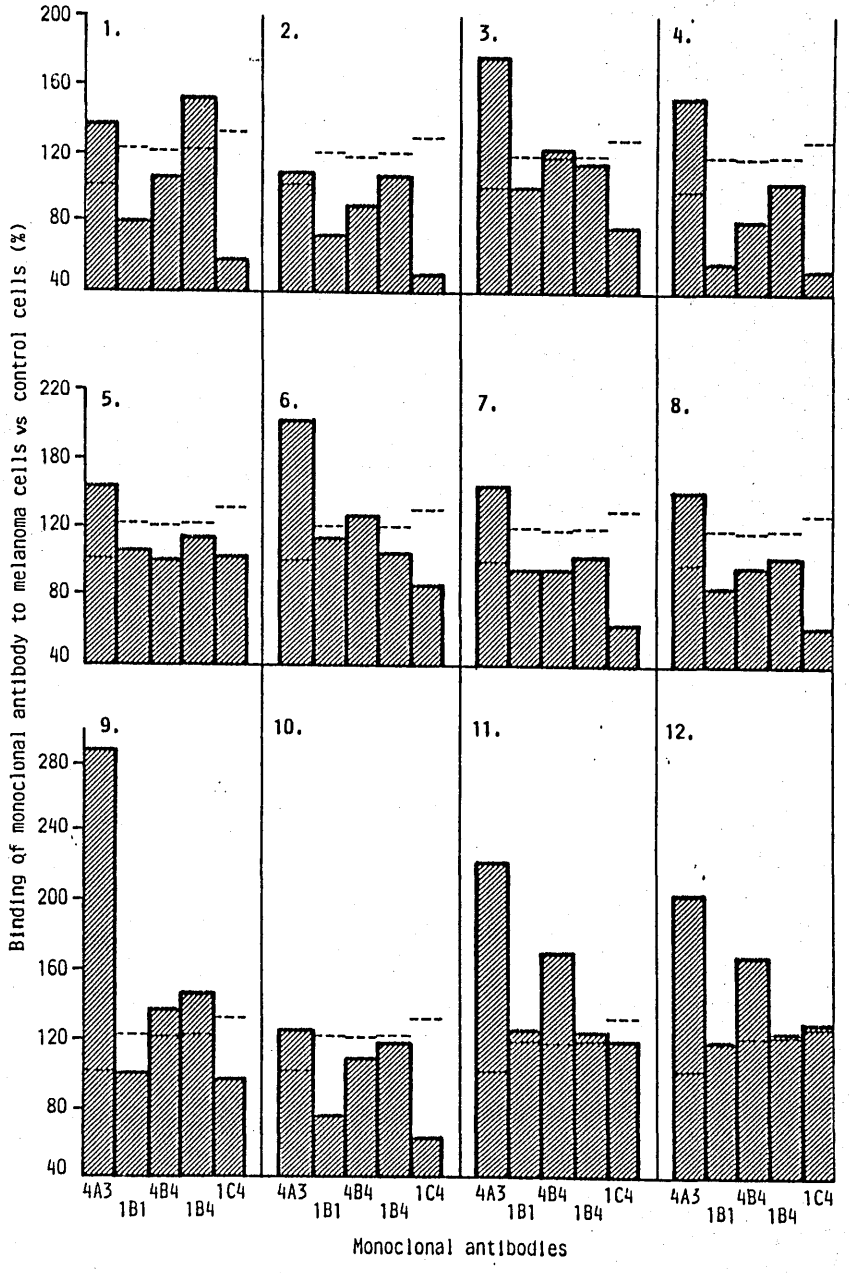

Binding of monoclonal antibody to melanoma cells vs control cells (%)
1.
2.
3.
4.
5.
6.
7.
8.
9.
10.
11.
12.
4A3
1B1
4B4
1B4
1C4
Monoclonal antibodies

Fig. 3.5

uveal melanomas. All antibodies reacted positively against the three tumours. After storage in liquid nitrogen, however, all the hybridomas became negative except for 4B4-2 which reacted with lymphocytes as strongly as with melanoma cells.

3.7 Antibody class of monoclonal antibodies

The antibody class of each monoclonal antibody was determined by the Ouchterlony technique (Section 2.7). All the monoclonal antibodies produced by fusion 18/2 were of the IgM class (Fig. 3.6). The monoclonal antibodies produced by fusion 53/1 were also of the IgM class, with the exception of 4B4-2 and 1C4 which were IgG2a.

3.8 Discussion

3.8.1 Reasons for using uncultured melanoma cells

Primary human uveal melanomas were used for the immunisation of rats and for the various immunoassays required in the course of this research. The use of uveal melanoma cell lines, first established in 1984 (Albert *et al* 1984), was avoided because the antigenic expression of cells in culture might be quite different from that of the primary tumour. Antigenic expression can be influenced by the type of calf serum used in the culture medium and antigenic shift can occur due to tumour differentiation *in vitro* (Sorg *et al* 1978). Monoclonal antibodies raised against melanoma cell lines have been found to react only with such cell lines and not with uncultured melanoma cells (Brüggen, Bröcker *et al* 1984). Furthermore, when cell lines are used for immunisation and in the screening

Figure 3.6 IgM Class of mAb 4A3 as determined by Ouchterlony technique. (i) Precipitation of monoclonal antibody with rabbit anti-rat IgM.

(a) Rabbit anti-rat IgM in centre well and doubling dilutions of mAb 4A3 in peripheral wells.

(b) Monoclonal antibody in centre well and doubling dilutions of rabbit anti-rat IgM in peripheral wells.

(ii) Lack of precipitation of monoclonal antibody with rabbit anti-rat IgG.

(a) Rabbit anti-rat IgG in centre well and doubling dilutions of mAb 4A3 in peripheral wells.

(b) Monoclonal antibody in centre well and doubling dilutions of rabbit anti-rat IgG in peripheral wells.

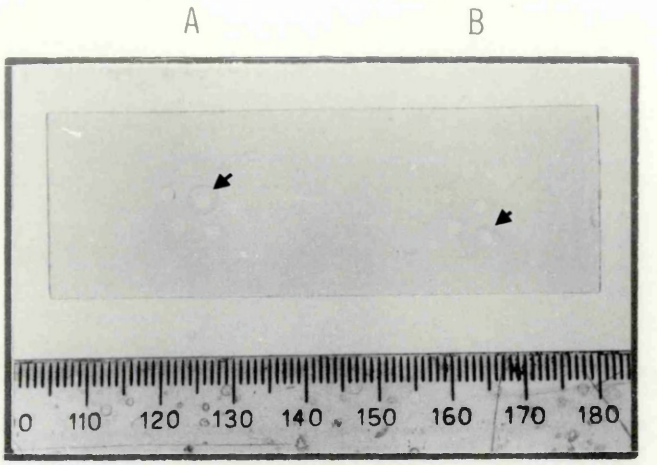


Fig. 3.6 (i)

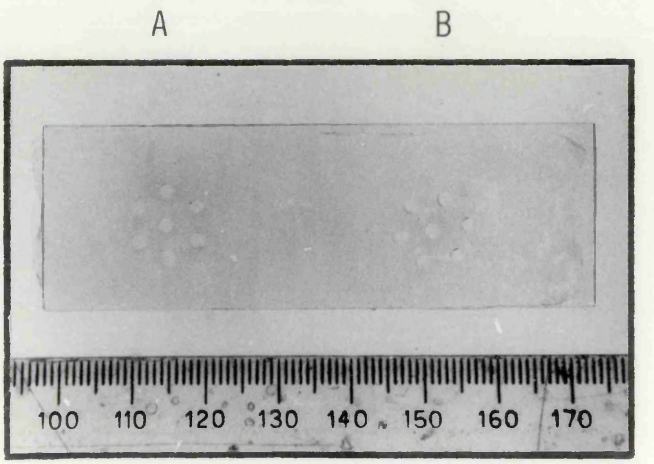


Fig. 3.6 (ii)

assays there is a small but significant risk of preparing monoclonal antibodies to the foetal calf serum in which these cells are grown. This is because foetal calf serum components can be incorporated into the membrane of cells grown in medium containing this supplement (Irie, Irie & Morton 1974; Yamaguchi et al 1987).

Sufficient amounts of tissue were available at all times for the purposes of this research. This is because of the relatively large numbers of patients with uveal melanoma referred from other parts of the country and from overseas for investigation and treatment at the Tennent Institute of Ophthalmology, University of Glasgow.

3.8.2 Fragility of uncultured melanoma cells

The uncultured uveal melanoma cells were much more fragile than myeloma cells obtained from long-term culture, and easily ruptured by manipulation and freezing. It was therefore essential to use fresh unfrozen tumour cells and to inspect the cells by phase contrast microscopy to ensure that their preservation was adequate for the preparation of ELISA plates.

3.8.3 Rat immunisation protocol

The total number of cells used for the animal immunisations ($6-15 \times 10^6$) was relatively small compared to the doses given by some other workers: Oi and associates, for example, used a total of approximately 4×10^7 cells for the animal inoculations (Oi et al 1978). Although such

vigorous immunisation might have resulted in higher frequencies of antigen-specific hybridomas, the amounts of tissue required for this would have exceeded our supply.

Initially, rats were immunised with melanomas from different patients because of fears of producing antibodies to antigens unique to the uveal melanoma of a single individual. When it became apparent that most of the monoclonal antibodies cross-reacted with lymphocytic antigens, this schedule was revised. Subsequently, rats were immunised and boosted with tumour cells obtained from the same patient and resulting hybrids were then assayed against different tumours.

The aim was to produce monoclonal antibodies recognising antigens expressed by all uveal melanomas and not by other tissues. Antigens expressed by all melanomas, however, might also be the least specific. It might, therefore, be more appropriate to use tumour cells from a single patient for all immunisation and screening procedures required for each fusion. This approach might be expected to increase the number of monoclonal antibodies reacting with histocompatibility antigens. Such antibodies could be excluded by screening hybridomas against lymphocytes taken from the same patient.

Rats immunised with whole uveal melanoma cells would inevitably have been sensitised to intracellular as well as membrane antigens because, although whole melanoma cells were used for the animal inoculations, the cells would undoubtedly have been destroyed by a foreign body reaction shortly after injection. Inoculation with cell membrane preparations was therefore considered but this

approach would have required large numbers of tumour cells and would have precluded the use of uncultured primary uveal melanoma tissue, which was thought preferable.

3.8.4 ELISA

3.8.4.1 Variation

The quality of the ELISA plates varied from batch to batch despite all the precautions that were taken. It was therefore necessary to test a sample from each new batch of plates with a range of known positive antibodies as well as negative controls consisting of complete medium. Inter-tumour antigenic heterogeneity in uveal melanomas was another complicating factor when screening the hybridomas. This was because a negative result could have been due to lack of expression of the relevant antigen by a uveal melanoma or to a lack of antibody production by the hybridoma. Screening assays were therefore performed simultaneously against a number of uveal melanoma specimens.

3.8.4.2 Number of cells per well

Other workers have used between $8x10^4$ cells/well (Douillard & Hoffman 1983) and $5x10^6$ cells/well (Heusser, Stocker & Gisler 1981) depending on the size of the cells and the method of preparation of the ELISA plates. The number of cells used for the screening of monoclonal antibodies is theoretically important. Many different antigens are present at different densities on melanoma cells so that if small numbers of cells are used, less abundant but potentially important antigens will give a

very weak signal on ELISA. On the other hand, if large numbers of cells are used to detect these antigens then low affinity antibodies to the more common epitopes may dominate the result. In the present investigation, pilot studies suggested that the best signal-to-noise ratios were were obtained with the use of 10^4 cells/well. The exact number of cells which actually attached in every well may have been different with each batch of plates.

3.8.4.3 Attachment of cells to plate

The plates were incubated with 0.1% poly-L-lysine to increase their electrostatic potential (Stulting & Berke 1973). Although this procedure is considered by some workers to be unnecessary and to cause high background (Douillard and Hoffman 1983), in the present study attachment of the cells to the plates was found to be uneven when prior incubation of the plates with poly-L-lysine was omitted.

3.8.4.4 Fixation of cells

Glutaraldehyde fixation of the cells in the ELISA plates was performed in order to strengthen the attachment of the cells to the plastic (Heusser, Stocker, and Gisler 1981) and also so that the plates could be stored at 4°C for long periods. The method used to fix the cells in this study differs from that described by Heusser, Stocker & Gisler (1981) who dipped the ELISA plates into 0.25% Glutaraldehyde at 4°C for five minutes. Instead, 100 µl 0.25% glutaraldehyde in PBS was gently added to each well at room temperature for three minutes. As the wells

already contained 100 µl PBS the effective glutaraldehyde concentration was 0.125%. Nevertheless, recent studies by other workers show that glutaraldehyde can produce artefacts even at such low concentrations (Drover & Marshall 1986)

3.8.4.5 Blocking of non-specific reactivity

Besides improving the adherence of the cells to the plastic, both poly-L-lysine and glutaraldehyde also increased the non-specific binding of the antibodies to the plate. This problem was partially resolved by the use of BSA, heterologous serum and glycine in the storage medium and of Tween 20 in the wash buffer as described in Campbell (1984) and Heusser, Stocker & Gisler (1981). Glycine was used because it binds to unreacted amino groups of glutaraldehyde, thereby inhibiting non-specific covalent linkage to antibodies. At first, plates were further blocked by incubation with a solution containing higher concentrations of BSA and heterologous serum overnight at 4°C. When the effect of this procedure was investigated by performing the same assay in duplicate, with and without pre-incubation with blocking solution, it was found that incubation of the wells in blocking solution did not significantly reduce background readings. This blocking step was therefore discontinued.

3.8.4.6 Interpretation of ELISA results

The results were assessed subjectively rather than by statistical analysis which was considered to be inappropriate, especially as the concentrations of the

monoclonal antibodies being tested varied according to the relative growth rate of the cells. These impressions were retrospectively confirmed by statistical analysis of a few representative assays.

3.8.4.7 Efficiency

The sensitivity of the ELISA was apparently sufficient for the detection of antibodies to uveal melanoma antigens in the hybridoma supernatants. The preparation of the ELISA plates was, however, laborious and expensive. In addition, both glutaraldehyde and poly-L-lysine are known to alter the antigenicity of cells. For example, other workers have shown that transplantation antigens and Moloney leukaemia virus-associated antigens, expressed by mouse fibroblasts, were almost completely destroyed by fixation with 0.25% glutaraldehyde for five minutes at 20°C (Gatti et al 1974), and that poly-L-lysine can alter the antigenicity of cells (Van Ewyk et al 1980). A 'dry' method of attaching cells to ELISA plates, first described by Effros and associates (1985), was, therefore, tried because it eliminated the need for poly-L-lysine, glutaraldehyde and storage media. This proved to be as efficient as the conventional techniques. With these plates the probability of detecting cytoplasmic rather than membrane antigens is said to be greater than with the conventionally prepared ELISA plates (Personal communication, B. Wright, Coralabs, Cambridge). In practice, however, there was probably little difference between the two methods.

The production of monoclonal antibodies to cytoplasmic antigens suggests that although whole melanoma cells were attached to the ELISA plates cytoplasmic antigens were also influencing the results and possibly dominating the selection of monoclonal antibodies. This could have occurred because the membranes of apparently intact cells were nevertheless permeable to antibodies or because rupture of even a small number of tumour cells exposed sufficient amounts of cytoplasmic antigens to interfere with the assay.

3.8.5 Choice of the rat hybridoma system

The rat hybridoma system was used because it has a number of advantages relative to the mouse system (discussed by Clark et al 1983; Campbell 1984). Firstly, rat hybridomas tend to be more stable in the early stages of cell culture. Secondly, rats are more easily handled than mice and yield larger amounts of serum. One potential disadvantage is that the Y3 myeloma cells can produce their own kappa light chain which can result in monoclonal antibodies of mixed specificities (discussed by Campbell 1984).

3.8.6 Screening of hybridomas

Despite the high sensitivity of the ELISA the selection of relevant hybridomas was not always straightforward, especially after very successful fusions. Initially, when screening primary hybridomas, unfused splenic lymphocytes and myeloma cells were used as negative controls. Retrospective evaluation, however,

demonstrated that the best negative controls were the negative hybridomas which usually predominated. Between five and ten hybridomas, which produced the highest results on ELISA, were therefore selected for further study.

Theoretically, 'weak positives' could have been directed against equally important although less abundant antigens. It was difficult, however, to distinguish such weak reactivity from experimental variation in the ELISA and, furthermore, it is unlikely that such rare antigens would subsequently have been detected by other methods such as Western Blotting or immunohistochemistry.

3.8.7 Loss of reactivity of hybridomas

In fusion 18/2, some of the primary hybrids which reacted with melanoma cells on initial screening were stored in liquid nitrogen but these became negative following such cryopreservation. In fusion 53/1, therefore, the positive primary hybrids were subcloned directly from the 96- or 24-well culture plate at densities of 1-5 cells/well. This was done in order to perform the primary subcloning as soon as possible after fusion and before the immunoglobulin-secreting hybrid cells were overgrown by more rapidly growing non-producing hybrid cells in the same well. Subcloned hybridomas which were positive prior to cryopreservation were again found to be negative after storage. There seemed to be little difference, therefore, between the two techniques.

3.8.8 Classes of monoclonal antibodies

Most of the monoclonal antibodies produced in this study were of the IgM class. This may be due to the immunisation protocol, with large final antigenic boosts being given four days prior to fusion. It may also be due to the selection procedure: lower affinity IgM antibodies by virtue of their multivalence may have been favoured by large amounts of closely spaced antigen on the selecting plates. Monoclonal antibodies of the IgM class have a number of theoretical disadvantages relative to IgG: because of their multivalence they are more likely to cross-react with non-specific antigens (Ghosh & Campbell 1986) and, *in vivo*, their large size may prevent sufficient access to the tumour.

3.8.9 Concluding remarks

Since each monoclonal antibody was considered to be a tool rather than an end in itself, further fusions were not performed until the potential of the antibodies already available had been investigated.

Chapter 4

STUDIES ON ANTIGENS DETECTED BY MONOCLONAL ANTIBODIES USING IMMUNOELECTROPHORETIC TECHNIQUES

4.1 Introduction

Western Blotting (Towbin et al 1979) was performed to provide an indication of the molecular weight and biochemical nature of the antigens recognised by the monoclonal antibodies produced in this study. This technique is described fully in Section 2.11. Briefly, proteins in the sample of uveal melanoma cells were separated according to their molecular weight by SDS-polyacrylamide gel electrophoresis (Fig. 4.1). They were then transferred to an immobilising matrix by electrophoretic elution so that the relevant antigens could be located by autoradiography following successive incubations with rat monoclonal antibody, rabbit anti-rat immunoglobulin and, finally, ^{125}I-labelled protein A. Standard markers were included in all gels.

4.2 Antigens detected by Western Blotting

Only mAb 4A3 and mAb 4B4-2 reacted with uveal melanoma antigens on Western Blotting. The 4A3 monoclonal antibody reacted positively with all ten melanomas tested. Most tumours demonstrated two bands with molecular weights of approximately 61kD and 58kD respectively (Fig. 4.2). Unlike the 61kD band, the 58kD band varied in intensity from tumour to tumour (Fig. 4.3). Monoclonal antibody 4B4-2 detected an antigen with a molecular weight of approximately 66kD (Fig. 4.4).

Figure 4.1 Silver nitrate stain of homogenised uveal melanoma cells separated by SDS-PAGE.

Lane 1. Homogenised uveal melanoma cells

Lane 2. Pharmacia low molecular weight protein standards

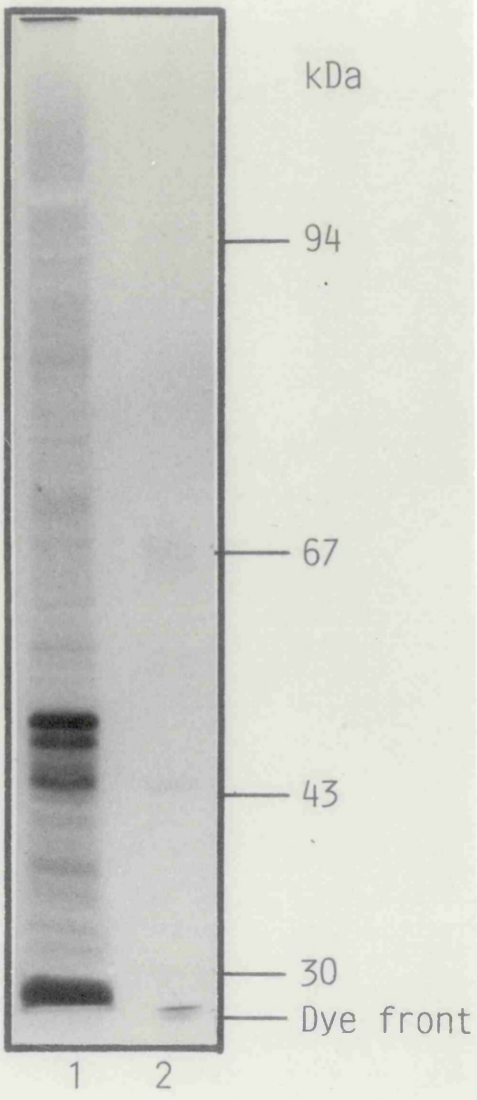


Fig. 4.1

Figure 4.2 Reactivity of mAb 4A3 with uveal melanoma on Western blotting. Homogenised uveal melanoma cells were separated by SDS-PAGE and blotted onto nitrocellulose paper. The cell proteins are demonstrated by the amido black stain (A). Antigens detected by mAb 4A3 were identified by autoradiography using rabbit anti-rat IgG (H & L) as the second antibody and the ^{125}I protein A detection system (B). The molecular weights of the 4A3 antigens are 58 kD and 55 kD.

A. Amido black

B. Autoradiograph

1. Pharmacia low molecular weight protein standards
2. Homogenised uveal melanoma cells

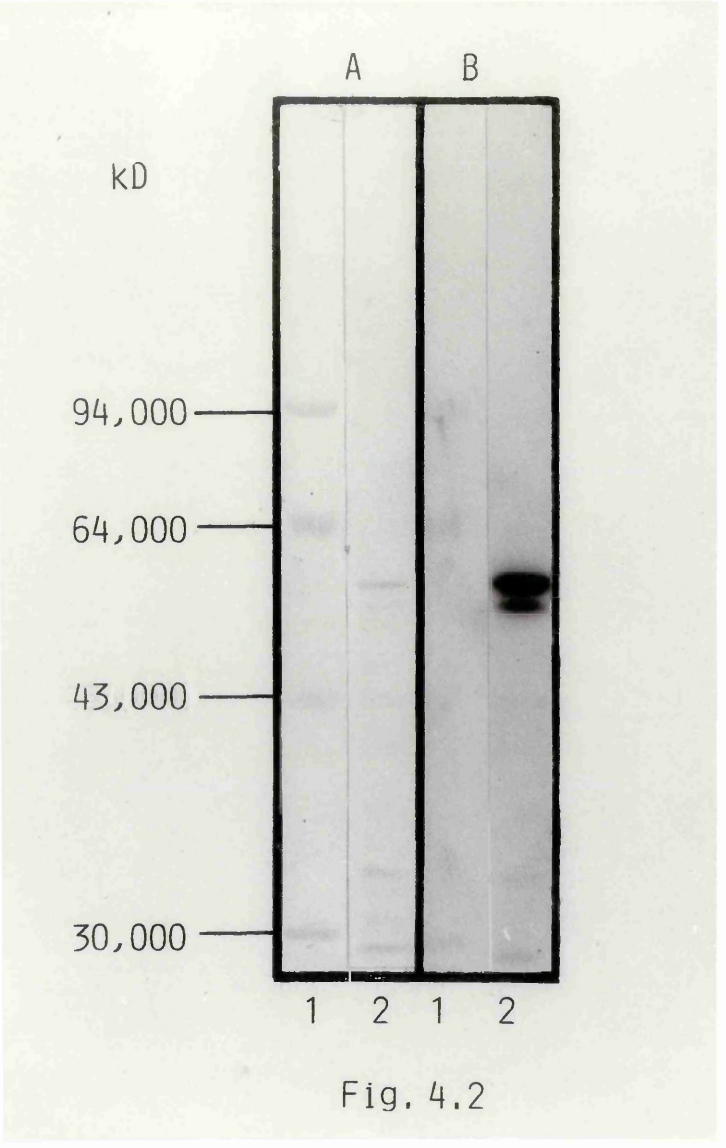


Fig. 4.2

Figure 4.3 Inter-tumour variation of the 4A3 antigen. (i) Homogenised cells from different uveal melanomas were separated by SDS-PAGE and blotted onto nitrocellulose paper (A). The antigens detected by the 4A3 monoclonal antibody were identified by autoradiography using the ^{125}I protein A system (B).

A. Amido black stain

B. Autoradiograph

Lane 1 Homogenised cells from uveal melanoma PE

Lane 2 Homogenised cells from uveal melanoma PO

Lane 3 Homogenised cells pooled from uveal melanomas 2,4 and 5.

Lane 4 Homogenised cells pooled from uveal melanomas 11 and 13

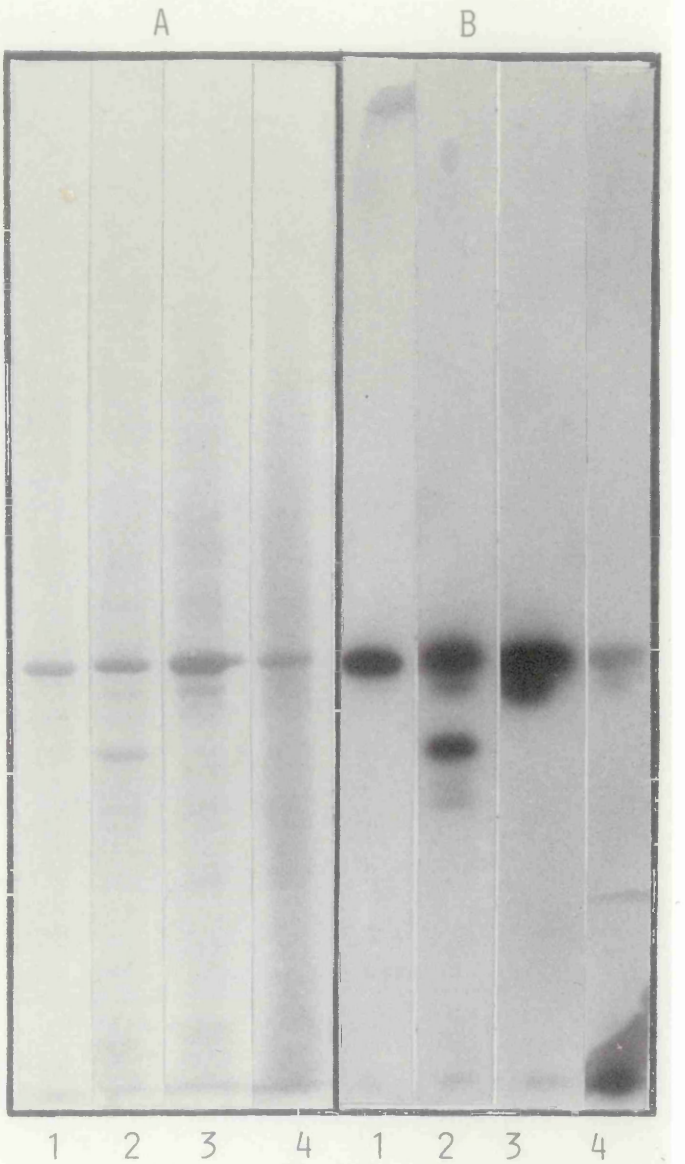


Fig. 4.3 (i)

Figure 4.3 Inter-tumour variation of the 4A3 antigen. (ii) Homogenised cells from three uveal melanomas were separated by SDS-PAGE and blotted onto nitrocellulose paper (A). The antigens detected by mAb 4A3 were identified by autoradiography using the ^{125}I protein A system (B). The first band is similar in each tumour, whereas the second band shows more variation.

