# STUDIES ON THE PRODUCTION AND APPLICATION OF MONOCLONAL ANTIBODIES FOR THE DETECTION AND TREATMENT OF CANCER.

A dissertation submitted for the degree of Ph.D.

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## ABSTRACT

Three aspects of the production and applications of monoclonal antibodies (mabs) were investigated. Two new cell lines (C23/11 and HET-37) were produced for use as potential fusion partners in human mab production. C23/11 was established from a blood sample obtained from a patient with myeloma. It was shown to have many of the cellular and antigenic properties associated with a lymphoblastoid cell line. HET-37 is a human/murine heteromyeloma cell line which was established after C23/11 cells were fused with murine NSO cells. Both C23/11 and HET-37 were characterised using electronmicroscopy, histology, immunophenotyping, isoenzyme analysis and DNA fingerprinting. In the second experimental section, three mab (HMFG-1, AUA-1 and H17E2) were labelled with the radioactive isotope, Indium-III. They were used for localisation of tumour in 21 patients with suspected ovarian carcinoma. <sup>In</sup>In-mab immunoscintigraphy was found to be a more sensitive technique for evaluation ovarian cancer than X-ray, CAT scan and ultrasound. <sup>III</sup>In-mab immunoscintigraphy was particularly useful in localising tumours in patients with serum CA-125 levels greater than 100 IU/ml. F(ab'), fragments of HMFG-1 were labelled with technetium and were used to determine the distribution of ovarian tumour in a patient with minimal residual disease. In the final experimental section, HMFG-1 was chemically conjugated to the photosensitiser hematoporphyrin, Hp. HMFG-1-Hp was shown to kill antigen-bearing MCF-7 cells in vitro after irradiation. The HMFG-1-Hp conjugate had no effect on EJ-138 cells under identical conditions. The EJ-138 cell line does not express the antigen to which the HMFG-1 antibody binds.

This is to state that the experiments described in this thesis are my own work. Where appropriate, the assistance of others in this work is acknowledged.

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To Brian and Bina Prosser, - eventually ....!

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#### PUBLICATIONS

Prosser, E., Carroll, K., O'Kennedy, R., Clarke, K., Grogan, L. and Otridge, B. (1989). 'Establishment and preliminary characterisation of a new myeloma cell line, C23/11. Biochem. Soc. Trans., 17, 1055-1056.

Prosser, E., Cox, D., O'Kennedy, R., Carroll, K. and Van der Putten, W. (1990). 'Effects of coumarins, haematoporphyrins and acridine orange on the viability of Landschütz ascites tumour cells, in the presence and absence of photoradiation'. Cancer Letts., 52, 71-77.

Carroll, K., Prosser, E. and O'Kennedy, R. (1990). 'Parameters involved in the in vitro immunization of tonsilar lymphocytes: Effects of rIL-2 and muramyl dipeptide'.

Hybridoma, 9, 81-89.

Egan, D., O'Kennedy, R., Moran, E., Cox, D., Prosser, E. and Thornes, D. (1990). 'The pharmacology, metabolism, analysis, and application of coumarin and coumarin-related compounds'.

Drug Metab. Rev., 22, 503-529.

Carroll, K., Prosser, E. and O'Kennedy, R. (1990). 'Human monoclonal antibodies'. Technology Ireland, 22, 47-50.

Prosser, E., Cahill, D. and O'Kennedy, R. (1991). 'Antibody-mediated in vivo detection and treatment of disease'. J. Biomed. Sciences, 1, 145-152.

Carroll, K., Prosser, E. and O'Kennedy, R. (1991). 'In vitro immunization of human tonsilar lymphocytes: Effects of PWM and rIL-6'. Hybridoma, 10, 229-239.

Prosser, E., Gleeson, C., Carroll, K. and O'Kennedy, R. (1991).

'An examination of the in vitro photodynamic effects of the monoclonal antibody-hematoporphyrin conjugated, HMFG-1'.

Biochem. Soc. Trans. (in press).

**Prosser, E.**, Pomeroy, M., Barker, F., Davis, M., Duffy, J., Fennelly, J.J. and O'Kennedy, R. (1991). 'The relationship between <sup>m</sup>In-labelled immunoscintigraphy and serum CA-125 levels in ovarian carcinoma patients post-chemotherapy'.

Biochem. Soc. Trans, (in press).

Prosser, E., Pomeroy, M., Barker, F., Duffy, G., Davis, M., Duffy, J., Fennelly, J.J. and O'Kennedy, R. (1991).

'The use of monoclonal antibodies and  $F(ab')_2$  fragments in the imaging of ovarian carcinoma after chemotherapy'.

J. Biomed. Sci. (in press).

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ADCC	Antibody-dependent cell-mediated cytolysis
ADEPT	Antibody-directed enzyme prodrug therapy
AG	Azaguanine
AGENT	Antibody-guided enzyme nitrite therapy
AMCA	Aminomethyl coumarin acetic acid
AUA-1	Arklie's unknown antigen-1
B-Cell	B lymphocyte cell
β-Gal	β-galactosidase
BCA	Bicinchoninic acid
BSA	Bovine serum albumen
BSOH	Bi-lateral salpingo-oophorectomy
CAT	Computerised axial tomography
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxicity
CDR	Complementarity-determining regions
DMF	N,N'-dimethylformamide
DMSO	Dimethylsulphoxide
DOTA	Tetraazacyclododecane-N-N',N"',N"'-tetraacetic acid
d.t.	Doubling time
DTE	Dithioerythritol
DTPA	Diaminotriethylenepenta-acetic acid
DTT	Dithiothrietol
DMEM	Dulbecco's modification of Eagle's medium
DMEM <sub>10</sub>	DMEM (supplemented with 10% (v/v) FCS)
EBV	Epstein-Barr virus
EDC	1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
F(ab)	F(ab) fragment of immunoglobulin
F(ab') <sub>2</sub>	F(ab') <sub>2</sub> fragment of immunoglobulin
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FDA	Food and Drugs Administration
FIGO	International Federation of Gynaecology and Obstetrics

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FITC	Fluorescein-isothiocyanate
FN	False negative
FP	False positive
G-418	Geneticin
GTP	Guanine phosphoribosyl transferase
G-6-P	Glucose-6-phosphate dehydrogenase
HAMA	Human anti-mouse antibody
НАНА	Human anti-human antibody
HAT	Hypoxanthine aminopterin thymidine
H/E	Hematoxylin and Eosin stain
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethane sulphonic acid])
HGPRT	Hypoxanthine guanine phosphoribosyl transferase
hmab	Human monoclonal antibody
HMFG	Human milk fat globule
Нр	Hematoporphyrin
HpD	Hematoporphyrin derivative
HPLC	High Pressure Liquid Chromatography
HRP	Horse radish peroxidase
HSA	Human serum albumen
HT	Hypoxanthine thymidine
hu-mab	Humanised monoclonal antibody
IC <sub>50</sub>	50% inhibitory concentration
IFO	Ifosfamide
Ig	Immunoglobulin
IL-1	Interleukin 1
IL-2	Interleukin 2
<sup>ID</sup> In	Indium-III
i.p.	Intra-peritoneal
IPDT	Immunophotodynamic therapy
i.v.	Intra-venous
LAL	Limulus amoebocyte lysate
LAT	Landschütz Ascites Tumour
LCL	Lymphoblastoid cell line
LDH	Lactate dehydrogenase
Leu-OME	L-leucine-0-Methyl ester
mab	Monoclonal antibody
2-ME	2-mercaptoethanol

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MDH	Malate dehydrogenase
MDP	Methylene diphosphonate
MPI	Mannose phosphate isomerase
n	Number of replicates
NaOH	Sodium hydroxide
N.D.	Not determined
NP	Nucleoside phosphorylase
N.S.	Non-secretors
OD	Optical density
OUA	Ouabain
ONPG	O-nitrophenyl galactopyranoside
Р	Passage number
PAGE	Polyacrylamide gel electrophoresis
PB	Peptidase B
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline (Dulbecco's A)
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PEG	Polyethylene glycol
PDT	Photodynamic therapy
РНА	Phytohemagglutinin
PLC	Phosopholipase C $\alpha$ -toxin
PWM	Pokeweed mitogen
r	Immunoreactivity fraction
ren	restriction endonucleases
грт	revolutions per minute
RPMI.S <sub>10</sub>	RPMI (supplemented with 10% v/v FCS)
s.d.	Standard deviation
SAC	Soyabean agglutinin
SAR	Structure activity relationship
SRBC	Sheep red blood cells
SCD	Soyabean Casein Digest Medium
SCID	Severe combined immune deficient
SDS	Sodium-dodecyl sulphate
[T]	Total radioactivity
Таа	Tumour-associated antigens
T <sub>c</sub>	Cytotoxic T cells

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T Cell	T lymphocyte cell
<sup>99М</sup> Тс	Technetium-99M
TEMED	$N, N, N^1, N^1$ -tetramethyl-ethylaminediamine
6-TG	6-Thioguanine
T <sub>G</sub>	Thioglycolate
Т <sub>н</sub>	Helper T cell
TLC	Thin layer chromatography
TN	True negative
TNF	Tumour necrosis factor
TRIS	Tris(hydroxymethyl)methylamine
Ts	Suppressor T cell
w.t.	weight

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## UNITS

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cpm	counts per minute	
hr	hours	
kD	kiloDalton	
keV	kilo-electron Volts	
mCi	milliCurie	
MBq	MegaBequerel	
MeV	Mega-electron Volts	
mg	milligram	
min	minute	
ml(s)	millilitre(s)	
M	Molar	
mM	millimolar	
mW	milliWatt	
uF	microFaraday	
110	microgram	
nM	microMolar	
no	nanogram	
o	nanometer	
 v/v	Volume per volume	
vi v	Weight per volume	
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## SECTION 1 : INTRODUCTION

4.

1.1.

If there was a way to direct drugs and other molecules *selectively* to target cells, many diseases could be treated without affecting non-diseased tissues. Such a selective therapy would allow the drug dosage to be reduced, the harmful side-effects to be minimised and thus, the efficacy of the drug could be improved. Selective delivery systems would have wide applications, including use against viral infections such as AIDS and hepatitis, against autoimmune diseases such as rheumatoid arthritis, and they would be especially useful in locating and perhaps treating cancers.

Conventional cancer therapies exploit the fact that neoplastic cells and tissues are more rapidly dividing and usually more vascular than normal cells. However, other healthy cells such as liver cells, bone marrow and germ cells also divide rapidly and are often killed non-selectively as a side-effect of such therapies. An alternative therapeutic approach is to exploit the differences in the proteins and carbohydrate antigens which are present on the surface of cancerous and healthy cells. One such approach would be to use highly specific monoclonal antibodies (mabs) which would selectively recognise these altered protein or carbohydrate antigens on tumour cells and could, therefore, form the basis of both selective diagnostic and therapeutic systems. Researchers involved in monoclonal antibody technology are currently looking at new ways of generating murine, human and genetically engineered (i.e. recombinant) monoclonal antibodies. Research groups are also looking at novel ways of treating diseases, such as cancer, with mabs alone and also with mabs which are chemically and genetically conjugated to drugs, toxins and isotopes (Figure 1.1.).

In this thesis, three separate aspects of monoclonal technology will be explored:

- (a) The establishment of a human myeloma-derived cell line (C23/11) and a human-murine heteromyeloma cell line (HET-37) which could be used to immortalise human antibody producing B-cell and ultimately produce human mabs (hmabs). This is discussed in detail in Section 3.
- (b) The use of therapeutic-grade murine mabs, which are conjugated to radioactive isotopes to diagnose ovarian carcinomas in patients with suspected disease (Section 4).
- (c) The production of an immunoconjugate for use in immunophotodynamic therapy. A mab was conjugated to a photoactive drug (hematoporphyrin) and was used to selectively kill antigenbearing tumour cell lines (Section 5).

These experimental topics are described in separate sections. However, in the remainder of this section, the broader aspects of mab technology and the current and future uses of mabs in oncology are critically evaluated.

2



Figure 1.1: Summary of the major areas of research in the production and application of monoclonal antibodies for the detection and treatment of cancer. The grey boxes indicate areas in which experimental work was undertaken.

Murine monoclonal antibodies (mabs) are secreted by immortalised antibody producing B-cells called hybridomas. Murine hybridomas secreting specific antibodies were first produced by Köhler and Milstein in 1975. They discovered that normal immunised murine B-lymphocytes (which do not grow in cultures) could be established as continuous cell lines when fused with malignant plasma cells or myelomas. Certain drug-resistant myeloma cells, were isolated and used for fusion. Myeloma cells, which are resistant to purine-antimetabolites such as 8-azaguanine or 6-thioguanine, are also deficient in the enzyme hypoxanthine-guanosine-phosphoyribosyl-transferase (HGPRT) and must, therefore, produce purines by the purine salvage pathway. The purine salvage pathway can be blocked by the addition of aminopterin to the medium. Therefore, myeloma cells which are grown in medium containing aminopterin and which are also lacking the enzyme HGPRT cannot produce purines. This can form the basis of a cell selection system. The myeloma cells will die in culture, normal B-cells will not grow in vitro, while B-cell-myeloma hybrids, or hybridomas having taken up the genes for purine synthesis, can grow in medium containing hypoxanthine, aminopterin and thymidine (HAT medium).

Following the selection of hybridomas which secrete the required antigen-specific antibodies, the cells were diluted to a density of <u>one cell</u> per well and grown. All resultant cells were of the same parental origin and are therefore monoclonal. The ability to grow large numbers of hybridomas <u>in vivo</u> as murine ascites tumours as well as <u>in vitro</u>, has led to the production of a wide range of antigen-specific murine mabs. Examples of these antigens include lipids, enzymes, hormones, carbohydrates, viruses, bacteria and a plethora of antigens which are found in high concentrations on the surface of tumour cells called tumour-associated antigens (Taas). Taas are not *specific* for neoplastic cells (reviewed by Thurin, 1990). They are usually normal antigens, such as placental alkaline phosphatase, human milk fat globule and chorioembryonic antigen, which are expressed in elevated levels on cells which do not normally express this antigen. Using murine, human and recombinant mabs against Taas, which are unconjugated or conjugated to drugs, toxins and isotopes to target the appropriate cells, several researchers have detected and killed tumours.

However, murine monoclonal antibodies have not been as successful as initially forecasted in detecting and treating cancer. Because of the "foreignness" of murine antibodies in humans, patients have been found to develop human-anti-mouse antibodies (HAMA). This immune response is quite potent and results in the production of both anti-allotypic and anti-idiotypic antibodies (against the variable region of the antibody) (Shawler *et al.*, 1985). HAMA reactions may lead to anaphyaxis and immune complex formation (Schroff *et al.*, 1985). Anti-idiotypic antibodies (anti-id) mirror the target antigen and these

1.2.

anti-id antibodies compete with the antigen for the injected antibodies (i.e. anti-Taa), and reduce the amount of anti-Taa antibody bound to the tumour which reduces cell killing and the quality of the the diagnostic images which can be obtained (Herlyn *et al.*, 1986). Murine mabs fail to recognise some human antigens, and mice will raise antibody responses against human antigens which are not conserved between the mouse and human species. Taas sometimes differ from normal antigens in only amino acid substitutions or in a small number of carbohydrate moieties (Tsuruo and Hamada, 1990) and human-derived mabs are likely to be more subtle in recognising these altered antigens than murine antibodies (Larrick and Bourla, 1986).

Aside from the reduced immunogenicity, regulatory agencies such as the FDA are more willing to licence human-derived rather than non-human derived products (Brown, 1990). These limitations have lead to the production of human-derived mabs, bifunctional antibodies and genetically engineered antibody fragments such as  $F_v$  and F(ab) (Figure 1.2). The production of these new mabs and their use in cancer research is a vast and potentially exciting area of research which will be further outlined.



**Figure 1.2:** Structure of an antibody. An antibody consists of heavy (H) and light (L) [either  $\kappa$  or  $\lambda$ ] chains which are joined together by disulphide linkages. The antibody can be further divided into variable (V) and constant (C) regions. Various antibody subunits or fragments can be produced, including the F<sub>e</sub> fragment which does not bind to the antigen F(ab')<sub>2</sub>, F(ab) and F<sub>v</sub> fragments which can bind to the antigen. Individual V<sub>H</sub> or V<sub>L</sub> regions are known as single domain antibodies or dabs.

Studies by Croce and his colleagues in 1980 showed that human hybridomas producing monoclonal antibodies (hmabs), could be produced by fusing human B-cells which secreted the required antibodies, with human myeloma fusion partners. However, hmab technology has taken longer to establish than the murine technology described in Section 1.2. Hurdles which have limited the development of hmabs include the source of B-lymphocytes, immunisation strategies used, techniques used to immortalise the immunised B-cells, screening methods and problems encountered with the large-scale production of hmabs. In fact, every aspect of hmab production has presented new challenges to researchers, and, the results have added to the general understanding of human immunity and antibody production.