A. Amido black stain

B. Autoradiograph

Lanes 1-3 Homogenised cells from three different uveal melanomas

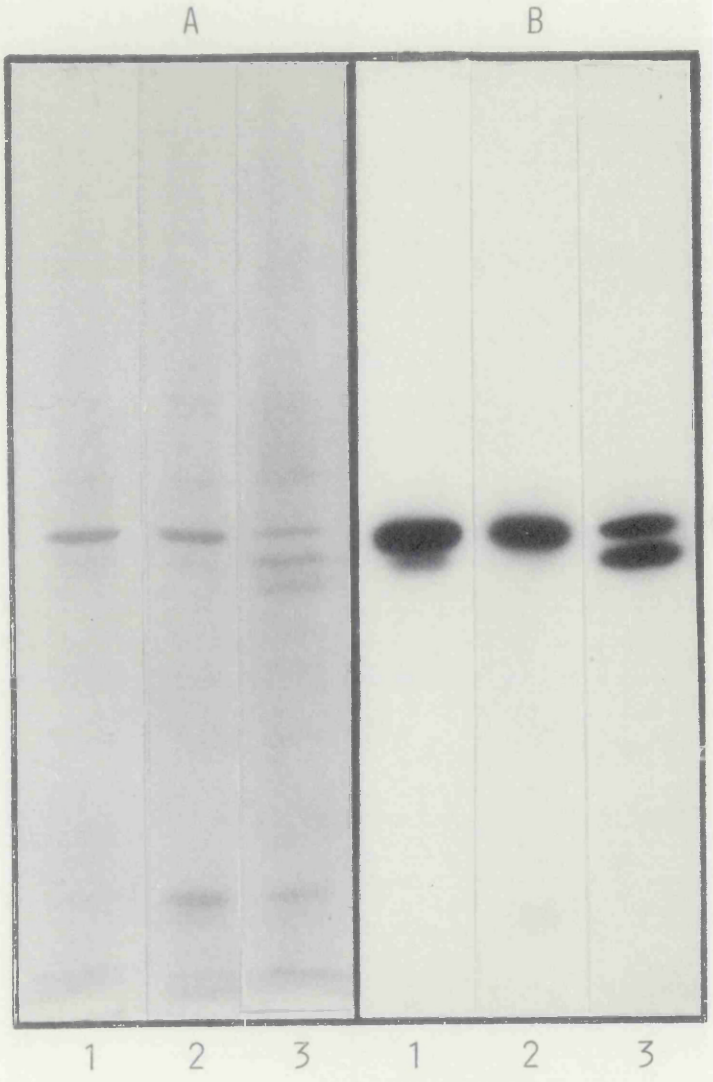


Fig. 4.3 (ii)

Figure 4.4 Reactivity of mAb 4B4-2 with uveal melanoma, demonstrated by Western blotting. Homogenised uveal melanoma cells were separated by SDS-PAGE and blotted onto nitrocellulose paper (A). The antigen detected by the 4B4-2 monoclonal antibody was identified by autoradiography using the ^{125}I protein A system (B). The molecular weight of the antigen is approximately 66 kD.

A. Amido black stain
B. Autoradiograph

1. Pharmacia low molecular weight protein standards
2. Homogenised uveal melanoma cells

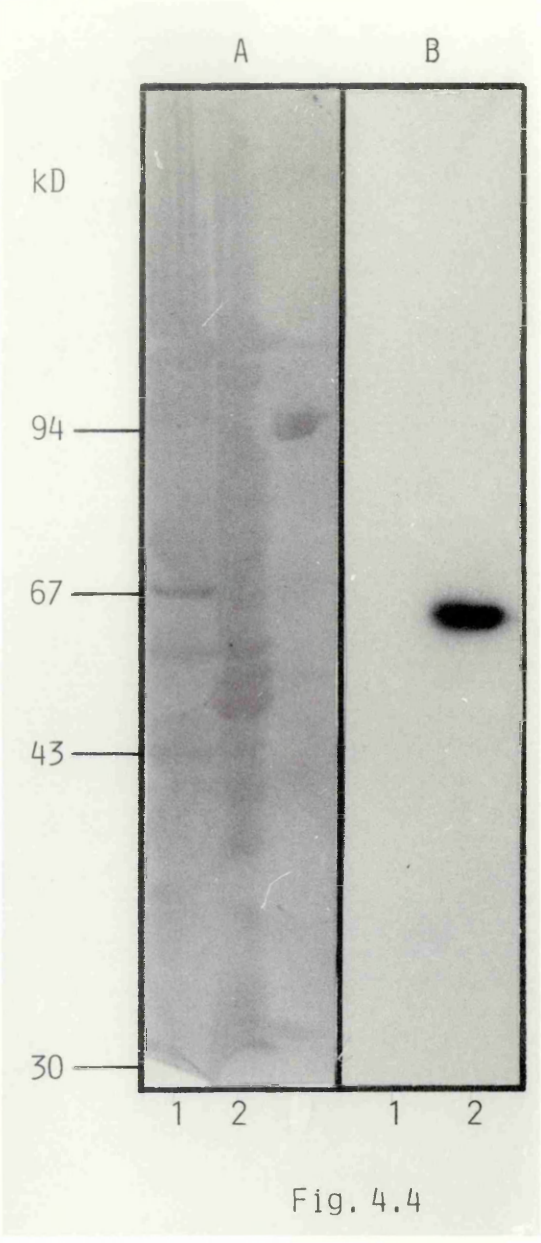


Fig. 4.4

4.3 4A3 antigen in cell membrane and nucleus

A blot was performed to investigate the presence of antigen 4A3 in a cell membrane preparation and in a nuclear preparation, both from cells pooled from a number of uveal melanomas as described in Section 2.3.3. The contents of each of these two preparations were examined by electron microscopy. The cell membrane preparation consisted of fragments of plasmalemma as well as intracellular organelles (Fig. 4.5i) whereas the nuclear preparation consisted mostly of nuclei (Fig 4.5ii). Melanin granules and amorphous material, suggestive of cytoplasm, were seen in both preparations. The effects of mercaptoethanol, which destroys disulphide linkages, and of boiling, on the 4A3 antigen were also investigated.

The results are shown in Figs. 4.6 and 4.7. Mercaptoethanol fragmented high molecular weight molecules (range 134-218kD) into a variety of smaller components, ranging from less than 20kD to 54kD, whereas boiling had little effect, only resulting in the disappearance of 96kD and 46kD molecules in the membrane and nuclear preparations respectively. The nuclear preparation contained more of the low molecular weight 4A3 antigen than did the membrane preparation (Fig. 4.7).

Figure 4.5 Components of membrane and nuclear preparations, made from uveal melanoma cells, demonstrated by electron microscopy. (i) Cell membrane preparation. Membranous structures and cytoplasmic debris (x 4000). (ii) Nuclear preparation. Nuclei and cytoplasmic debris (x 9900).

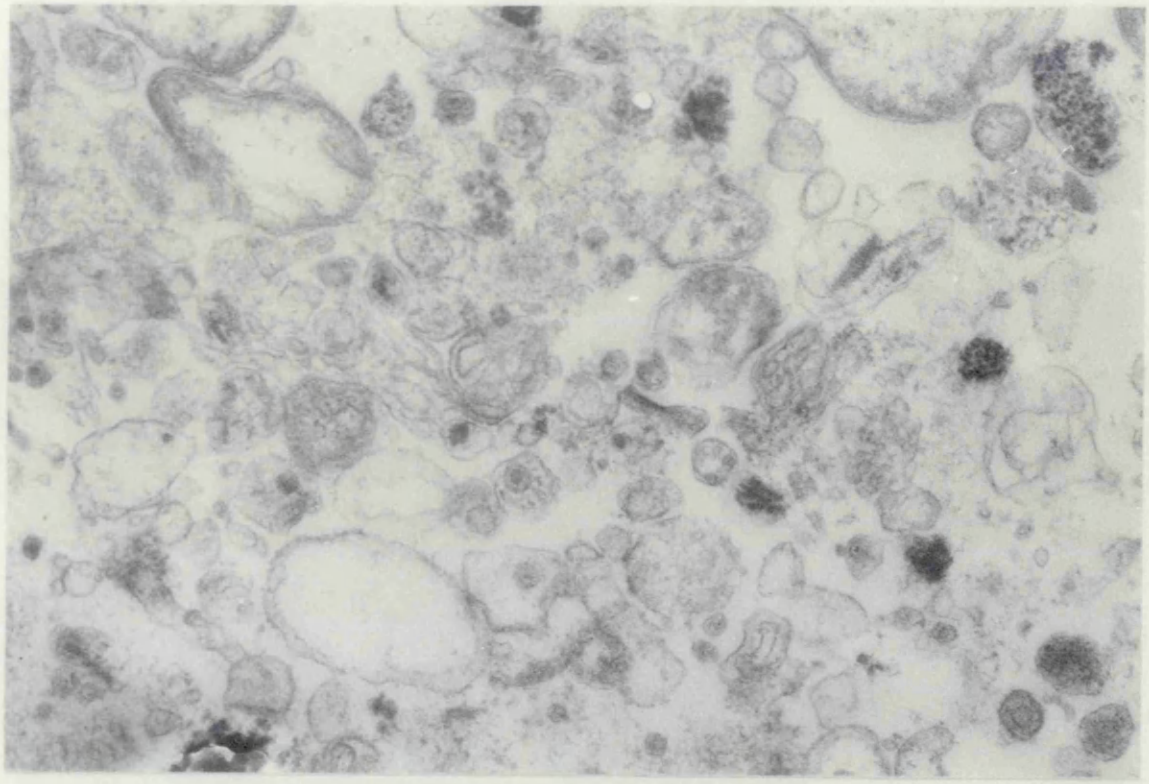


Fig. 4.5 (i)

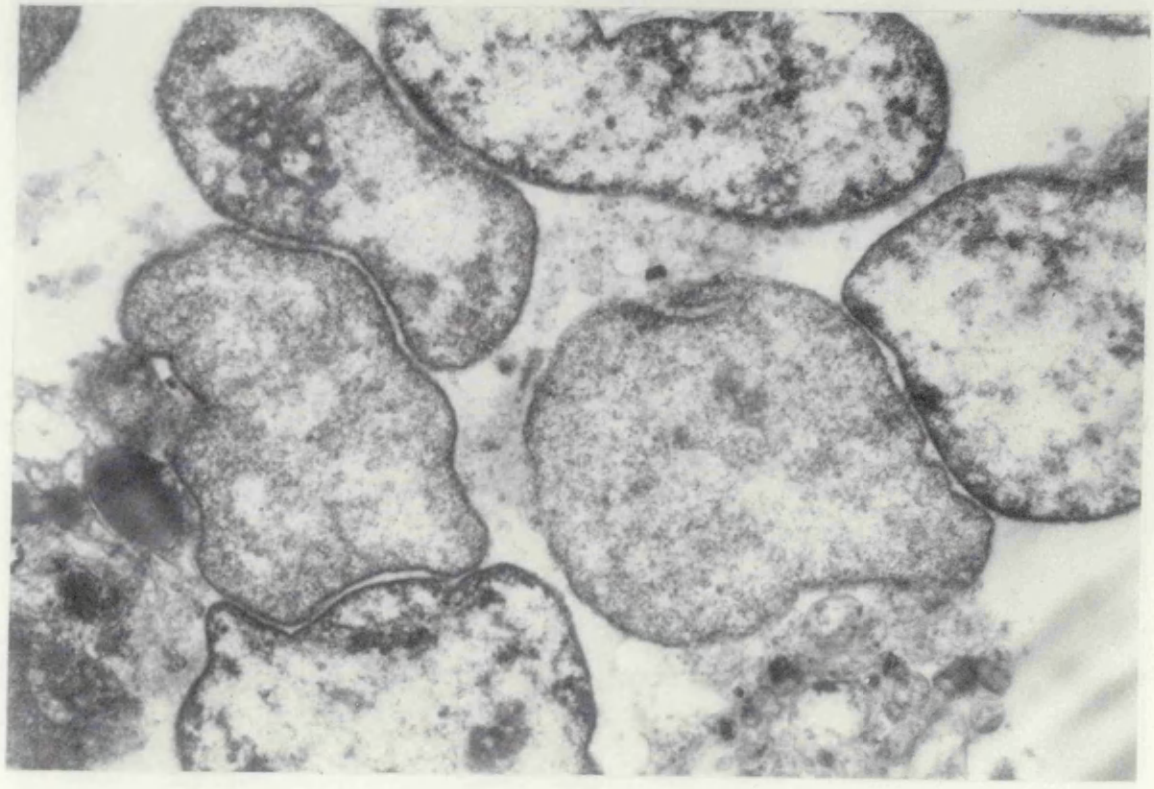


Fig. 4.5 (ii)

Figure 4.6 Antigens detected by mAb 4A3 in a membrane preparation made from uveal melanoma cells. Membrane preparations from pooled uveal melanomas were separated by SDS-PAGE directly, or after treatment with mercaptoethanol, or boiling, or both. The electrophoretic strips were transferred to nitrocellulose paper (A) and the 4A3 antigens were identified by autoradiography using the ^{125}I protein A system (B). Whereas boiling had little apparent effect, mercaptoethanol fragmented the high molecular weight molecules into a number of small components.

A. Amido black stain

B. Autoradiograph

Lane 1. No mercaptoethanol; no boiling

Lane 2. Boiling only

Lane 3. Pharmacia low molecular weight protein standards

Lane 4. Mercaptoethanol only

Lane 5. Mercaptoethanol and boiling

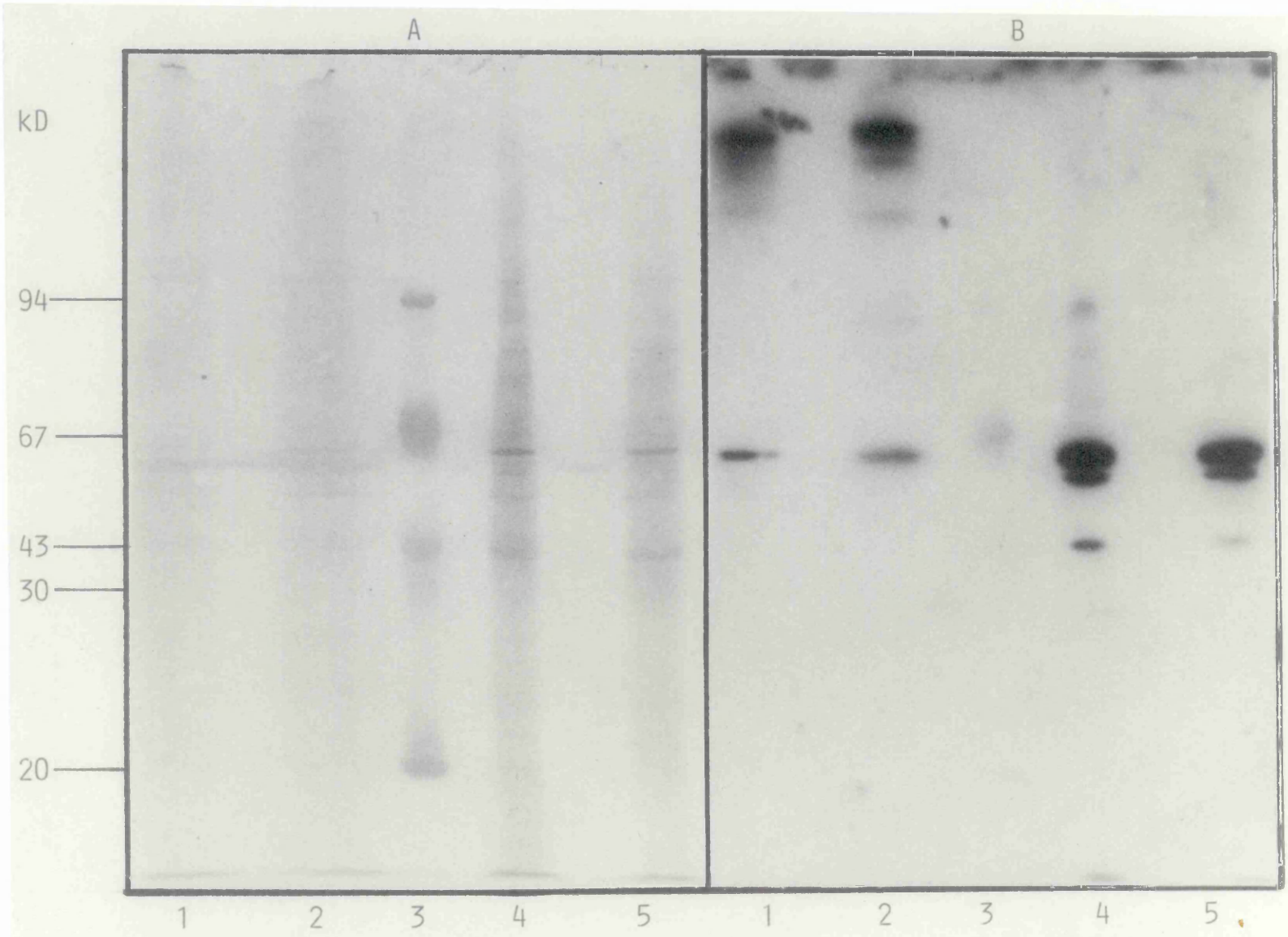


Fig. 4.6

Figure 4.7 Antigens detected by mAb 4A3 in a cytoplasm/nucleus preparation made from uveal melanoma cells. Extracts from several tumours were separated by SDS-PAGE directly, or after treatment with mercaptoethanol, or boiling, or both. The electrophoretic strips were transferred to nitrocellulose paper (A) and the 4A3 antigens were identified by autoradiography using the ^{125}I protein A system (B). The nuclear preparation contained more low molecular weight 4A3 antigens than did the membrane preparation.

A. Amido black stain

B. Autoradiograph

Lane 1. No mercaptoethanol; no boiling

Lane 2. Boiling only

Lane 3. Pharmacia low molecular weight protein standards

Lane 4. Mercaptoethanol only

Lane 5. Mercaptoethanol and boiling

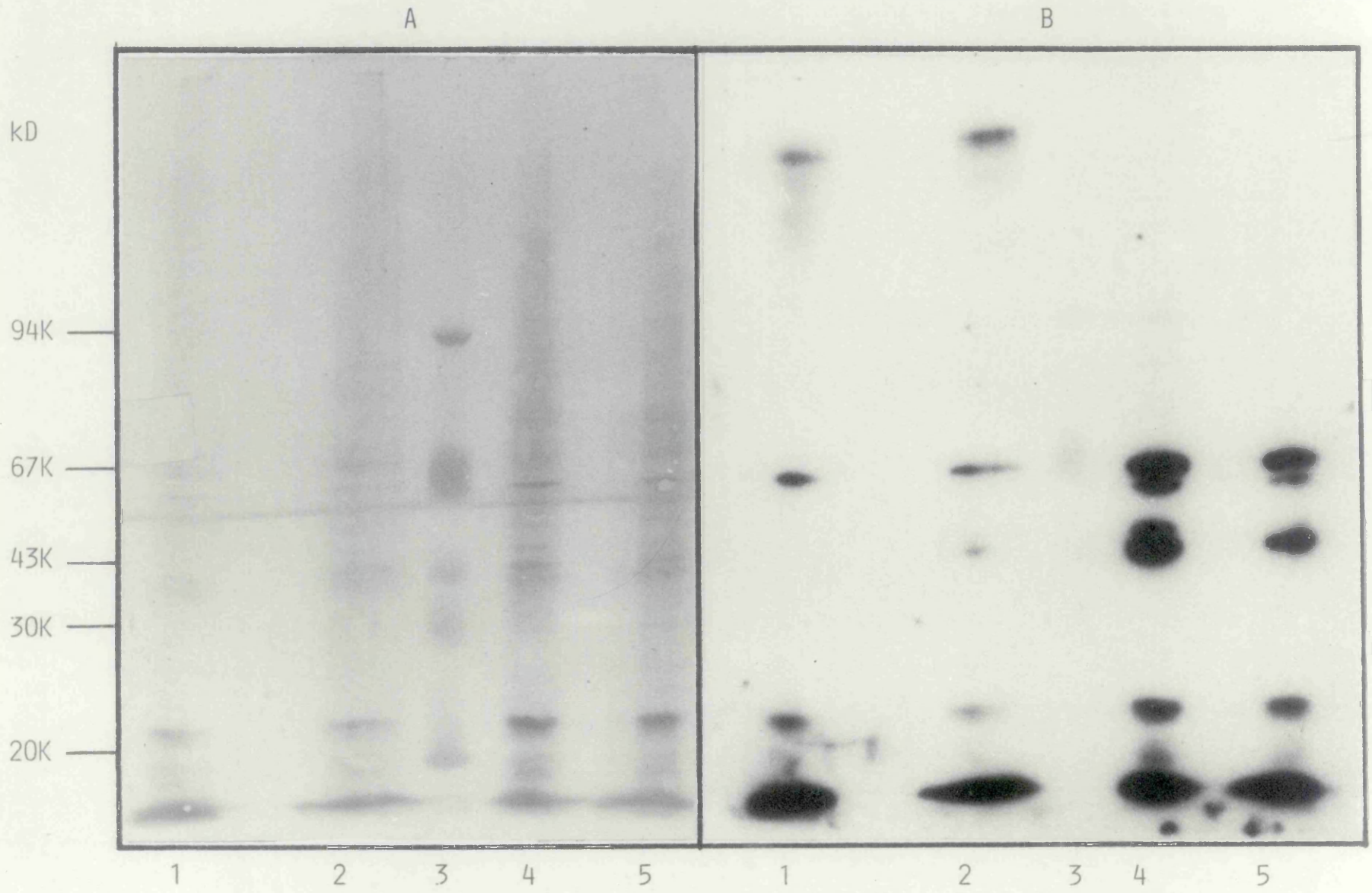


Fig. 4.7

4.4 Disulphide linkages in the 55-62 kD 4A3 antigen

The 61kD antigen was further investigated by a modification of two dimensional electrophoresis (Cleveland et al 1977). The uveal melanoma cell proteins were separated by SDS-gel electrophoresis; the band containing the 4A3 antigen was then excised from the gel and, after incubation with mercaptoethanol, placed sideways in a second gel (Fig. 4.8). The molecular weights of this antigen, 61 and 58.5kD, were found to be unaltered by the mercaptoethanol.

4.5 Enzymatic degradation of the 4A3 antigen

A uveal melanoma membrane preparation was divided into four aliquots which were incubated in neuraminidase or PBS, both with and without mercaptoethanol. Western Blotting showed that the neuraminidase did not significantly alter the 55-57kD 4A3 antigen (Fig. 4.9).

In a different experiment a preparation of fresh uveal melanoma cells was divided into six aliquots which were incubated with pepsin, chymotrypsin, trypsin, endoglycosidase, neuraminidase or PBS respectively. The 58.5kD 4A3 antigen was only visible in the control specimen, suggesting that each of the enzymes tested had digested the antigen which contained the epitope (Fig. 4.10).

Figure 4.8 Effect of mercaptoethanol on 4A3 antigen, demonstrated by two-dimensional blot. Uveal melanoma proteins were separated by SDS-PAGE in duplicate. The bands containing the 4A3 antigen doublet were excised. After incubating the samples in mercaptoethanol or transfer buffer the gel fragments were placed sideways in a second gel and further separated by SDS-PAGE. The proteins were transferred to nitrocellulose paper (A) and the 4A3 antigens identified by autoradiography using the ^{125}I protein A system. The mercaptoethanol has not apparently affected the 61 kD antigen.

A. Amido black

B. Autoradiograph

Lane 1 No mercaptoethanol

Lane 2 Mercaptoethanol

Lane 3 Pharmacia low molecular weight protein standards

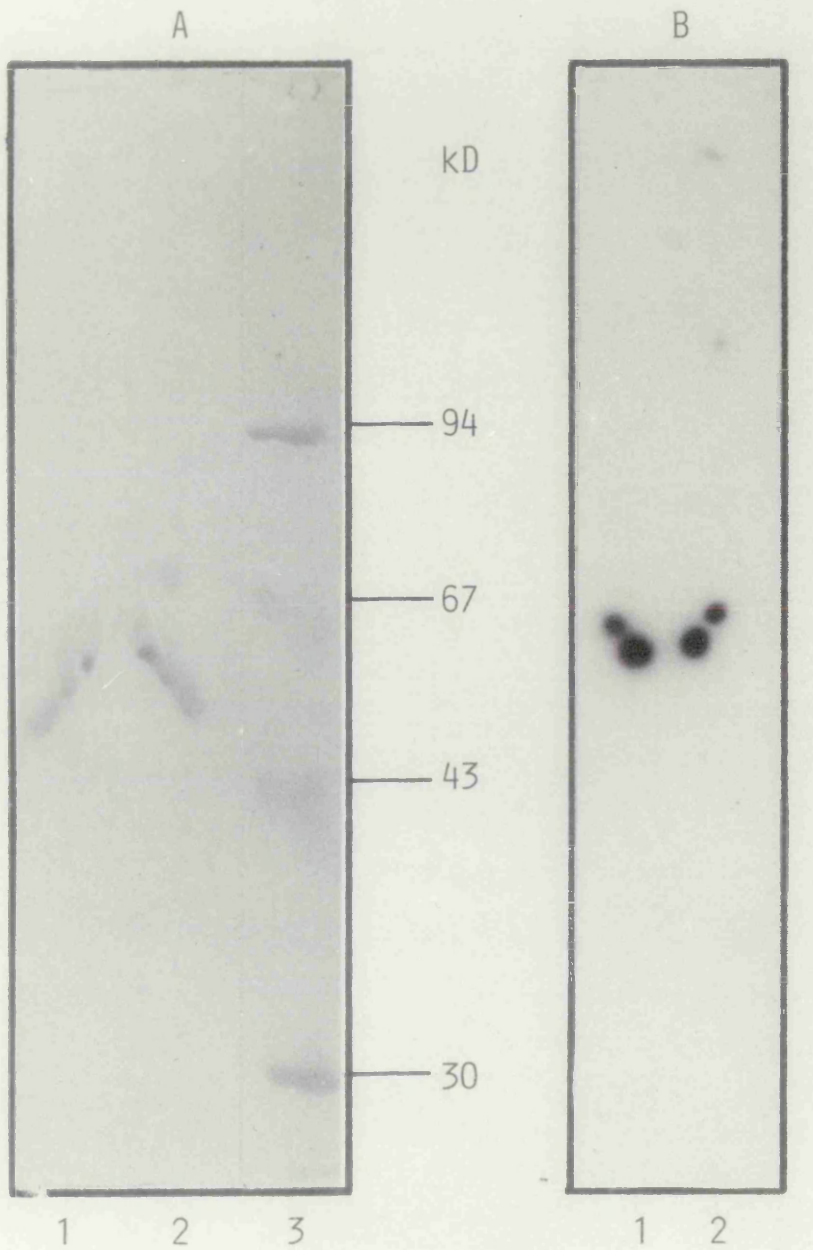


Fig. 4.8

Figure 4.9 Effect of neuraminidase and/or mercaptoethanol on the 4A3 antigen. A membrane preparation made from pooled uveal melanomas was divided into four aliquots which were incubated in neuraminidase or PBS, both with and without mercaptoethanol. The proteins were then separated by SDS-PAGE and transferred to nitrocellulose paper (A). The 4A3 antigen was identified by autoradiography using the ^{125}I protein A system.

A. Amido black stain

B. Autoradiograph

Lane 1. Pharmacia low molecular weight standard proteins

Lane 2. No neuraminidase; mercaptoethanol

Lane 3. No neuraminidase; no mercaptoethanol

Lane 4. Neuraminidase; no mercaptoethanol

Lane 5. Neuraminidase and mercaptoethanol

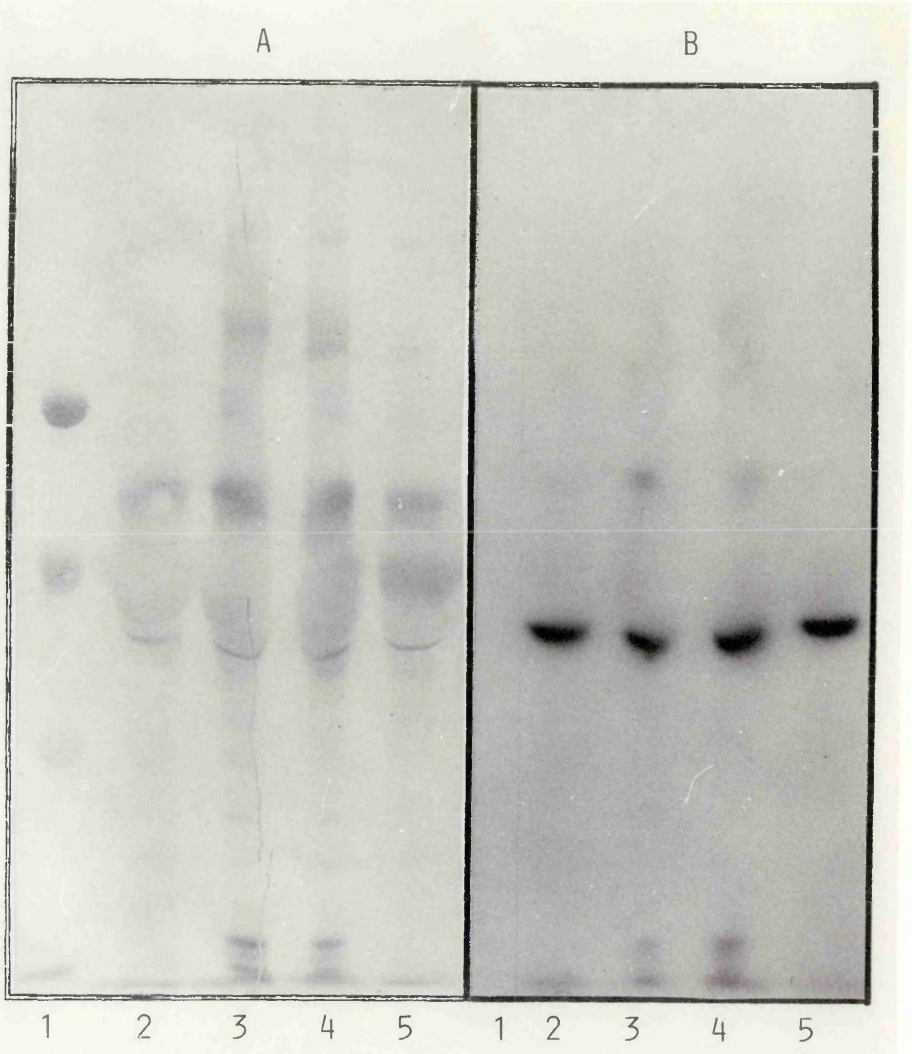


Fig. 4.9

Figure 4.10 Effect of neuraminidase on the 4A3 antigen. A fresh uveal melanoma was divided into six aliquots which were incubated with neuraminidase, endoglycosidase, PBS, trypsin, chymotrypsin or pepsin. The samples were then separated by SDS-PAGE and transferred to nitrocellulose paper (A). The 4A3 antigen was identified by autoradiography using the ^{125}I protein A system (B). The 4A3 antigen was only visible in the control specimen (Lane 3).

A. Amido black stain

B. Autoradiograph

Lane 1 Neuraminidase

Lane 2 Endoglycosidase

Lane 3 PBS

Lane 4 Trypsin

Lane 5 Chymotrypsin

Lane 6 Pepsin

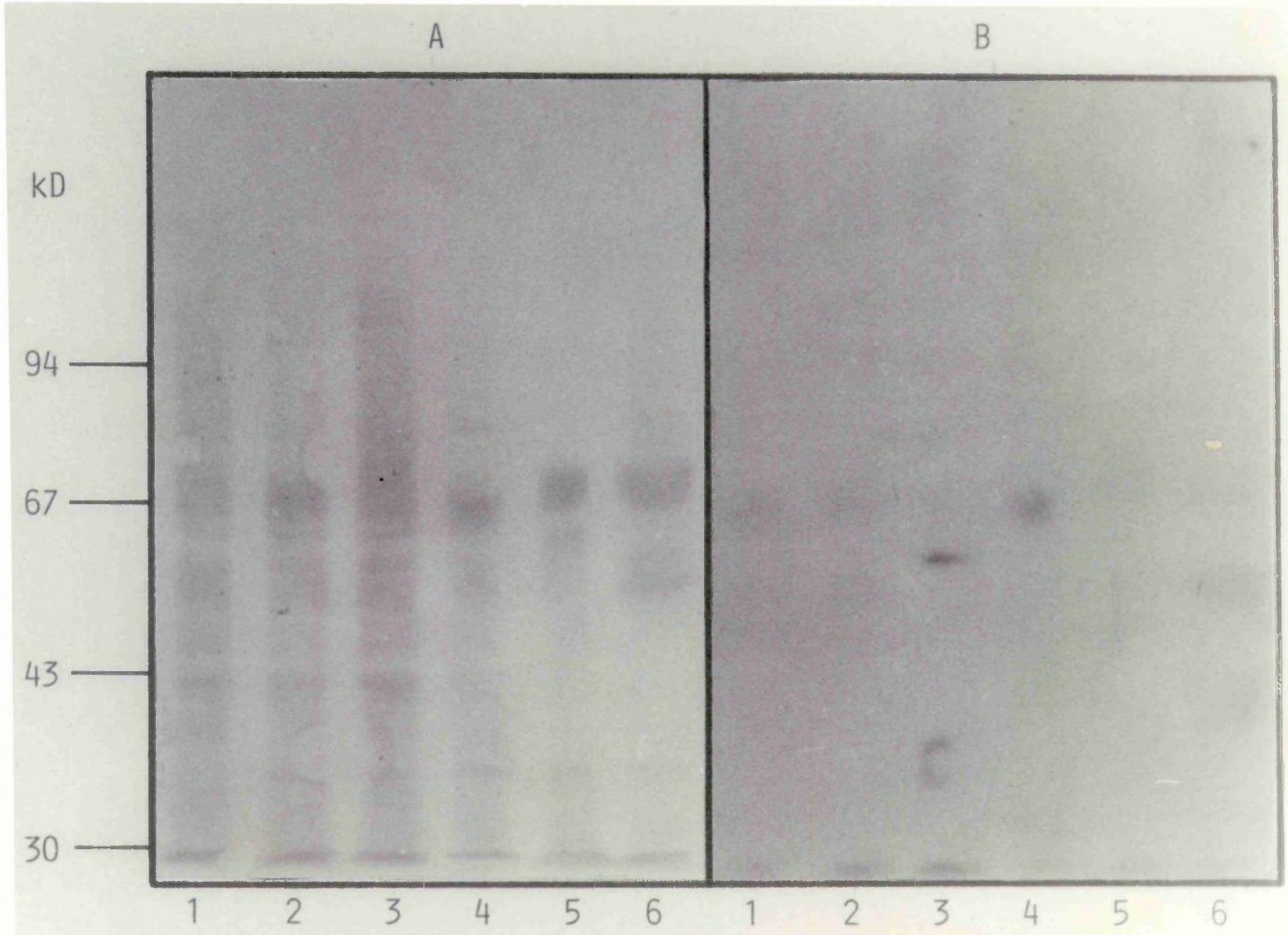


Fig. 4.10

4.6 Discussion

4.6.1 Methods

4.6.1.1 SDS-PAGE

Homogenised uveal melanoma cells were first heated with mercaptoethanol and SDS in order to split disulphide bonds and to solubilise large proteins. The SDS provides the proteins with a uniform electrostatic charge so that they migrate in the gel according to their molecular weight. Some proteins can, however, show anomalous migration because they are heavily glycosylated or because they are not fully unfolded by the SDS treatment. The porosity of a polyacrylamide gel is inversely related to its acrylamide concentration. In the present study, 8.75% w/v total acrylamide gels were found to be suitable. The discontinuous system was used in order to increase the resolution of the system.

4.6.1.2 Western Blotting

Instead of directly applying the antibodies to the gels the proteins were transferred to nitrocellulose paper (Western Blotting). This method is preferable because it obviates the need for chemical fixation of the proteins, and because it is possible for antibodies of all classes to penetrate the porous membrane to reach the antigens.

4.6.1.3 Protein A

Radiolabelled protein A was used to localise the monoclonal antibodies because it is more simple and efficient than the use of radiolabelled primary or

secondary antibodies. Rabbit anti-rat IgG (H & L) second antibody, which binds to the light chains of IgM, was required because protein A does not show sufficient affinity to rat IgM.

4.6.1.4 Determination of the molecular weight of antigens

Although six marker proteins were used only four of these were usually visible in the electrophoretic strips. Correlation of these standards with BSA in a preliminary experiment demonstrated that the missing antigens were Soyabean trypsin inhibitor (20kD) and a-lactalbumin (14.4kD) which had usually migrated with the dye front. The molecular weight of the relevant tumour-associated antigens was determined mathematically using regression analysis and not by plotting a graph. This was done to prevent subjective bias. Inaccurate results occasionally occurred when the marker proteins were indistinct or when the proximal edge of the strip was poorly localised.

4.6.2 Results

4.6.2.1 Antigens detected by Western Blotting

Only two antigens, 4A3 and 4B4-2, survived the immunoblotting procedure. The remaining antigens could represent denatured protein conformational epitopes or gangliosides and glycolipids which require different chromatographic techniques for their analysis. The 4B4-2 antigen, however, was shown by ELISA to be non-specific relative to lymphocytes and was therefore of little interest.

4.6.2.2 The 4A3 antigen

The antigen recognised most consistently by the 4A3 monoclonal antibody had a molecular weight of 61-62kD. This antigen was demonstrated in all uveal melanomas tested. A number of uveal melanomas also expressed a second antigen with a molecular weight of 57-59kD. This may represent a separate epitope on non-identical proteins, differential glycosylation, or the same protein with a different degree of proteolytic digestion.

The expression of the 4A3 antigen in a membrane preparation of pooled uveal melanomas was compared to its expression in nuclear preparations of the same cells. Both preparations contained the 61-62kD 4A3 antigen, possibly because of unavoidable contamination of both samples with cytoplasmic material. The two preparations otherwise showed significant differences in that the nuclear extracts contained larger amounts of low molecular weight epitopes, less than 20kD in size. The possibility that the 61-62kD antigen might contain more than one 4A3 epitope, linked together by disulphide bonds, was excluded by two dimensional electrophoresis of the 61-62kD antigen. It therefore seems likely that these small antigens were derived from the high molecular weight molecules which were visible only when the incubation with mercaptoethanol was omitted. A possible reason for the different bands might be multispecific reactivity of the 4A3 monoclonal antibody which is of the IgM class.

The degradation of the 4A3 antigen by pepsin, chymotrypsin, trypsin, and endoglycosidase suggests that

this antigen is a glycoprotein. Neuraminidase appeared to digest the 4A3 antigen in the fresh melanoma tissue but not when melanoma cells had been treated with mercaptoethanol and SDS. It is possible that these substances destroyed the neuraminidase, producing a false negative result so that the 4A3 antigen does indeed contain sialyl residues. Attempts were made to further characterise the 4A3 antigen using lectins such as wheat germ agglutinin (Triticum vulgaris) and lentil lectin (Lens culinaris) but these did not produce conclusive results.

Chapter 5

STUDIES ON 4A3 ANTIGEN IN SUBRETINAL FLUID

5.1 Introduction

Uveal melanomas are usually associated with an exudative retinal detachment which occurs because the hydrodynamic forces responsible for the apposition of the retina to the choroid are disturbed. The presence of the 4A3 antigen in subretinal fluid was investigated by Western Blotting in patients with uveal melanoma and in patients undergoing surgery for retinal detachment secondary to retinal tears (ie, rhegmatogenous retinal detachment).

5.2 4A3 Antigen in subretinal fluid

In a pilot study a 1/100 dilution of the subretinal fluid in 0.1 M Tris solution was shown to be adequate for the preparation of satisfactory gels. By means of Western Blotting (Section 2.11) the 4A3 antigen was detected in the subretinal fluid of all of five patients with uveal melanoma tested and in one out of two valid samples from patients with rhegmatogenous retinal detachment (Fig. 5.1, i & ii).

Figure 5.1 4A3 Antigen in subretinal fluid, demonstrated by Western blotting. (i) The proteins of subretinal fluid samples from two patients with uveal melanoma and two patients with rhegmatogenous retinal detachment were separated by SDS-PAGE and transferred to nitrocellulose paper (A). The 4A3 antigen was detected by autoradiography using the ^{125}I protein A system (B). This antigen was present in samples from both patients with uveal melanoma, but not in the sample from a patient with rhegmatogenous retinal detachment. (Lane 3 is considered invalid because of insufficient sample on the nitrocellulose paper).

A. Amido black

B. Autoradiograph

Lane 1. Pharmacia low molecular weight protein standards

Lanes 2,5 Subretinal fluid from eyes with uveal melanoma (ie, Lane 2: Gat; Lane 5: Mon)

Lanes 3,4 Subretinal fluid from eyes with rhegmatogenous retinal detachment.
(ie, Lane 3: Min; Lane 4: Cam)

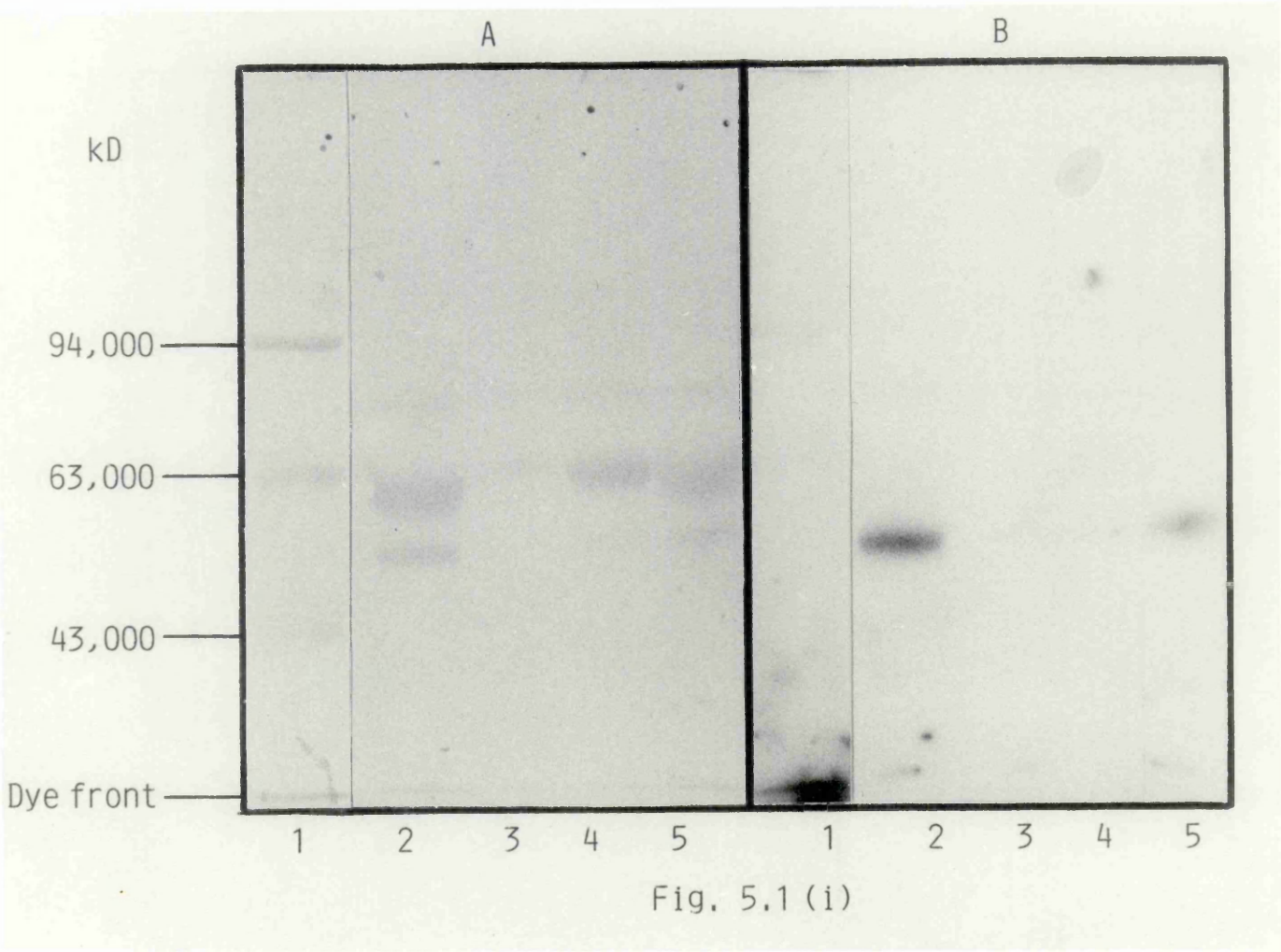


Fig. 5.1 (i)

Figure 5.1 4A3 antigen in subretinal fluid demonstrated by Western blotting. (ii) The proteins of subretinal fluid samples from patients with uveal melanoma and patients with rhegmatogenous retinal detachment were separated by SDS-PAGE and transferred to nitrocellulose paper (A). The 4A3 antigen was detected by autoradiography using the ^{125}I protein A system (B). Antigen 4A3 is demonstrable in all three samples from patients with uveal melanoma, and in one of two valid samples from patients with rhegmatogenous retinal detachment.

A. Amido black

B. Autoradiograph

Lane 1. Pharmacia low molecular weight protein standards

Lanes 3,5,7,8 Subretinal fluid from eyes with uveal melanoma
(Lane 3: Haff; Lane 5: Swa; Lanes 7,8: Eva)

Lanes 2,4,6 Subretinal fluid from eyes with rhegmatogenous retinal detachment
(Lane 2: Raff; Lane 4: Cam; Lane 6: McD)

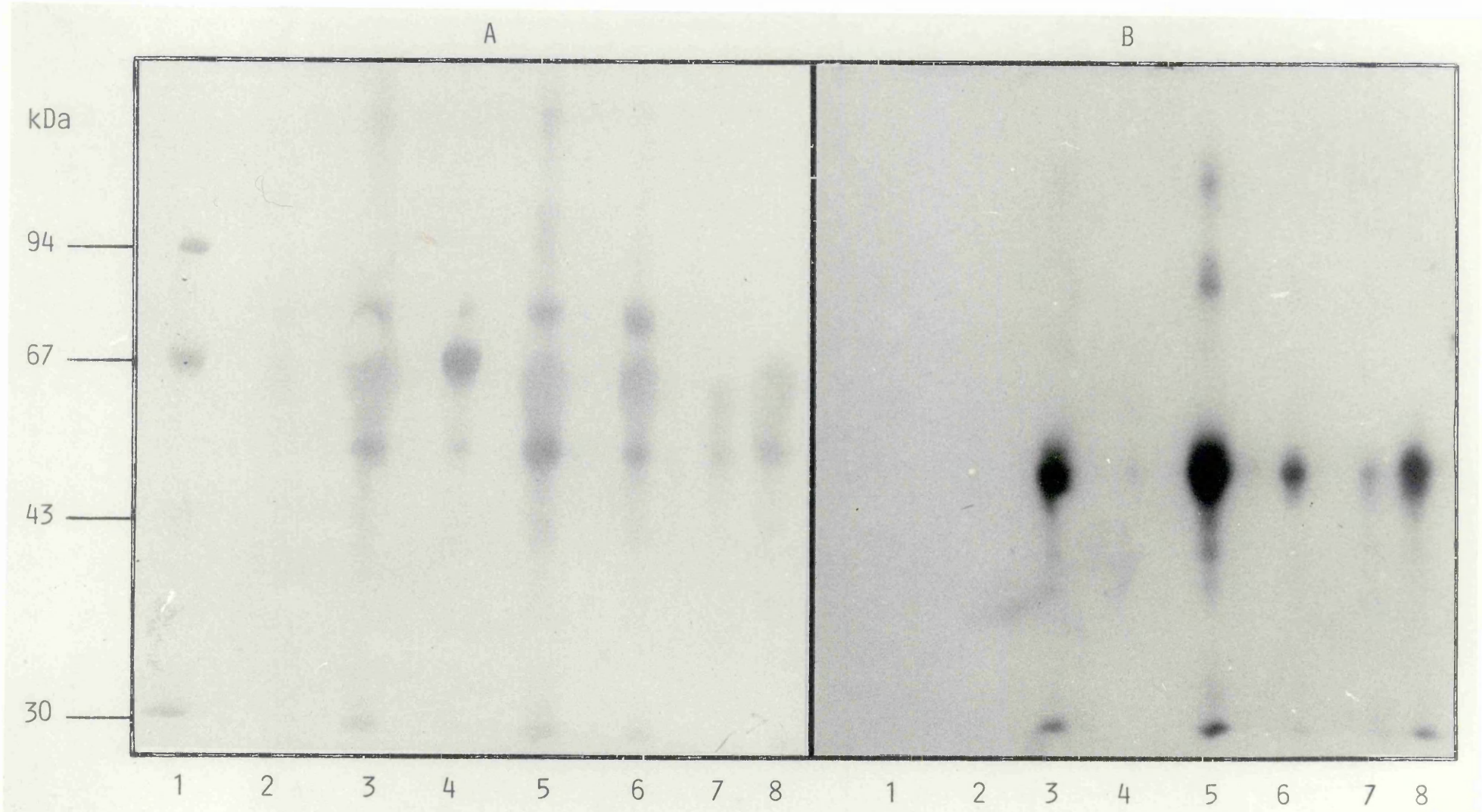


Fig. 5.1 (ii)

5.3 Discussion

The 4A3 antigen was detected in the subretinal fluid of all five patients with uveal melanoma. Gels stained with Coomassie Blue indicated that the subretinal fluid of patients with rhegmatogenous retinal detachment was different from the subretinal fluid of patients with uveal melanoma. Subretinal fluid from patients with exudative retinal detachments might have provided better controls but such samples were not available. Whether the positive reaction seen in one of the control samples was genuine or whether it was due to contamination of the sample with fluid from an adjacent well is uncertain. More extensive studies are required, although samples of subretinal fluid for this work become available very infrequently.

It is interesting that another melanoma marker, S-100 protein, has also been detected in the subretinal fluid of patients with uveal melanoma (Cochran et al, UCLA Medical Centre, USA, British Journal of Ophthalmlogy, in press). Testing of subretinal fluid for melanoma-associated antigens is not likely to have a clinical application because paracentesis of subretinal fluid is technically difficult and because different methods of biopsy are already established as a reliable, if not routine, procedure in specialised centres. The detection of the 4A3 antigen in subretinal fluid suggests that attempts to develop methods for detecting this and other melanoma-associated antigens in the serum might be worthwhile.

Chapter 6

STUDIES ON THE REACTIVITIES OF MONOCLONAL ANTIBODIES USING IMMUNOPEROXIDASE AND IMMUNOFLUORESCENCE MICROSCOPY

6.1 Introduction

The reactivity of some of the monoclonal antibodies was studied using immunoperoxidase and immunofluorescence techniques.

6.2 Immunohistochemistry

The immunohistochemical techniques used are described in detail in Section 2.3.6. Briefly, fixed or frozen tissue sections were incubated successively with blocking solution, monoclonal antibody, peroxidase-labelled rabbit anti-rat antibody and substrate, which consisted of either diamino benzidine or amino-ethyl carbazole.

6.2.1 Frozen-tissue sections

Monoclonal antibody 4A3 reacted strongly with all cells in each of a small number of primary uveal (Fig. 6.1) and cutaneous (Fig. 6.2) melanomas and in a metastatic cutaneous melanoma (Fig. 6.3). This antibody apparently cross- reacted with cells of a cutaneous naevus (Fig. 6.4). Positive reactivity was also seen with vascular endothelial cells, myo-epithelial cells, fibroblasts and some interfollicular lymphocytes (Fig. 6.5).

Initial ELISA studies had indicated that mAbs 1C4, 4B4-2, and 1B1 were non-specific. The preparation of sufficient amounts of mAb 1C4 for immunohistochemical

Figure 6.1 MAb 4A3 reactivity with primary uveal melanoma demonstrated by immunohistochemistry on frozen section. All melanoma cells show a positive reaction (AEC, x 230).

Figure 6.2 MAb 4A3 reactivity with primary cutaneous melanoma demonstrated by immunohistochemistry on frozen section. (AEC, x 92).

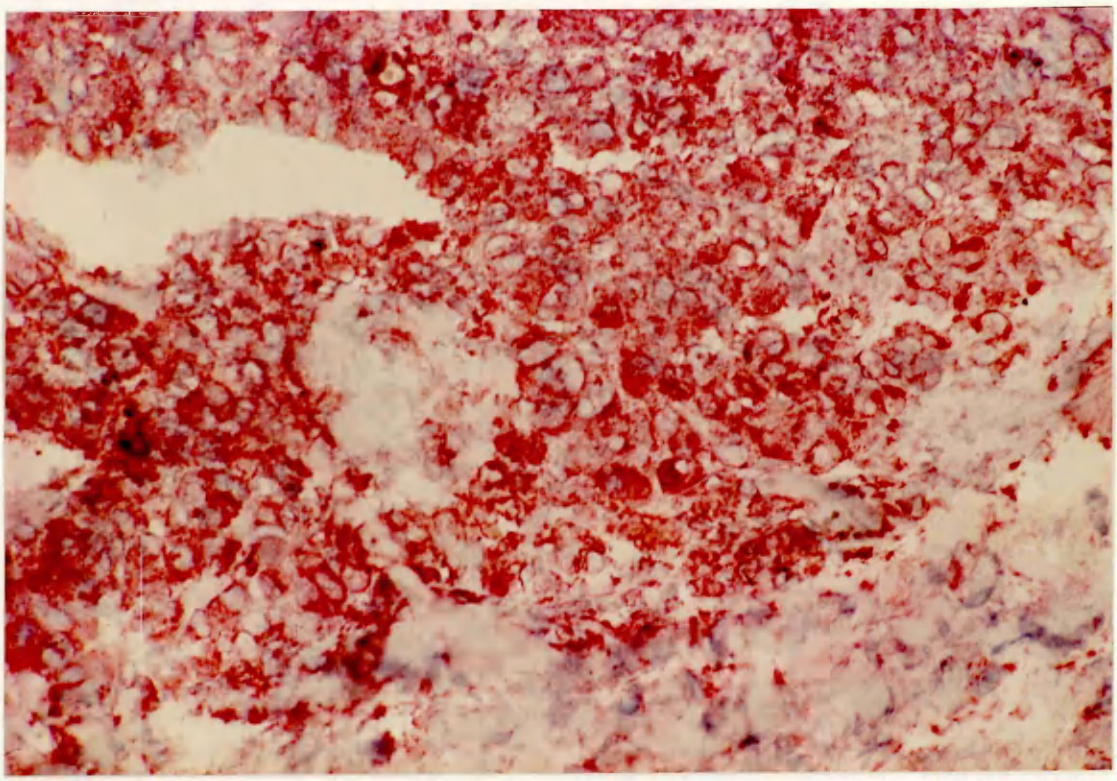


Fig. 6.1

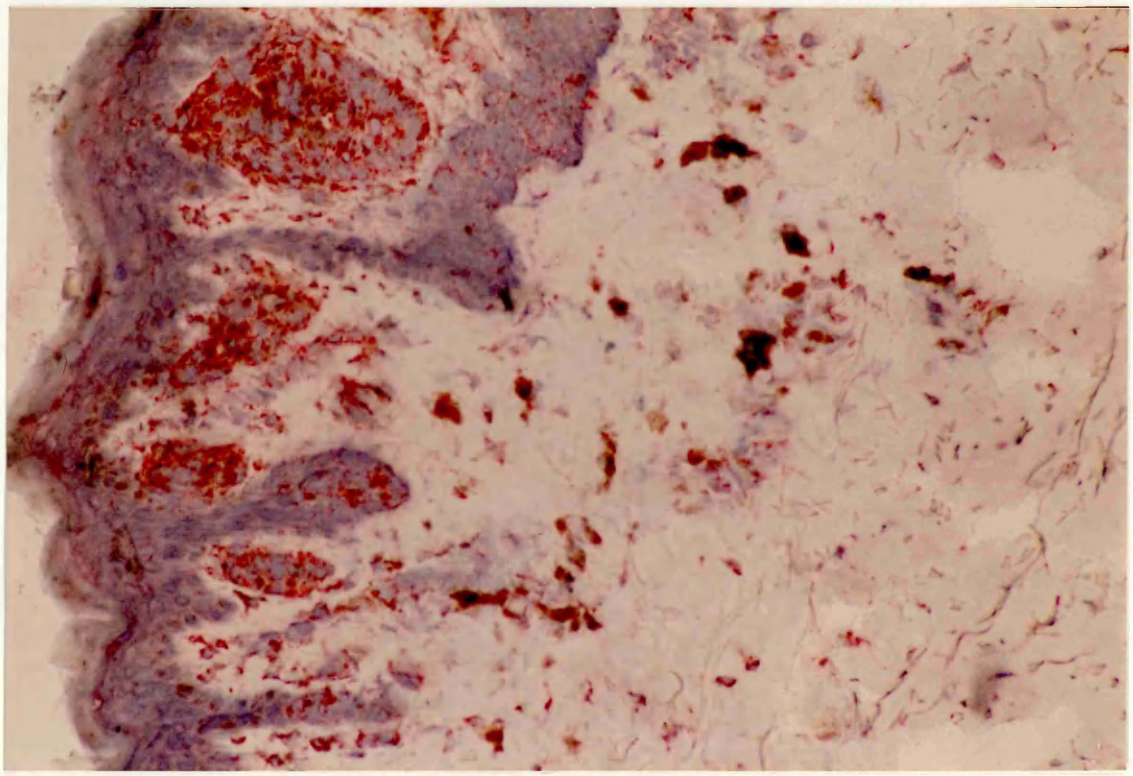


Fig. 6.2

Figure 6.3 MAb 4A3 reactivity with intradermal metastasis from primary cutaneous melanoma demonstrated by immunohistochemistry on frozen section. (DAB, x 92).