## 1.3.1. Sources of B-Lymphocytes.

Human peripheral blood lymphocytes, (PBLs), spleen, tonsils, lymph nodes, and bone marrow cells, are the main sources of cells used in hmab production. Researchers have also used lymphocytes from pleural effusions and from placental and turnour infiltrations as sources of pre-immunised B-cells (Glassy and Dillman, 1988). The advantages and disadvantages of each B-cell type are summarised in Table 1.3.1.

Patients who have suffered from a viral disease such as Herpes simplex (Evans *et al.*, 1984), cytomegalovirus (Matsumoto *et al.*, 1986), rubella (Van Meel *et al.*, 1985) and hepatitis B have immunised B-cells which require no further immunisation. However, PBLs, lymph nodes and tumour-infiltrating lymphocytes have been of limited use in generating hmabs against Taas (reviewed by Borrebaeck *et al.*, 1990). The major limiting factors are thought to be the T cell to B-cell ratio (T : B), and the fact that these cells are mature and, therefore, not in exponential phase of growth for use in fusion studies. Where immature or naïve lymphocytes from spleen and tonsil cells have been used, a significant anti-Taa response was reported. This effect can be attributed to the low levels of cytotoxic T cells (Tc) and suppressor T cells (Ts) in these tissues which inhibit the immune response both <u>in vitro</u> and <u>in vivo</u>.

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B-cell Source	Advantages	Disadvantages	Reference
Peripheral Blood	Availability.	Ratio T:B cells.	Glassy et al., 1988
Lymphocytes	Repeated use.	Lack of naïve B-cells.	Borrebaeck, 1986
	IgG response.		
Splean Calls	Correct T:R ratio	Availability	Lowe at al. 1084
Spicen Cens	Collect 1.B fallo.	Availability.	Oleson and Kaplan
		Steroids.	1984
		IgM response.	1901
Tonsil Cells	Naïve B-cells.	IgM response.	Lagace and Brodeur,
	Correct T:B cell ratio.		1985
	Availability.		Carroll et al., 1990
Tumour Infiltrating	Antigen primed.	Drug effects and	Sikora et al., 1982;
Lymphocytes	IgG response.	myelotoxicity.	1983
	Availability.		Paulie et al., 1984
Lumph Node	Drimed antigan	Augilakility	TT-L-L' /
Lympn Node	rinned anugen.	Availability.	Hironasni et al.,
	lgG response.	Incorrect 1:B ratio.	1983
Воле Маттом	Committed B-cells.	Availability.	Seigneurin et al
	IgG production.		1983

Table 1.3.1: Sources of B-lymphocytes for use in human monoclonal antibody production.

## 1.3.2. In Vitro Immunisation.

It is both ethically and practically impossible to immunise human beings against most antigens. Similarly, it is often desirable but difficult to make human antibodies that react with self-antigens. In vitro immunisation has been used to obtain B-cells with the desired specificity, affinity and, in some cases, antibody isotype (Table 1.3.1.). Where in vivo immunisation is not possible, the source of Bcells used, the cytokines and mitogens used, as well as the T : B ratio, are vital components for successful in vitro immunisation (Borrebaeck, 1988). While tonsil and spleen cells provide rich sources of naïve B-cells which can be activated in vitro, the antibodies produced following short term in vitro immunization are usually of an IgM isotype. IgM mabs are generally of lower specificity than IgG antibodies and are involved in many multi-specific antibody interactions (Ghosh and Campbell, 1986). Moreover, restimulation of more mature B-cells from tumour-infiltrating lymphocytes has been used to produce an IgG response in vitro. Gallatin et al. (1986) argued that PBLs are indeed a good source of B-cells producing anti-tumour antibodies, provided the T : B ratio is altered and the correct soluble factors are supplied before restimulating of the lymphocytes. Borrebaeck et al., (1988 (b)) have suggested the inclusion of the lysosomatropic agent L-leucine-methyl-ester (LEU-OME) in the medium for in vitro immunisation. LEU-OME has been shown to accumulate in lysosomes in human monocytes, macrophages and cytoxic T cells. The LEU-OME is metabolised to free amino acid and alcohol and this causes the death of macrophages and natural killer cells and it has also been shown to inhibit CD4<sup>+</sup> and especially CD8<sup>+</sup> cytotoxic T cells which inhibit immunisation. PBLs can be an excellent source of B-cells. The T : B cell ratio varies from 1 : 2 to 1 : 5, depending on the pretreatment method used to reduce Ts and Tc cells (Borrebaeck, 1988 (a)). Other methods used to alter the T:B ratio are summarised in Table 1.3.2.

Several other factors have been shown to influence in vitro immunisation. T cell growth factors contained in thymus-conditioned medium (TCM) are required for in vitro immunisation. Lymphokines such as IL-4 (Ho, 1988), IL-2 (Rosén *et al.*, 1988) and IL-6 (Carroll *et al.*, 1990; 1991) and mitogens such as pokeweed mitogen (PWM), phytohemagglutinin (PHA), concanavailin A, lipopolysaccharide (LPS), and soyabean agglutinin (SAC) have been used to activate various cell types in vitro (Borrebaeck, (1988 (a)). Interestingly, PWM has been added to the medium in a time-delayed manner to reduce T cell activation and improve B-cell responses (Carroll *et al.*, 1991). Muramyl dipeptide (MDP) is known to improve immune responsiveness but it is not a polyclonal mitogen (Watson and Whitlock, 1978). The purity, solubility and concentration of the antigens used affects the success of immunisation. Matsumoto *et al.* (1986), showed that high doses of antigen may actually *inhibit* immunisation, while Ho *et al.* (1985), showed that the solubility and the purity of the antigen used influenced the isotype which is secreted from the cell. They showed that insoluble or particulate antigens were shown to favour the production of IgG antibodies.

Table 1.3.2:	Techniques	used to deplet	e supressor T-cell	s (Ts) from	lymphoid samples.
				· ·	

Method	Reference	
Sheep red blood cell (SRBC) rosetting	Teng et al., 1985	
Separation on nylon wool.	Garzelli <i>et al.</i> , 1984 Ho <i>et al.</i> , 1985 Yamura <i>et al.</i> , 1985	
L-leucine-methyl ester	Borrebaeck et al., 1988(b)	
Sephadex G-10 column	Hoffman and Hirst, 1985	
Lysis by OKT8, (Ts-specific mab)	Lagace and Brodeur, 1985	
Cimetidine	Ho et al., 1985	

## 1.3.3. Immortalisation.

A variety of fusion partners including human myeloma cells, human lymphoblastoid cells, murine myeloma cells and mouse-human hybrid cells have been used to immortalise human B-cells. Continuous B-cell lines have also been established by viral transformation and by cloning of the Ig genes into suitable host cell lines. These techniques will be discussed in more detail in Section 3.1.

## 1.3.4. Large Scale Production of hmabs.

After identifying immortalised B-cells which secrete the desired antibodies, it is then necessary to grow them at large scale level. Considerable difficulties have been reported in producing stable antibodysecreting clones by limiting dilution or by soft-agar cloning techniques (Glassy and Dillman, 1988). Further difficulties were encountered when these cells were grown by large scale fermentation. A number of different systems have been employed such as hollow fibre reactors (Altshuter *et al.*, 1986), agarose beads (Scheirer *et al.*, 1984), stirred tank reactors (Kitano *et al.*, 1986) and in air-lift fermenters (Birch *et al.*, 1985), but have met with limited success. This area of large scale hmab culture has recently been reviewed by Glad and Nilsson (1990). Attempts to grow hmabs in vivo as ascites in irradiated pristane-primed nude mice (Kozbar *et al.*, 1985; Kosinski and Hämmerling, 1986), or when mixed with human skin fibroblasts, have been of very limited use to date. In a recent review by Borrebaeck *et al.* (1990), it was reported that hmab were produced in Severe Combined Immun-Deficient (SCID) mice. These mice are deficient in both T and B cells. However, the amounts of hmab produced are still much lower than those obtained with murine mabs. The difficulties which are encountered in producing stable human hybridomas prompted several researchers to 'by-pass' hybridoma technology and, in some instances, animal immunisation altogether. The use of mouse-human chimaeric monoclonals, humanised mabs (h-mabs) and the production of mabs in non-myeloma cells, yeast, bacteria and even plants has renewed interest in monoclonal technology.

## 1.4.1. Chimaeric Antibodies.

Chimaeric antibodies are part-human and part-mouse mabs. From Figure 1.4.1., it can be seen that these chimaeric antibodies are produced by attaching a human antibody constant region to murine antibody variable regions (reviewed by Morrison and Oi, 1989). Therefore, chimaeric antibodies retain the specificity of the original murine antibody but should be less immunogenic than intact murine antibody. The presence of an intact human Fc portion in these antibodies allows their use to treat patients by stimulating both complement-mediated cytotoxicity (CMC) and antibody-dependent cellmediated cytotoxicity (ADCC), and will be discussed in Section 1.11.2. However, the intact Fc portion reduces the effectiveness of mabs in tumour imaging (Section 4.4.5.). A number of genetic engineering techniques have been used to produce chimaeric mabs. Most of the initial studies transferred human genes coding for Fc region DNA into murine myeloma cells using plasmids. pSV2 plasmids which contained heavy (and/or) light chain genes and drug-resistant markers, such as xanthine-guaninephosphoribosyltransferase (gtp), were constructed. These drug selection genes can then be used to select cells that have been successfully transfected with the plasmid. These cells are called transfectomas. Protoplast fusion, using protoplasts from cultures of *Escherichria coli* which contain a high plasmid copy number have been successfully transfected into cells by using polyethylene glycol (Gilles et al., In other studies, chimaeric antibodies which were prepared by calcium phosphate co-1983). precipitation (Boulianne et al., 1984) have been shown to have low transfection efficiencies (1 x 10<sup>4</sup> to 1 x 10<sup>-5</sup> transfected cells) (reviewed by O'Kennedy and Roben, 1991). Electroporation has resulted in the production of more stably-transfected clones, which can be isolated with frequencies of  $10^{-3}$ transfected cells (Boulianne et al., 1984; Potter et al., 1984). These transfectomas can be cloned and cultured in the same way as murine hybridomas, and, they have been shown to produce a range of stable mabs, with good antigen-binding capacities as well as the correct human isotypes for use in therapy (Morrison and Oi, 1989).

Rather than constructing transfectomas, Neuberger (1985) attempted to produce chimaeric antibodies in <u>*E. coli*</u>. However, the V<sub>H</sub> and V<sub>L</sub> chains (Figure 1.2) did not recombine properly and they formed and insoluble aggregate within the bacterium. These V<sub>H</sub> and V<sub>L</sub> fragments were not glycosylated by



**Figure 1.4.1:** General structure of recombinant monoclonal antibodies. Messenger RNA (mRNA) is prepared from murine hybridomas or EBV transformed cells. First-stand copy DNA (cDNA) synthesis is performed and primers corresponding to the human framework region are used to initiate PCR amplification (O'Kennedy and Roben, 1991). Chimaeric antibodies are constructed by grafting the murine hypervariable or CDR regions onto human framework regions. Effector hybrid mabs are constructed by attaching antibody binding regions to effector molecules such as enzymes and toxins (Section 1.4.). Bifunctional antibodies (BFAs) may be produced chemically, by cell hybridisation or by genetic manipulation (Section 1.5.) for use as targeting or effector mabs [adapted from Larrick *et al.*, 1990].

<u>*E. coli*</u> and chemical recombination of the purified  $V_H$  and  $V_L$  regions was both complex and inefficient. Strategies which have been used to overcome these problems will be explored in Section 1.4.3.

## 1.4.2. Humanised Monoclonal Antibodies.

The genes coding for  $V_{\rm H}$  and  $V_{\rm L}$  regions contains six complementarity-determining regions (CDRs) embedded in a framework region (FR1 - FR4), as shown in Figure 1.4.1. Reichmann and his colleagues (1988), designed a recombinant '*humanised*' mab (hu-mab) in which *murine* CDR regions were embedded in human-framework regions. Unlike chimaeric antibodies, the Fc portion and most of the F(ab')<sub>2</sub> portions of the antibody are human. Hu-mabs are of use in ADCC and CMC but they are less immunogenic and have longer circulating half-lives when administered to patients (Section 4.4.5.). Certain residues in the original murine framework make key contacts with murine CDRs that help to maintain the 3-dimensional conformation of the antibody binding site. This 3-D structure determines the antibody binding site and, therefore, the specificity of the antibody for a given antigen. When murine framework regions are replaced with human sequences, those CDR contacts are altered which alters the affinity of the hu-mab for the specified antigen. Co *et al.* (1991 (a)) have used human V regions that are particularly homologous in sequence to the murine V regions. These hu-V regions have minimised so-called 'framework shift affinity changes'. Computer modelling technology was used to locate the CDR-FR contacts and to stabilise hu-mabs against the gB and gD glycoprotein herpes simplex virus antigens.

Both chimaeric and humanised monoclonal antibodies have been produced to therapeutic grade and used <u>in vivo</u>. The results of these clinical applications of mabs will be discussed in more detail in Section 4.4.5.

## 1.4.3. Second Generation Recombinant Mabs.

Chimaeric and hu-mabs have been termed the first generation recombinant mabs by Clackson, (1991). In this review, he also describes second generation recombinant mabs, where the immunoglobulins are produced in bacteria.

First-generation recombinant mabs used murine hybridoma DNA as a source of  $F_v$  and CDR DNA. Instead of producing hybridomas or immunising animals, these recombinant mabs are constructed from DNA derived from primary human tissue. In the case of anti-tumour antibodies, mRNA is isolated from a cancer patient's bone marrow, spleen or tumour infiltrating lymphocytes. These patients should have mRNA in their plasma cells which gives rise to antibodies against the tumour as well as other mRNA. All the mRNA is translated into DNA by use of the reverse transcriptase enzyme. The DNA is cut by restriction endonucleases and only those DNA fragments which code for  $V_H$  (or  $V_L$  when DNA is cut with a separate set of restriction endonucleases) are isolated and amplified using the polymerase chain reaction (PCR).  $V_H$  and  $V_L$  genes have been cloned into a range of vectors which includes the



Schematic view of a technique described by Clackson, (1991), for producing Figure 1.4.2: genetically engineered Fv fragments in E. coli using the phagemid library technique. mRNA is extracted from a patient's bone marrow and its cDNA amplified by PCR. DNA coding for V<sub>H</sub> (or V<sub>L</sub>) fragments is isolated by cutting with restriction endonucleases (ren's) and the resulting V<sub>H</sub> segments can be ligated to the phagemid vector (1). This vector (2) is then transfected into E. coli by electroporation and grown to large scale. Following lysis of the bacteria the vector is cut with separate rens (which had cut the VL fragments) and these new vectors (3) are electroporated into E. coli. The phagemid vector used in this technique is deficient in some of the genes needed to form a phage coat, or capsid. Capsid formation (which allows the expression of Fy fragments) can proceed if these genes are borrowed from a helper phage. Capsids expressing the correct antigen specific Fy fragments can then be isolated. In the next step, the phage DNA is cut out from the vector by a further set of rens and the plasmid which contains the functional Fv fragment can be transfected into E. coli and grown to large scale. Following lysis of these bacteria, Fy fragments can be purified.

**1.5. BIFUNCTIONAL ANTIBODIES.** 

Bifunctional antibodies (BFAs) are bi-specific, that is they bind to two separate antigens. Each arm or F(ab) fragment binds to a different antigen (Figure 1.4.1.). BFAs have been produced chemically, by hybridoma technology and, more recently, by genetic engineering techniques.

Chemically produced BFAs are produced by synthesising an interchain disulphide bond between two different types of F(ab) fragments (Brennan *et al.*, 1985; Glennie *et al.*, 1987). These BFAs have two functional binding sites and are produced with a higher overall yield than biologically produced BFAs.

BFA can be produced by hybrid hybridomas or quadromas or, in some cases, triomas. Quadroma cells are formed when two hybridoma cells are fused, while trioma cells are formed when a hybridoma is fused to an immunised B-cell. These hybrid cells produce 16 different random combinations of heavy and light chains. Only one of these functional Igs contains the correct heavy and light chain recombination to produce a bispecific or bifunctional antibody (Martinis *et al.*, 1982; Milstein and Cuello, 1984). This recombination of heavy and light chains is not entirely random. There is a bias towards the recombination of homologous (i.e. hybridoma 1 x hybridoma 1) rather than heterologous (i.e. hybridoma 1 x hybridoma 2) antibodies. Milstein and Cuello (1984), have estimated that there is an overall ratio of 1 heterologous to 12 homologous antibodies formed following cell fusion. The vast array of functional immunoglobulins which are produced do not react with correct combination of antigens and this neccessitates screening of the large number of clones, which creates both screening and purification problems. Techniques such as Protein A-Sepharose and hypoxyapatite column - HPLC have simplified this selection process (Nolan and O'Kennedy, 1990).

Genetically engineered recombinant BFA fragments have also been produced. Constructs in which two single chain  $F_v$  fragments with anti-CD3 and anti-DNP specificities were linked and expressed in bacteria. However, further work is needed to obtain an <u>active</u> refolded single-chain BFA (Fanger *et al.*, 1991).