Figure 6.4 MAb 4A3 reactivity with compound cutaneous naevus, demonstrated by immunohistochemistry on frozen section (DAB, x 92).

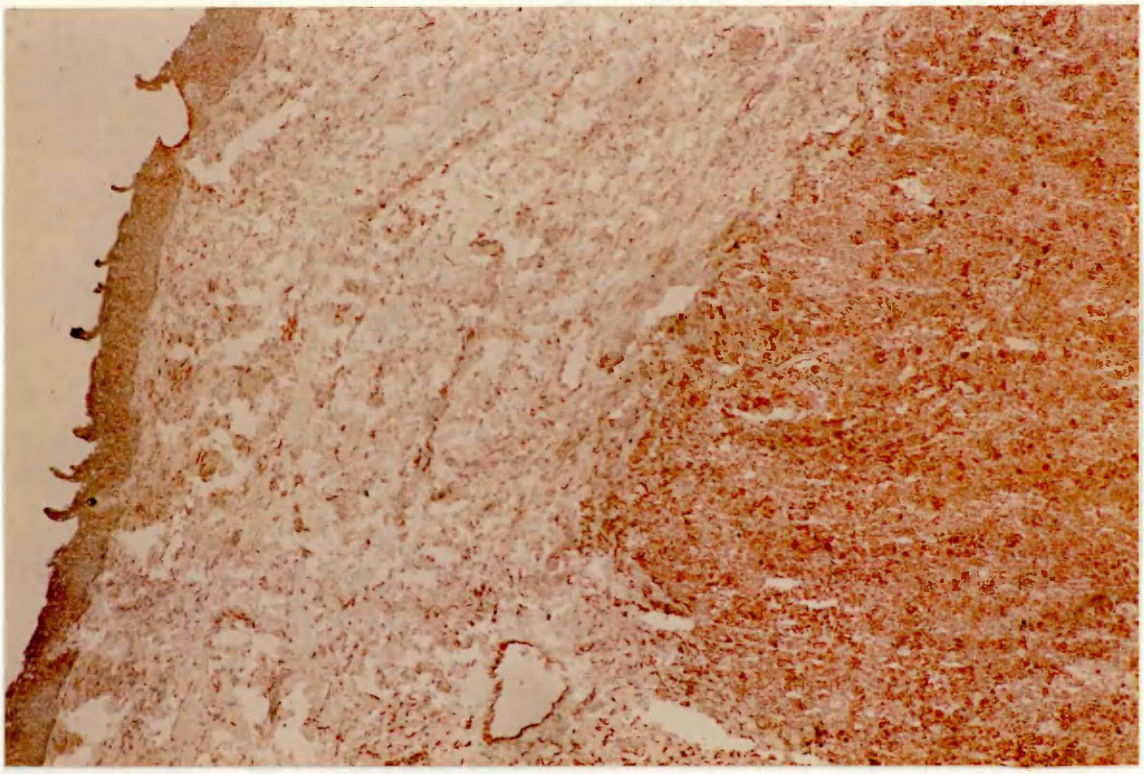


Fig. 6.3

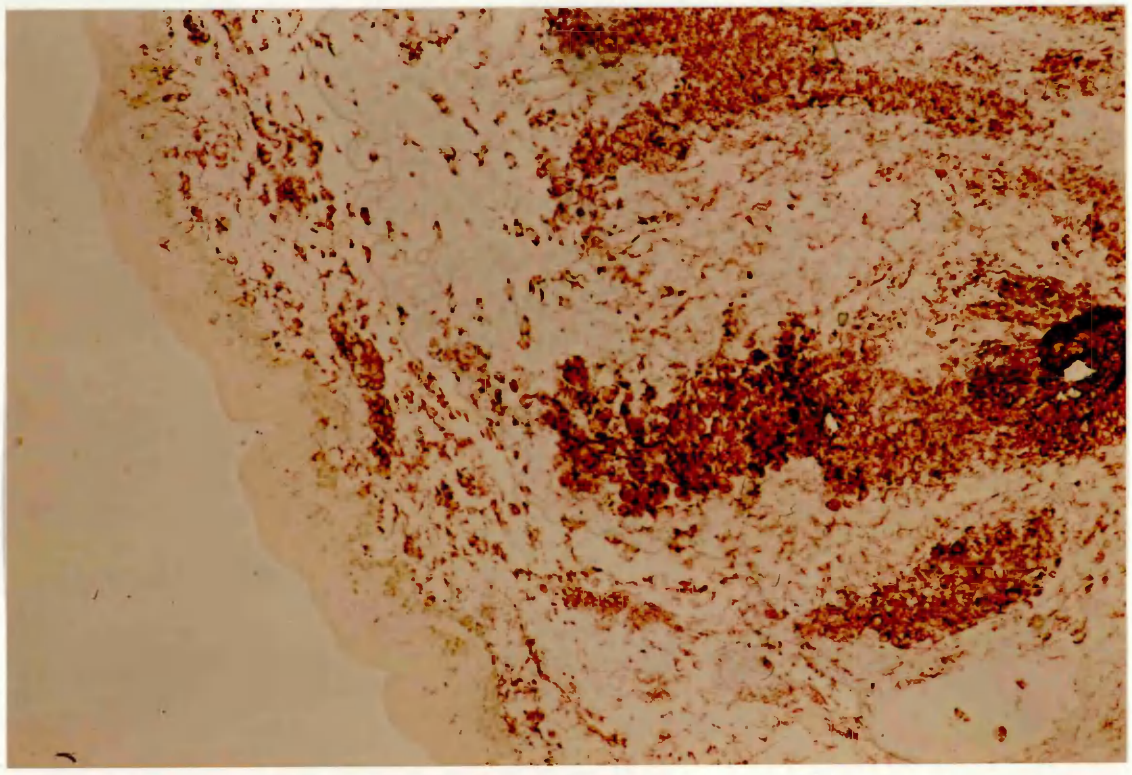


Fig. 6.4

Figure 6.5 MAb 4A3 reactivity with lymphocytes and vascular endothelial cells (arrows), demonstrated by immuno-histochemistry on frozen section. Human tonsil (DAB, x 92).

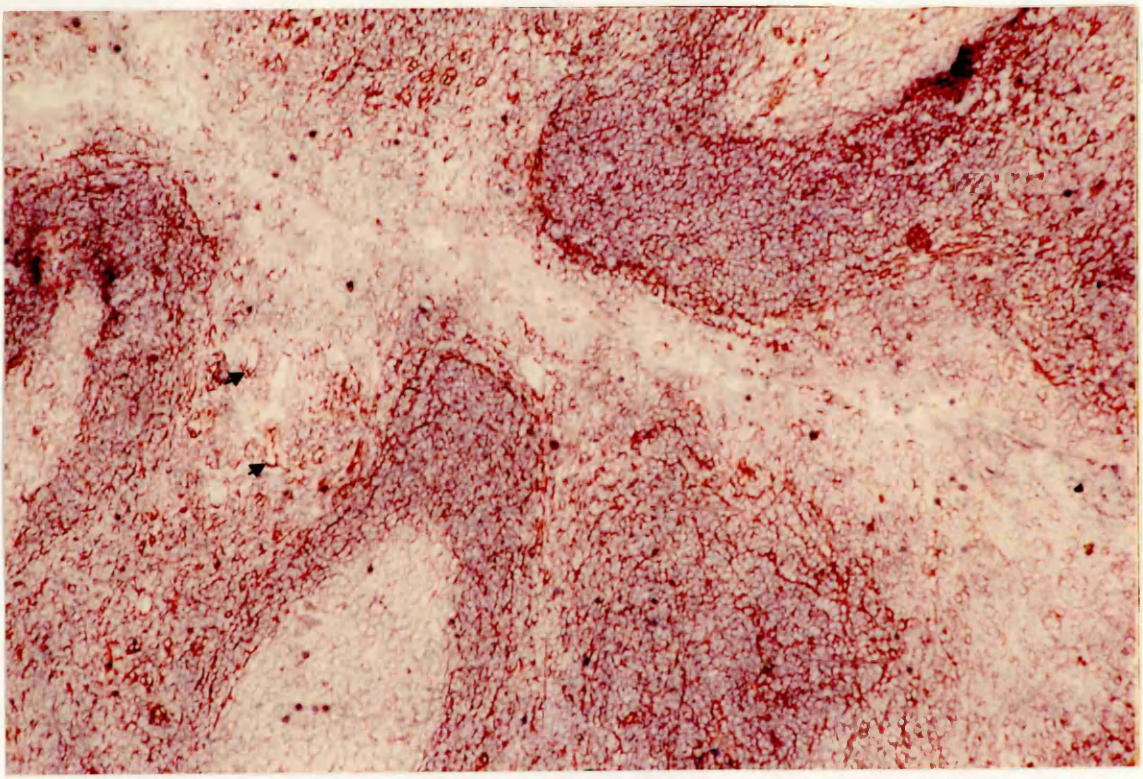


Fig. 6.5

investigation was, therefore, not considered worthwhile. MAb 1B1 reacted against nuclear antigens (Fig. 6.6). MAb 4B4-1 cross-reacted with epithelial cells in sweat glands (Fig. 6.7) and in tonsil (Fig. 6.8) but not with primary and secondary cutaneous melanomas. Figure 6.9 shows an intradermal metastasis from a primary cutaneous melanoma. The melanoma cells are negative whereas the adjacent sweat glands are strongly positive (Fig. 6.10).

6.2.2 Fixed tissue sections

Mab 4A3 was tested against a specimen of uveal melanoma tissue which had been fixed in glutaraldehyde and then in formaldehyde before being embedded in paraffin (Fig. 6.11). The melanoma cells in the centre of the tumour showed strong cytoplasmic staining. Melanin pigment was easily distinguishable from DAB staining by its dark and granular appearance. Lymphocytes and macrophages were negative. The 4A3 antibody showed no reactivity with the peripheral portions of the tumour, which had been fixed more strongly (Fig. 11 iii).

MAb 4A3 was tested against specimens taken from a wide range of normal and neoplastic tissues which had been fixed in formaldehyde and paraffin-embedded. Positive reactivity of weak to moderate intensity was seen with breast carcinoma cells (Fig. 6.12) and vascular endothelial cells (Fig. 6.13).

Figure 6.6 MAb [B] reactivity with cell nuclei, demonstrated by immunohistochemistry on frozen section. Cutaneous melanoma (DAB, x 230).

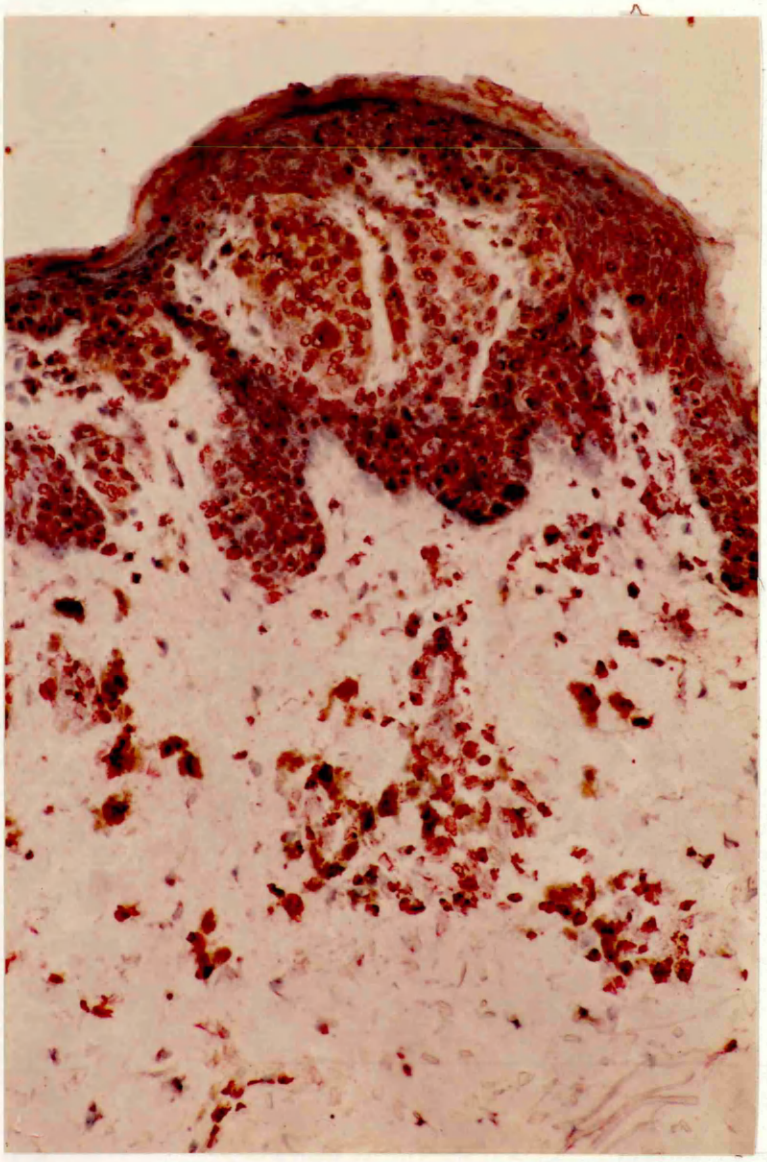


Fig. 6.6

Figure 6.7 MAB 4B4-1 reactivity with sweat gland epithelium demonstrated by immunohistochemistry on frozen section (AEC, x 230).

Figure 6.8 MAB 4B4-1 reactivity with tonsillar epithelium demonstrated by immunohistochemistry on frozen section (DAB, X 92).

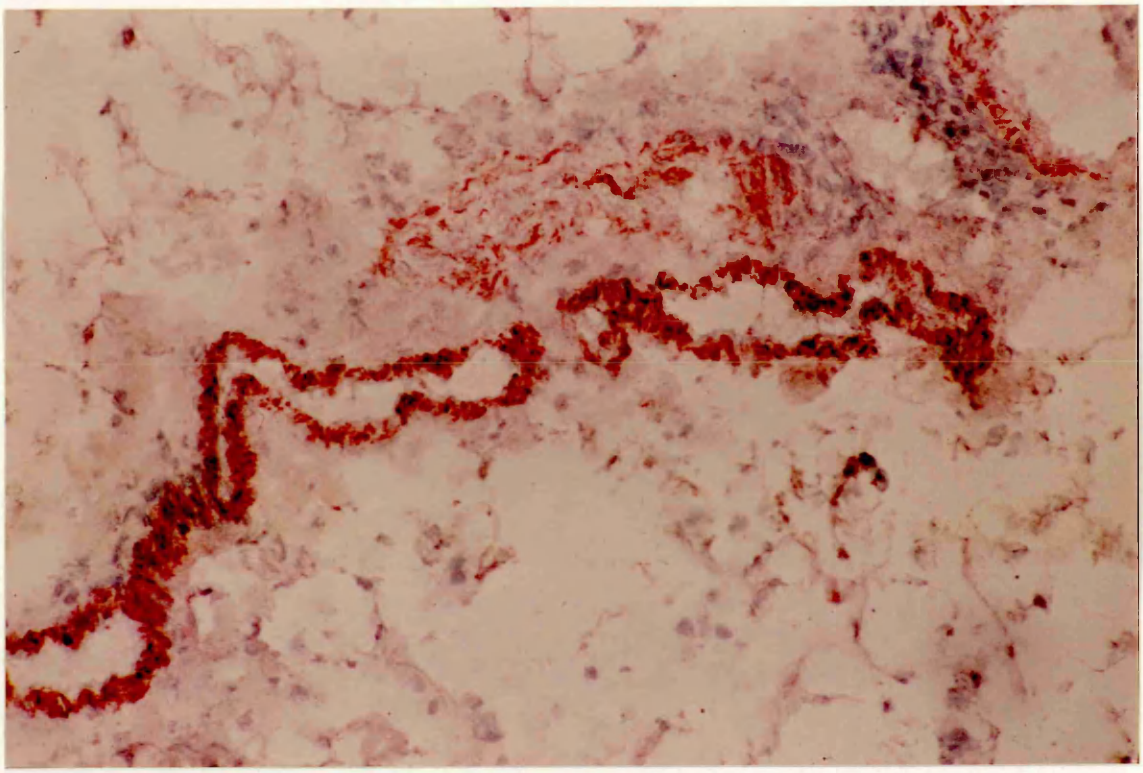


Fig. 6.7

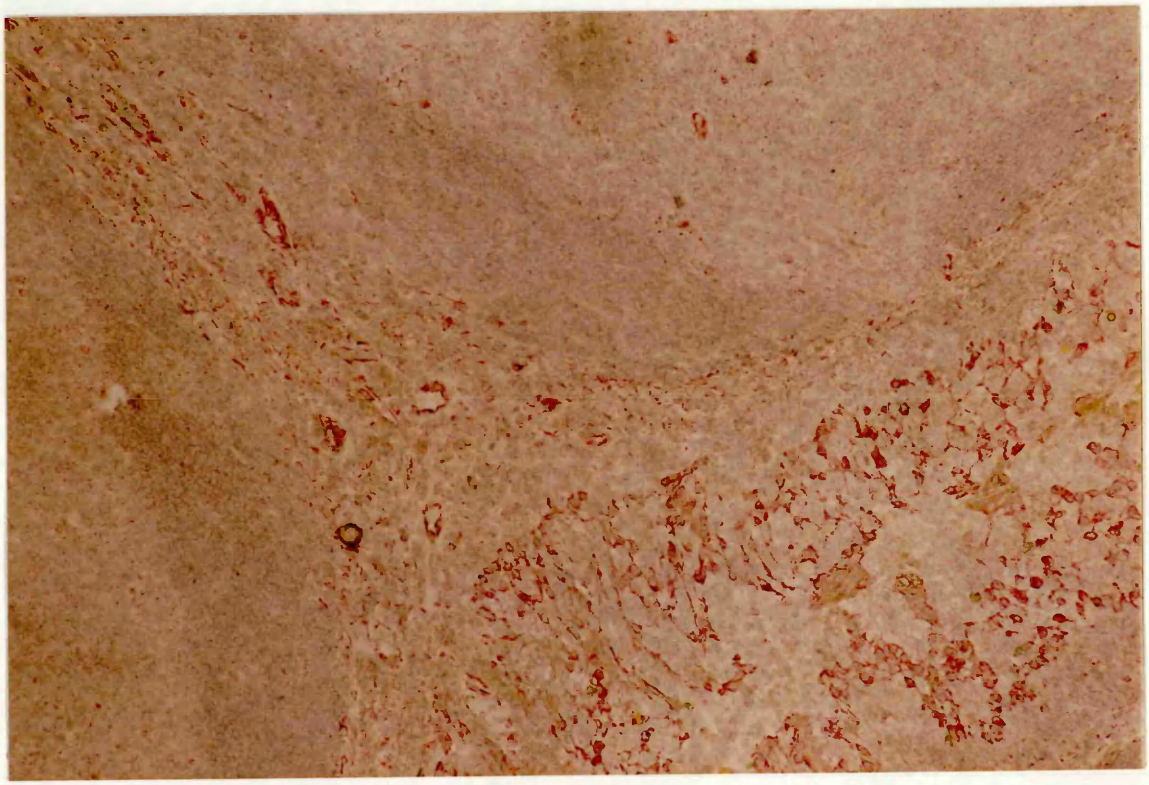


Fig. 6.8

Figure 6.9 Lack of reactivity of MAB 4B4-1 with intradermal metastasis from a primary cutaneous melanoma demonstrated by immunohistochemistry on frozen section. (AEC, X 92).

Figure 6.10 MAB 4B4-1 reactivity with intradermal glandular structures displaced by metastatic lesion shown in Fig. 6.9 demonstrated by immunohistochemistry on frozen section. (i) (AEC, X 230).

Fig. 6.9

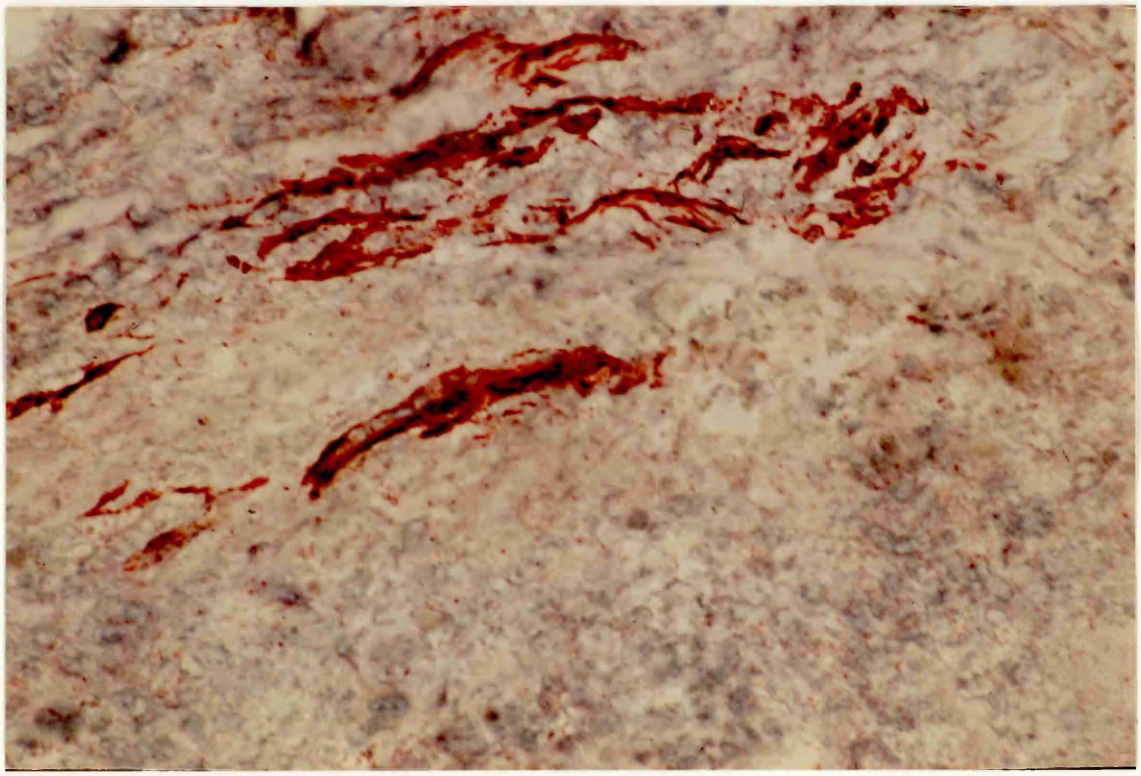


Fig. 6.10

Figure 6.11 MAb 4A3 reactivity with fixed uveal melanoma tissue. (i) Immunoperoxidase staining. Tumour cells, but not lymphocytes and macrophages, are positive (DAB, x 230). (ii) Negative control (DAB, x 230). (iii) Surface of specimen. Staining was weak where there was rapid primary fixation and stronger where there was a delay in primary fixation (DAB, x 230). (iv) Haematoxylin-eosin of same tumour (x 230).