Human BFA production has been hindered by the limited success of human hybridoma technology to date. It appears that the developments in genetic engineering will be the most practical method of obtaining human BFAs for clinical use (Section 4.4.5.).
#### 1.6. CATALYTIC ANTIBODIES.

The specificity and speed of antibody-antigen interactions are very similar to those observed in enzymesubstrate reactions. Affinity constants of up to  $10^{-12}$  M<sup>-1</sup> which were observed for some antibodyantigen reactions, led some researchers to suggest that they may have some enzyme-like catalytic activity. The discovery that antibodies *can* catalyse a range of reactions such as acyl-transfer and peptide-bond formations has lead to renewed interest in catalytic antibody or 'abzyme' technology (reviewed by Green and Tawfik, 1989; Harris, 1991).

Green and Tawfin (1989) outlined several roles for catalytic antibodies. They can act as catalysts in organic synthesic and in sterospecific synthesis. Catalytic antibodies may also be used as biosensors and as cleavage systems in protein engineering. In addition, catalytic antibodies have several potential clinical applications. These applications include:

- (a) Catalysis of the breakdown of bacteria and toxins. It was suggested that catalytic antibodies with proteolytic activity would act more speedily and with lower overall dose than unconjugated antibodies (Section 1.11.).
- (b) Similarly, catalytic antibody 'vaccines' may passively immunise against toxins, bacteria, virus and potentially cancer cells.
- (c) Catalytic antibodies may be used to replace enzymes which are deficient in a patient.
- (d) These antibodies may replace mab-enzyme conjugates in prodrug activation (reviewed in more detail in Section 1.10.2.).

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Soon after the discovery of monoclonal antibodies, it became apparent that the exquisite sensitivity and selectivity of these molecules would be of medical importance. Mabs have been used to diagnose and treat a variety of diseases. Mabs have been used to treat autoimmune disorders such as rheumatoid arthritis (Sany, 1990), they have been used as vaccines to treat malaria (reviewed by Coppel, 1986) and they have also been used as anti-viral agents (Co *et al.*, 1991 (b)). Mabs have also been used to diagnose non-specific infections and myocardial necrosis (Strauss *et al.*, 1991). However, particular interest has been expressed in the application of mab technology to the detection and treatment of cancer.

Several factors influence the type of mab or mab-conjugate which can be used in a particular clinical application (Table 1.7.1.). These factors include the intended usage of the antibody (i.e. for detection or therapy), the antibody unit used (i.e. IgG,  $F(ab')_2$  or  $F_v$  fragment), the antigen target and the distribution and pharmokinetics of the mab when administered to patients.

While tumour detection (Section 4) and a novel method of tumour immunotherapy (Section 5.5.5.) will be discussed in some depth in later sections the aim of this review is to outline the clinical applications of mab technology to date. By summarising the results of studies already conducted and examining the problems they have encountered and overcome, it may be possible to find new applications for recombinant mabs, to develop more effective immunoconjugates and to make mab therapy more accurate and disease-specific in the 1990s.

1.7.

## Table 1.7.1: Factors affecting the choice of monoclonal antibody for use in vivo.

Factor	Choices and Limitations		
Intended usage	delivery system vs effector molecule		
Molecular Species used	whole antibody, $F(ab')_2$ , $F(ab)$ , $F_v$ murine, human, humanised or recombinant monospecific, bifunctional		
Specificity	monospecific <u>vs</u> multispecific mabs extracellular <u>vs</u> intracellular antigens minimal cross-reactivity with non-specific antigens		
Affinity	immunoreactivity ( $k_q > 10^{10} L/M$ )		
Antigen	antigen density (> 10 <sup>6</sup> sites per cell) internalisation of antibody, capping metabolism/excretion of radiolabeled antibody presence of antigen in blood/tumour		
Biodistribution	route of administration (iv, ip, etc.) pharmacokinetics, vascularity of target area, size of target, rate of blood clearance and non-specific mab uptake by liver, spleen etc.		

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Drugs, toxins, biological response modifiers and radioisotopes have been conjugated to mabs. These toxic or localising immunoconjugates have been used to target particular cells which express antigens.

The most stable immunoconjugates have been produced following the covalent linkage of the compound to the mab. The most accessible sites for covalent attachment to mab polypeptide chains are the  $\varepsilon$ -amino groups of the lysine residues (approximately 90 lysines in a mab). Both drugs and toxins have been conjugated to intact mab and mab fragments by stable amide linkages (Pieters, 1990).

Regardless of the covalent binding technique which is used, the following points are of importance:

- (a) The attachment of the drug (or toxin or isotope) must not alter the specificity or immunoreactivity of the antibody.
- (b) The chemical linkage system must not interfere with the toxicity of the drug.
- (c) The drug should be covalently attached to the antibody. Non-specific absorption of the drug to antibody, which may be reversible, should be minimised.
- (d) The conjugate should be prepared under conditions that will minimise the formation of large molecular weight complexes which would increase the immunogenicity of the conjugate.
- (e) The conjugate protocol should be capable of scale-up to production level. This production must be carried out under sterile and pyrogen-free conditions, especially if the products are for administration to immunocompromised patients.

These general guidelines refer to all immunoconjugates. In the next sections, the production and applications of specific immunoconjugates, such as chemoimmunoconjugates, immunotoxins, immunocytokines and radio-labelled mabs will be discussed in more detail.

#### 1.8.1. Chemoimmunoconjugates.

Most of the drugs currently used in cancer treatment (Table 1.8.2.) kill all growing cells nonspecifically. The side effects of these drugs are also well characterised. However, these cytotoxic drugs are not ideal agents for use in immunotherapy. Firstly, many of these drugs do not have a chemical

A. CHEMOIMMUNOCONJUGATES	Reviewed by:		
DNA binding and intercalating agents			
Daunomycin, Adriamycin, Bleomycin and Macromycin	Pietersz, (1990)		
Alkylating agents	Reisfeld and Schrappe, (1990)		
Chlorambucil, Melphalan, Mitomycin C, Trenomin,	Bagshawe, (1989)		
cis-Platinum, Phenylenediamine Mustard	Pietersz et al., (1987)		
Microtubule inhibitors			
Vindesine, Deacetyl colchicine			
Anti-metabolites			
Fluorouracil, Methotrexate, Cytosine arabinoside			
B. IMMUNOTOXINS	<u>Reviewed by</u> :		
Ribosomal protein synthesis inhibitors			
Ricin, Gelonin, Abrin, Modeccin, Saporin, Purothionine,	Spooner and Lord, (1990)		
$\alpha$ -Amanitin, Pokeweed anti-viral protein	Bjorn and Villemez, (1988)		
Elongation factor 2 inhibitor	Vitetta et al., (1987)		
Diphtheria toxin, Pseudomonas endotoxin			
C. IMMUNOCYTOKINES	Reviewed by:		
Local response			
Interleukin (IL-2, IL-1), Interferon	Ferrini et al., (1990)		
Complement activation	Figlin, (1989)		
C3b, Cobra venom factor (C3/C5 convertase)	Foon, (1989)		
Inflammatory response	Cavagnaro, (1987)		
TNF			

## Table 1.8.1: Summary of Main Immunoconjugates.

group which is suitable for conjugation well separated from the active site. The presence of such binding groups will allow covalent linkage of the drug to the antibody without affecting the drug potency or toxicity. Several researchers have overcome this problem by introducing new active groups and by using carrier molecules such as dextran, polyaminoacids or human serum albumen (Pietersz, 1990) to minimise reduction in drug potency.

In order to deliver a potent dose of drug to the selected cell, each mab usually has several drug molecules attached to it. Even when very active drug immunoconjugates are targeted to the most drugsensitive tumour cells, between  $10^6$  to  $10^7$  drug molecules must be accumulated in a tumour area to mediate tumour killing (Reisfeld and Schrappe, 1990). Johnson *et al.* (1987) showed that many of the commonly used drugs (Table 1.8.1.) lose much of their activity following binding to proteins. This loss in activity has been attributed to steric hindrance, altered mechanisms of cellular uptake and reduction in the drug half-life following linkage.

Many researchers have abandoned the use of conventional drugs and are beginning to produce new drugs specifically designed for mab-conjugation. These drugs have been termed structure-activity-relationship (SAR) compounds, and have been designed with both the potency of the drug and the immunoreactivity of the antibody in mind. The developments of new SAR drugs such as KS1/4 - DAVLB [a vinblastine derivative] (Koppel, 1991), and LY203625 (Laguzza *et al.*, 1989) a new synthetic drug mark the beginning of SAR immunoconjugates. Further developments in prodrug chemistry (Section 1.10.2.), the use of chemoimmunoconjugates to by-pass multidrug resistance in cell lines (Tsuruo and Hamada (1990), and lysosome-sensitive linkages, which allow the release of free-drug on internalisation of the complex (Trouet *et al.*, 1982), will maximise tumour cell killing by chemoimmunoconjugates.

#### 1.8.2. Immunotoxins.

Toxins were proposed as suitable agents for immunotherapy because of their extreme cytotoxicity. The main plant and bacterial toxins which have been conjugated to mabs are summarised in Table 1.8.1. Toxins are protein molecules which are found in plants and bacteria and they can be easily conjugated to mabs by using heterobifunctional reagents (reviewed by Spooner and Lord, 1990). Toxins may act enzymatically on the cell membrane. (Bjorn and Villemez, 1988). Ricin and other lectin toxins contain an A chain which is non-specifically toxic, and a second sugar-binding B chain. Most immunotoxins consist of the toxin A chain conjugated to the mab. This immunotoxin is able to bind to antigen bearing cells in a selective manner. The toxin portion of the immunotoxin is activated after the mab binds to the cell. Because immunotoxins are often much more cytotoxic than drug-antibody conjugates, non-specific uptake by liver, bone marrow and other cells must be avoided. The use of  $F(ab')_2$ ,  $F_y$  and

smaller single domain antibodies, may reduce non-specific uptake in the liver, spleen and other organs. Recombinant anti-Tac effector mabs, with genes coding for toxin fused in frame with the antibody DNA, have been produced (Section 1.4.4.), but may not be of practical use <u>in vivo</u> until these problems of toxin uptake by non-antigen-bearing cells have been overcome.

#### 1.8.3. Immunocytokines.

Several researchers have looked at the body's natural mechanisms for fighting disease and have tried to redirect these natural cellular molecules called biological reponse modifiers (BRMs) to kill cancer cells.

BRMs such as interferons (Weiner *et al.*, 1988), interleukins (IL-1, IL-2), colony-stimulating factors, erythropoietin, human granulocyte colony-stimulating factor and tumour necrosis factor (TNF) (Reisfeld and Schrappe, 1990) have all been conjugated to mabs and used in cancer treatment.

Foon and Fanning (1990) reported that antibody-IL-2 conjugates were used to activate LAK cells and tumour-infiltrating lymphocytes which expressed the CALLA antigen. Similarly, Chovnick *et al.* (1991) used a recombinant anti-Tac-IL-2 fusion protein which bound to the IL-2 receptor of cells bearing the CALLA antigen and inhibited protein synthesis.

In a recent article by Ferrini and his colleagues (1990), it was noted that immunologists are only *beginning* to unravel the complex reactions of BRMs, cytokines, lymphokines and growth factors. They also noted that many of these molecules have pluripotent effects on many cells of the immune system (Section 1.11.2.) and, therefore, the efficacy of mab-BRM conjugates on tumour is more difficult to monitor than mab-drug, mab-toxin or mab-isotope conjugates. Research aimed at directing BRMs to specific cells may help to elucidate these pluripotent effects.

Isotopes conjugated to mabs have two distinct clinical applications, either in diagnosis by immunoscintigraphy or treatment by radioimmunotherapy. Both of these applications will be outlined in this section and discussed in more detail in Section 4 and Section 5.

#### 1.9.1. Radioimmunotherapy.

The aim of radioimmunotherapy (RIT) is to irradiate and kill antigen-bearing tumour deposits throughout the body with minimal radiation damage to normal tissues. A large variety of  $\beta$ -emmitting and  $\alpha$ -emmitting isotopes and a smaller number of electron capture systems and fissionable nuclides have been used in RIT to date (summarised in Table 1.9.1.). The limited success of RIT in vivo has mainly been attributed to the inability to target sufficient doses of isotope to the tumour. In a recent review by Britton and colleagues (1991(a)), they reported that one day after the administration of mabisotope, approximately 30% of the administered radioactivity has been excreted. They also reported that an average of 1% of the administered activity is actually located to the tumour site, while 69% accumulates in normal tissues (liver, kidneys, bone marrow and with Iodine compounds, the thymus). This leads to extensive lethal irradiation of non-specific tissues. In many cases less than 1% of the administered radioimmunotherapy. Similar factors govern the success of tumour imaging or immunoscintigraphy and these will be discussed in detail in Section 1.9.

#### (a) Tumour Selection:

The size of a solid tumour is a critical parameter in radioimmunotherapy (and in all other immunoconjugate therapy regimes). Since the blood supply to a tumour is from the surface of the tumour inwards, cells lining the superficial tumour capillaries which are more permeable than normal cells and if these cells express the relevant target antigen they will bind the antibody more rapidly than cells in the centre of a tumour mass. Additionally, the core of the tumour is often less oxygenated, and therefore, less sensitive to radiotherapy, so that the deeper parts of larger tumours not only receive less radiolabelled antibody, but the radiation they receive is less effective (Hagan *et al.*, 1986). An antibody which binds to an antigen on the surface of a tumour may not bind to necrotic tissue in the centre of an anoxic tumour. These necrotic tumour cells often express different non-tumour antigens such as TNF, and the inclusion of such a mab-conjugate (i.e. anti-TNF) in a cocktail of mabs may increase access to the tumour and bind to the centre of larger tumours (Epstein *et al.*, 1990).

1.9.

Radioisotope	Half Life (days)	Particulate Energy (MeV)	Comment	Reference
A. β-Emitters				Reviewed by:
Iodine-131	8	0.6	Readily available, cheap too-long half-life	Britton et al., 1991(a)
Rhenium-188	0.7	2.1	Tc-99m chemistry stable in vivo too short half-life	Levy and Miller, 1991
Rhenium-186	3.5	1.1	Energy suitable for both imaging and therapy	Foon and Fanning, 1990
Copper-67	2.5	0.4 - 0.6	Suitable energy, very expensive	
Yttrium-90	2.5	2.3	Indium-III chemistry myelotoxicity	
Phosphorus-32	14.3	0.695	Pure negatron emitter	
Scandium-46	3.4	0.162	High energy $\beta$ emitter, expensive	
B. α-Emitters				
Astatine-211	0.35	5.9	Short range, iodine-	
Bismuth-212	1 hour	6.1	High energy emission	
c. Electron Capture				
Iodine-125	2.5	7.5	Dehalogenation	
D. Fissionable Nuclides				Mafane <i>et al.</i> , (1987)
Boron-10	-	-	Requires external source of neutrons, delivered to tumours	

## Table 1.9.1: Radioisotopes for Use in Radioimmunotherapy.

# <u>Table 1.9.2</u>: Comparison of Factors Affecting the Effectiveness of Radioimmunotherapy and Immunoscintigraphy.

	Radioimmunotherapy	Immunoscintigraphy	
Cell binding	Difficulties in targeting to cells in the centre of the turnour	only a fraction of cells are required to bind to surface of tumour	
Isotopes used	$\alpha$ and $\beta$ emitters, electron capture, fissionable nuclide	$\gamma$ emitters, some $\beta$ emitters	
Isotope half-life	long life (days)	short life (hours)	
Access to tumour site	large doses required (> 40 mg) to increase penetration	small doses required (0.5 - 2 mg)	
Tumour geometry	size-limited (i.e. diffusion limited), can kill seedlings	larger tumours imaged more easily	
Administration	multiple fractionated doses, regional delivery, intravenous	single dose, usually intravenous	
Repeated administration	HAMA toxicity, myelotoxicity	lower immunogenicity	
Immunoconjugate internalisation	not required to kill cells	not required to image cell	

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#### (b) Route of Administration:

In order to increase the amount of radiolabel which could attach to the tumour and decrease non-specific uptake, various researchers have demonstrated that malignancies which recur in the peritoneum, pericardium or pleural cavity are more effectively treated by regional therapy to these areas. Recent results by Hird *et al.* (1990) showed at intraperitoneally administered <sup>90</sup>Y-labelled monoclonal antibodies (80 mg/kg/24 hr) were successfully used to treat recurrent ovarian carcinoma. The antibody used was <sup>90</sup>Y-HMFG-1 and this antibody was also used to locate tumours in these patients (the therapeutic and diagnostic potential of this antibody will be discussed in Section 4.4.5.). In this study, free EDTA was administered with the <sup>90</sup>Y-HMFG-1 to help remove non-specifically bound antibody. The results obtained were superior to those found with intraveneously administered antibody and gave reduced myelotoxicity.

Various strategies are also being used to minimise HAMA reactions. These reactions are particularly useful where up to 500 mg of mab are administered to each patient. The strategies which have been developed to minimise HAMA reactions are summarised in Table 1.9.3. Radiation protection for both patient and hospital personnel is an important factor in RIT. Improvements in catheters and continuous infusion systems to avoid spillages will be necessary in order to satisfy many staff and administrators that this type of therapy is suitable for all hospitals with basic Nuclear Medicine departments.

It appears that RIT is an effective method of treating locally recurrent regional disease such as peritoneal and pleural effusions, especially where the tumours are small in size and the patient can tolerate high doses of antibody without developing an immune response against the injected antibody.

#### 1.9.2. Immunoscintigraphy.

Immunoscintigraphy has been the most useful and successful application of immunoconjugates in oncology. Immunoscintigraphy aims to detect tumours in vivo rather than to kill these tumours. Table 1.9.2. shows that immunoscintigraphy uses lower levels of radiation (gamma  $\gamma$ ), low energy isotopes such as <sup>m</sup>In and <sup>99M</sup>Tc and smaller doses of radio-labelled mab conjugate than in RIT. Typically, patients are administered between 0.5 - 2.0 mg of radiolabel for diagnostic purposes, but are administered with up to 500 mg of radiolabelled-mab for therapeutic purposes (Britton *et al.*, 1991(b)). While radioimmunotherapy is more successful on very small and diffuse tumours, immunoscintigraphy has been most effective on large tumours with larger surface areas to bind antibodies. A detailed evaluation of techniques currently used in immunoscintigraphy, with particular emphasis on imaging ovarian carcinoma using <sup>m</sup>In and <sup>99M</sup>Tc fragments, is given in Section 4.

	Approach	Treatment	Comments
Ι	Immune Supression	azathioprine, cyclophosphamide, cyclosporin A, anti-CD3 mab,	HAMA only partially suppressed, potential toxicity from immune suppression
II	Reduce mab Immunogenicity	F(ab') <sub>2</sub> , F <sub>v</sub> , hmabs, hu-mabs	Anti-idiotypic responses can occur
III	Induction of Tolerance	anti-T-cell mab, total lymphoid irradiation, hyperimmunisation	Anti-idiotypic responses, tolerance is still difficult to achieve

# <u>Table 1.9.3</u>: Strategies to Overcome Human-Anti-Mouse Antibody (HAMA) Reactions following Immunotherapy.

[Adapted from Dillman, (1987); Di Maggio et al. (1990)].

Chemoimmunoconjugates, immunotoxins and radioimmunoconjugates have all been of limited success in vivo, mainly due to non-specific uptake of conjugate by non-tumour tissues. Several researchers have tried to overcome this non-specific uptake by using pre-treatment with EDTA (Goodwin *et al.*, 1986) and by lactosamination, biotin-avidin and anti-mouse IgG antibodies (reviewed by Klibanov *et al.*, 1988). Other researchers use two step systems, administering the conjugate to the patient and allowing the clearance of mab from the bloodstream before activation of the drug or isotope. Novel therapy systems, including immunophotodynamic therapy, antibody-directed enzyme prodrug therapy and radio-avidin-biotin therapy, may be better solutions to the problem of non-specific tumour uptake.

#### 1.10.1. Immunophotodynamic Therapy.

A group of photoactive drugs termed photosensitisers produce singlet oxygen when activated by light in the presence of oxygen. These photosensitisers are relatively harmless in the absence of light. By administering photosensitisers such as hematoporphyrin, hematoporphyrin derivatives, phthalocyanines, chlorines and benzoporphyrins (reviewed by Nelson *et al.*, 1988) and some more unusual compounds such as methyene blue (Yu *et al.*, 1990) and rhodamine-123, to patients, and then irradiating those areas of their body with tumour masses using light focused from a Xenon-Arc lamp, a laser or another light source, significant reductions in the mass of these tumours have been observed. The use of photosensitisers and light to kill tumour cells is known as photodynamic therapy (PDT), and this area has been reviewed extensively (Dougherty, 1984; Ash and Brown, 1988). However, PDT is not a selective treatment, i.e. when a patient with a bladder tumour is administered with a photosensitiser. wile *et al.* (1984) argued that the increased blood flow to the tumour increases the amount of photosensitiser in the tumour and decreases the blood flow to the surrounding tissue but has only partial selectivity. By coupling photosensitisers to monoclonal antibodies the immunophotosensitiser should be delivered to the target tumour tissue, with minimal uptake by the normal cells.

STEP 1: Administration of immunophotosensitiser to the tumour cell followed by clearance of conjugate which has not been taken up by antigen-bearing cells.

STEP 2: Irradiation of tumour area by laser or focused light leading to death of tumour cells.

Immunophotodynamic therapy (IPDT) is particularly selective for tumours than many other immunotherapies. Areas such as the liver, spleen and bone marrow, which take up antibody non-

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specifically are <u>not</u> damaged, because these areas are not irradiated. In Section 5, initial studies on the preparation of an immunophotosensitiser where hematoporphyrin was conjugated to the anti-HMFG antibody will be described. The potential of IPDT as a method of overcoming non-specific drug uptake will be discussed Section 5.5.4.

#### 1.10.2. Antibody-directed Enzyme Prodrug Therapy, ADEPT.

Antibody - enzyme conjugates (or recombinant antibody-enzyme molecules) can be used to cleave inactive precursors or prodrugs into their active form in vivo using two separate administration steps.

- *STEP 1:* The injection, selective localisation and extracellular retention of an antibody-enzyme conjugate at the tumour site.
- STEP 2: Conversion of the prodrug with a low toxicity to a highly toxic drug by enzymatic conversion takes place after non-specifically bound antibody has been cleaved from the blood pool.

ADEPT systems which have been used to date in tumour cell killing include:

- 1. Anti-hCG-carboxypeptidase B and the prodrug bis-chloro-benzoic acid, which was converted to the active agent bis-chloro-mustard (Searle *et al.*, 1986).
- 2. Mab-β-lactamase converts the prodrug cephalosporin-DAVLBHYD, to an active DAVLBHYD.
- 3. Anti-CEA-beta-glucuronidase converts prodrugs etoposide or methylumbelliferone to the active form (Bosslet *et al.*, 1991 (a)).

The enzymes used in ADEPT are usually not human in origin. This has been shown to cause some problems, especially with increased immunogenicity in vivo (Bagshawe, 1989). Other enzyme systems used include alkaline phosphatase, glucouronide oxidase and phospholipase C (reviewed by Di Maggio *et al.*, 1990) while ifosfamide (IFO) (Ceny *et al.*, 1990) and tetrachloro-(d,1-trans)-1,2-diaminocyclohexaneplatium (IV) (tetraplatin) (Chaney *et al.*, 1991) have recently been proposed as prodrugs which may be of use in cancer treatment.

#### 1.10.3. Further Two-Stage Techniques.

Paganelli et al. (1991), have recently reviewed a series of two-stage or pretargeting techniques,

including:

(a) The Avidin-Biotin System:

Avidins are a family of proteins which have both a high affinity (KD ~  $10^{15}$  M) and specificity for biotin. Each avidin molecule (or streptavidin molecule) can bind 4 biotin residues. This reaction is used to <u>amplify</u> mab reactions both <u>in vitro</u> and <u>in vivo</u> (Section 2.4.3.). Various combinations of mab-biotin and avidin-labelled drugs, toxins and radiolabels have been proposed (Britton *et al.*, 1991(a)). One such two-step immunotherapy procedure has been proposed by Paganelli *et al* (1991).

STEP 1: Mab-biotin antibodies are administered intravenously.

STEP 2: Radiolabelled avidin or strepavidin is injected into the peritoneal cavity of the patient after 2 to 3 days and the resulting mab-biotin-avidin-radiolabel complexes may be visualised.

The use of mab-avidin-biotin immunotherapy will be discussed in more detail in Section 5.5.5.

(b) Bifunctional Antibodies:

A number of two-stage therapies involving BFAs with affinities for both tumour/long-lived radionuclides (Bosslet *et al.*, 1991(b); Britton *et al.*, 1991(a)) and for the iodinated-Bolton-Hunter reagent (Bator and Reading, 1990) will be discussed in more detail in Section 4.4.5.

To date, few clinical studies have been carried out using two-stage immunotherapy strategies. The more conventional one-stage immunotherapy procedures have resulted in significant toxicity and damage to non-tumour cells. These two-stage strategies do not eliminate these non-tumour effects, but they may *minimise* the unfavourable effects of immunotherapy.

Unconjugated antibodies are often forgotten in the race to find effective immunoconjugates. These mabs can play a vital role in vivo by stimulating and manipulating the immune system by mimicing a patient's own antibodies. Unconjugated mabs can achieve an anti-tumour response by five different mechanisms, (reviewed by Mellstedt, 1990). Each mechanism will be described in more detail.

#### 1.11.1 Complement-Dependent Cytotoxicity (CDC).

An activated terminal complement complex can attack a cell membrane and induce lysis and destruction of target cells. Mabs have been used to activate the cascade that causes the production of the complement complex. In this system, the Fc portion of the mab (Figure 1.2.) which contains the Clq binding site of complement cascade mediates the activation of some components of complement. Murine mabs have been less effective than human mabs in mediating complement reactions (Dillman, 1987). Human IgM is the most efficient complement-binding antibody, followed by IgG1, IgG3 and IgG2a. Intact mabs (including the Fc portion) are needed for CDC. Human IgM antibodies (which are of limited use in immunoconjugate production), have been particularly useful in inducing the regression of metastatic melanoma, following the administration of a complement-fixing IgG3 mab against the GD3 ganglioside antigen (Vadham-Raj *et al.*, 1988).

#### 1,11.2. Antibody-Dependent-Cell-Medicated-Cytotoxicity (ADCC).

A wide range of cells called effector cells have been shown to produce cytotoxic reaction when activated by antibodies. These effector cells include killer (K) cells, macrophages, NK cells, neutrophils and even non-immune cells such as platelets (reviewed by Di Maggio *et al.*, 1990). Each of these cell types expresses a receptor for the Fc portion of the intact mab, (Fc R). In circulating mabs, the Fc portion of the mab is not accessible to the Fc R receptors on effector cells, however, when the mab binds to the target antigen, the Fc R binds the Fc region and effector cells are brought to the membrane of the tumour cell to cause cell lysis. This process has been shown to be stimulated in vivo when biological response modifiers such as IL-2,  $\alpha$ -interferon and M-CSF are administered to the patient (Masucci *et al.*, 1989; Tang *et al.*, 1989). BFAs are particularly useful in replacing the role of the Fc regions in bridging different cells and can bind tumour directly to the effector cell. Fanger *et al.* (1991) have recently reviewed this area in more detail. The use of BFA fragments, without the Fc portion may also reduce the immunogenicity of current-intact IgG ADCC treatments. Fanger and his colleagues also described the production of a CD4 - Fc fusion protein which binds to HIV infected cells expressing the gp-120 antigen and thereby mediates ADCC.

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#### 1.11.3 Interaction with Receptors on Malignant Cells.

Receptors such as platelet-derived growth factor (PDGF) receptors, epidermal growth factor receptor (EGF), transferrin receptor and several oncogene-related receptors are known to cause the proliferation of target cells (reviewed by Thurin, 1990). Antibodies such as anti-TAC (Waldmann *et al.*, 1988), and anti-ECF, (Mendelsohn, 1990), have been used to block these receptors and to retard the proliferation of their target cells.

#### 1.11.4. Anti-Idiotypic Antibodies.

When antibodies are repeatedly injected into a patient, the patient develops an immune response against the antibody, and anti-idiotypic (or anti-antibody) antibodies are produced. When this anti-idiotypic antibody (anti-id ab) response is against the <u>variable</u> region of the injected antibody, the anti-id ab is said to be the mirror-image of the antigen. These anti-id ab can then be used to mount a <u>second</u> antiidiotypic antibody response (anti-id<sup>2</sup>), against the mirror antigen. These anti-id<sup>2</sup> antibodies can crossreact with antigen on a patient's tumour and can act as an anti-idiotypic vaccine. Anti-id<sup>2</sup> antibodies reacting with the antigens HMFG-2 and HMFG-1 have been used to treat patients with ovarian carcinoma by Courtenay-Luck *et al.*, (1988) and they showed that patients with low volume, residual cancers were particularly sensitive to treatment by anti-id<sup>2</sup> therapy.

#### 1.11.5. Non-Specific-Effector Cell Activation.

Mellstedt (1990) has argued that non-specific activation of effector cells by mabs has a pleiotrophic effect on cell regulation. Mab immunotherapy has been reported to stimulate apoptosis, or programmed cell death, of tumour cells (Trauth *et al.*, 1989). As more is learned about cytokines/lymphokines and apoptosis, the potential role of mabs in this process will become clearer.

In the 1970s, mabs were produced by Köhler and Milstein, and in the 1980s, chimaeric and humanised monoclonal antibodies became available. The 1990s is the era of immuno-genetics. Recombinant mabs, BFAs and fusion proteins are becoming readily available. Epitope mapping technology (Holsford *et al.*, 1991) will help define better antibodies to more defined targets. The use of two-stage immunotherapy, and strategies to overcome HAMA and HAHA reactions will be investigated and may have major clinical implications. Above all, it is important to decide which types of tumours are suitable for immunotherapy and immunoscintigraphy and to use the combined advances in immunology, genetics and nuclear medicine to find effective routine clinical uses for mabs.

The future of diagnostic and therapeutic monoclonal antibodies has been aptly summarised by Britton *et al.* (1991):

'Provided the regulatory authorities do not do permanent damage to this approach to cancer therapy, the bullet may yet perform its magic'.

# SECTION 2 : MATERIALS AND METHODS

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Reagents used in experimental work were of analytical grade and were obtained from Sigma Chemical Co., Poole, Dorset, England; BDH Chemicals Ltd., Poole, Dorset, England and Riedel de Häen, AG, Seelze, Hannover, Germany. Cell culture media, supplements, sera and disposable plastic-ware were supplied by Flow Laboratories, Woodcock Hill, Harefield Road, Hertz, WD3 1PQ, U.K. Stains used for cytogenetic and histological analysis were obtained from BDH Chemicals Ltd., Poole, Dorset, England. A number of specific reagents, which vary from supplier to supplier are listed in Table 2.1.1.

Several experimental kits were also used for diagnostic and analytical purposes; these are summarised in Table 2.1.2.

The animal cell lines used are summarised in Table 2.1.3, while the antibodies used in immunocytochemistry, ELISA and FACS analysis are listed in Table 2.1.4.

Reagent	Source	Address	
BriClone	Dr. Kenneth Carroll	BioResearch Ireland,	
Coumarin	Kodak Eastman	Rochester, New York, U.S.A.	
Dimethylsulphoxide (Sure Seal)	Aldrich Chemical Co.	Gillingham, Dorset, U.K.	
Hoechst 33258	Calbiochem	Behring Diagnostics, La Jolla, CA 92037, U.S.A.	
Isoton II	Coulter Electronics	Northwell Drive, Luton, LU3 3RH, U.K.	
Indium-Chloride	Mallinckrodt Diagnostica DV	Petten, Holland.	
Polyethylene Glycol 1500	Boehringer Mannheim	Hannover, Germany.	
Taab 812 Resin	Taab Laboratory Equipment Ltd.	3 Minerva House, Aldermaston, Berks, RG7 4QN, U.K.	
Technetium	Mallinckrodt Diagnostics DV	Petten, Holland.	
Water for Injection Water for Irrigation (Sterile)	Boots Hospital Products	Same Rd., Nottingham, NG2 3AA, U.K.	

### Table 2.1.1. Sources of Reagents

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Name	Source	Address
Amerscan Medronate II Kit (MDP Bone Kit)	Amersham International	Amersham, Bucks., HP7 9NA, U.K.
Authentikit (Isoenzyme Dialysis Kit)	Corning Laboratories	Palo Alto, CA 94306, U.S.A.
Bicinchonic Acid (BCA Protein Assay Kit) Coomassie Protein Assay Reagent	Pierce Chemical Co.	Rockford, Illinois, 61105, U.S.A.
CoATest (Endotoxin Kit)	KabiVitrum Ltd.	KabiVitrum House, Riverside Way, Middlesex, UB8 2YF, U.K.
Vectastain (ABC Staining Kit)	Vector Laboratories	30 Ingold Street, Burlingam, CA 94010, U.S.A.

# Table 2.1.2. Summary of Diagnostic Kits Used

Cell		Cat.	
Line	Cell Туре	Number	Supplier
J82	Human, Bladder Carcinoma	НТВ 1	ATCC,
MCF-7	Human, Breast Adenocarcinoma	НТВ 22	12301 Park
LOVO	Human, Colon Adenocarcinoma	CCL 229	Lawn Drive,
A431	Human, Epidermal Carcinoma	CRL 1555	Rockville,
NRK	Rat, Normal Kidney	CRL 6509	Maryland,
			20861, USA.
RT112/84	Human, Bladder Carcinoma	85061106	ECACC,
EJ-138	Human, Bladder Carcinoma	8511412	PHLS,
NSO	Mouse, Balb/c Plasmacytoma	84112004	Porton Down,
RPMI-8226	Human, Myeloma	CCL 155	Salisbury,
			Wiltshire,
			SP4 0JG,
			U.K.
T24	Human, Bladder	-	Dr. J. Tager,
ĺ			University of
			Amsterdam,
			1105 AZ,
			Amsterdam,
			Netherlands.
LAT	Mouse, Landschütz ascites	÷.	From DCU
	tumour cells		stocks.