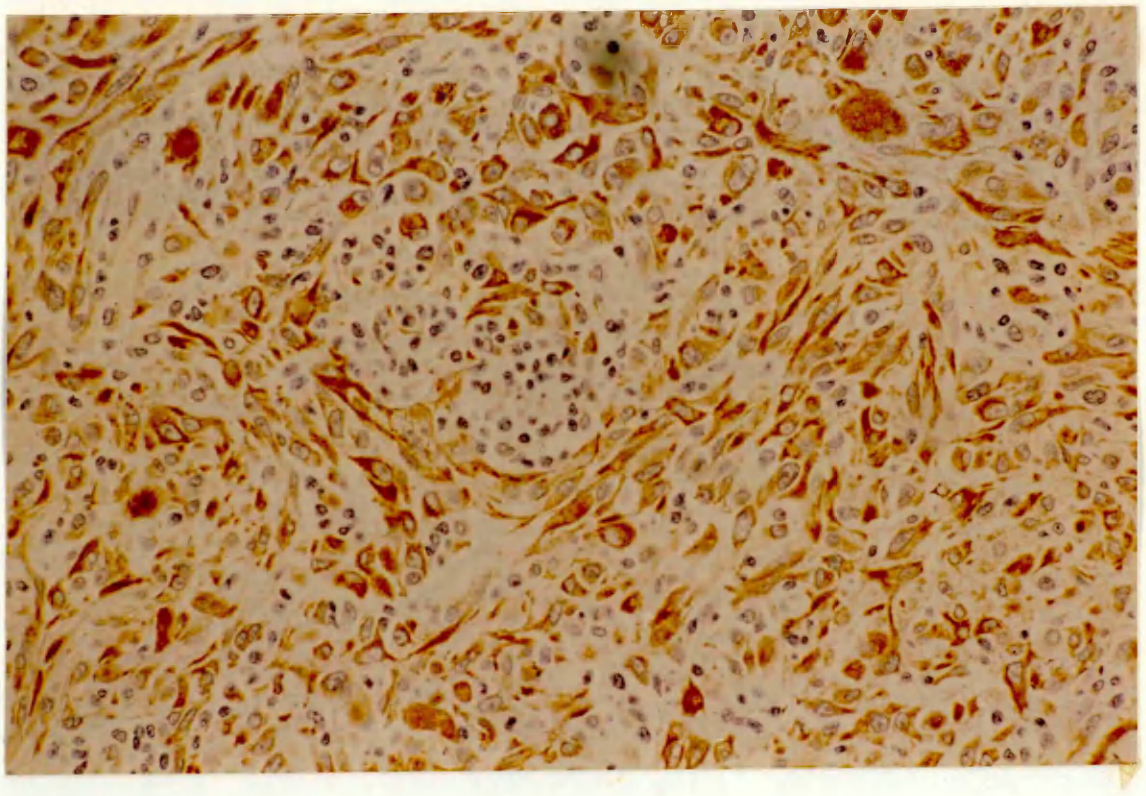


Fig. 6.11 (i)

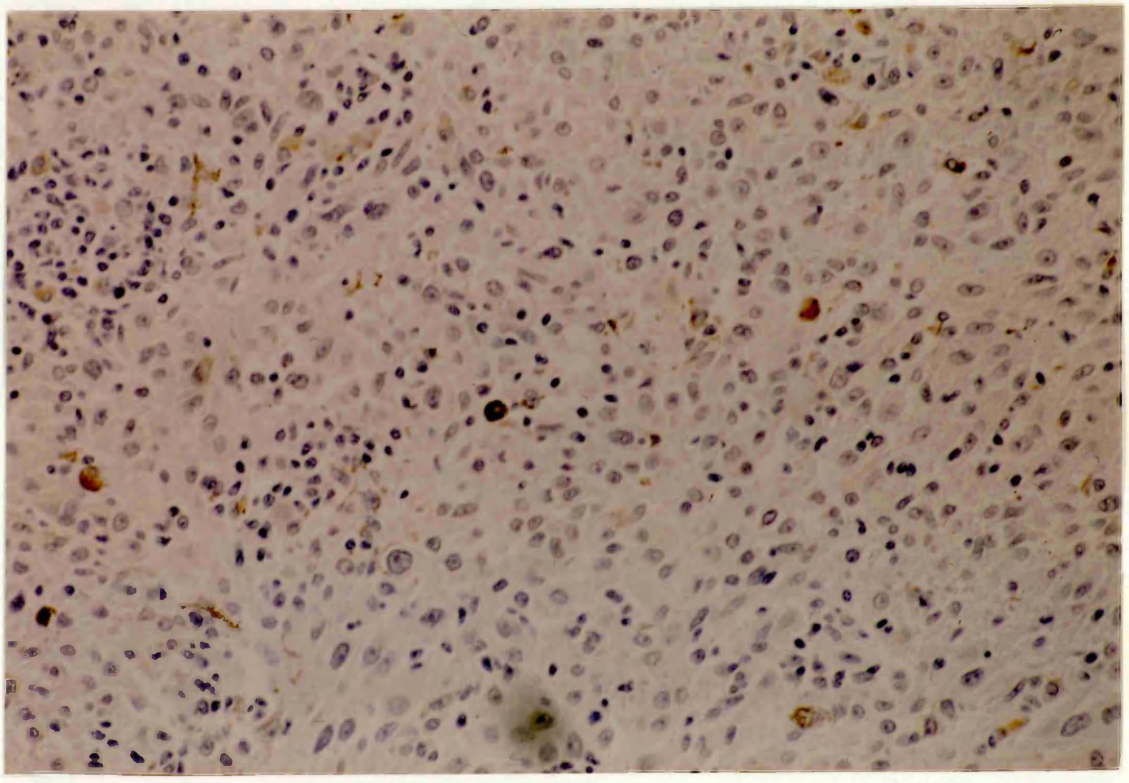


Fig. 6.11 (ii)

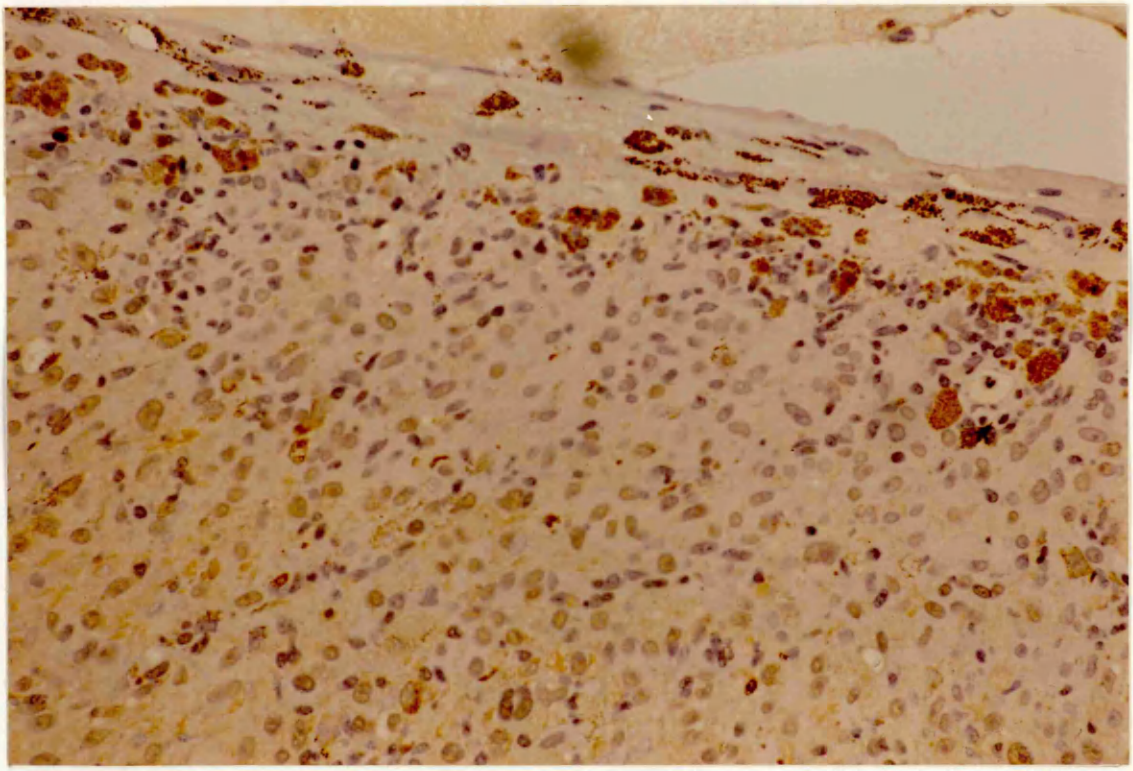


Fig. 6.11 (iii)

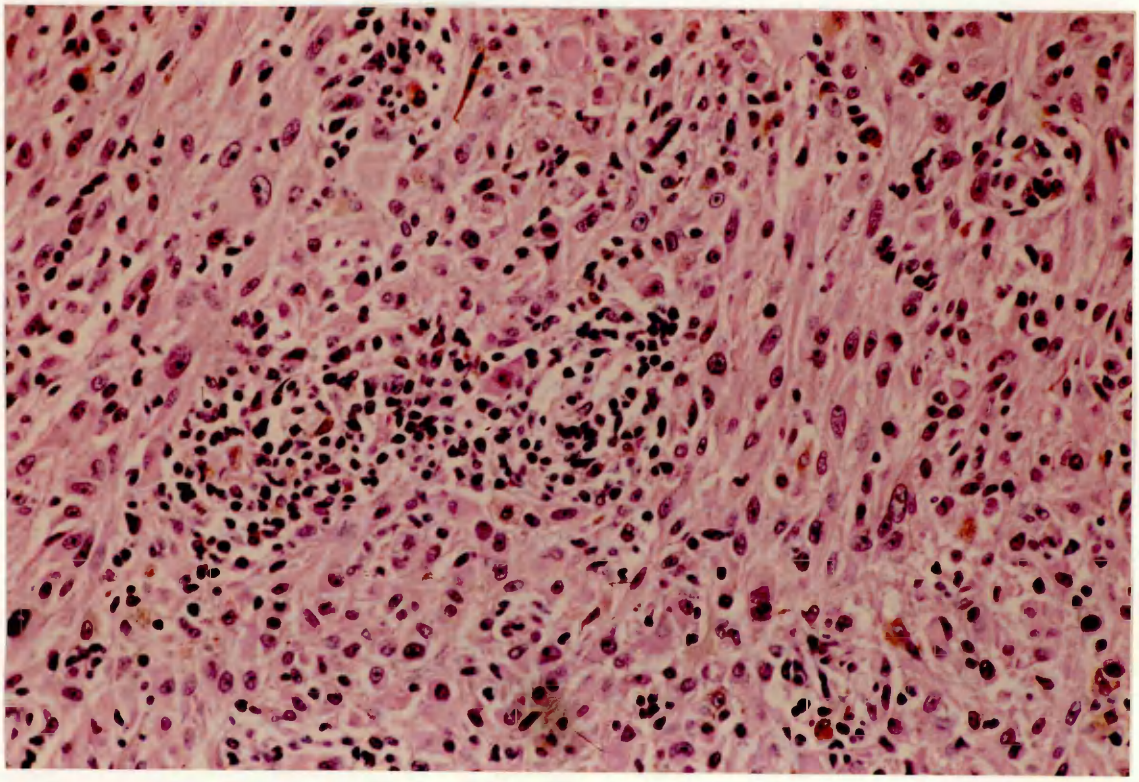


Fig. 6.11 (iv)

Figure 6.12 MAb 4A3 reactivity with breast carcinoma, demonstrated by immunohistochemistry on fixed tissue preparation. (i) Monoclonal antibody (DAB, x 230). (ii) Negative control showing non-specific staining (DAB, x 92).

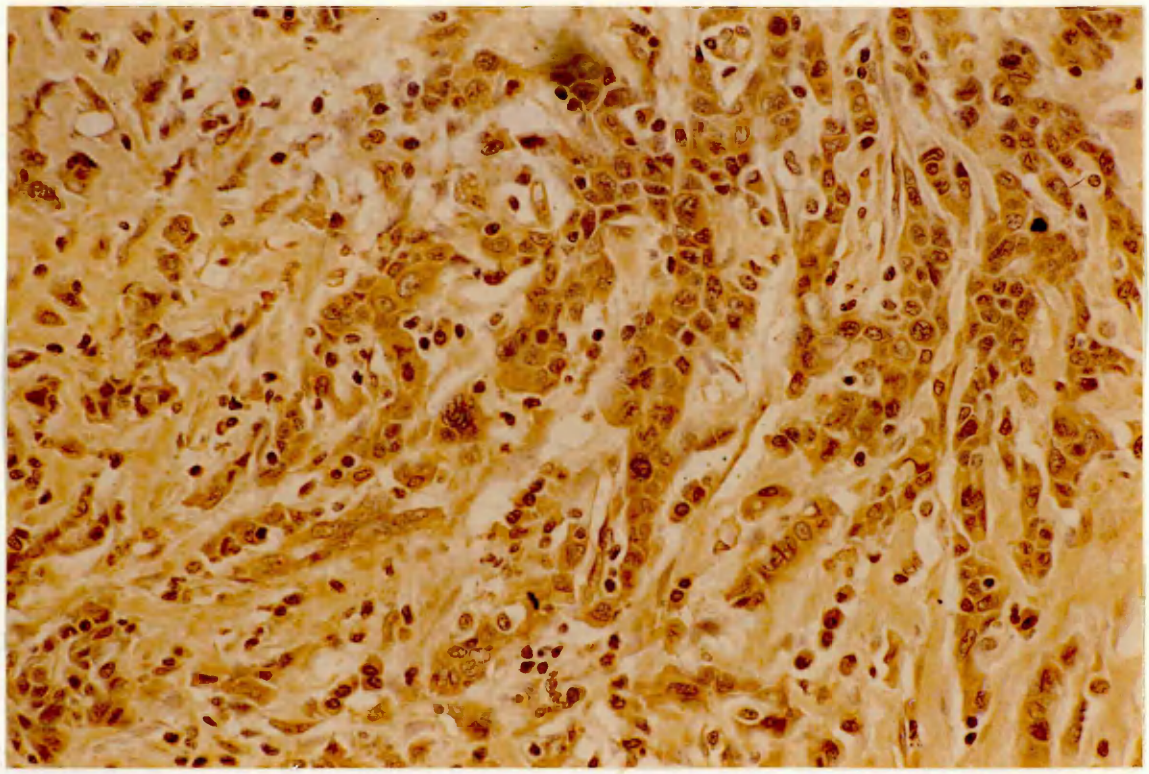


Fig. 6.12 (i)

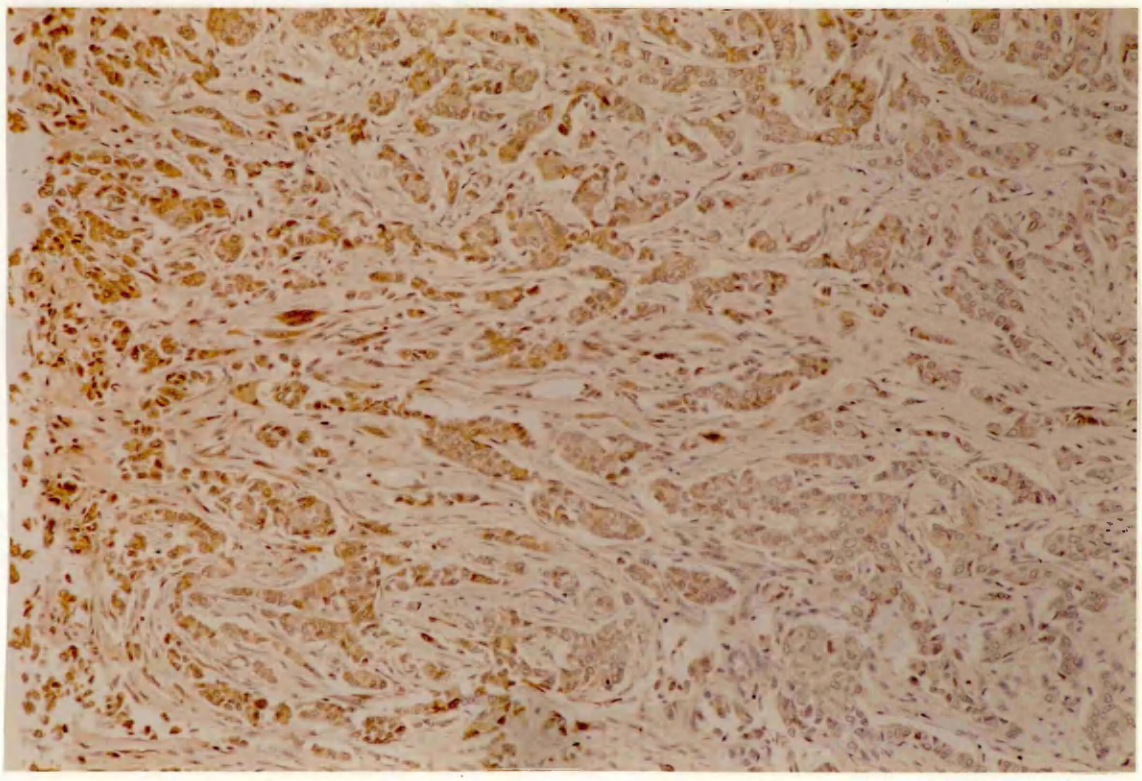


Fig. 6.12 (ii)

Figure 6.13 MAb 4A3 reactivity with vascular endothelial cells, demonstrated by immunohistochemistry on fixed tissue preparation. (i) Sections of renal carcinoma incubated with monoclonal antibody (ii) Negative control (DAB, Magn. (i) and (ii) x 230).

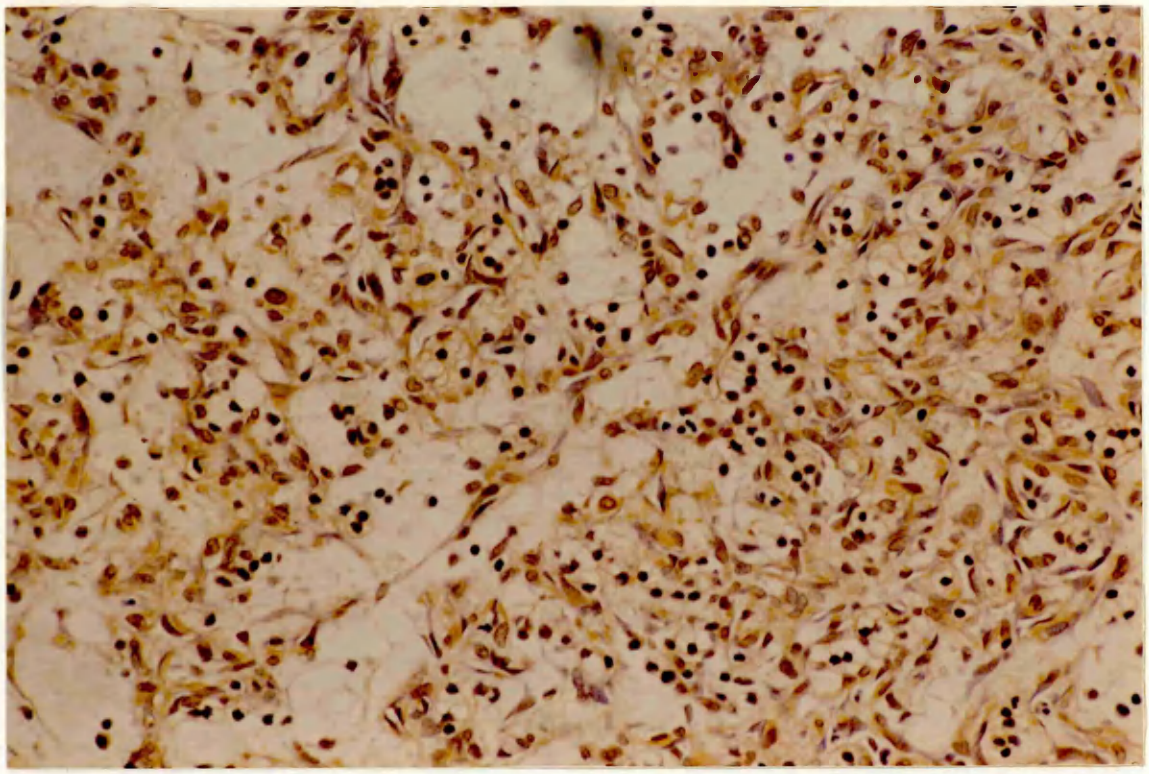


Fig. 6.13 (i)

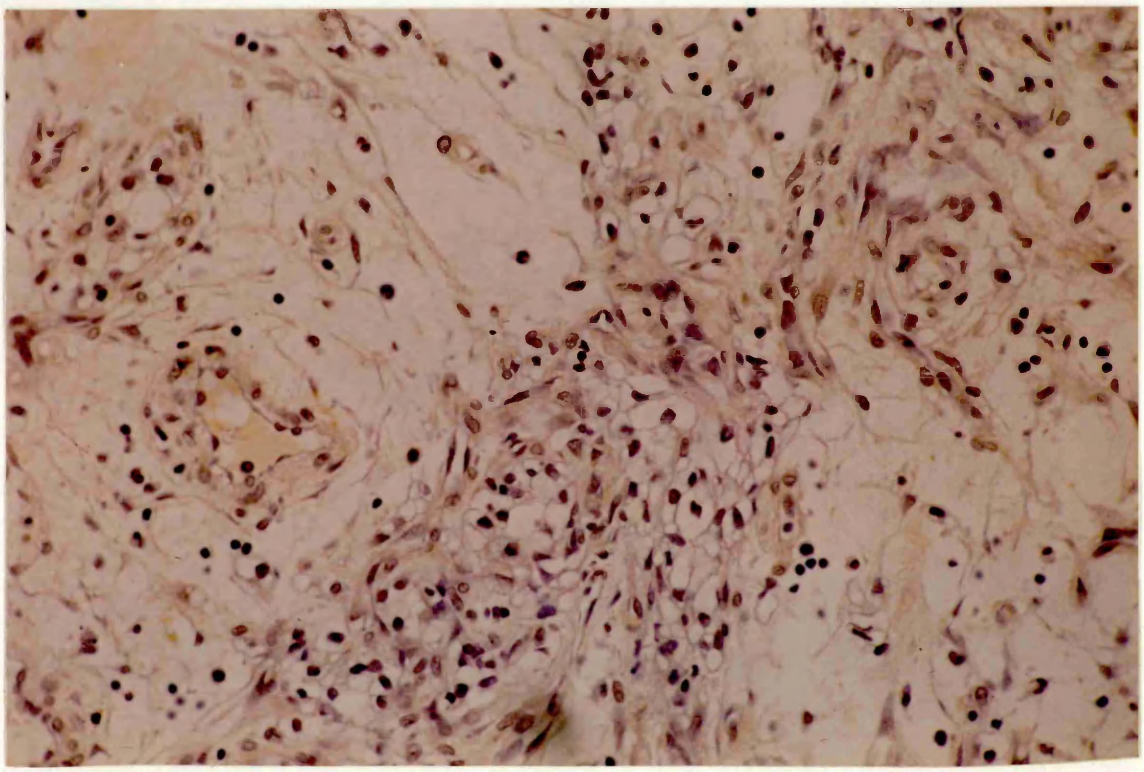


Fig. 6.13 (ii)

6.3 Immunofluorescence microscopy

The methods used are described in Section 2.3.7. Briefly, the cells were attached to plastic slides, incubated successively with monoclonal antibody and FITC-labelled rabbit anti-rat IgG (H&L) and then examined microscopically in ultraviolet-blue light.

MAb 4A3 reacted strongly with all cultured B008 melanoma cells but only if these had been fixed with alcohol or acetone (Fig. 6.14); there was no staining of live unfixed melanoma cells. The antigen appeared to be concentrated in the peri-nuclear region. Like the 4A3 antibody, Mab 4B4-2 reacted with all fixed melanoma cells but tended to show diffuse cytoplasmic staining (Fig. 6.14).

Whereas monoclonal antibody 4A3 reacted equivocally with lymphocytes, definite reactivity was seen with HeLa cells (Fig. 6.15).

Attempts were made to stain melanoma cells using FITC labelled Mab 4A3. Although the antibody was labelled successfully, there was no staining of the tumour cells.

Figure 6.14 Reactivity of mAbs 4A3 and 4B4-2 with cultured B008 melanoma cells, demonstrated by immunofluorescence microscopy. (i) Reactivity of mAb 4A3 showing fluorescence in the perinuclear region (ii) Reactivity of mAb 4B4-2 showing diffuse cytoplasmic staining. (iii) Negative control. (iv) Phase contrast micrograph of a representative field (x 370).

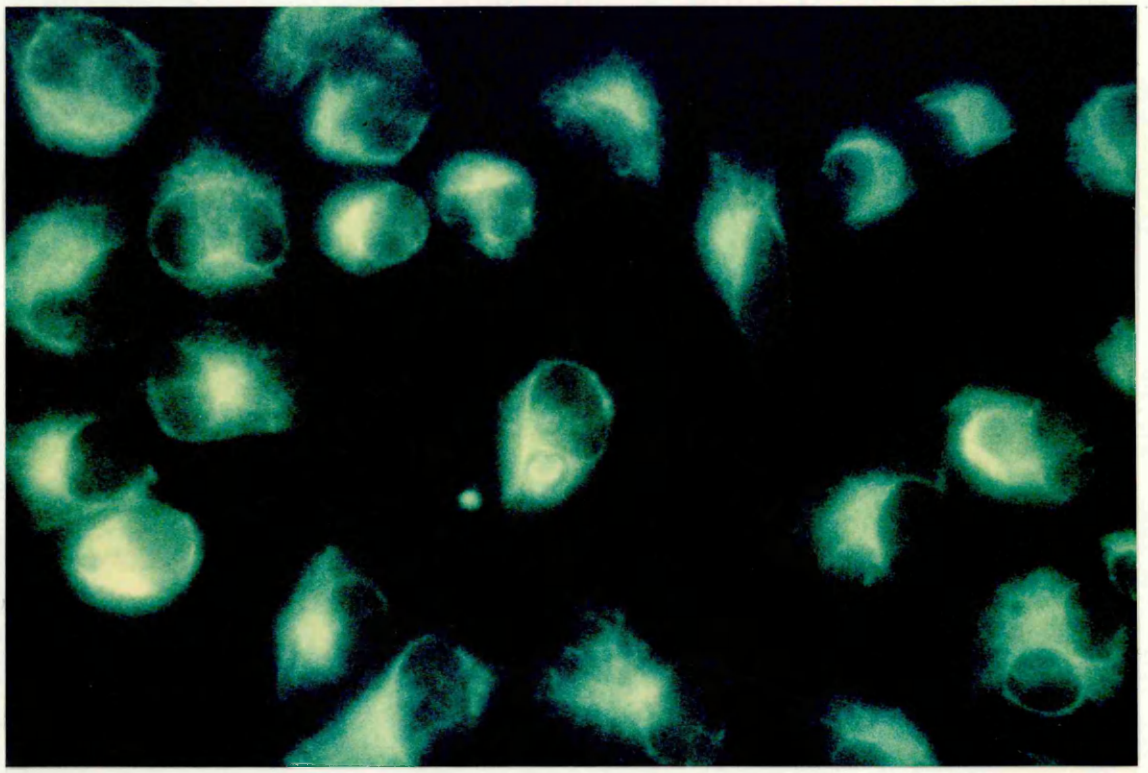


Fig. 6.14 (i)

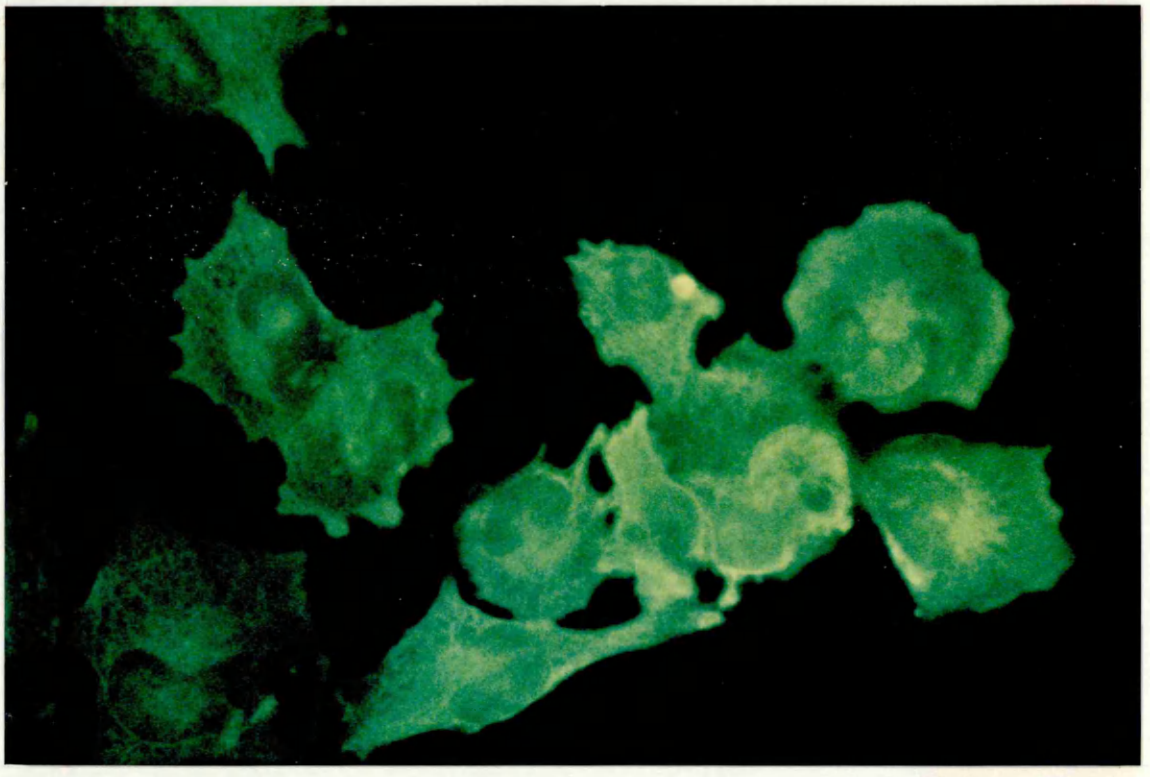


Fig. 6.14 (ii)