# Table 2.1.4. Monoclonal Antibodies used in

Antigen	Label	Dilution	Code	Supplier
Mouse anti-CD-38	•	1/20	MCA 376	Serotec, 22 Bankside, Oxford, UK.
Mouse anti-EBV	•	1/20	ROE176	Seralab, Crawley Down, Sussex, RH104FF.
Sheep anti-human Igκ light chain Sheep anti-human Igλ light chain		1/200	PC015	The Binding Site 97 Vincent Dr., Edgbaston, Birmingham, B15 25Q, UK.
Goat anti-mouse Ig	HRP	1/500	083-67	ATAB, PO Box 60, Scarborough, MG 04074, USA.
Goat anti-mouse Ig		-	M-8642	Sigma Chemical Co., Poole, Dorset, UK <u>.</u>
Mouse anti-human HLA-DR	-	-	<b>M</b> 704	Dakopatts, Denmark <u>.</u>
Mouse anti-human IgG Mouse anti-human CD19 Mouse anti-human CD25 Goat anti-mouse F(ab') <sub>2</sub> Goat anti-sheep F(ab') <sub>2</sub> Swine anti-goat F(ab') <sub>2</sub>	- FITC - FITC FITC FITC	- - 1/50 1/50 1/50	5140 M0524 7640 M0848 P1604 P2170	Becton Dickinson, 2350 Quem Dr., San José, CA 95131-1812, USA.
Sheep anti-mouse IgG (F(ab') <sub>2</sub> )	β-gal.	1/1000	NA 8310	Amersham Int., Amersham Place, Little Chalfont, Bucks. UK.

### Immunocytochemistry, ELISA and FACS Analysis

A Nikon type 104 microsocpe was used for fluorescence and phase contrast microscopy. Cells in culture were examined using a Nikon type ELWD 0.3 inverted phase contrast microscope. Photographs were taken with Nikon F-301 and Nikon FX-35WA cameras. A Nikon HFX-IIA automatic exposure unit was used to calculate exposure.

Electron microscopy studies were performed using an Hitachi H-600 electron microscope.

A model DN coulter counter was supplied by Coulter Electronics and was used to enumerate cells in growth rate studies.

Slides of cells in suspension were prepared using a Shandon Programmable Cytospin Unit, with disposable inserts used for each slide prepared.

Fluorescence Activated Cell Sorting was undertaken using Becton Dickinson FacScan, using a Consort 10 Software System.

Electroporation of myeloma cell lines was carried out on a ProGenetor Pulse Controller from Hoefer Scientific Instruments, which was fitted with a Gene Pulse Cuvette Unit (Bio-Rad).

Ascites tumours, isolated from the peritoneum of Schofield mice, were dried using a High Temperature Vacuum Oven, Furkamp, D.V.

A Hereaus Christ Labofuge 600 and Labofuge GC were used to centrifuge universal tubes (1 - 20 mls).

A Hereaus Biofuge A was used for smaller volumes (0.5 - 1.5 mls).

A Hetosicc CD-52 freeze drier was used to lyophilise both HPD and HMFG-1-Hp conjugates.

A LKB 2050 Midget Electrophoresis Unit was used in the preparation of SDS-PAGE gels.

Patients were imaged following immunoscintigraphy with a General Electrics GE 400 AC Gamma Camera. Low levels of gamma isotopes were detected using a Nuclear Enterprises NE Scalar-Ratemeter.

An Applied Photophysics Xenon Arc Lamp and a Coherent Light Meter were used for irradiation in the photoradiation therapy studies reported.

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#### 2.3.1. Routine Culture of Cells in Suspension

NSO, C23/11, RPMI-8226, HET-37, Sp2/0 and LAT cells were maintained in RPMI.S<sub>10</sub> [RPMI-1640 medium (Flow) supplemented with L-glutamine (2mM) Hepes (1mM) and 10% (v/v) FCS]. Cultures were initiated at cell densities of 2 x 10<sup>5</sup> cells/ml using 12 ml of medium per 75 cm<sup>2</sup> culture flask. Cells were harvested when ~75% confluent by flushing them from culture surfaces using a sterile pasteur pipette. All animals cells in culture were incubated in a humid, 5% CO<sub>2</sub> atmosphere at 37°C.

#### 2.3.2. Routine Cell Culture of Adherent Cells

A431, LOVO, EJ-138 and T24 cells were maintained in DMEM-S<sub>10</sub> (DMEM medium supplemented with L-glutamine (2mM), Hepes (1mM) and FCS 10% (v/v)). Media for the MCF-7 breast adenocarcinoma cell line was further supplemented with sodium pyruvate (1mM). The A431, LOVO and EJ-138 cell lines are strongly adherent and required trypsinization to release them during harvesting and subculturing procedures. All of the culture medium was decanted and 4 ml of trypsin:EDTA solution (Flow Laboratories; 0.25% (w/v) trypsin with 0.02% (w/v) EDTA in PBS) was added to the flasks. After 1 minute, a further 3 - 4 ml of trypsin:EDTA solution was added and incubated at 37°C for 10 minutes. The cell suspension was decanted into 5 - 10 ml of complete culture medium to inactivate the trypsin and centrifuged at 1,200 rpm for 10 minutes in universal tubes. Cells were resuspended in 36 ml of culture medium and divided into 3 x 75 cm<sup>2</sup> sterile flasks. The MCF-7 cell line does not adhere to plastic as strongly as the above cell lines and, therefore, requires a more dilute trypsin solution (0.05 (w/v) trypsin; 0.02% (w/v) EDTA in PBS) to minimise surface antigen loses (Sasaki, *et al.*, 1981).

#### 2.3.3. Cell Counts and Viability Staining

Cell counts were performed on an improved Neubauer Hemocytometer slide. Acridine orange/ethidium bromide and/or trypan blue were used routinely to determine cell viabilities (Mishell *et al.*, 1980). The acridine orange/ethidium bromide stain solution contained 0.1 mg acridine orange and 0.1 mg ethidium bromide in 100 mls PBS. One volume of cells was added to an equal volume of stain and examined by UV fluorescence microscopy. Live cells stain green while dead cells stain orange. For trypan blue staining, one volume of cells was

2.3.

added to an equal volume of stain (0.2% (w/v) [Sigma]), left for one minute and examined within five minutes by light microscopy. Longer incubation periods with stains leads to non-specific uptake of dye and inacurate results. Live cells excluded the dye and remained white while dead cells stained blue.

#### 2.3.4. Long Term Storage and Recovery of Animal Cells

Cryopreservation methods described by Reid and Cour (1985) and Price (1985) were used. Stocks of cells were maintained in liquid-nitrogen freezers. Washed cells were resuspended to the desired concentration (usually 1 x  $10^6$ /ml) in FCS supplemented with 5% (v/v) dimethylsulphoxide (DMSO; cryoprotectorant). One ml aliquots were placed in sterile cryotubes (Flow) and frozen at a rate of approximately -1°C per minute to -70°C on a freezing tray (Union Carbide) before being immersed in liquid nitrogen. Recovery of cells from liquid nitrogen was achieved by thawing the cells rapidly to 37°C and then washing them twice in medium, or PBS, as appropriate. Cells recovered from liquid nitrogen were approximately 90% viabile, with the exception of C23/11 cells which retained approximately 80% overall viability.

#### 2.3.5. Mycoplasma Detection - Hoechst 33258 Fluorescence Assay

The DNA-intercalating agent bisbenzimid (Hoechst 33258) was employed to detect the presence of mycoplasma in cellular cytoplasm. The detection mechanism used was further described by Carroll et al. (1988). Cultures of mycoplasma-free NRK cells were established on glass coverslips using 5 x  $10^3$  cells in 1 ml of DMEM containing 6% (v/v) FCS and cultured overnight. Supernatant from cultures to be tested was then added to the NRK cells, which were reincubated for a further 3 to 4 days or until the cells were approximately 70% confluent. Duplicate NRK cultures were fed with fresh media and used as a negative control. Positive controls, previously prepared and stored at 4°C were included. The coverslips were then washed three times in PBS (taking care to keep the cells on the uppermost surface at all times) and fixed for six minutes in 1:1 methanol:acetone solution at -20°C. The coverslip preparations were then rinsed three times in PBS and stained for 10 minutes in Hoechst 33258 (0.05 µg/ml in PBS). The stain was washed from the cells and the coverslips air-dried and mounted on clean slides. Slides were stored in the dark until viewed under UV light on a Nikon fluorescence microscope at 100 X magnification with a B2 combination filter (Nikon). The membranes of contaminated NRK cells had areas of fluorescence DNA which could be associated with mycoplasma, while no such fluorescence was found in the negative and control slides.

#### 2.3.6. Cloning of Suspension Cells by Limited Dilution

Suspension cultures in exponential growth phase were cloned out by limiting dilution in 96 well plates in order to ensure monoclonality of cells. Prior to cell cloning, 96 well plates were seeded with 100 µl of culture medium supplemented with 10% (v/v) FCS and 5% (v/v) BriClone. Cells were removed from culture flasks, 24 well plates or 6 well plates and adjusted to a concentration of 4 x  $10^3$  cells/ml. Using an octapipette (Costar), 100µl of cell suspension (approximately 400 cells) was added to each of the 8 wells in Row A of the 96 well plate. The contents of each well of Row A were mixed thoroughly and 100 µl transferred to each well of Row B (approximately 200 cells). This dilution procedure was continued across the microtitre plate until eventually a row of wells existed containing approximately one cell/well. The 96 well plates were incubated for 5-6 days and then examined microscopically for clone formation. Wells containing one clone per well were allowed to grow to confluence, expanded, and the cloning procedure was repeated to ensure monoclonality. Eventually, following sequential cloning by limited dilution the cells were expanded into 25 cm<sup>2</sup> flasks and frozen in liquid nitrogen (Section 2.3.4.).

#### 2.3.7. Determination of the Growth Rate of Cells Grown in Suspension

Cells were subcultured (Section 2.3.1.) the day prior to growth rate determination to ensure exponential growth. Four mls of RPMI- $S_{10}$  was placed in each well of a 6 well plate to which 1 ml of a 1 x 10<sup>5</sup> cell/ml suspension was added.

Following a 24 hour incubation period, the contents of duplicate wells were harvested, resuspended in 0.5 mls of tissue culture medium and added to 19.5 ml of Isoton II (Coulter Electronics). Cells were subsequently counted in a coulter counter (Model DN, Coulter Elec.). This procedure was repeated at 24 hour intervals, over a period of 5-7 days and the doubling time was determined. All determinations were carried out in duplicate.

#### 2.4. ESTABLISHMENT AND CHARACTERISATION OF C23/11 & HET-37

#### 2.4.1, Primary Cell Culture of Myeloma Cell Lines.

White blood cells were isolated from human peripheral whole blood by differential centrifugation as described by Alley and MacDermott, (1980). Heparinized whole blood and bone marrow aspirates were collected from patients with myeloma. The heparinized blood was diluted 1:1 with PBS while the bone marrow aspirates were diluted in RPMI-1640 containing 10% (y/y) FCS (RPMI-S<sub>10</sub>). Two parts of the cell rich sample were then layered over one part of lymphoprep (Ficoll-Metrizoate;  $\rho = 1.077$  g/ml) in a sterile universal tube and centrifuged at 1,000 rpm for 30 minutes at room temperature in a swinging bucket rotor (Heraeus Christ Labofuge 600). The blood cells separated into two distinct fractions: (a) red blood cells, which form a pellet; and (b) the white cells which form a "buffy layer" between the lymphoprep and the plasma. The white cells were carefully withdrawn using a sterile pasteur pipette, avoiding the unnecessary uptake of Ficoll-Metrizoate. Cells were washed three times in RPMI- $S_{10}$  and subsequently resuspended in 6 mls of  $RPMI-S_{10}$ . Cells were fed at three day intervals according to the method described in Section 2.1.1, with the exception that these cells were resuspended in 3 mls of fresh RPMI-1640 and 3 mls of supernatant from the previous passage. In this way, growth factors and metabolic intermediates were conserved in the medium. Primary myeloma samples were maintained for 6-8 weeks and were then discarded if cell growth was not observed.

#### 2.4.2. Histological Staining of Myeloma Cells

Haematoxylin and Eosin (H/E) and Giemsa stains were used to examine the cellular and nuclear morphology of myeloma cells. Slides of C23/11, NSO and HET-37 cells in suspension were prepared using a Shandon Programmable Cytospin. Slides were then stored at -20°C and thawed for 30 minutes prior to staining. Cytospin preparations were fixed in 95% (v/v) alcohol for one minute and then washed thoroughly with tap water. When using the H/E stain, cell nuclei were stained blue with hematoxylin (0.15% (w/v); Gurr, BDH) for one minute, washed in tap water, and the cytoplasm was counterstained with eosin (0.1% (w/v) [in distilled water], BDH). The stained cells were subsequently dehydrated in 95% (v/v) alcohol followed by 100% alcohol and finally cleared in xylene (BDH) before mounting in DPX (BDH). Alternatively, cells were stained with Giemsa (5% (v/v)), washed in tap water and then dehydrated and mounted as outlined above.

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#### 2.4.3. Immunocytochemical Analysis of Myeloma Cells

Air-dried cytospin preparations of C23/11, NSO and HET 37 were prepared using a Shandon Programmable Cytospin. Cells were tested for the presence of human kappa ( $\kappa$ ) and lambda  $(\lambda)$  light chains using the avidin-biotin, ABC Vectastain Kit System (Vector Laboratories). All blocking sera, Avidin DH, biotinylated Horseradish peroxidase and the biotinylated goat antimouse antibody were supplied in kit form. Slides were blocked with 100 µl diluted goat serum for 20 minutes and the excess was removed. Slides were then stained with primary antibody (anti-human kappa chain antibody or anti-human lambda chain antibody) for 30 minutes at room temperature and then washed for 10 minutes in PBS. Slides were then incubated with the biotinylated anti-mouse antibody (100µl) for 30 minutes, washed again in PBS and incubated with Avidin-DHO; biotinylated Horseradish peroxidase, (HRP solution) which contained excess avidin for 30 minutes. This allowed binding between the antibody containing biotin and the unreacted biotin sites on the avidin. Following a 10 minute wash period the bound HRP was visualised by a 5 minute incubation with substrate (1:1 solution of 0.02%  $H_2O_2$ : 0.1% diaminobenzidine tetrahydrochloride (DAB) in 0.1 M Tris buffer, pH 7.2). The reaction was stopped by washing the slide in water and the cells were counterstained with 0.3% (w/v) methyl green (BDH) for one minute. Cells were then washed in PBS, dehydrated in 95% and 100% alcohol, cleared in xylene and mounted in DPX.

#### 2.4.4. Electron Microscopy of Cell Lines in Cell Culture

Pelleted cells (Section 2.3.1.) were preserved according to the method of McDowell and Trump (1976). Cells were then washed three times in 0.1 M phosphate buffer (pH 7.2) post-fixed in 1% (v/v) osmium tetroxide (BDH) for two hours and stained in 2% aqueous uranyl acetate for one hour. Dehydration was completed by serial incubations of 30 minutes in 50%, 70%, 95%, 100% alcohol and 100% ethanol. The dehydration process was continued, by incubation in propylene oxide (BDH) for 20 minutes and propylene oxide/Taab 812 resin (50:50) for 60 minutes at room temperature. The cell pellet was then incubated in 100% resin for 24 hours at 60°C. Ultra-thin sections were then examined with an Hitachi H-600 Electron microscope.

#### 2.4.5. Isoenzyme Analysis of Cell Lines

The electrophoretic isoenzyme profiles of a cell line have been used to establish the species of origin of lines and have been used for comparison of inter and intraspecies variations which exist between different cell lines (Halton *et al.*, 1983). Isoenzyme analysis was carried out using an "Authentikit" (Corning) system; all buffers, agarose gels, substrates, stain and

electrophoresis apparatus were supplied in kit form. Cell lysates were prepared from cultures containing > 3 x 10<sup>6</sup> cells/ml in PBS. Samples of 1.2 mls of this suspension were centrifuged at 3,000 rpm for 5 minutes and the resulting pellet was resuspended in 300 µl PBS. Cells were further disrupted by three freeze-thaw cycles of  $-20^{\circ}$ C to room temperature, followed by centrifugation (Hereaus Labofuge) at 5,000 rpm for 5 minutes. The cell lysate was stored at  $-20^{\circ}$ C. The lysate was separated by agarose gel electrophoresis. This was carried out by loading 1.0 µl of control lysate or sample lysate specimens into the appropriate pre-cut sample well. The loaded agarose film was placed in the cassette base with Universal barbital buffer (pH 8.6). Separation was achieved using 90 V for 35 minutes from a LKB power supply unit. The resulting isoenzyme profiles were stained using the appropriate substrate linked to a formazine dye and migration distances were calculated. The kit contains formazine dyes for the detection of seven different isoenzymes; purine Nucleoside Phosphorylase, (NP), Glucose-6-phosphate dehydrogenase (G-6-P), Malate dehydrogenase (MDH), Mannose phosphate isomerase (MPI), Peptidase B (PB), Aspartate aminotransferase (AST) and Lactate dehydrogenase (LDH).

#### 2.4.6. Determination of Surface Antigens on Cell Lines using FACS Analysis

The presence of antigens on the surface of myeloma and heteromyeloma cell lines and the effect of conjugation procedures on the immunoreactivity of monoclonal antibodies was determined by fluorescence activated cell sorting. A 5 x 10<sup>6</sup> cell/ml suspension was prepared in PBS containing 1% (w/v) BSA and 0.1%( w/v) sodium azide and placed at 4°C. Cell suspension (200 µl) was pipetted into each Falcon FACS analysis tube to which 5 µl undiluted antibody (or an appropriate volume of dilute antibody) was added. Antibody binding took place over 10 minutes at 4°C to prevent internalisation of the antibody. The cells were then washed twice in cold PBS containing BSA and sodium azide (as above). While some of the antibodies used in FACS were pre-labelled with FITC (Table 2.1.4.), other antibodies required a second FITC-linked antibody to visualise the non-FITC labelled primary antibody. Fluorescein-conjugated F(ab')<sub>2</sub> fragment of rabbit immunoglobulin was pipetted into sample and control tubes, incubated at 4°C for 30 minutes, washed twice, resuspended in 500 µl of filtered 1% (v/v) paraformaldehyde and stored at 4°C for up to seven days. Analysis was performed using a Becton Dickenson FACScan, with Consort 10 software.

#### 2.4.7. Generation of G-418 Resistant Cell Lines

A subclone of the myeloma cell line which was resistant to the antibiotic geneticin (G-418), was generated by insertion of the pSV2 *neo* plasmid containing the gene for G-418 resistance

into the cell line by electroporation. C23/11 cells were electroporated using a variation on the protocol outlined by Winterbourne *et al.* (1988). C23/11 cells (100 µl) at a concentration of 1 x 10<sup>6</sup> cells/ml were placed in a gene pulser cuvette (Bio-Rad) to which 200 µl of 1mM Tris, 0.1mM EDTA, pH 8.0, was added. Plasmid DNA, pSV2 *neo* was (20 µg/ml) was added and the suspension was incubated at 4°C for 10 minutes. Electroporation was achieved using a capacitance of 25 µFD and a constant voltage (0.75 V) in a ProGenetor pulse controller (Hoefer Scientific Instruments). Cells which had successfully acquired the G-418 resistance gene were selected by their ability to grow in medium supplemented with 200 - 1000 µg/ml geneticin (Sigma). Continued resistance to G-418 was maintained by the subsequent culturing in selective media containing 200 µg/ml geneticin.

#### 2.4.8. Heteromyeloma Cell Production by PEG Mediated Cell Fusion

NSO and C23/11 cells were maintained in mid-log phase of growth by frequent subculturing for seven days prior to fusion. The NSO cell line was pre-tested for HAT sensitivity by proving their inability to grow in RPMI-S<sub>10</sub> containing HAT (100 µM hypoxanthane, 1µM aminopterin and 60 µM thymidine). Similarly, C23/11 cells were pretested for their inability to grow in RPMI-S<sub>10</sub> containing 1 x 10<sup>5</sup>M ouabain. Both NSO and C23/11 were tested for the presence of mycoplasma (Section 2.3.5.). Before fusion C23/11 cells were pretreated with neuraminidase (Sigma) at a concentration of 1 unit/ml in RPMI-S<sub>10</sub> for 45 minutes at 37°C and washed three times in RPMI before use (Igarashi and Bando, 1990). The pretreated C23/11 cells were combined with the harvested NSO cells in sterile universal containers. This cell mixture was then washed twice in RPMI medium without FCS. Cells were centrifuged at 4,000 rpm for 10 minutes and the supernatant was fully decanted. The cells were fused in PEG-1500 (Boehringer Mannheim). One ml of the PEG solution was added to the pelleted cells over one minute with gentle mixing. This suspension was left for a further minute while the container was rotated with a continual gentle swirling motion. The PEG was then slowly diluted by the dropwise addition of 3 mls of RPMI (serum-free) over three minutes, followed by 8 mls of medium over a further 10 minutes. The cells were pelleted and finally resuspended at a concentration of  $2 \times 10^{5}$  cells/ml of RPMI-S<sub>10</sub> supplemented with 5% (v/v) BriClone. One ml of this suspension was added to each well of a 24 well plate and incubated at 37°C in 5% CO<sub>2</sub>. After 24 hours, one ml of RPMI-S<sub>10</sub> (supplemented with 5% BriClone, 2 x HAT (Flow) and 2 x 10<sup>5</sup>M ouabain) was added to each well. Cells were then left undisturbed for seven days and subsequently fed in RPMI-S10 containing HAT and 1 x 10<sup>-5</sup>M ouabain. Clones developed after 3-4 weeks. The HAT medium was replaced by HT medium after 4 weeks. After 6 weeks the cells were cultured and expanded in RPMI-S<sub>10</sub> without HAT, HT or ouabain.

#### 2.4.9. Chromosome Analysis Studies

Several methods have been investigated to differentiate between human and mouse chromosomes (Schroder, 1985). G-11 banding and Hoechst 33258 procedures were used. The Hoechst 33258 procedure gave the most reproducible stains and an adaption of the procedure developed by Kozak *et al*, (1977) was used. On the day prior to chromosome spreading cells were subcultured with 8-10 mls of fresh medium in order to promote maximum growth. Metaphase was induced by the addition of 0.5 ml of colcemid (0.1 µg/ml) overnight at 37°C. Following cell harvesting and centrifugation, cells were incubated for 15 minutes in a hypotonic solution of 0.075 M KCL at 37°C. The enlarged cells were fixed by the gentle addition of fresh fixative (methanol glacial: acetic acid, 3:1, at 4°C) for incubation periods of one hour, 5 minutes and one minute. The final pelleted cells were prepared for light microscopy by staining in 5% (v/v) Giemsa (BDH) or Hoechst 33258 staining. The fluorescent intensity of the chromosomes was assessed by staining spreads in a freshly prepared solution of 0.04 µg/ml of Hoechst 33258 stain in saline for 10 minutes. Following rinsing in distilled water and mounting in glycerol, slides were examined using a Nikon Type 104 fluorescence microscope with a UV combination filter system.

#### 2.4.10. In Vivo Growth of Tumour in Nude Mice

In vivo growth of C23/11 myeloma cell line was investigated using the MF1-nu/nu/old/Hsd mouse strain. 10 week old female mice were maintained under 'near sterile' conditions in an Bio-Breed isolator unit. All food, water and bedding were sterilized at 121°C for 15 minutes prior to use. Tumours were established in vivo following injection of  $1 \times 10^7$  cells in 0.5 mls PBS. Animals were injected in either the interclavical or intraperitoneal regions. Animals were checked daily and maintained for 8 weeks or until the appearance of tumours. Tumours were removed aseptically and re-established in culture (Section 2.3.1.). Samples of these tumours were frozen and further samples embedded in parafin for H/E staining (Section 2.4.2).

#### 2.5.1. Antibodies Used in Immunoscintigraphy

Three therapeutic grade monoclonal antibodies were kindly donated by Unipath Ltd., Bedford, U.K. The HMFG-1 antibody was first described by Taylor-Papadimitriu *et al.* (1981). HMFG-1 is an IgG<sub>1</sub> immunoglobulin and it reacts primarily with human milk fat globulin-1.  $F(ab')_2$  fragments of HMFG-1 were also used in this study. The second antibody used was termed AUA-1, a murine IgG antibody which was first described by Arklie *et al.* (1980). The third antibody, H17E2, a mouse IgG1 against the placental alkaline phosphatase (PLAP) antigen which was first reported by McDicken *et al.* (1983).

#### 2.5.2. Sterile, Pyrogen and Metal Free Treatment of IgG & F(ab'), Fragments

The use of deionised water (injection grade, Boots), sterile and depyrogenated plastics and the depyrogenation of tubing, magnetic stirrers and gel filtration apparatus was essential, as these materials were for human use. Tubing and magnetic stirring bars were rendered metal-free by soaking overnight in 0.1 N nitric acid followed by extensive washing with water (injection grade) under sterile conditions. Ultrafiltration devices were kindly sterilised by gamma irradiation by Baxter (Castlebar) Ltd. The use of deionised water and the absence of metal or glass materials was necessary as DTPA will chelate any metal in the water prior to labelling with the appropriate metal radionuclide, thus resulting in poor specific labelling efficiency.

#### 2.5.3. Conjugation of DTPA to IgG and F(ab'), Fragments

A bifunctional chelator, diethyltriaminepentacetic acid (DTPA), was attached to IgG and used to chelate free Indium-III to the DTPA-IgG conjugate (Hnatowich *et al.*, 1983). Antibody was dialysed against HEPES buffer (0.05 M, pH 8.0) in the presence of free DTPA (approximately 100 µg). Dialysis tubing was boiled in 100 mM EDTA and rinsed in sterile water for injection. Sterilised dialysis clips and magnetic stirrers were used (Section 2.5.2.). Following dialysis, the antibody was concentrated to > 10 mg/ml by centrifugal ultrafiltration (Centrifugal Ultrafree, Millipore). DTPA was dissolved in anhydrous dimethyl sulphoxide, DMSO (Aldrich) at a concentration of 10 mg/ml, immediately prior to use. Sufficient DTPA was added to the antibody to give a molar ratio of 3.5:1, DTPA : IgG1 or 1.5:1, DTPA : F(ab')<sub>2</sub>. The DTPA was added slowly to the antibody, mixed for 30 seconds and left at room temperature for 15 minutes. A 100 µl sample was removed [i.e. in order to find the number of DTPA molecules which were bound to each mab (Section 2.3.5.)]. Separation of the DTPA-mab from free DTPA was achieved by gel filtration column (PD-10 column containing Sephadex G-25, Pharmacia) with 0.1 M sodium acetate (anhydrous) buffer, pH 5.5, under gravity filtration. 1 ml fractions were collected using an LKB Redirac 2112 Fraction Collector. Protein determination was carried out (Section 2.5.6.) and aliquots of 1-2 mg of antibody conjugate were filter sterilised with a 0.22 µm filter and stored at 4°C.

#### 2.5.4. Reduction of F(ab')<sub>2</sub> Fragments for Technetium-99M Labelling

Antibody  $F(ab')_2$  fragment was labelled with technetium (<sup>99M</sup>Tc) following the reduction of intrinsic disulphide bonds with the reductant 2-mercaptoethanol (2-ME). The reduced  $F(ab')_2$  was labelled with <sup>99M</sup>Tc in the presence of methylene diphosphonate (MDP), a weak competing ligand, as described in detail by Mather and Ellison (1990). One ml of  $F(ab')_2$  fragment (Section 2.3.1.) was concentrated by ultrafiltration to approximately 10 mg/ml in 0.9% sodium chloride for injection (Baxter). The  $F(ab')_2$  fragment was reduced by reaction with a molar excess of 2-mercaptoethanol to  $F(ab')_2$  of 1000:1 at room temperature for 30 minutes. It was then purified by gel filtration (PD-10 column, Pharmacia) using saline as the eluent.  $F(ab')_2$  was recovered in the void volume and was stored under sterile and pyrogenfree conditions. The radioactive labelling procedure was undertaken on the day of administration to the patient and will be described in Section 2.5.14.

### 2.5.5. Determination of the Labelling Efficiency of DTPA Coupled Monoclonal Antibodies Using Indium-III and Technetium-99m.

A thin layer chromatographic (TLC) technique was used to determine the number of DTPA molecules bound to each monoclonal antibody as a percentage of the total number of DTPA molecules initially added. This percentage is termed the labelling efficiency of DTPA per antibody. Using the sample of DTPA-IgG and free DTPA described in Section 2.3.3., 10 µl of sample was added to Indium-III which had been previously reacted with 5M sodium acetate, pH 5.0 (detailed in Section 2.5.15.). This was mixed thoroughly and left at room temperature for 15 minutes. Strips of TLC - DC cards (10 cm Riedel-de-Häen) were cut. A spot of water soluble ink was placed 0.5 cm from the bottom of the card to mark both the solvent front and the application line. Using a micropipette, 1 µl of the IgG bound and free DTPA mixtures were spotted onto the centre of the application line on the pre-marked card. All used radioactive materials were disposed of into a suitable waste container. An equal volume of free In-III was also spotted on the card as a control marker. The TLC solvent consisted of 10mM EDTA and 0.1M sodium acetate, pH 5.5, (1:1). The developed TLC plate was imaged
using a GE 400 AC Gamma Camera. The level of radioactivity recorded at the base and top of the card represent gamma emitter bound to DTPA-Ig and free DTPA, respectively, from which the labelling efficiency can be calculated. This procedure was also used to determine the gamma emitter labelling efficiency of  $F(ab')_2$  -DTPA - In<sup>III</sup> and  $F(ab')_2$  - <sup>99M</sup>Tc binding.

#### 2.5.6. Protein Concentration Determination

Two methods were used to determine the concentration of protein in a sample: a miniaturized Bradford Assay (Bradford, 1976) and a miniaturized Bicinchoninic Acid (BCA) Protein Assay (Smith *et al.*, 1985). The reagents for both assay systems were obtained in kit form from Pierce Chemical Co.

#### (a) Bradford Coomassie Protein Reagent Kit Procedure

A miniaturized version of the assay kit was developed in order to minimise the amount of sample required. The assay had the advantage of providing a rapid indication of the protein level. However, many of the samples to be analysed contained the bifunctional chelator, DTPA, which interferes with the accuracy of the assay by complexing in the acid environment. Aliquots of 10 µl of diluted standard (0.01 - 0.1 mg BSA) or unknown protein samples were pipetted into a 96 well plate (Flow) to which 200 µl of Pierce Protein Assay Reagent was added. The plate was mixed and the absorbance read immediately at 595 nm on a Titertek Twin Reader Plus (Flow).

#### (b) BCA Protein Assay Reagent Kit Procedure

In this assay Cu<sup>++</sup> combines with protein under alkaline conditions to form Cu<sup>+</sup> which reacts with BCA to give a coloured product. Two separate reagents are supplied: Reagent A, an alkaline bicarbonate buffer and Reagent B, 4% (w/v) copper sulphate solution. The BCA protein assay working reagent was made up by mixing 50 parts of Reagent A with 1 part of Reagent B, and was stable for one week at room temperature. Two different miniaturized protocols were followed. One used incubation at 37°C for 30 minutes for expected protein concentrations of  $0.02 \cdot 1.5$  mg/ml. The second method used incubation at 60°C for 30 minutes for expected protein concentrations in the range of 0.001 - 0.25 mg/ml. In both cases, a standard BSA stock solution of 2 mg/ml was prepared to give a range of suitable standards on dilution with PBS. Then 10 µl of each standard or unknown protein sample was pipetted into a well of a 96 well plate and 200 µl of BCA working reagent was added to each well. For control wells, 10 µl of diluent was used. The tubes were incubated at the selected temperature and time and absorbance readings taken at 562 nm on a Titertek TwinPlus Reader. All samples and standards were prepared in duplicate. The BCA gave more accurate protein readings than the Bradford Assay, as less interference was obtained from detergents and chelating agents. However, the assay system required incubation at pre-set temperatures and results were not as rapid as the Micro-Bradford Assay.

# 2.5.7. Immunoreactivity Testing of Radiolabelled IgG and F(ab')<sub>2</sub> Fragments Using the Lindmo Cell Binding Assay

Lindmo et al. (1984) described a method of assessing antibody reactivity following radiolabelling based on the amount of radioactivity bound to cells expressing the relevant antigen. A constant amount of radiolabelled antibody is incubated with serial doubling dilutions of antigen. The amount of radiolabelled antibody is chosen such that the antigen is always in excess, even at low dilution. MCF-7 cells were used to test the immunoreactivity of both intact HMFG-1 antibody and its F(ab')<sub>2</sub> fragment. A431 and LOVO cells were used to test AUA-1 and HI7E2. Dilutions of unlabelled antibody (250 µg/ml) and labelled antibody (30 - 50 ng/ml) were prepared in PBS containing 1% BSA (w/v). All procedures were carried out behind lead-perspex shielding and all waste was placed in a radioactive disposal unit. Cells were prepared at a concentration of 1 - 5 x  $10^6$  cells in 1% BSA (w/v) in PBS. Duplicate dilutions of neat, 1/2, 1/4, 1/8, 1/16 and 1/32 were prepared in 1% BSA (w/v) in PBS. Two extra vials of the neat cell suspension were also prepared as non-specific binding controls, to which 50 µl of unlabelled antibody was added and incubated for 30 minutes at 4°C. Following the incubation of the unlabelled control antibody, 250 µl of radiolabelled antibody was added to both the test and non-treated control tubes, placed at 4°C, mixed and incubated for 4 to 6 hours. Cells were subsequently centrifuged in a Biofuge A (Hereaus) for 5 minutes at 3,000 rpm, washed three times in PBS containing 1% BSA(w/v), and finally resuspended in 500 µl of PBS containing 1% BSA (w/v). The gamma ray emission at each cell dilution was counted by a NE Scalar-Ratemeter SR5. This procedure was adapted for use with technetium labelled F(ab')<sub>2</sub> fragments.