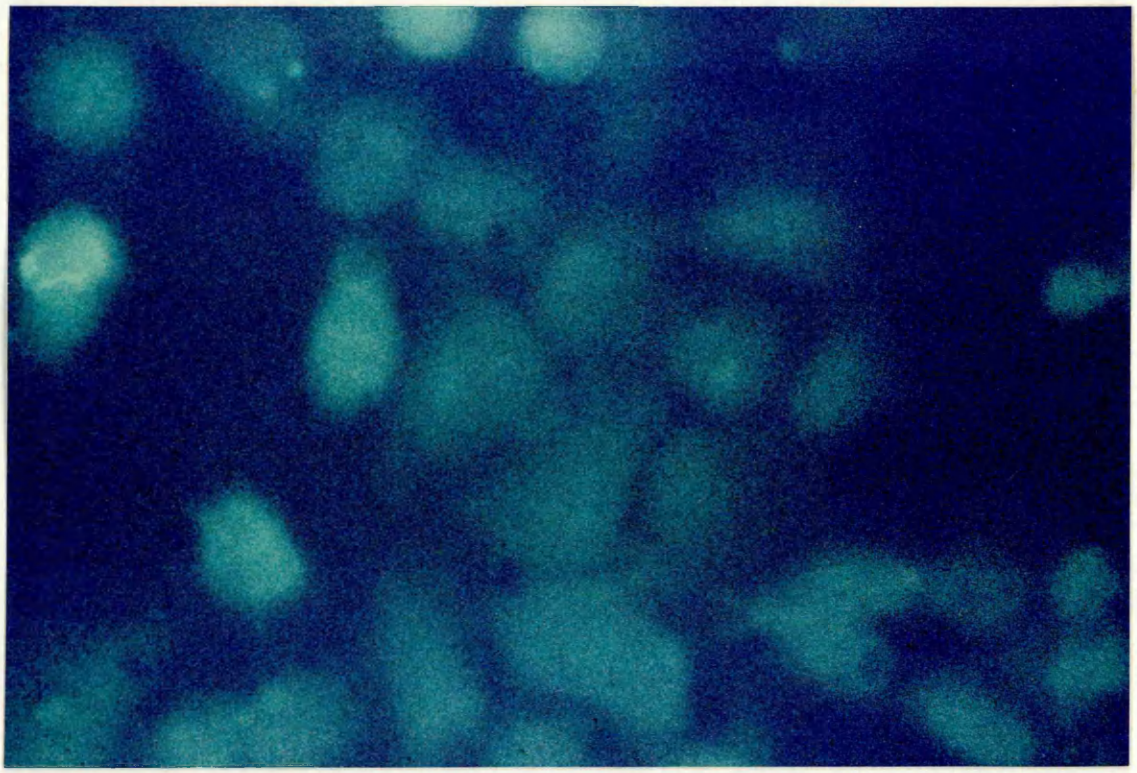


Fig. 6.14 (iii)

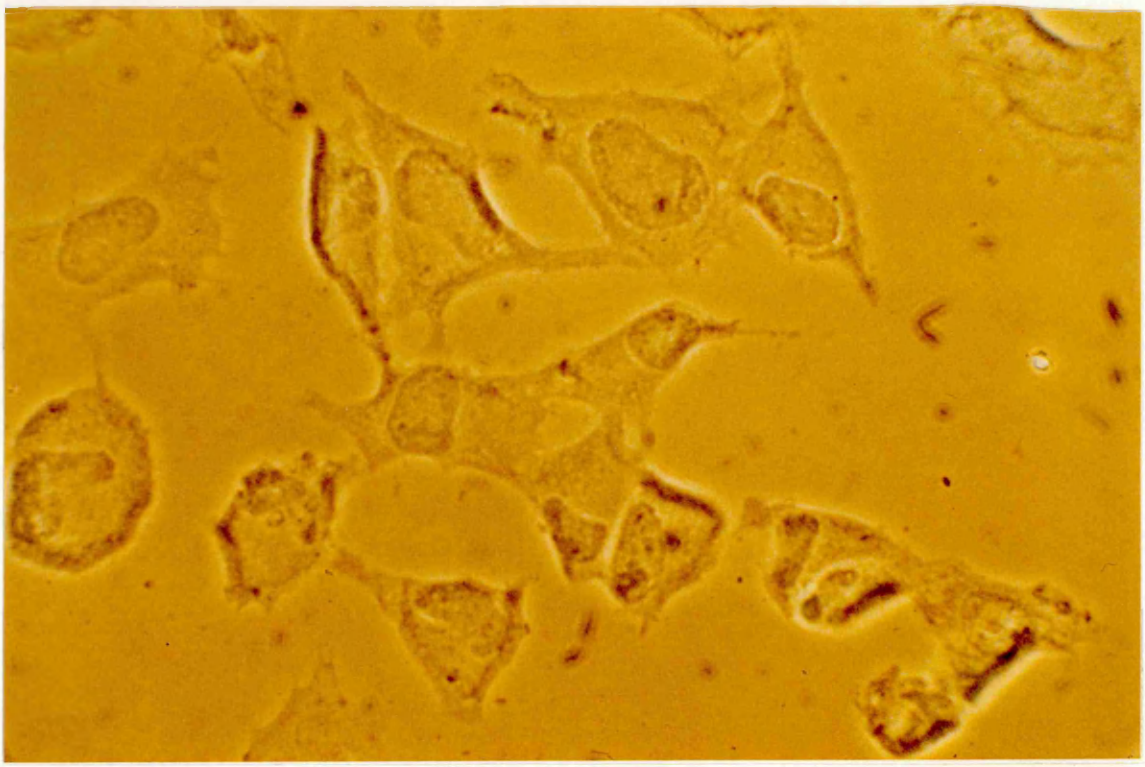
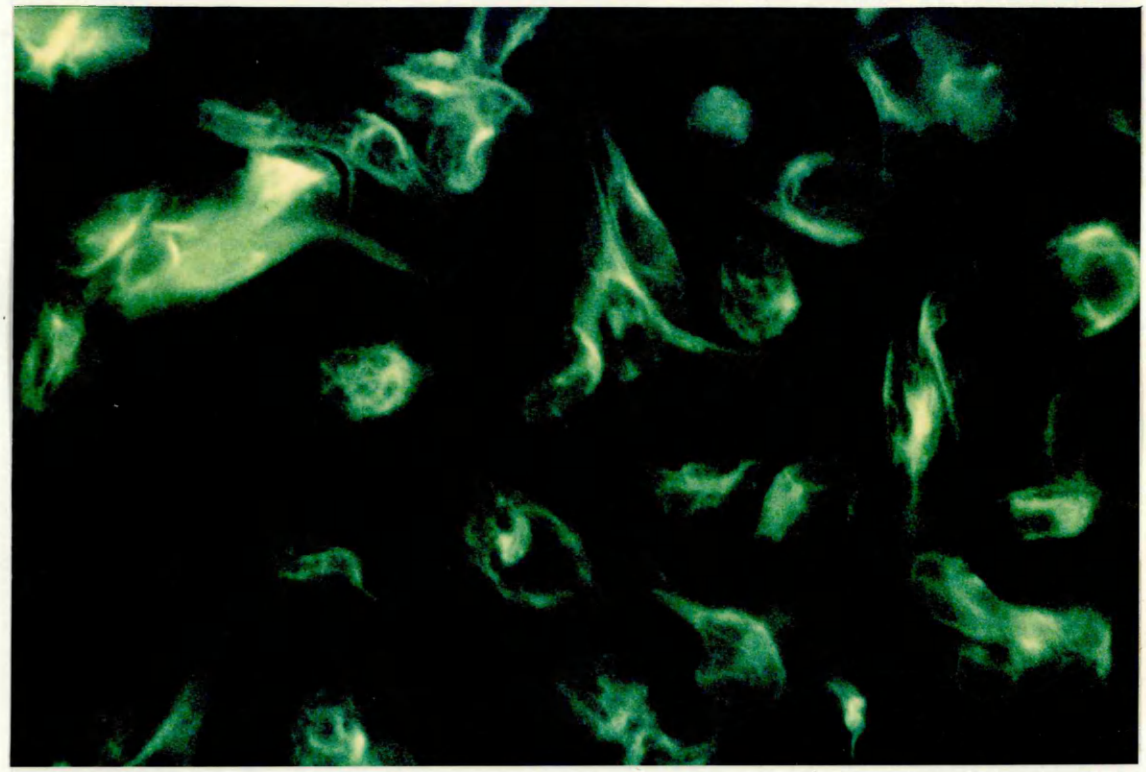
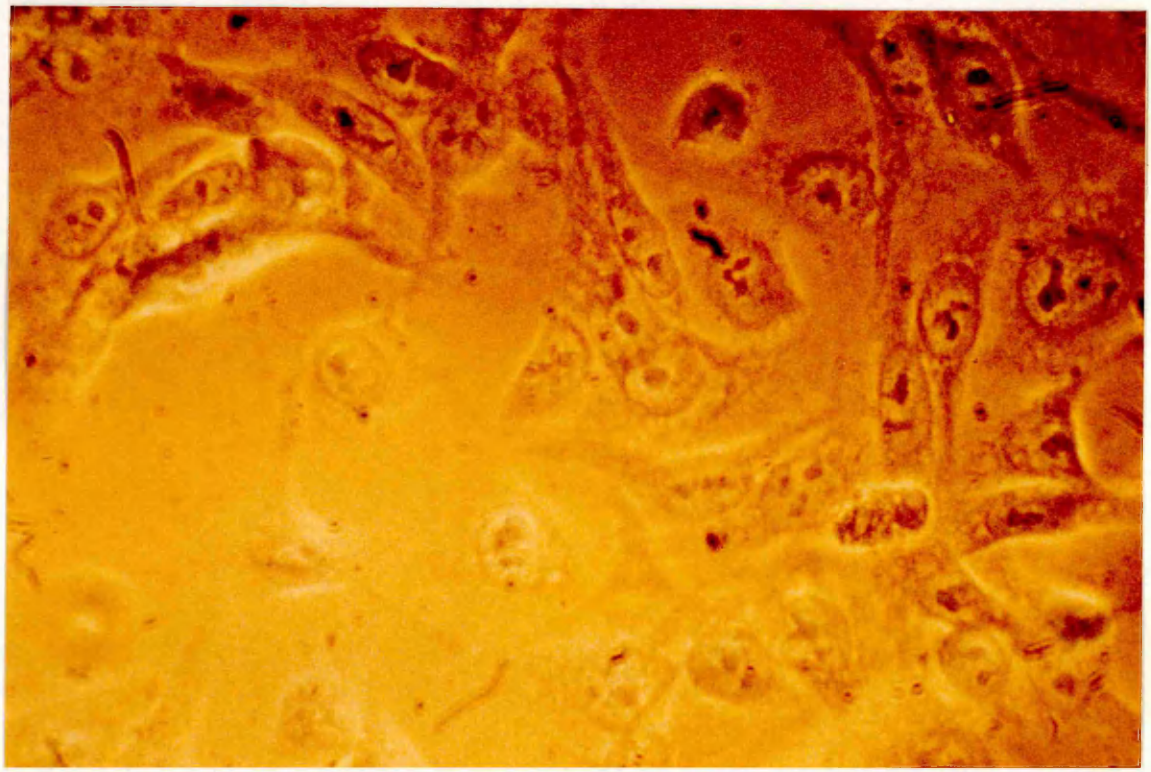


Fig. 6.14 (iv)

Figure 6.15 MAb 4A3 reactivity with HeLa or melanoma cells, demonstrated by immunofluorescence microscopy. (i) HeLa cells (ii) Melanoma cells (a) Fluorescent light (b) Phase contrast (x 370).



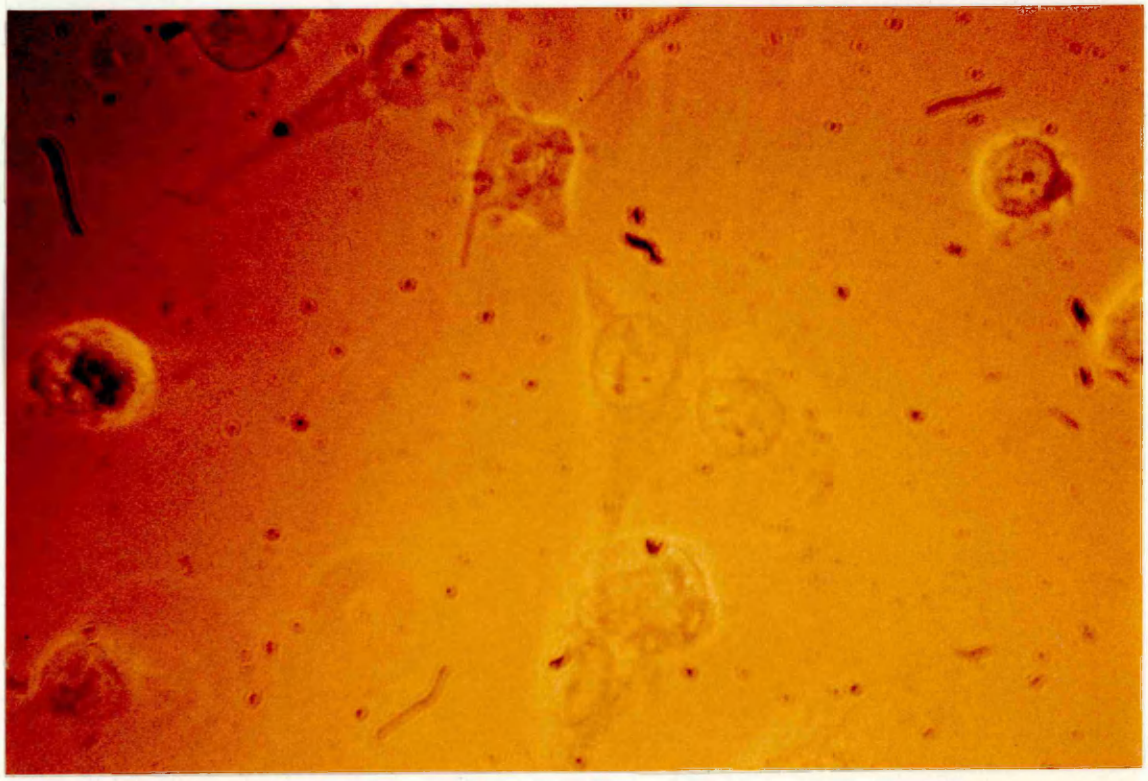
(a)



(b)

Fig. 6.15 (i)

(a)



(b)

Fig. 6.15 (ii)

6.4. Discussion

6.4.1 Immunohistochemistry

Monoclonal antibody 4A3 did not react specifically with melanoma cells on frozen sections, cross-reacting with fibrocytes, some lymphocytes, vascular endothelial cells, and naevus cells. With fixed tissues this antibody did not seem to react with lymphocytes and macrophages, although breast carcinoma cells and vascular endothelial cells were positive.

Different methods of tissue fixation and of performing the immunohistochemistry, produce different results. (Judd & Britten 1982; Van Ewijk *et al* 1984; Warford & Ketchin 1986; Hancock & Atkins 1986; Ciocca & Bjercke 1986). If further studies are performed to confirm the preliminary results that were obtained, preliminary investigations would be necessary to determine which protocol is most suitable.

6.4.2 Immunofluorescence microscopy

Immunofluorescence microscopy demonstrated that both 4A3 and 4B4-2 react with cytoplasmic antigens. The 4A3 antigen appeared to be situated in the peri-nuclear region. Immuno-electron microscopy would be required to locate the antibody more precisely. Positive staining of the HeLa cells is further evidence that the antigen recognised by the 4A3 monoclonal antibody is not specific for melanoma. The staining pattern of the 4B4-2 antigen suggests that this antigen is related to cytoskeletal elements.

It was hoped that by immunofluorescence microscopy, using the 4A3, 4B4-2 and other antibodies, it would be possible to investigate intra-tumour antigenic heterogeneity in uveal melanomas. It was found, however, that the rodent monoclonal antibodies tested stained either all melanoma cells or none at all, probably because of the way in which the hybrids were selected.

Chapter 7

STUDIES ON SERUM ANTIBODIES TO MELANOMA-ASSOCIATED ANTIGENS USING AN INHIBITION ELISA

7.1 Introduction

Until standardised preparations of purified melanoma-associated antigens become available, antigens for assays of humoral immunity to uveal melanoma will need to consist of autologous or allogeneic melanoma cells. The use of whole cells as antigens in such assays has certain practical disadvantages. Firstly, cells prepared from uveal melanomas of different individuals differ in their antigenic expression, thereby preventing reproducible results from being obtained in assays using such antigens. Secondly, the results of these assays might be dominated by the antigens which are present in the largest amounts rather than by those which are more relevant or specific. An inhibition ELISA was developed in the present study in the hope of overcoming these problems. The rationale of the assay was as follows: if the binding of human serum antibodies to a particular antigen could be measured by the extent to which they inhibit the binding of a xenogeneic monoclonal antibody to that antigen, then it should theoretically be possible to obtain reproducible results using whole cells from different uveal melanomas. The use of a monoclonal antibody as the probe should also make it possible to identify and measure serum antibodies reacting with an antigen whose specificity, structure and biological significance might be well known.

The methods are described in detail in Section 2.3.5.4. Briefly, binding of the monoclonal antibodies to uveal melanoma cells was measured in the usual fashion after prior incubation either with patient serum or with control serum from healthy volunteers.

7.2 Inhibition ELISA

The results of inhibition assays using four different monoclonal antibodies are shown in Fig. 7.1. Serum from four out of five patients with uveal melanoma apparently inhibited the binding of monoclonal antibody 4A3 to allogeneic melanoma cells. The same sera produced very similar results with each of three other monoclonal antibodies (1B1, 1B4 and 4B4-1). These results did not appear to correlate with the levels of IgG, IgA and IgM, total protein and albumin respectively in the serum samples (Fig. 7.2). The experiment was repeated using a different set of serum samples, with similar results (Fig. 7.3).

When the inhibition ELISA was performed using doubling dilutions of human serum, the undiluted control samples tended to give the highest Multiscan readings (Fig. 7.4). This suggested that the horseradish-linked rabbit anti-rat IgG (H&L) antibody was reacting non-specifically with antibodies or other factors in the human serum thereby interfering with the results of the inhibition ELISA. This hypothesis was supported by the results of a chequerboard ELISA (Fig. 7.5): serial doubling dilutions of the patient serum and monoclonal antibody (4A3) were dispensed vertically and horizontally

Figure 7.1 Inhibition of binding of monoclonal antibodies to melanoma cells on ELISA by human serum. ELISA wells, coated with melanoma cells, were incubated with serum from healthy individuals (1-4) and patients with uveal melanoma (A-E), prior to performing the assay. Similar results were obtained with different monoclonal antibodies.

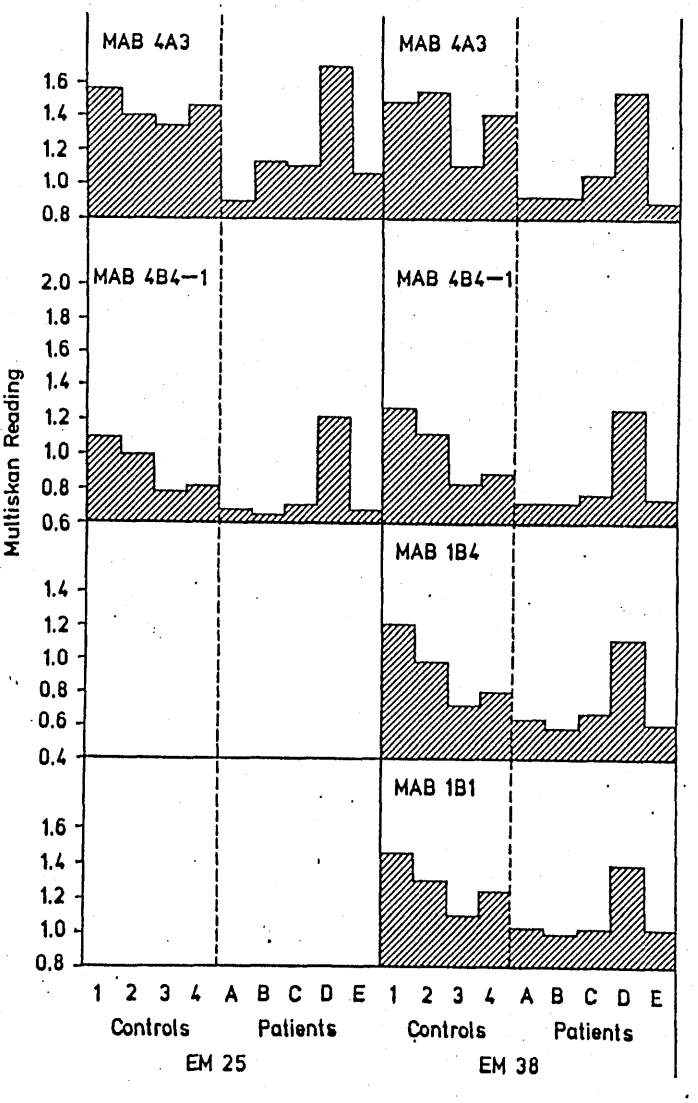

MAB 4A3
MAB 4A3
MAB 4B4–1
MAB 4B4–1
MAB 1B4
MAB 1B1
Multiskan Reading
1 2 3 4 A B C D E 1 2 3 4 A B C D E
Controls
Patients
Controls
Patients
EM 25
EM 38

Fig. 7.1

Figure 7.2 Levels of immunoglobulins, protein and albumin in serum samples used for inhibition studies summarised in Fig. 7.1. Measurements were performed in a routine hospital laboratory.

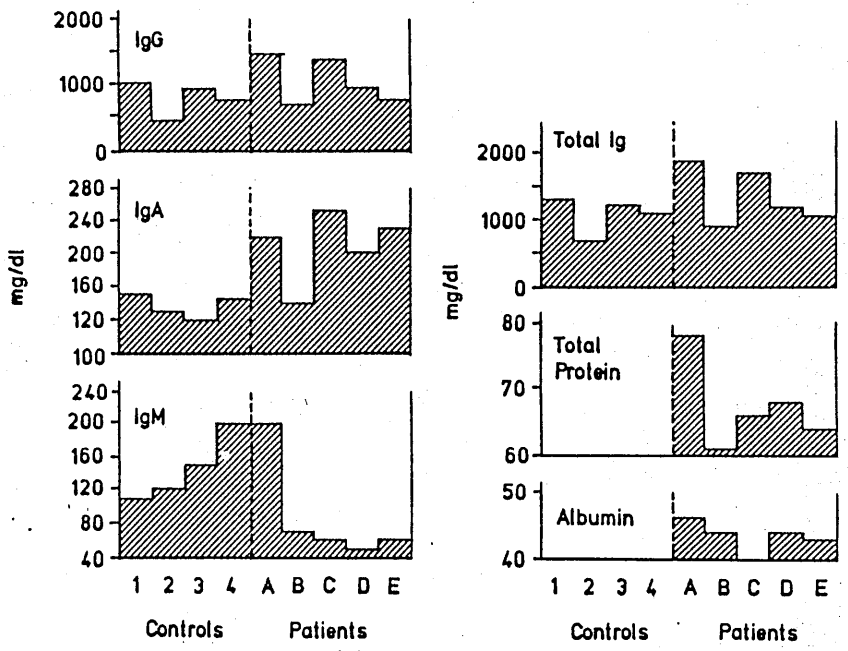

IgG
IgA
IgM
Total Ig
Total Protein
Albumin
mg/dl
mg/dl
2000
1000
0
280
240
200
160
120
100
240
200
160
120
80
40
80
70
60
50
40
1 2 3 4 A B C D E
Controls
Patients
1 2 3 4 A B C D E
Controls
Patients

Fig. 7.2

Figure 7.3 Inhibition of binding of mAb 4A3 to melanoma cells on ELISA by further samples of human serum taken from patients with uveal melanoma (F-L) and healthy individuals (5-8). The samples were different from those in Fig. 7.1.

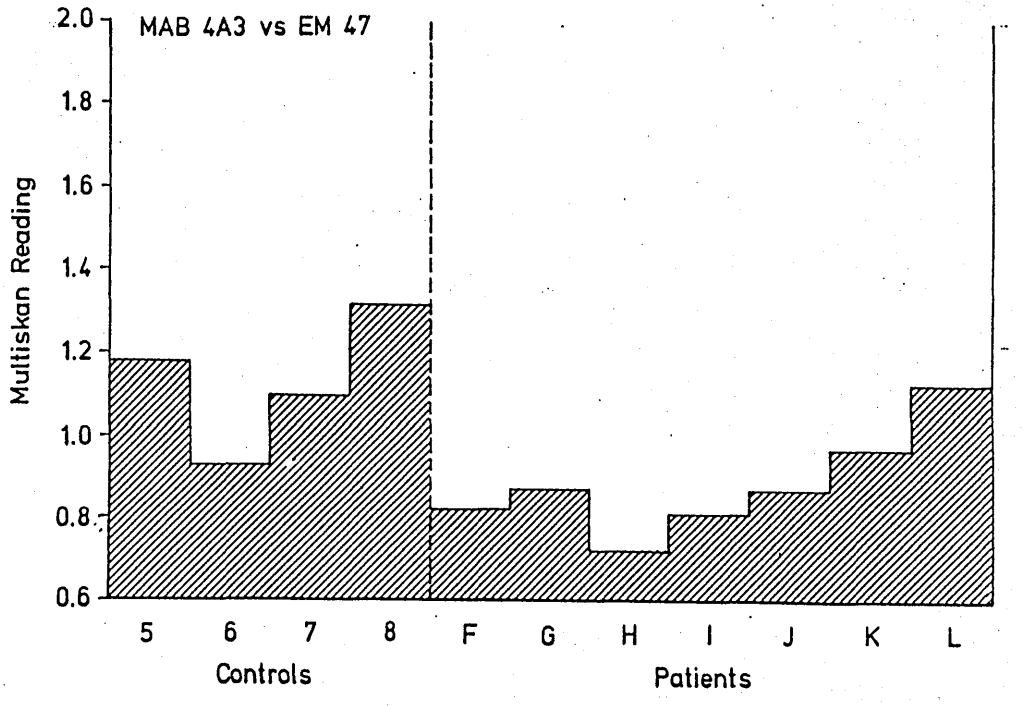

MAB 4A3 vs EM 47
Multiskan Reading
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
5
6
7
8
F
G
H
I
J
K
L
Controls
Patients

Fig. 7.3

Figure 7.4 Reactivity of mAb 4A3 with uveal melanoma cells after incubation with dilutions of human serum. (i) With serum from healthy individuals, multiskan readings are highest with neat serum, suggesting that the second antibody is reacting with constituents of the human serum. The data were obtained from the experiment summarised in Fig. 7.1).

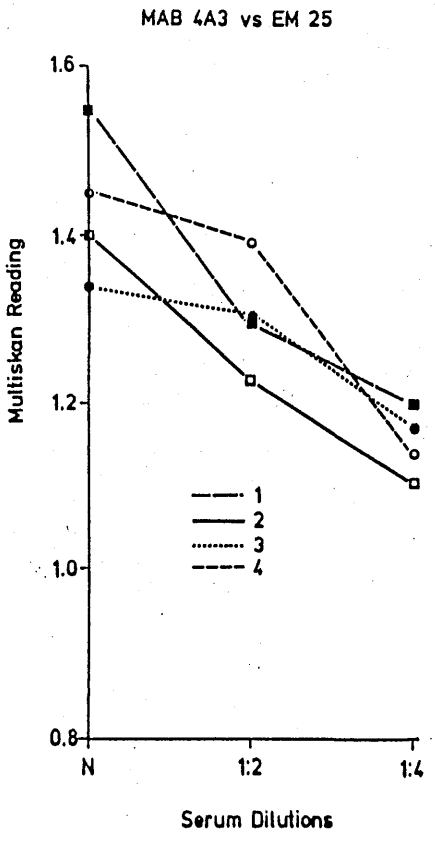

MAB 4A3 vs EM 25
Multiskan Reading
1.6
1.4
1.2
1.0
0.8
1
2
3
4
N
1:2
1:4
Serum Dilutions

Fig. 7.4 (i)

Figure 7.4 Reactivity of mAb 4A3 with uveal melanoma cells after incubation with dilutions of human serum. (ii) With serum from patients with uveal melanoma, four out of the five samples apparently inhibited binding of mAb 4A3 relative to the control samples.

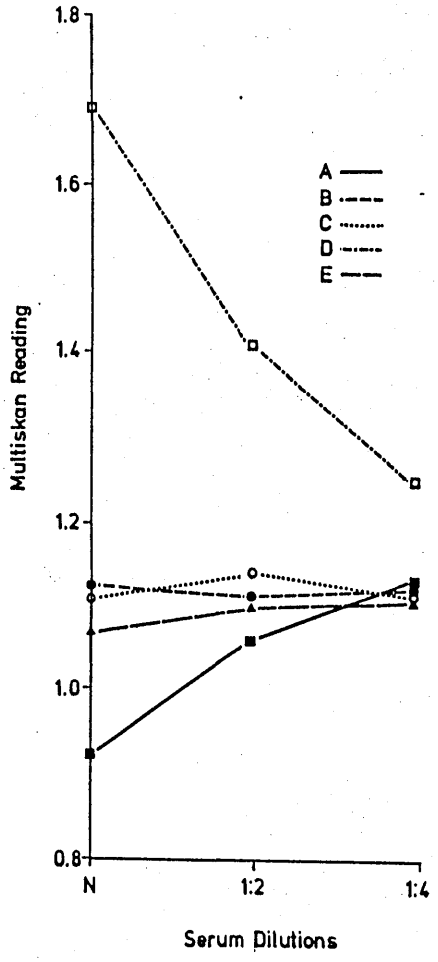

1.8
1.6
1.4
1.2
1.0
0.8
Multiskan Reading
A
B
C
D
E
N
1:2
1:4
Serum Dilutions

Fig. 7.4 (ii)

Figure 7.5 Chequerboard ELISA showing effect of different dilutions of serum on different dilutions of monoclonal antibody. The ELISA plate, coated with uveal melanoma cells, was incubated with doubling dilutions of serum taken from a single patient with uveal melanoma (rows A-H) before the addition of doubling dilutions of mAb 4A3 (rows 2-12). Even at very low concentrations of monoclonal antibody, the multiskan reading is highest with undiluted serum. This suggests that the rabbit anti-rat IgG (H & L) antibody cross-reacts with serum constituents.

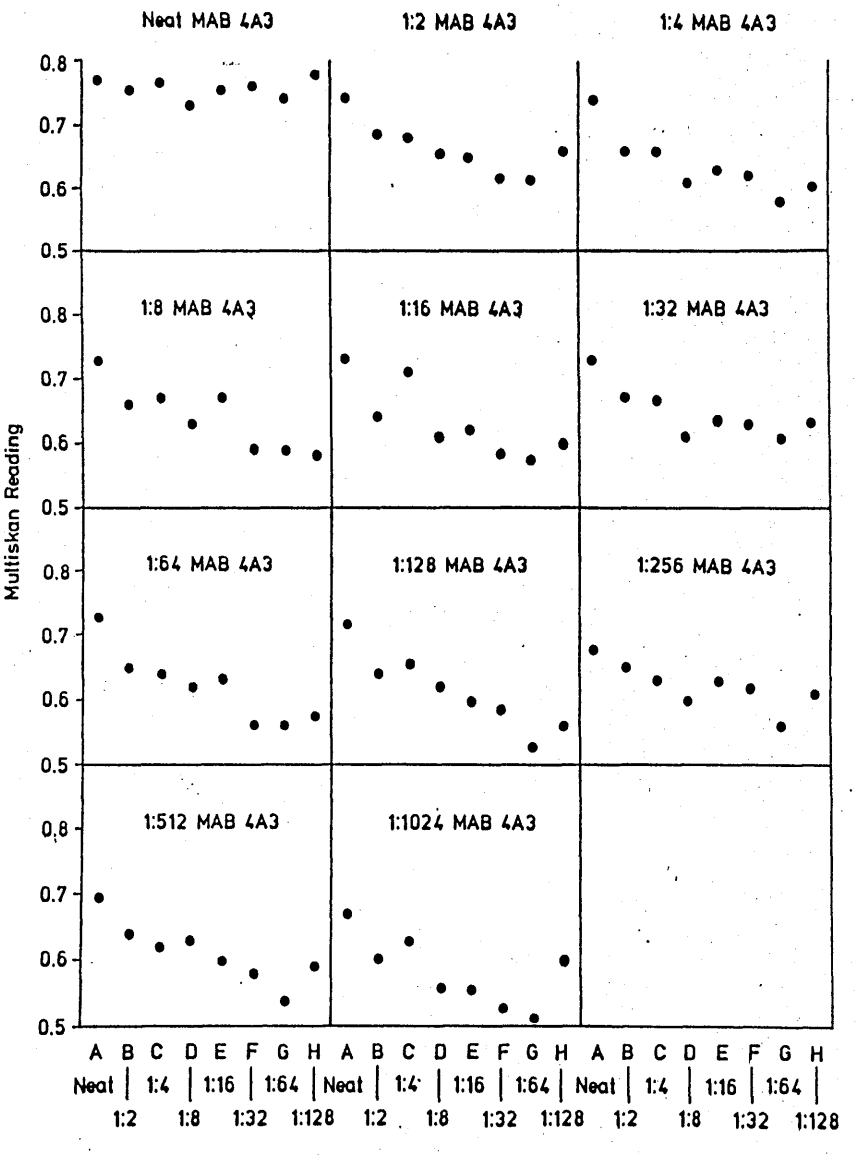

Neat MAB 4A3
1:2 MAB 4A3
1:4 MAB 4A3
1:8 MAB 4A3
1:16 MAB 4A3
1:32 MAB 4A3
1:64 MAB 4A3
1:128 MAB 4A3
1:256 MAB 4A3
1:512 MAB 4A3
1:1024 MAB 4A3
Multiskan Reading
0.8
0.7
0.6
0.5
A B C D E F G H
Neat | 1:4 | 1:16 | 1:64 |
1:2 1:8 1:32 1:128

Fig. 7.5

respectively on the ELISA plate. At high concentrations of patient serum (ie, dilutions less than 1:8), high multiscan readings were obtained even at low concentrations of monoclonal antibody (ie, dilutions greater than 1:2).

7.3 Discussion

This study suggests that serum from most of the patients with uveal melanomas inhibited the binding of the monoclonal antibodies to the corresponding antigens. The results, however, were similar with each of the monoclonal antibodies used suggesting that the binding of the monoclonal antibodies was inhibited in a non-specific manner by the patient sera. The lack of correlation with the immunoglobulin levels suggests that other serum constituents may have caused these results. Another possibility is that the different sera contained similar proportions of antibodies to the different antigens.

The fact that prior incubation with the control serum samples increased rather than diminished the Multiscan readings, and the results of the chequerboard assay, suggest that the second antibody cross-reacted with antibodies or other factors in the human serum. It is therefore possible that the sensitivity of the inhibition assay would be increased by using a more specific second antibody.

There are a number of potential problems associated with the measurement of serum antibodies to uveal melanomas by means of an inhibition assay. False positive results might be caused by inhibition of the binding of

the monoclonal antibodies by non-specific antibodies or other factors. False negative results could occur if the human antibodies are dissociated from their antigens by monoclonal antibodies of higher affinity. Alternatively, serum antibodies to tumour-associated antigens might not bind to the melanoma cells on the ELISA plate because they are sequestered within the patient in complex with either the antigen or anti-idiotypic antibodies. In the case of anti-idiotype interactions it has been suggested that the problem could be overcome by dissociating such complexes under acidic conditions (Sjögren et al 1971).

Despite all the potential difficulties the results of these preliminary studies are encouraging and suggest that the use of an inhibition ELISA for the detection of serum antibodies to uveal melanoma merits further investigation.

Chapter 8

STUDIES ON EBV TRANSFORMATION OF PERIPHERAL B LYMPHOCYTES FOR MEASURING HUMORAL IMMUNITY AND FOR THE PRODUCTION OF HUMAN HYBRIDOMAS TO UVEAL MELANOMAS

8.1 Introduction

Quantitative measurements of anti-tumour antibodies in the serum could provide a false impression of the extent of an anti-tumour humoral immune response. There are two reasons for this: the first is the general background non-specific binding observed in all serum samples, and the second is that the antibodies might not be detected if they are already bound to tumour antigens or anti-idiotypes (Section 7.3). Some workers have therefore suggested that it might be better to assess the number of B lymphocytes which are secreting the anti-tumour antibodies (Cote *et al* 1983; Houghton *et al* 1983; Campbell *et al* 1986). Such an approach has recently become possible with the development of techniques for activating and immortalising B lymphocytes *in vitro* by infection with Epstein-Barr virus (Steinitz *et al* 1979, reviewed by Roder, Cole & Kozbor 1986). This technique has another potential advantage at least in theory: relevant B lymphocytes could be propagated indefinitely *in vitro* for the production of human monoclonal antibodies and these antibodies could then be used to investigate the nature of the tumour-associated antigens that are stimulating the immune response.

This chapter gives the results of a preliminary investigation into the scope of using Epstein-Barr virus transformation of peripheral blood lymphocytes, firstly, as a means of studying the humoral immune response to uveal melanomas in patients with this disease and, secondly, as a means of obtaining sufficient numbers of lymphocytes for fusion. The methods have been described in Section 2.5.2. Briefly, B lymphocytes were extracted from a 20 ml sample of peripheral blood, transformed by infection with Epstein Barr virus and dispensed into 60 wells of a cell-culture plate. After two to three weeks of *in vitro* culture with cyclosporin A, the supernatants of the transformed lymphocytes (henceforth referred to as 'transformants') and controls consisting of 20% complete medium were then simultaneously tested by ELISA against autologous or allogeneic uveal melanoma cells and against control cells consisting of autologous or allogeneic lymphocytes. A transformant was considered to show significant reactivity with target cells if its Multiskan reading was greater then three standard deviations over the mean of six controls. The proportion of transformants that reacted significantly was expressed as a percentage and was considered to be an indication of the level of humoral immunity to antigens expressed by the target cell.

As mentioned above, the possibility of producing human monoclonal antibodies to uveal melanomas was also investigated. Transformants which reacted with the uveal

melanoma cells by ELISA were expanded and fused with cells from a human lymphoblastoid cell line as described in Section 2.6. Hybridomas were then screened by ELISA for reactivity with melanoma cells.

8.2 Measurement of humoral immunity to uveal melanoma by EBV transformation of peripheral blood lymphocytes

8.2.1 Preliminary studies

A series of experiments was performed in order to develop an appropriate protocol. These showed that the techniques used for lymphocyte transformation were almost invariably successful, producing transformants in almost all wells after every transformation. Apparently similar growth of transformants occurred whether the T lymphocytes were separated by rosetting with sheep RBC's or whether the T lymphocytes were killed by adding Cyclosporin A to the culture medium. The latter procedure was adopted because it was less time-consuming.

The ELISA was performed using air-dried cells. Simultaneous testing of supernatants for IgM and IgG antibody production showed that the vast majority of the transformants produced IgM antibody (Table 8.1).

The reproducibility of the ELISA was investigated by simultaneously testing transformants in duplicate with two plates coated with allogeneic melanoma cells obtained from a single tumour. The same result was obtained in 87% of all wells (Table 8.2).

Table 8.1 IgM and IgG in EBV lymphocyte transformant supernatants. Supernatants of EBV transformed B lymphocytes of a patient with uveal melanoma (IS) were simultaneously tested by ELISA in duplicate, using two ELISA plates coated with autologous melanoma cells. Reactivity was detected using anti-human mu and anti-human gamma chain rabbit antibodies labelled with horseradish peroxidase. Results greater than the mean +3SD of six control readings are considered to be positive and are indicated by an asterisk.

1. Optical density readings of control wells incubated with rabbit anti-human mu chain antibodies
2. Optical density readings of 60 transformants incubated with rabbit anti-human mu chain antibodies
3 Optical density readings of control wells incubated with rabbit anti-human gamma chain antibodies
4 Optical density readings of 60 transformants incubated with rabbit anti-human gamma chain antibodies

	1	2		3	4
	0.280	0.312*		0.317	0.276
	0.256	0.601*		0.303	0.251
	0.288	0.750*		0.328	0.299
	0.262	0.708*		0.334	0.305
	0.276	0.615*		0.341	0.335
	0.271	0.449*		0.327	0.264
Mean :	0.272	0.405*	Mean :	0.325	0.291
3SD :	0.035	0.490*	3SD :	0.040	0.270
M+3SD:	0.307	0.308*	M+3SD:	0.365	0.291
		0.304			0.308
		0.530*			0.299
		0.729*			0.285
		0.447*			0.282
		0.522*			0.271
		0.553*			0.288
		0.409*			0.316
		0.407*			0.301
		0.482*			0.277
		0.831*			0.298
		0.530*			0.292
		0.489*			0.316
		1.715*			0.325
		0.450*			0.325
		0.730*			0.289
		0.431*			0.282
		0.775*			0.292
		0.572*			0.299
		0.829*			0.316
		0.681*			0.304
		0.578*			0.291
		0.631*			0.317
		0.461*			0.298
		0.740*			0.331
		0.784*			0.325
		0.424*			0.320
		0.391*			0.314
		0.438*			0.298
		0.555*			0.293
		0.666*			0.304
		0.465*			0.303
		0.442*			0.318
		1.999*			0.285
		0.492*			0.285
		0.347*			0.262
		0.435*			0.314
		0.879*			0.293
		0.412*			0.306
		0.440*			0.312
		0.475*			0.305
		0.377*			0.279
		0.762*			0.320
		0.485*			0.298
		0.535*			0.335
		0.475*			0.308
		0.543*			0.302
		0.481*			0.294
		0.527*			0.316
		0.588*			0.323
		0.452*			0.340
		0.452*			0.312

Table 8.1

Table 8.2 Reproducibility of ELISA. Supernatants of EBV transformed lymphocytes of a patient with uveal melanoma were simultaneously tested by ELISA in duplicate, using two ELISA plates coated with melanoma cells from a different patient. Results greater than the mean +3SD of six control readings are considered to be positive and are indicated by an asterisk. Twenty three positive results were obtained with each assay, although these did not show absolute correlation. The same result was obtained in 87% of all wells.

1. Optical density readings of 6 control wells in plate 1
2. Optical density readings of wells in plate 1
3. Optical density readings of 6 control wells in plate 2
4. Optical density readings of wells in plate 2
5. Percentage ratio of reading in plate 2 to reading in plate 1

		1	2		3	4	5
1		0.544	0.55		0.610	0.564	107.882
2		0.546	0.68		0.505	0.662	102.419
3		0.664	0.61		0.584	0.575	99.1674
4		0.605	0.738		0.597	0.595	84.8187
5		0.635	0.608		0.557	0.568	98.2824
6		0.544	0.658		0.510	0.636	101.686
7	Mean	:0.590	0.571	Mean	:0.560	0.572	105.388
8	3SD	:0.158	0.607	3SD	:0.134	0.663	114.909
9	M+3SD	:0.748	0.659	M+3SD	:0.695	0.912*	145.593
10			0.619			0.68	115.571
11			0.666			0.655	103.466
12			0.525			0.558	111.816
13			0.63			0.597	99.693
14			0.76 *			0.641	88.731
15			0.721			0.821*	119.795
16			0.629			0.738*	123.435
17			0.818*			0.755*	97.1012
18			0.674			0.652	101.77
19			0.76 *			0.659	91.2227
20			0.636			0.661	109.339
21			0.809*			0.764*	99.3518
22			0.745			0.63	88.9642
23			1.098*			0.754*	72.2437
24			0.542			0.613	118.985
25			0.812*			0.747*	96.7822
26			0.845*			0.669	83.2914
27			0.927*			0.822*	93.2874
28			0.768*			0.658	90.1355
29			1.021*			0.672	69.2428
30			0.642			0.6	98.3212
31			0.76 *			0.736*	101.881
32			0.697			0.629	94.9399
33			0.879*			0.996*	119.207
34			0.564			0.55	102.592
35			0.857*			0.801*	98.3292
36			0.736			0.637	91.0526
37			0.641			0.747*	122.601
38			0.678			0.59	80.8205
39			0.757*			0.852*	118.406
40			0.65			0.654	105.851
41			0.879*			0.717*	85.8146
42			0.63			0.572	95.5183
43			0.795*			0.946*	125.186
44			0.726			0.642	93.0314
45			0.879*			0.731*	87.4902
46			0.703			0.737*	110.292
47			0.788*			0.646	86.2457
48			0.603			0.572	99.7952
49			0.89 *			1.04 *	122.935
50			0.612			0.58	99.7028
51			1.213*			0.677	58.7163
52			0.762*			0.742*	102.442
53			0.803*			0.889*	116.471
54			0.636			0.672	111.159
55			0.748			0.983*	138.256
56			0.561			0.738*	138.396
57			0.836*			0.663	83.4331
58			0.637			0.615	101.57
59			0.741			0.754*	107.049
60			0.539			0.619	120.818

Table 8.2

The reproducibility of the lymphocyte transformation was investigated by transforming a single blood sample in duplicate and simultaneously testing all supernatants against cells from the same pair of allogeneic uveal melanomas. The results were highly reproducible, varying by only 1% and 5% respectively (Table 8.3). EBV lymphocyte transformation was performed on the same patient using another blood sample taken a day later. The results were similar to those obtained with the first two transformations (Table 8.4). Table 8.3 also demonstrates that when the same transformants were simultaneously tested against different uveal melanomas, different results were obtained. Whenever possible, therefore, transformants were tested against autologous cells.

Occasionally when ELISA was simultaneously performed using plates coated with lymphocytes and melanoma cells respectively, much higher background readings were obtained with the lymphocyte preparations. This was probably due to the second antibody reacting with the B lymphocytes. The reason why this high background did not occur with each batch of ELISA plates is uncertain.

8.2.2 Reactions of patients with uveal melanoma with autologous cells

The reactivity of the peripheral B lymphocytes with autologous uveal melanoma cells was tested in six patients. Three patients, GM, IS and MT, were each tested on two separate occasions. In five patients the

Table 8.3 Reproducibility of EBV lymphocyte transformation. EBV transformation was performed in duplicate on a single blood sample taken from a patient with uveal melanoma. Supernatants from both transformations were tested simultaneously against cells from two different uveal melanomas, (a) MT, and (b) YS respectively. Positive results, considered to be those greater than the mean+3SD of six controls, are indicated by an asterisk. Table 8.3a shows that 21 (35%) and 24 (40%) transformants showed significant reactivity with MT. Table 8.3b shows that 4 (7%) and 5 (8%) transformants showed significant reactivity with YS.

a. ELISA reactivity of transformants with cells from uveal melanoma MT
b. ELISA reactivity of transformants with cells from uveal melanoma YS

1. Optical density readings of control wells in transformation 1
2. Optical density readings of 60 transformants from transformation 1
3. Optical density readings of control wells in transformation 2
4. Optical density readings of 60 transformants from transformation 2

	1	2		3	4
	0.523	0.567		0.586	0.671
	0.586	0.564		0.648	0.704
	0.578	0.596		0.625	0.751*
	0.513	0.681*		0.652	0.771*
	0.557	0.567		0.617	0.576
	0.547	0.616		0.686	0.701
Mean :	0.551	0.538	Mean :	0.636	0.812*
3SD :	0.087	0.602	3SD :	0.1029	0.789*
M+3SD:	0.638	0.627	M+3SD:	0.739	0.694
		0.574			0.631
		0.546			0.682
		0.522			0.633
		0.575			0.736
		0.666*			0.844*
		0.610			0.833*
		0.732*			0.763*
		0.554			0.889*
		0.550			0.740*
		0.707*			0.660
		0.666*			0.699
		0.661*			0.802*
		0.577			0.774*
		0.599			0.867*
		0.606			0.794*
		0.687*			0.732
		0.640*			0.641
		0.767*			0.753*
		0.639*			0.706
		0.551			0.766*
		0.595			0.739
		0.722*			0.648
		0.610			0.707
		0.707*			0.758*
		0.641*			0.723
		0.589			0.812*
		0.584			0.670
		0.637			0.673
		0.621			0.878*
		0.747*			0.734
		0.578			0.677
		0.647*			0.714
		0.568			0.734
		0.644*			0.806*
		0.638			0.829*
		0.701*			0.745*
		0.595			0.742*
		0.642*			0.712
		0.551			0.714
		0.612			0.833*
		0.620			0.716
		0.654*			0.729
		0.529			0.692
		0.663*			0.720
		0.565			0.618
		0.634			0.776*
		0.593			0.680
		0.678*			0.687
		0.543			0.672
		0.594			0.730
		0.562			0.617

Table 8.3a

	1	2		3	4
	0.399	0.634		0.459	0.760
	0.541	0.586		0.595	0.554
	0.551	0.486		0.540	0.695*
	0.535	0.630		0.456	0.497
	0.539	0.531		0.435	0.485
	0.517	0.513		0.445	0.551
Mean :	0.514	0.527	Mean :	0.488	0.469
3SD :	0.172	0.664	3SD :	0.193	0.603
M+3SD:	0.685	0.533	M+3SD:	0.681	0.560
		0.509			0.523
		0.520			0.512
		0.555			0.578
		0.540			0.536
		0.617			0.587
		0.701*			0.955*
		0.617			0.690*
		0.546			0.472
		0.545			0.618
		0.656			0.510
		0.680			0.590
		0.600			0.545
		0.706*			0.512
		0.547			0.606
		0.619			0.572
		0.648			0.509
		0.664			0.625
		0.657			0.648
		0.494			1.059*
		0.576			0.506
		0.444			0.482
		0.592			0.513
		0.688*			0.649
		0.582			0.614
		0.553			0.629
		0.649			0.543
		0.522			0.572
		0.649			0.609
		0.652			0.845*
		0.646			0.488
		0.521			0.557
		0.579			0.510
		0.551			0.540
		0.610			0.592
		0.565			0.547
		0.634			0.538
		0.596			0.526
		0.503			0.581
		0.522			0.513
		0.579			0.536
		0.550			0.516
		0.709*			0.579
		0.565			0.524
		0.570			0.582
		0.626			0.519
		0.568			0.662
		0.570			0.540
		0.606			0.520
		0.624			0.511
		0.628			0.538
		0.596			0.563

Table 8.3b

Table 8.4 Reproducibility of patient lymphocyte reactivity with allogeneic uveal melanoma cells. EBV lymphocyte transformation on blood sample taken from same patient as Table 8.3 one day later. Supernatants were tested by ELISA against cells from YS. Four (7%) transformants were positive (ie, optical density reading greater than 2SD of the mean of six controls). The results are similar to those shown in Table 8.3.

1. Optical density readings of six control wells
2. Optical density readings of 60 transformants

	1	2
	0.440	0.563
	0.498	0.610
	0.579	0.581
	0.509	0.620
	0.526	0.528
	0.437	0.556
Mean :	0.498	0.566
3SD :	0.162	0.571
Mean+3SD :	0.660	0.516
		0.599
		0.572
		0.582
		0.587
		0.652
		0.584
		0.601
		0.579
		0.630
		0.688*
		0.585
		0.590
		0.599
		0.676*
		0.576
		0.536
		0.700*
		0.581
		0.569
		0.676*
		0.575
		0.597
		0.575
		0.551
		0.537
		0.637
		0.550
		0.566
		0.513
		0.546
		0.539
		0.616
		0.490
		0.501
		0.576
		0.556
		0.531
		0.600
		0.471
		0.657
		0.551
		0.559
		0.545
		0.653
		0.519
		0.518
		0.576
		0.601
		0.543
		0.568
		0.493

Table 8.4

transformants were simultaneously tested against autologous lymphocytes. The results, summarised in Fig. 8.1, suggest that there is positive reactivity with lymphocytes as well as with uveal melanoma cells.

8.2.3 Reactions of patients with uveal melanoma with allogeneic cells

The reactivity of the peripheral B lymphocytes with allogeneic cells was tested in six patients, five of whom were different from those mentioned in the previous section. Three patients were tested on two separate occasions: one of these three patients was mentioned in Section 8.2.1. In five transformations, supernatants were simultaneously tested against uveal melanoma cells and, in some cases, lymphocytes. The two types of cells were obtained from different patients. The results are shown in Fig. 8.2 and are similar to those obtained with autologous cells.

8.2.4. Reactions of healthy individuals with melanoma cells

The reactivity of the peripheral B lymphocytes of four healthy individuals was simultaneously tested against uveal melanoma cells and, in some cases, lymphocytes. The two types of target cell were obtained from different individuals in three of the four experiments. The results are summarised in Fig. 8.3 and are similar to those obtained with patients with uveal melanoma.

Figure 8.1 ELISA reactivity of EBV transformed B lymphocytes of patients with uveal melanoma with autologous tumour cells and lymphocytes. The histogram shows the percentage of transformants with optical density readings greater than the mean+3SD of six controls. Significant reactivity with both types of cells is apparent in three patients. (Autologous lymphocytes of patient MR were not available).

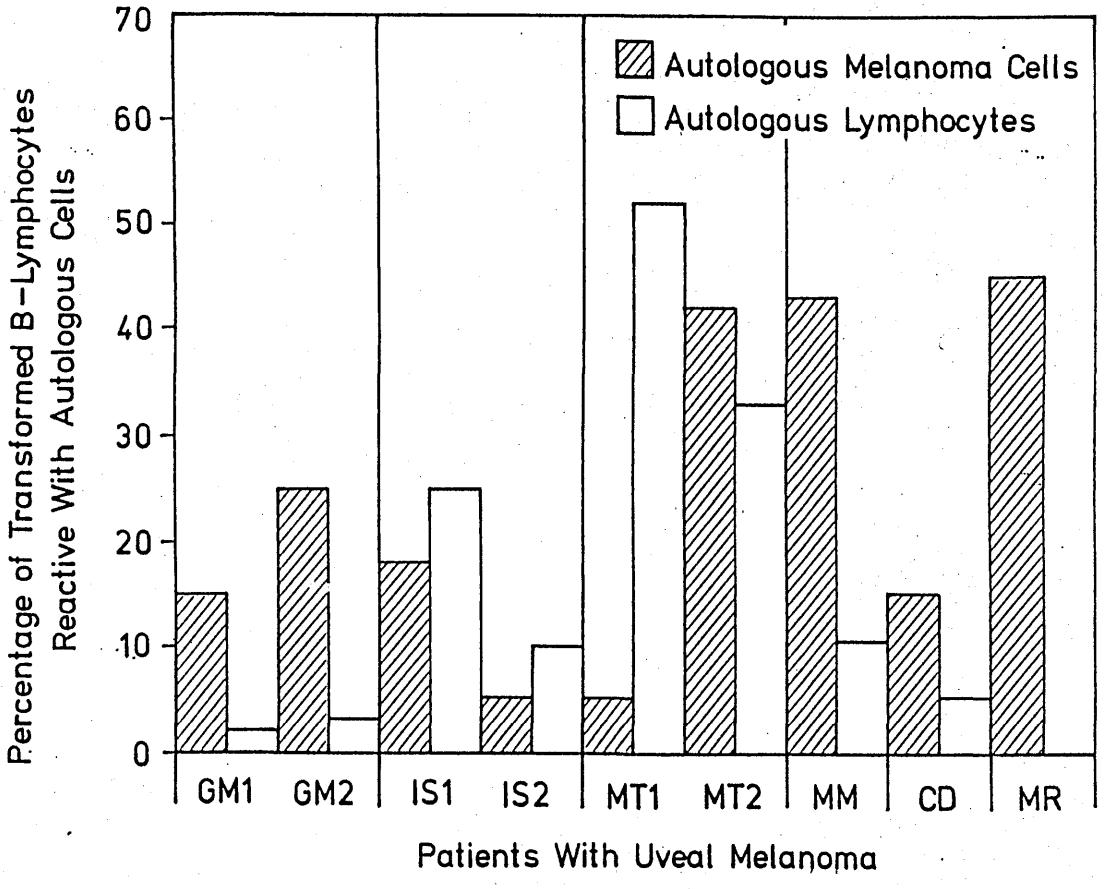

Percentage of Transformed B-Lymphocytes Reactive With Autologous Cells
70
60
50
40
30
20
10
0
Autologous Melanoma Cells
Autologous Lymphocytes
GM1
GM2
IS1
IS2
MT1
MT2
MM
CD
MR
Patients With Uveal Melanoma

Fig. 8.1

Figure 8.2 Reactivity of EBV transformed B lymphocytes of patients with uveal melanoma with allogeneic melanoma cells and lymphocytes. The histogram shows the percentage of transformants with optical density readings greater than the mean+3SD of six controls. Results are similar to those seen in Fig. 8.1. Reactivity with lymphocytes could not be tested in patients RB, AA, MR and, on the second occasion, HC.