#### 2.5.8. Immunofluorescence Analysis of Antibody Conjugates

Pleural effusion and ascites fluid specimens were obtained from patients with ovarian carcinoma. Tumour samples were collected into 0.9% (w/v) sodium chloride and stored at 4°C. Cells were isolated from the suspension by centrifugation at 1,200 rpm for 10 minutes. These cells were then washed three times in PBS containing 0.02% (w/v) sodium azide. A portion of the tumour specimen was frozen according to the method outlined in Section 2.3.4. Approximately 4 x  $10^6$  cells were added in duplicate to ependorph tubes. Samples of ovarian tissue which had been previously shown either to express or not to express the antigen, were

included as positive and negative controls. 100 µl of antibody (0.25 mg/ml) was added to each tube and incubated at room temperature with gentle shaking. The same antibody was used for both immunofluorescence analysis and immunoscintigraphy of the tumour. The cells were then washed twice and incubated for 10 minutes with 100 µl fluorescein isothiocyanate (FITC) conjugated anti-mouse antibody (1/300 dilution, Dakopatts). Labelled cells were washed twice and resuspended in 90% glycerol. Cells were counted on a Neubauer haemocytometer and fluorescent cells were viewed on a Nikon Type 104 fluorescence microscope with B2 combination filter.

#### 2.5.9. Enzyme-Linked Immunosorbent Assay, ELISA

ELISA was used to determine whether or not the conjugation procedures used on antibodies had altered their ability to bind to target tumour cells in culture. MCF-7, A431 and LOVO cells were used to test the HMGF-1, AUA-1 and H17E2 antibodies, respectively. Cells were prepared in medium and 5 x 10<sup>3</sup> cells were dispensed into 96 well plates. Cells were then grown to approximately 75% confluency under aseptic conditions. The medium was then removed and the wells were washed with PBS containing 0.1% (v/v) Tween-20 (wash solution). Non-specific binding sites were blocked with 1% (w/v) BSA in PBS (100 µl/well) for one hour at 37°C. The plates were then washed and 50 µl of β-galactosidase-linked sheep anti-mouse Ig,  $F(ab')_2$  fragment (Amersham) was added and incubated at 37°C for one hour. After a thorough washing to remove unbound conjugate, 100 µl of substrate (ONPG; onitrophenyl-β-galactopyranoside) was added to each well. When sufficient colour had developed, the reaction was stopped by the addition of 50 µl of 1 M sodium carbonate; this step also enhances the colour in positive wells. The absorbance was recorded at 405 nm on a Titertek TwinPlus Microplate Reader (Flow).

#### 2.5.10. SDS-Polyacrylamide Gel Electrophoresis

Conjugated and unconjugated monoclonal antibodies and their fragments were subjected to sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) using modifications on the method of Laemmli (1970). Proteins were separated on a resolving gel containing 7.5% (w/v) acrylamide, 0.33% (w/v) bis-acrylamide, 0.75 M Tris, 0.2% (w/v) SDS, 0.1% (v/v) TEMED and 0.1% (w/v) ammonium persulphate, pH 8.8. A stacking gel containing 3.5% (w/v) acrylamide 0.08% /(w/v) fresh ammonium persulphate, pH 6.8, was used. The electrode buffer contained 0.025 M Tris, 0.192 M glycine and 0.1% (w/v) SDS in distilled water, pH 6.8. Samples for electrophoresis were resuspended in a buffer containing 0.062 M Tris, 2.0% (w/v) SDS, 10% (v/v) glycerol and 0.001% (w/v) bromophenol blue. 2-mercaptoethanol (0.5%

(v/v) was included in the sample buffer only when reducing conditions were required. The samples were prepared at a concentration of 0.1 mg/ml in the loading buffer and boiled for 3 minutes at 100°C before being applied to the gel (5 - 10 µl volumes were used). Electrophoresis was carried out at a constant current of 40 mA for about 30 minutes at 4°C using an LKB 2050 Midget Electrophoresis Unit. Following electrophoresis, the gels were fixed by immersion in a methanol:distilled water (1:2) solution containing 11.4% (w/v) trichloroacetic acid and 3.4% (w/v) sulphosalicylic acid, for one hour. They were then stained with 0.25% (w/v) Coomassie Brilliant blue R-250 in a 5:1:1 solution of methanol, acetic acid and distilled water (1:3:6).

#### 2.5.11. Sterility Testing of Immunoconjugates

The presence of bacterial contamination was monitored by the use of microbial tests outlined in the US Pharmacopoeia (reviewed by Brown, 1990). Test tubes containing 10mls of either fluid thioglycollate medium (TG:Oxoid) or Soyabean-Casein-Digest Medium (SCD:Oxoid) were inoculated in duplicate with 1 ml of test antibody which had been previously diluted in sterile water for injection. The tubes were incubated for 30 days at 30 - 35°C for TG medium and 20 - 25°C for those samples in SCD medium under aerobic conditions. Turbidity after 30 days indicated the presence of microbial contamination and the exclusion of the antibody from clinical use.

# 2.5.12. Quantitative Endotoxin Testing Using a Limulus Amoebocyte Lysate (LAL) Assay System

The CoAtest/Endotoxin kit (KabiVitrum) was used to detect the presence of bacterial pyrogens from gram negative bacteria. These pyrogens may lead to inflammatory and febrile reactions in patients after the conjugates are administered. This test must be carried out under sterile and pyrogen-free conditions. Glassware and aluminium foil were rendered pyrogen free by heating at 180°C for 4 hours. It is important to adhere exactly to the incubation times and temperatures in the assay in order to obtain an accurate result. Endotoxin standards containing between 1.0 - 12.5 pg/ml were prepared in sterile water for injection (Boots). Freeze-dried limulus amoebocyte lysate (LAL) was carefully reconstituted and incubated at room temperature for 15 minutes in order to rehydrate the enzymes present in the lysate and then placed in an ice bath. Standards and test samples (50 µl) were placed in a 96 well plate at 25°C for 3 - 5 minutes. LAL solution (50 µl) at 37°C was added to each well and the plate was incubated for exactly ten minutes. The substrate which converts the activated LAL into

a colourimetric product was added (100  $\mu$ l) and the reaction was stopped after three minutes by the addition of 4% (v/v) acetic acid and the absorbance at 405 nm was obtained using TwinPlus Plate Reader (Flow). The degree of colour produced was directly proportional to the concentration of endotoxin present in the sample.

#### 2.5.13. Labelling of DTPA Conjugated IgG and Indium-III

Sterile pyrogen free DTPA labelled monoclonal antibody (Section 2.3.3.) was brought to room temperature and added to Indium chloride (In-III) (Mallinckrodt Diagnostica). <sup>III</sup>InCl<sub>3</sub> had been activated by the addition of 5 µl 5M sodium actetate pH 5.0 per 1.0 ml <sup>III</sup>InCl<sub>3</sub> at room temperature for 15 minutes. The IgG-DTPA-<sup>III</sup>In solution was mixed and left at room temperature for 30 minutes before separation on a 10 ml sterile Sephadex G-25 gel filtration column (PD-10, Pharmacia). IgG-DTPA-In-III fractions were eluted in 0.9% sodium chloride containing 1% human serum albumen (HSA:Albumar). 1 ml fractions were collected and their radioactive counts determined. Radiolabelled antibody fractions were combined, filter sterilized through a 0.22 µM filter (Flow) and diluted to 50 mls in 0.9% sodium chloride, which contained 1% HSA for subsequent administration. Thin layer chromatography was used to confirm antibody binding (Section 2.3.5.).

#### 2.5.14. Labelling of Reduced F(ab')<sub>2</sub> Fragments with Technetium

On the day of administration, one vial (1 mg) of 2-mercaptoethanol- reduced  $F(ab')_2$  fragments was thawed to room temperature (Section 2.3.4.). The fragment was labelled with <sup>99M</sup>technetium (<sup>99M</sup>Tc) using a methylene diphosphate (MDP) bone scintigraphy kit, [Amerscan Medronate II (Amersham)]. The MDP vial was reconstituted with 0.9% saline(w/v) for injection. MDP solution (50 µl) was added to the antibody, mixed and to this solution approximately 700 MBq of [<sup>99M</sup>Tc] - pertechnetate was added. The conjugation between  $F(ab')_2 - {}^{99M}Tc$  was allowed to proceed for 10 minutes before the solution was filtered through a 0.22 µM filter and subsequently diluted to 50 mls in 0.9% sodium chloride with 1% human serum albumen. The amount of  ${}^{99M}Tc$  bound to antibody was assessed by thin layer chromatography, as outlined in Method 2.5.5.

#### 2.5.15. Clinical Administration and Imaging of Immunoconjugate

Patients were premedicated with hydrocortisone (100 mg iv) by a physician, before iv infusion of immunoconjugate over 30 - 60 minutes in 100 mls of 0.9% sodium chloride(w/v) containing 1% human serum albumen (w/v). Planar images were obtained after one hour on the day of

administration of DTPA-<sup>III</sup>In immunoconjugates (Day 0). Subsequent images were obtained from these patients on Day 1, Day 3 and Day 6. Tomographic images were obtained on Day 3 and Day 6, where required. Planar images were recorded after 1 hour, 6 hours and 24 hours for patients who had received techetium-labelled  $F(ab')_2$  (<sup>99M</sup>Tc- $F(ab')_2$ ) fragments. Images were recorded by the attendant physicist using a General Electric Gamma Type 400 AC Camera.

All procedures involving photoactive compounds were carried out in conditions of minimal natural light in order to minimise the non-specific activation of these photoactive components.

#### 2.6.1. **Preparation of Hematoporphyrin Derivative**

Hematoporphyrin Derivative (HpD) was derivatized from hematoporphyrin dichloride according to the protocol described by Bonnet (1981) under conditions of minimal natural light. Hp (345 mg) was slowly added to 15 mls of 5% (v/v) sulphuric acid in acetic acid at room temperature. HpD was then precipitated following the addition of 3% (v/v) sodium acetate trihydrate (19 volumes). The resulting precipitate was collected by centrifugation and washed twice in distilled water. The liquid was redissolved in a minimum volume of distilled water and freezedried using a Heotosicc CD-52 freeze drier (Heto). The HpD was frozen at -20°C and protected from light.

#### 2.6.2. Preparation of Hp-Monoclonal Antibody Conjugates

Hematoporphyrin dichloride (Sigma) was conjugated to the monoclonal antibody HMFG-1 (Section 2.3.1.) according to the method outlined by Mew et al., (1983). All procedures were carried out in darkened conditions. Hp (20 mg) in 1.2 ml of distilled water and 0.8 ml of N',N',-dimethylformamide (DMF) was added to 20 ml of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCL (EDC) in 0.6 ml of  $H_2O$ . The mixture was then mixed with 15 mg HMFG-1 to give a molar ratio of 3.5:1 (EDC : Hp) and 300:1 (Hp : HMFG-1). The pH was maintained between pH 6 and 7. Ethanolamine (50 µl) was added to stop the reaction. The mixture was then dialysed extensively against PBS at 4°C in order to remove unconjugated Hp. The antibody-Hp conjugate was then lyophilised using a Hetosicc CD-52 freeze drier and stored at -20°C. The Hp content of conjugate was determined spectrophotometrically from a standard curve (2 - 10 µg/ml) which had been measured at an absorbance of 383 nm. The protein content of the conjugate was assessed by the BCA assay outlined in Section 2.3.6.

#### 2.6.3. Preparation of Stock Hematoporphyrin and Coumarin Compounds

A stock solution of 5 mM coumarin was dissolved in 1 ml of ethanol and diluted with 99 ml PBS. This 5 mM solution was filtered through a 0.2 µM filter prior to use. A 5 mM stock solution of 7-hydroxycoumarin was prepared in PBS and autoclaved at 2.7 bar for 30 minutes.

Stock hematoporphyrin (0.1 mg/ml) was prepared in 1 ml of ethanol to which 99 ml of PBS was added. HMFG-1-Hp conjugate (1 mg/ml) was prepared in DMEM and filter sterilised by filtration through a 0.22  $\mu$ M filter.

# 2.6.4. <u>In vitro</u> Assay to Assess the Toxicity and Phototoxicity of Coumarin and Hematoporphyrin Compounds

Landschütz acites tumour cells (LAT cells) were grown overnight in RPMI-1640 containing 10% FCS to a density of 1 x 10<sup>6</sup> cells/ml at 37°C. Drug solutions of Hp and HpD (0.01 - 0.1 mg/ml), acridine orange (0.05 - 1.0 mg/ml), coumarin, 7-hydroxycoumarin and AMCA (0.1 mM - 1.0 mM) were prepared from stock concentrates (Section 2.6.4.) in RPMI medium. Cells were incubated for one hour and irradiated with 200 mW of broadband light from a Xenon Arc Lamp (Applied Photophysics) for 20 minutes. UV wavelengths were minimised by the polypropylene lid on the tissue culture plates. Cells were then incubated for 18 hours at 37°C in 5% CO<sub>2</sub> and the final cell viability was assessed by ethidium bromide/acridine orange and/or trypan blue dye exclusion (Section 2.3.3.). Controls containing PBS, PBS containing ethanol (1% v/v) and RPMI were also included. All control experiments and test experiments were also carried out without irradiation, to monitor any non-specific irradiation effects generated in vitro.

#### 2.6.5. Effect of HMFG-1-Hp as an Immunophotosensitiser

The HMFG-1 monoclonal antibody binds strongly to the human milk fat globule antigen which is expressed on the surface of the MCF-7-human breast adenocarcinoma cell line but it does not bind to the EJ-138 human bladder carcinoma cell line. Therefore, MCF-7 was used to determine if the HMFG-1Hp conjugate specifically caused cell death while EJ-138 cells were used as controls to monitor non-specific effects. MCF-7 cells (or EJ-138 cells) were added to 6 well plates at a density of 1 x 10<sup>5</sup> cells/ml and incubated overnight. Concentrations of HMFG-1-Hp conjugate (0.02 - 1.0 mg/ml), Hp (0.01 - 0.025 mg/ml), HMFG-1 (1.0 mg/ml) and combined free Hp and free HMFG-1 (0.01 and 1.0 mg/ml, respectively) were prepared in DMEM containing 10% (v/v) FCS and added to the cells for a 30 minute incubation period at 37°C and then removed. Cells were washed twice in medium. Cultures were then irradiated for 20 minutes with 200 mW of broadband light and were incubated for 36 hours to allow cells to divide. Two further control assays were undertaken. In the first control experiment, the test samples and control samples were not removed prior to irradiation. This determined if Hp conjugated to HMFG-1 behaved in the same manner as unconjugated Hp. It was also necessary to investigate whether the HMFG-1-Hp conjugate bound non-specifically to the EJ- 138 cell line. A second control experiment, in which the cells were not irradiated was also undertaken. All experiments were carried out in duplicate.

## 2.6.6. Assessment of Cell Viability by Cell Transplantability in Schofield Mice

The ability of Landschütz ascites tumour cells to grow in the intraperitoneal cavity of Schofield mice has been used to assess and confirm their viability (Smyth, *et al.*, 1977). An inoculum of treated cells (1 x  $10^5$  cell/ml) was prepared in PBS, and injected i.p. into Schofield mice. The resultant tumour was harvested after 12 days and dry weights of the tumour were recorded.

SECTION 3 : MYELOMA-DERIVED CELL LINES

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Normal human B-cells can be immunised in vivo and in vitro to express antibodies which react with specific antigens or immunogens. These B-cells do not grow indefinitely in vitro and techniques such as viral transformation, recombinant antibody technology and fusion to myeloma or lymphoblastoid cells, have been developed to immortalise these cells. Once immortalised and cloned to grow from a single B-cell, these cells can secrete human monoclonal antibodies (hmabs) (reviewed in more detail in Section 1.3).

In 1967, Moore and his colleagues reported that normal or somatic human lymphocytes could be grown in culture when *transformed* by the Epstein-Barr virus, EBV. This technique was used by Steinitz *et al.* (1979) to make hmabs which reacted with a group A streptococcal carbohydrate antigen. B-cells are transformed by EBV in the supernatant of B95-8 marmoset leukaemia cells, or, alternatively by active EBV present in a patient's blood (Lewin *et al.*, 1990). However, the hazards of using a transforming virus, the difficulties in producing EBV transformed cells which are stable in vitro and the unwillingness of regulatory authorities to licence products of viral origin, have led to the development of other immortalisation strategies.