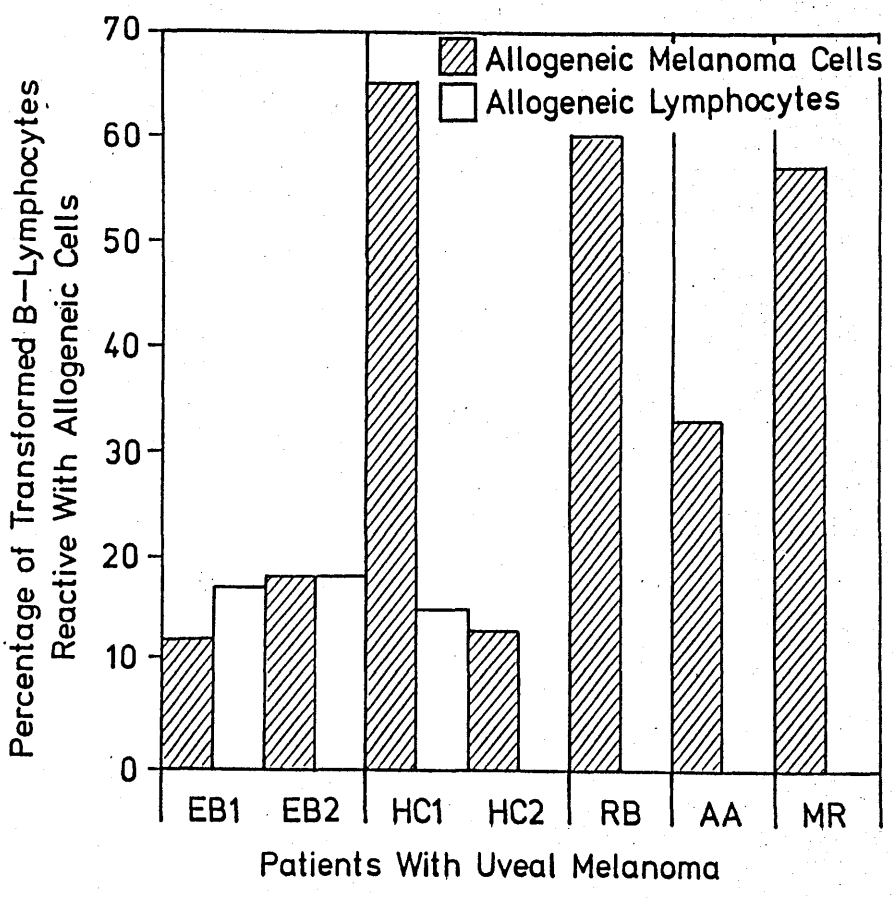

Allogeneic Melanoma Cells
Allogeneic Lymphocytes
Percentage of Transformed B-Lymphocytes Reactive With Allogeneic Cells
70
60
50
40
30
20
10
0
EB1
EB2
HC1
HC2
RB
AA
MR
Patients With Uveal Melanoma

Fig. 8.2

Figure 8.3 Reactivity of EBV transformed B lymphocytes of healthy individuals with uveal melanoma cells and lymphocytes. The histogram shows the percentage of transformants with optical density readings greater than the mean+3SD of six controls. Results are similar to those obtained with patients with uveal melanoma.

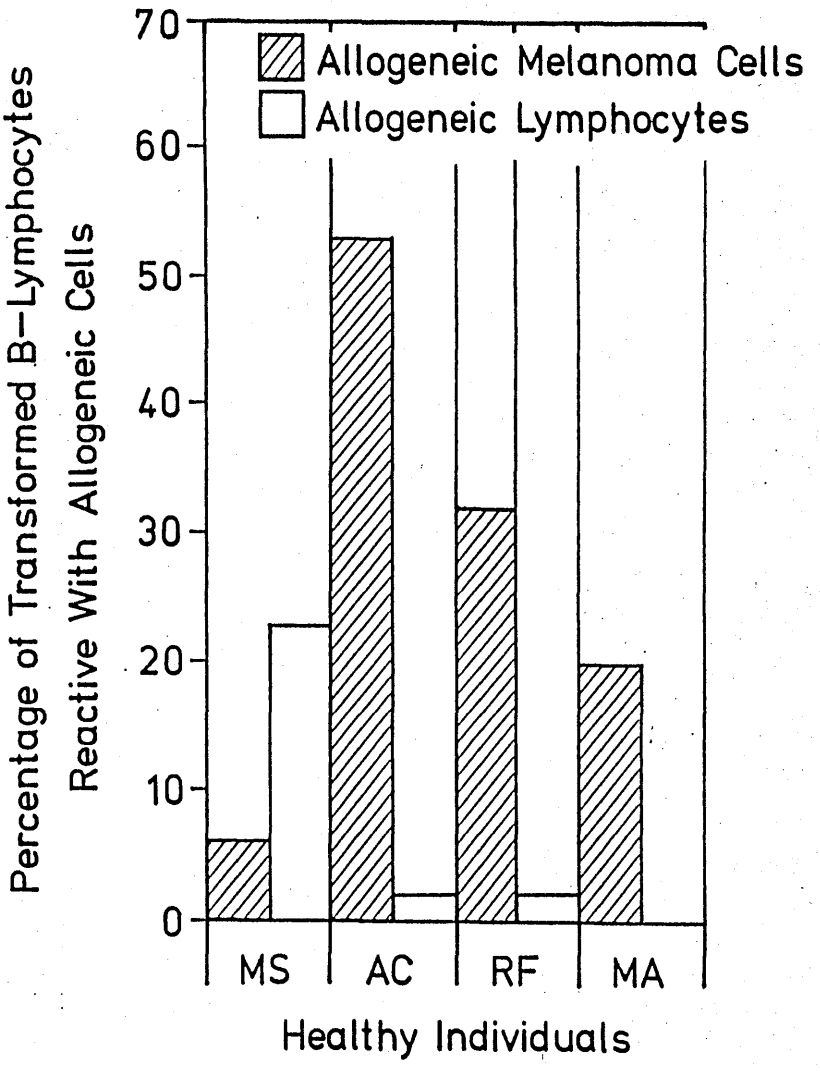

Allogeneic Melanoma Cells
Allogeneic Lymphocytes
Percentage of Transformed B-Lymphocytes Reactive With Allogeneic Cells
70
60
50
40
30
20
10
0
MS
AC
RF
MA
Healthy Individuals

Fig. 8.3

8.3. Production of human monoclonal antibodies

Transformants were selected for fusion if they produced antibodies reactive with melanoma cells but not lymphocytes. Initially, these positive transformants were expanded individually but the reactivities of the supernatants diminished during the long period of culture necessary for obtaining sufficient numbers of cells for fusion. Since this was thought to be due to domination of the cultures by more rapidly growing non-secretory transformants, the delay before fusion was reduced by pooling positive transformants so that a sufficient number of cells for fusion was achieved more quickly. Two backfusions were performed but the hybrids reacted weakly with the melanoma cells.

8.4. Discussion

Both patients and healthy controls produced similar results. In all three groups significant reactivity also occurred with lymphocytes. Although more transformants appeared to react with melanoma cells than with lymphocytes, the validity of such a comparison was often doubtful because the lymphocytes gave higher background readings, probably due to their immunoglobulin content.

Other workers have previously demonstrated that specific antibody to a variety of antigens can be obtained from unimmunised healthy individuals (Winger *et al* 1983, reviewed by Shoenfeld & Witz 1986). Serum antibodies apparently reactive with melanoma-associated antigens

have been reported in normal individuals (Houghton et al 1980; Cote et al 1986). Whether such results represent a true biological phenomenon or whether they are due to laboratory artefact is uncertain.

In the present study the high proportion of transformants apparently reactive with tumour cells on ELISA is unlikely to be genuine. Other workers have suggested that antibodies, especially of the IgM class, can react non-specifically with irrelevant antigens, especially those with a densely packed highly repeating molecular structure (Ghosh & Campbell 1986). If these concepts regarding the 'multispecific' behaviour of antibodies are correct, then assays of humoral immunity using whole tumour cells or crude tumour extracts as antigens are of doubtful significance.

The reactivity of the lymphocyte transformants tended to be weak relative to rat hybridomas. If this occurred because the antibodies were reacting with antigens expressed by small sub-populations of tumour cells then ELISA might not be appropriate for screening transformants. Antibodies to the less common antigens could be the ones that are the most specific for uveal melanoma and those with the greatest potential for clinical use.

Apart from the problem of detecting and counting peripheral B lymphocytes secreting antibodies to uveal melanoma antigens, the use of the EBV technique for measuring humoral immunity to tumours has other deficiencies. This technique appears to transform only a small proportion of human B cells, which are mostly those

of the IgM class (Steel *et al* 1977, Chan *et al* 1986). EBV transformation, therefore, appears to introduce a significant degree of bias so that even if a good method of screening the transformants were available the results would not be representative of the *in vivo* situation.

The second aim of this project was to investigate the feasibility of producing monoclonal antibodies from positive EBV transformants. This objective was not achieved for three reasons. Firstly, the reactivities of the transformants against uveal melanoma cells tended to be weak relative to those seen with rodent hybridoma supernatants and, in addition, tended to diminish over a few weeks. Both these difficulties have been acknowledged by other workers, with much experience in this field (Roder, Cole & Kozbor 1986). Secondly, during the course of this investigation other studies revealed that the vast majority of human monoclonal antibodies detected intracellular antigens (Cote *et al* 1986; Campbell *et al* 1986). This is probably because such intracellular antigens dominated the assays either because the cell membranes of apparently intact cells were, nevertheless, permeable to antibodies or because rupture of even a small number of cells was sufficient to release large amounts of cytoplasmic antigen. Thirdly, the value of ELISA as a tool for screening EBV transformants and hybrids became even more doubtful when it apparently indicated that both healthy individuals and patients with uveal melanoma reacted equally strongly with this tumour. In the absence

of a reliable method of screening monoclonal antibodies to relevant antigens on the cell membranes of uveal melanoma cells, there was no scope for performing further human fusions.

In conclusion, current methods of detecting and immortalising circulating B lymphocytes ostensibly responsible for the humoral immune response to uveal melanoma antigens are highly inefficient.

Chapter 9

DISCUSSION

9.1 Introduction

When this project was initiated only seven years after monoclonal antibodies were first produced (Köhler & Milstein 1975), hybridoma technology was already proving useful in many areas of biology and clinical medicine. The main aim of the present study was to investigate the feasibility and the scope of developing monoclonal antibodies to uveal melanomas.

The original plan was to produce rat and human monoclonal antibodies to cell-membrane antigens expressed by all uveal melanomas and not by other tissues. This strategy was based on the prevailing hypothesis that human neoplasms express cell membrane antigens which are phenotypically distinctive for various specific types of tumour, and that monoclonal antibodies to these antigens would open the way to new immunodiagnostic and immunotherapeutic applications (Kaplan, Olsson & Raubitschek 1982). The doubtful nature of tumour-specific antigens (Old 1981) and the phenomena of antigenic heterogeneity (Fidler & Hart 1982) and modulation (Rosenthal, Tompkins & Rawls 1980) were already recognised at the onset of this study, but it was believed at the time that such obstacles could be overcome by the development of 'cocktails' of monoclonal antibodies. Such early optimism has since been diminished by the disappointing results of other studies using monoclonal antibodies, and by recent discoveries regarding oncogenes

and their translational products (reviewed by Gordon 1985). These entities, previously thought to be unique to cancer cells, now appear to be very similar to components which are essential for the regulation of mitosis in normal cells. To date, oncogenic products of tumour cells have been found to bear partial identity to normal growth factors such as platelet-derived growth factor (Waterfield et al 1983), or to show only minor structural differences. Also, the majority of oncogene products are intracellular. For example, the c-H-ras oncogene in bladder carcinoma cells differs from its normal homologue by only a single amino acid (Seeburg et al 1984) and is located on the inner surface of the cell membrane (discussed by Darnell, Lodish & Baltimore 1986). Such findings suggest that antigenic differences between normal and neoplastic cells are likely to be quantitative in nature rather than qualitative, so that prospects for producing highly specific monoclonal antibodies to tumours are not as good as previously envisaged.

9.2 Rodent monoclonal antibodies to uveal melanoma

9.2.1 Use of uncultured uveal melanoma cells

A large number of monoclonal antibodies have been produced against cutaneous melanomas, mostly by large teams working in specialised cancer research laboratories in the United States. Not all monoclonal antibodies to cutaneous melanomas react with uveal melanomas (Mitchell et al 1980, Atkinson et al 1985). Such findings suggest

that the two different anatomical origins of these neoplasms confer a different antigenic profile. It is therefore justifiable to generate monoclonal antibodies to uveal melanomas.

Prior to this study, monoclonal antibodies had not been produced to uveal melanomas. Donoso and associates (Donoso, Folberg, Edelberg et al 1985) later produced monoclonal antibodies reactive with uveal melanoma by immunising mice with mixtures of viable uveal melanoma cells and cultured cutaneous melanoma cells. The hybrids were screened by immunohistochemistry using formalin fixed tissues but the monoclonal antibodies that were produced were not very specific, reacting with other kinds of tumour and with normal tissues. In the present study the production of monoclonal antibodies was achieved using only tissue from uncultured uveal melanomas. The use of such material gave rise to a number of problems. Firstly, this tissue also contained lymphocytes, endothelial cells, macrophages and, in some cases, retinal pigment epithelial cells. Although the number of such cells was small in comparison with the malignant melanocytes it may have been enough to contribute to the cross-reactivity of the monoclonal antibodies with these types of cells. Secondly, the uncultured tumour cells were very fragile. Thirdly, the proportions of spindle and epithelioid cells varied between melanomas. The problem of antigenic heterogeneity is discussed more fully in the next section.

9.2.2 Antigenic heterogeneity

As previously stated, the aim was to generate antibodies recognising antigens expressed by all uveal melanomas rather than antigens unique to a single patient. Cells from different uveal melanomas were, therefore, used successively for the animal immunisations and for the screening of hybridomas.

This approach was complicated by inter-tumour antigenic heterogeneity. Because of this phenomenon, inconsistent results were obtained when cells from different uveal melanomas were used to assay a single batch of hybridoma supernatants. Unless assays were performed simultaneously against a variety of uveal melanomas it was not possible to decide whether a negative result indicated lack of expression of the antigen by the tumour cells, or lack of production of the antibody by the hybridoma. This problem could be overcome by using cells from a single tumour for all assays required for the selection of a single hybridoma.

Because of inter-tumour antigenic heterogeneity, this protocol (ie, the use of one tumour per fusion) could also improve the specificity of the monoclonal antibodies generated. Simultaneous screening of the hybridomas against lymphocytes obtained from the same patient would be required to identify antibodies recognising histocompatibility antigens.

A relatively specific monoclonal antibody may react with only a small proportion of uveal melanomas and with only a minority of cells within a melanoma. Such an antibody would probably not be very useful as a targeting

agent, unless it formed part of a cocktail of antibodies. It could, however, recognise an antigen which has prognostic significance.

9.2.3 Screening of hybridomas and subclones

Successful production of monoclonal antibodies to uveal melanomas is dependent on an efficient assay for the screening of hybridomas and subclones. The original strategy was to identify by ELISA the antibodies which reacted strongly with uveal melanoma cells but not with lymphocytes, and to perform further specificity studies against a wider variety of tissues, normal and abnormal, by means of immunohistochemical techniques. Lymphocytes were selected for these purposes because these cells expressed histocompatibility and other non-specific antigens and because they were readily available. The choice of ELISA followed by immunohistochemistry for the selection of hybrids is conventional, and dictated by the need for the rapid screening of large numbers of supernatants followed by more thorough evaluation of a smaller number of interesting antibodies.

The type of ELISA selected, using whole melanoma cells fixed gently with glutaraldehyde, was described as a method for detecting membrane antigens (Cobbold & Waldmann 1981). Such claims seem to have been over-optimistic. Despite the precautions taken to preserve the morphology of the melanoma cells, all the monoclonal antibodies produced in this study recognised cytoplasmic or nuclear antigens. It is likely that such intracellular antigens dominated the ELISAs. This could be because the cell

membranes of apparently intact cells were nevertheless permeable to antibodies, or because rupture of even a small number of cells was enough to release large amounts of cytoplasmic antigens onto the plate. Apart from these considerations, it is now recognised that glutaraldehyde can give rise to false positive and negative results on ELISA (Drover & Marshall 1985). Furthermore, this assay does not take into account the phenomenon of intra-tumour antigenic heterogeneity. More suitable methods of screening hybridomas might therefore be immunofluorescence microscopy (Boucheix et al 1986) or immunohistochemistry (Ciocca & Bjercke 1986).

A preliminary investigation into the use of immunohistochemistry for the investigation of monoclonal antibodies to uveal melanomas produced inconsistent results, which varied according to the manner in which the tissues were prepared. For example, the 4A3 antibody reacted with lymphocytes on frozen, but not on fixed, tissue sections. Although the extensive literature on immunohistochemical techniques can provide useful suggestions, it is not possible to predict which of the numerous protocols available is the most suitable (Hancock & Atkins 1986; Ciocca & Bjercke 1986). An empirical evaluation is, therefore, required of all aspects of the procedure before definitive assessment of the specificities of the antibodies can be undertaken.

9.2.4 Class of monoclonal antibodies

While most of the anti-melanoma antibodies described in the literature are of the IgG class, most of the monoclonal antibodies produced in the present study have been of the IgM class. This preponderance of IgM monoclonal antibodies may be due to chance. Alternatively, the immunisation and selection protocols have favoured epitopes which are closely spaced and which would therefore have an increased affinity for IgM, which is multivalent. It may be more appropriate to use an IgG probe which does not detect the antibody light chains of IgM. Antibodies of the IgM class may be less useful than IgG antibodies as their large size could reduce their penetration into tumour tissue and because their multivalent nature could reduce their specificity (Ghosh & Campbell 1986). For example, even if the 4A3 antigen were specific to melanoma, the 4A3 monoclonal antibody, being multivalent, might nevertheless react with other tissues in a 'multispecific' fashion.

9.2.5 Specificity of monoclonal antibodies

None of the antibodies produced in this study recognise antigens which are totally specific to melanoma. Whilst this is disappointing, a review of the literature reveals that the vast majority of 'anti-melanoma' monoclonal antibodies produced by other workers show preferential rather than specific reactivity with this type of tumour. Although some monoclonal antibodies were reported to be specific such claims have subsequently been found to be false. The apparent lack of specificity of

the monoclonal antibodies could reflect deficiencies in the assay techniques used to screen the hybridomas or a genuine lack of highly specific antigens. Despite lack of total specificity, monoclonal antibodies could have useful applications in research and clinical practice.

9.2.6 Nature of antigens recognised by monoclonal antibodies

The most consistent form of the antigen recognised by mAb 4A3 had a molecular weight in the region of 60K. Melanoma-associated antigens with a similar molecular weight have previously been reported (Mitchell *et al* 1980; Dippold *et al* 1980; Akutsu & Jimbow 1986). Many different proteins, however, are in the molecular weight range 40-70 kD; albumin is a common example. In order to establish that the monoclonal antibodies produced in the present study detect novel antigens, these antibodies would need to be tested against monoclonal antibodies produced in other laboratories, using competitive immunoassays. At present, there does not seem to be scope for such an investigation.

9.2.7 Human monoclonal antibodies to uveal melanoma

EBV-transformation of peripheral B lymphocytes of patients with uveal melanoma, followed by fusion with human lymphoblastoid cells, was investigated in the hope of obtaining human monoclonal antibodies to tumour-associated antigens (Chapter 8). Nearly all of the lymphocyte transformants, however, produced IgM antibody, and the reactivity of their supernatants was weak and

directed against intracellular antigens. This phenomenon is well recognised and discussed by Olsson (1985). The disappointing results of this investigation are similar to those of other workers who have tried a similar approach (Cote et al 1986). At present, the prospects for producing monoclonal antibodies to uveal melanoma are better with the rodent than with the human hybridoma system.

9.3 Potential applications of monoclonal antibodies reactive with uveal melanoma

The second main aim of the present study was to investigate the potential applications of monoclonal antibodies to uveal melanomas in research and clinical ophthalmology.

9.3.1 Diagnosis of the primary tumour

Most intraocular melanomas can be reliably diagnosed by ophthalmoscopy supplemented by appropriate investigations. An immunological diagnostic test would, therefore, be useful in only a small proportion of cases. Such a test would need to be non-invasive, highly reliable and practicable in the clinical situation even when not performed frequently.

Other workers have unsuccessfully based their immunological diagnostic assays on the patient's cellular and humoral immune response to the uveal melanoma. There may be more scope, however, for developing assays which are based on the detection on tumour antigens in the blood or urine. The detection of the 4A3 antigen in the

subretinal fluid of patients with uveal melanoma by Western Blotting is further evidence that antigens are shed by this tumour.

Imaging techniques using radioisotopes labelled to monoclonal antibodies generated against cutaneous melanoma are currently under intense investigation. In view of the antigenic differences that are known to exist between cutaneous and uveal melanoma further knowledge of the antigenic nature of uveal melanomas is required before such antibodies could usefully be applied to intraocular tumours.

Immunohistochemistry using monoclonal antibodies to melanoma-associated antigens could provide support for microscopic examination of excised tissue, particularly following conservative forms of therapy when it is difficult to differentiate surviving tumour cells from melanomacrophages or activated fibroblasts.

9.3.2 Diagnosis of metastatic disease

From a clinical point of view, the scope of immunodiagnosis of subclinical metastases would depend on how this information influenced the management of the primary tumour and on the efficacy of any available treatment for extraocular disease. Early detection or, preferably, accurate prediction of metastatic disease could improve the evaluation of different forms of treatment of the primary tumour.

Such aims could be achieved in various ways. Firstly, the primary tumour could be examined by immunohistochemistry for the expression of antigens which

are known to have prognostic significance. It would be preferable to prepare monoclonal antibodies reactive with fixed tissues for this purpose so that retrospective studies would be feasible.

9.3.3 Treatment of the primary tumour

An immunological tool for the treatment of the primary tumour could in the future be used as an alternative or adjunct to other forms of conservative treatment, none of which guarantees success. The treatment of tumours with monoclonal antibodies attached to radioactive isotopes and toxins is limited by the risk of damage to other organs, such as the liver and kidney, in which monoclonal antibodies tend to accumulate. This problem is likely to be less serious with photodynamic agents, which are toxic only when exposed to light. It may be possible, therefore, to use monoclonal antibodies to enhance the accumulation of haematoporphyrin derivatives in uveal melanoma, thereby reducing the systemic phototoxicity that currently complicates the use of such agents. The possibility of adversely influencing the presumed immunological defences of the host against the tumour would need to be assessed in relation to the uncertain immunological consequences of all the other therapeutic modalities, including enucleation.

9.3.4 Prevention of metastatic disease

The prevention of metastatic disease from uveal melanoma is one of the most urgent problems in ophthalmic oncology. The fact that only about half of all patients

develop metastatic disease following enucleation could indicate that metastatic disease might be prevented if its pathogenesis were better understood. Hybridoma technology offers the best means of providing the appropriate antigens and antibodies required for relevant studies of uveal melanoma antigens and of the immune responses to them.

In the absence of purified melanoma-associated antigen preparations, a preliminary investigation was performed to investigate the possibility of measuring serum antibodies to melanoma-associated antigens by means of an inhibition ELISA. The initial results were encouraging in that the patient sera appeared to inhibit the binding of the rat monoclonal antibodies to the melanoma cells relative to the sera of the healthy controls. Such inhibition seemed to occur in a non-specific fashion so that the same results were obtained whichever monoclonal antibody was used.

A potential problem, common to all methods of measuring serum antibodies to tumour-associated antigens, is that such antibodies might not be detected if they already form part of antigen-antibody complexes or if they are bound to anti-idiotypes. Although this difficulty could, theoretically at least, be overcome by dissociating the complexes under acid conditions (Sjögren et al 1971), a more recent approach has been to 'freeze' the humoral immune response by performing EBV-transformation on peripheral blood lymphocytes (Campbell et al 1986). Peripheral B lymphocytes apparently producing antibodies to antigens expressed by melanoma cells were demonstrated

in patients with uveal melanomas, but such cells were also identified in healthy individuals. As with previous studies such results may be artefactual and due to the use of impure antigens in the assays.

9.3.5 Treatment of metastatic disease

The high morbidity and mortality of established metastatic disease justify further research into all forms of therapy, including those using monoclonal antibodies.

9.4 Conclusions

The following conclusions are based on the results of original experimental research and on a review of the current literature (September 1987).

1. This study shows that it is technically possible to prepare monoclonal antibodies using only uncultured uveal melanoma cells for the immunisation of animals and the screening of hybrids.

   Despite the availability of large number of monoclonal antibodies that react with cutaneous melanoma, there appears to be scope for producing monoclonal antibodies to uveal melanoma because of the significant antigenic differences that are known to exist between the two sub-types of tumour.

Since melanoma cells are known to undergo significant antigenic changes during *in vitro* culture, it would seem to be preferable to prepare monoclonal antibodies to uveal melanomas using uncultured cells obtained from primary tumours.

Like the vast majority of 'melanoma-associated' antigens recognised by monoclonal antibodies from other laboratories, the antigens detected in the present study have, at best, been only partially specific for uveal melanoma cells. Further studies may establish whether these results are due to inadequate methodology or to a genuine paucity of specific tumour-associated antigens in melanoma cells.

2. The ELISA is inadequate for selecting antibodies to membrane-antigens expressed by uveal melanomas. Despite early claims that this assay could be used for cell surface antigens, it tends to be dominated by intracellular antigens. In addition, it is unlikely to detect potentially useful antibodies recognising antigens expressed by small minorities of cells. Depending on the intended use of the monoclonal antibodies, hybridomas should be screened by immunohistochemistry using fixed or frozen tissue, or against viable tumour cells by immunofluorescence microscopy.

The inter-tumour antigenic heterogeneity of uveal melanomas demonstrated by ELISA using a panel of monoclonal antibodies has implications not only for the application of monoclonal antibodies but also for the production of these reagents, especially when uncultured cells are used in the screening assays. Instead of using cells from different uveal melanomas for the immunisation and screening procedures required in each fusion it would be better to use only one tumour per fusion.

3. The presence of the 4A3 antigen in samples of subretinal fluid from patients with uveal melanoma is interesting but of limited significance until it becomes possible to test samples from patients with other causes of serous retinal detachment. This finding, however, suggests that with an appropriate assay it may be possible to detect similar antigens in the blood or urine.

4. The binding of monoclonal antibodies to uveal melanoma cells on ELISA tends to be inhibited by serum from patients with this tumour.

5. It is not possible to measure humoral immunity to uveal melanoma by performing the ELISA on supernatants of cultured EBV-transformed peripheral B lymphocytes of patients with this disease.

6. Human-human fusions using EBV-transformed peripheral B lymphocytes obtained from patients with uveal melanoma do not appear to be an efficient method of producing human monoclonal antibodies to membrane antigens expressed by these tumours if ELISA is used for screening transformants and hybridomas.

7. The production of monoclonal antibodies to tumour-associated antigens is still in its infancy and has hitherto been based on concepts which now appear to have been too simplistic. Techniques of hybridoma production and selection are, however, becoming more efficient and awareness of the limited antigenic nature of many neoplasms and of the cross-reactive behaviour of monoclonal antibodies is improving.

In the light of these considerations, there would seem to be much scope for further efforts at producing monoclonal antibodies to uveal melanoma.

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