Human antibody fragments have been produced in myeloma cells, yeast, bacteria and plants using genetic engineering techniques. The methods used to produce these recombinant hmabs and the impact of this technology are discussed in more detail in Section 1.4.

Functional recombinant human mabs are not yet available for clinical use, while the more conventional human hybridoma technology has been used by many researchers. Recombinant human mab technology is a rapidly developing area of research (Winter and Milstein, 1991). Mab technology has been revolutionised by the ability to produce a library of hmab fragments which can then be expressed in  $\underline{E}$ . Coli. However, the ability to produce such fragments for use in vivo has neither been licenced nor commercialised to date.

Most of the human hybridomas produced to date have been immortalised by fusion with myeloma cells. Human, mouse and human x mouse heteromyelomas have all been used for this purpose (reviewed by James and Bell, 1987). Myeloma is classified as a tumour of monoclonal plasma cells (Millar *et al.*, 1988). These cells typically have well-defined cytoplasmic inclusions, abundant rough endoplasmic reticulum and well developed golgi-apparatus (Kozbor and Croce, 1985). It is these characteristics that allow myeloma cells to secrete high levels of immunoglobulin light and heavy chains of 'M' proteins both <u>in vitro</u> and <u>in vivo</u>. These properties also allow the myeloma cell, when fused to a B-cell, to \*secrete large quantities  $(1 - 10 \mu g/ml)$  of a single type of antibody. Human myeloma cells which are used as fusion partners include drug-resistant variants of RPMI-8226 (Matsuoka *et al.*, 1967) and SKO-007 (Olsson and Kaplan, 1980). The difficulties encountered in establishing myeloma cells in culture and the patent restrictions which already limit the availability of myeloma cells established in culture, have severely hindered the production of stable hmabs.

Human lymphoblastoid cell lines (LCLs) have also been used as fusion partners for human B-cells. LCLs show a constant association with EBV, have numerous free polyribosomes, less well developed rough endoplasmic reticulum and secrete less immunoglobulin than myeloma cells. Unlike myeloma cells, LCLs are easily maintained in vitro and generally have doubling times between 17 - 30 hr (Kozbor and Croce, 1985). Human lymphoblastoid cell lines, such as GM1500 (Croce *et al.*, 1980), LICR-LON-HMy-2 (Edwards *et al.*, 1982) and UC729-6 (Glassy *et al.*, 1988) have been used to produce human hybridomas.

Murine myeloma cell lines have also been used as fusion partners in both heterohybridoma and heteromyeloma cell line production, but many researchers have found that these mouse-human hybrid cells preferentially shed human chromosomes, making stable antibody production difficult (Jahn *et al.*, 1987). Teng *et al.* (1983) described the use of a human-mouse hybrid myeloma, or heteromyeloma to produce human monoclonal antibodies. They constructed a heteromyeloma between a human lymphoblastoid cell line (FU-266) and a murine myeloma (Ag-8) and isolated a non-secreting TG-resistant, HAT-sensitive clone into which the pSV2*neo* plasmid with G-418 resistance was transfected. Fusion of this heteromyeloma with human lymphocytes gave rise to stable monoclonal antibody production. The major advantage of these heteromyeloma cell lines is their fast doubling time in vitro and the ability to culture large quantities of heterohybridomas for production purposes (Foung *et al.*, 1984).

In this section, the development of myeloma cell lines for use as potential fusion partners in monoclonal antibody production will be described. Two main experimental aims were explored:

- (a) To establish a human myeloma-derived cell line for use in hmab production studies. The C23/11 cell line was established from a patient with multiple myeloma. This cell line was characterised by its morphology, cellular ultrastructure, antigenic expression, isoenzyme analysis and DNA fingerprinting studies.
- (b) In the second experiment, a heteromyeloma cell line, HET 37, was established following the fusion of C23/11 with the mouse myeloma cell line NSO. The characterisation of HET-37 and a comparison of C23/11 and NSO with HET 37 was also undertaken.

#### 3.2.1. Patient Samples.

Lymphocytes were isolated from a total of 106 blood samples and 5 bone marrow aspirates taken from patients with myeloma (56), multiple myeloma (44) and plasma cell leukaemia (11), using the method outlined in Section 2.4.1. The lymphocytes were placed in RPMI-S<sub>10</sub> and were maintained in culture for 40-80 days. Only 3/111 samples were still growing after this initial stage in culture and only one sample maintained viability after 5 sequential subcultures or at p = 5. The cell line was called C23/11, and was established from a patient with multiple myeloma. At the time of removing the blood sample, this patient had a serum protein level less than 0.15 g/l and had no Bence-Jones proteins in the urine. A quantitative paraprotein determination showed elevated levels of  $\beta_2$  globulin. C23/11 was grown in vitro up to passage number 67 and is, therefore, considered to be an established cell line.

#### 3.2.2. Growth of C23/11 In Vitro and In Vivo.

Though C23/11 was established from a patient with multiple myeloma it grows with lymphoblastoid growth characteristics in vitro. The clump-like morphology in vitro is shown in Figure 3.2.1.(a). C23/11 has a cell doubling time of between 16 - 18 hours, calculated for C23/11 [passage numbers less than 10], while a slower doubling time of between 20 - 23 hours was observed in later passage numbers (p = 42). As C23/11 was established in vitro with the aim of using this line for hmab production, the growth of the cell line in nude mice was particularly relevant. Figure 3.2.1.(b) shows that C23/11 (p = 4) grew as solid tumours following subcutaneous injection. However, C23/11 did not form ascitic tumours in MF1-nu-nu-old-Hsd mice.

#### 3.2.3. Morphological Characteristics of C23/11.

Despite the lymphoblastoid-like growth of C23/11 in vitro, this cell line has a typical plasma-cell-like morphology. Figure 3.2.2. shows C23/11 cells (p = 10) stained with Giemsa (Section 2.4.2.). This diagram shows that C23/11 has an excentric nucleus with coarse heterochromatin and extensive golgi apparatus, which are features of plasma cells. Both Giemsa staining and haematoxylin and eosin (H/E) staining showed a number of mitotic cells. Further analysis of the ultrastructure of C23/11 was undertaken using transmission electron microscopy, which will be described in Section 3.



Figure 3.2.1:In vitro and in vivo growth of C23/11 when grown (a) in vitro in<br/>RPMI-1640 supplemented with 10% FCS, and (b) transplanted<br/>subcutaneously into an athymic MFI-nu-nu-old/Hsd mouse.



 Figure 3.2.2:
 C23/11 cells stained with Giemsa stain, showing immature plasma cell

 morphology such as excentric nucleus, extensive golgi apparatus and

 coarse heterochromatin.

Antibody Used	C2	3/11
	P = 6	P = 42
Polyclonal anti-human $\lambda$	+	-
Polyclonal anti-human κ light chain	-	-
Monoclonal anti-human $\lambda$ light chain	•	-
Monoclonal anti-human Ig light chain	-	-
anti-EBVNA	+	+

# Table 3.2.1: Immunohistochemical Analysis of C23/11

#### 3.2.4. Immunohistochemical Analysis of C23/11.

Repeated immunohistochemical analysis of C23/11 with polyclonal antibody showed that at early passage numbers, the cell line retained some human  $\lambda$  light chains (Table 3.2.1.). The patient from which this sample was established also expressed  $\lambda$  Bence-Jones proteins in urine and  $\lambda$  chains on electrophoresis, at the time of initial diagnosis. These proteins were not seen in later passage numbers.

However, the monoclonal antibody to human anti- $\lambda$  light chains did not show any reactivity with C23/11 at either passage number 6 or passage number 42. The polyclonal antibody did not react with FCS or albumen when added to negative controls. It was thought that C23/11 may express an altered  $\lambda$  chain. Such abnormal antibody secretion may be associated with EBV transformation (Wang *et al.*, 1990). This would be consistent with the presence of the Epstein-Barr viral antigen (EBVNA) on the surface of both early and late passages of C23/11. Further confirmation of the presence of EBVNA was provided by DNA probing performed with specific probes for EBV mRNA (analysis performed by D. Purtilo, University of Nebraska, USA).

#### 3.3.1. Generation of C23/11 Cells Resistant to G-418.

In order to produce successful hybrid cell lines, it is necessary to select the new hybrid cell lines from parent cell lines present in the culture. A drug-resistant variant of C23/11 was prepared by the electroporation of the plasmid pSV2*neo* which carries the gene for G-418 or geneticin resistance. Table 3.2.2. shows that a voltage of 0.75 mV resulted in a moderate reduction in cell viabilities, and, was chosen for all further experiments. Following electroporation, two separate cultures were initially selected in 200  $\mu$ g/ml geneticin and variants which were resistant to up to 1 mg/ml geneticin were obtained. However, these cells lost their G-418 resistance on reculturing following long-term storage in liquid nitrogen (Section 2.3.4.), and failed to establish following reculturing.

Voltage (mV)	Average Cell Viability (48 hours) %
0.0	95.7
0.5	100.0
0.75	78.3
1.0	52.6
1.25	43.3
1.50	55.5
1.75	37.5
2.0	25.0

Table 3.3.1: Optimisation of Electrical Conditions for Electroporation.

where: capacitance = 25  $\mu$ FD; time constant = 0.1 sec.

Cells were transfected with 0.5  $\mu g$  pSV2neo DNA /  $10^{5}$  cells.

Viability was assessed by trypan blue dye exclusion.

#### 3.4.1. Generation of the Mouse-Human Heteromyeloma, HET-37.

C23/11 cells fused with cells from the murine myeloma cell line NSO, to produce a heteromyeloma cell line, HET-37. Table 3.4.1. shows that pre-treatment of C23/11 cells with neuraminidase prior to fusion increased the number of clones formed following selection in both 96 well and 24 well plates. A total of eight heteromyeloma clones (Table 3.4.2.) were expanded into 24 well plates and into 25 cm<sup>2</sup> flasks. Both HET-A1 and HET-H8 were then cloned to monoclonality by limiting dilution (Section 2.3.6.). Four clones from HET A1 were re-cloned and of these monoclonal heteromyelomas HET-37 was found to be the fastest growing clone in RPMI-S<sub>10</sub> without BriClone as a media supplement.

#### 3.4.2. Characterisation of HET-37.

In order to determine (confirm) whether HET-37 was a true hybrid cell line and not a HAT or ouabainresistant variant of C23/11 or NSO, various characterisation studies were undertaken.

#### 3.4.2.2. Electron Microscopy.

The ultrastructural characteristics of a cell line can be used to determine the function of a cell and to classify cell types. Plasma cells and other lymphocyte types have defined ultrastructural features which represent stages of differentiation in B-cell lineage. Electron micrographs of C23/11 (p = 11) and HET-37 (p = 4) were prepared and their ultrastructural components were identified.

#### *C23/11*:

Figure 3.4.2. is an electron micrograph of C23/11 (p = 11). The nucleus of the cell is typical of an immature plasma cell, though the dispersed chromatin is more typical of a less differentiated lymphocyte. The invaginations or vacuole-like bodies, seen in the nucleus are more typical of a lymphoma cell than a plasma cell. In Figure 3.4.3., extensive arrays of endoplasmic reticulum in sheet-like morphology can be seen, which is typical of plasma cells (Klein *et al.*, 1977). The cytoplasm contained some ribosomes and mitocondria and this gave the cells their granular texture. Further electron micrographs showed both binucleated and trinucleated cells, which are again typical of myeloma cells. However, the irregular shape of many of the cells observed is typical of lymphoblastoid morphology (Bassan *et al.*, 1981).

# Table 3.4.1: The Effect of Neuraminidase on the Number of Clones Formed Following the Fusion of C23/11 and NSO Cells.

Neuraminidase treated C23/11 (no. of clones formed/plate $\pm$ s.d.)		Non-neuraminidase treated C23/11 (no. of clones formed/plate $\pm$ s.d.)		
24 well plate	96 well plate	24 well plate	96 well plate	
14.7 <u>+</u> 1.9	17.0 <u>+</u> 4.5	1.3 <u>+</u> 1.8	1.7 <u>+</u> 1.2	

Table 3.4.2: Growth Characteristics of Heteromyelomas.

Clone No.	Growth Morphology	Growth in Absence of BriClone
HET-B6	lymphoblastoid	slow
HET-A1	single cell	good growth
HET-D6	single cell	slow
HET-E7	single cell	very slow
HET-H8	single cell	good growth
HET-C10	lymphoblastoid	no growth



**Figure 3.4.1:** Chromosome preparation of (a) C23/11 cells (p = 11) in metaphase showing metacentric chromosomes, and (b) HET-37 cells (p = 3) showing two bi-armed chromosomes which may be of human origin. Chromosomes were stained with Giemsa stain 5% (v/v).



**Figure 3.4.2:** Electron Micrograph of C23/11 (p = 10) [magnification x 5,000] showing two mononucleated plasma-like cells with dispersed chomatin and granular cytoplasm containing ribosomes.



Figure 3.4.3: Electron Micrograph of C23/11 (p = 10) [magnification x 7,000] showing extensive rough endoplasmic reticulum and lysosomal inclusions containing ribosomes.



**Figure 3.4.4:** Electron Micrograph of HET-37 (p = 4) [magnification x 7,000] which shows a euchromatic nucleus, many mitochondria, endoplasmic reticulum and microvilli-like surface projection.

#### HET-37:

Figure 3.4.4. is an electron micrograph of HET-37 (p = 2). This cell shows both an accentric nucleus and prominent nucleolus, which may be plasma cell in origin. The giant mitochondrion is a feature of a cell with a rapid turn-over. The granular cytoplasm is comprised of ribosomes and some lysosomes. The projections seen on the surface of the cell are not usually seen in either plasma or lymphoblastoid cell lines and are more typical of a hairy cell - leukaemia cell.

## 3.4.2.3. Isoenzyme Analysis.

Cell lines and tissue types differ in enzyme activities. These differing enzyme activities can be compared qualitatively between cell strains or between clones of cells. Isoenzymes or isozymes may be separated chromatographically or electrophoretically and the distribution pattern obtained may be used as an enzyme 'finger print' for a species, tissue type or cell line (O'Brien *et al.*, 1977). The distribution patterns of isoenzymes in C23/11, HET-37 (p = 4) were determined (Table 3.4.2.3.).

#### 3.4.2..4 Immunophenotyping by FACS Analysis.

Faller *et al.* (1990) used immunophenotyping, or the profile of antigens expressed on a cell's membrane, to characterise a heteromyeloma cell line. In this experiment, FACS analysis was used to determine the immunophenotype of C23/11, NSO and HET-37 (p = 5) (Table 3.4.2.4.).

Isoenzyme	Electrophoretic Migration Distance (mm)			
	C23/11 HET-37			
Glucose-6-phosphate dehydrogenase	5.5	1.0	1.0	
Lactate dehydrogenase	-2.5, 0.5, 5.0, 10.0, 16.0	0.5, 1.0, 10.0	1.0	
Mannose phosphate isomerase	6.5	N.D.	N.D.	
Malate dehydrogenase	-3.0, 5.0	N.D.	N.D.	
Peptidase B	6.0	N.D.	N.D.	
Nucleoside phosphorylase	-	-	1.0	

## Table 3.4.2.3: Isoenzyme Profiles of C23/11, HET-37 and NSO Cell Lines.

(-) indicates a negative result; N.D. = not determined.

Surface Antigen	C23/11 (p = 10)	HET-37 (p = 3)	NSO	
control	-	-	<u> </u>	
IL-2 receptor	-	+	+	
human Ig	-	-	-	
mouse Ig	-	-	-	
HLA-DR	-	-	+	
CD 19	-	+	+	
CD 23	+	-	•	
CD 38	+	+	+	
EBVNA		-	•	

Table 3.4.2.4: Immunophenotype of C23/11, HET-37 and NSO.

+ indicates cells which express antigen, and, - indicates cells which do not express the antigen.

#### 3.4.2.5. DNA Fingerprinting.

DNA when treated with restriction endonucleases breaks down into smaller DNA fragments. These fragments are unique to a cell line. Similarly, each parent contributes a portion of their banding patterns to their offspring. These unique banding patterns are termed DNA fingerprints, and have found many applications in both forensic and basic science since their discovery (Jeffreys *et al.*, 1985). DNA fingerprinting was performed by Cellmark Diagnostics Ltd., Abingdon, U.K. DNA was extracted from each cell line and probed with the multi locus-probes 33.6 and 33.15, as well as a cocktail of the single locus probes, G3, MS43A and MSA.

The results in Table 3.4.2.5. suggest that HET-37 (p = 10) does not contain detectable human DNA, though some bands in HET-37 could not be attributed to either NSO or C23/11. Statistical analysis on the bands formed was performed by Cellmark Diagnostics Ltd., (Table 3.4.2.6.). This suggests that a portion of HET-37 DNA ( $\approx 3\%$ ) is not contained in the NSO cell line used in the fusion experiments. As DNA fingerprinting was performed a number of months after the fusion experiments, the extra bands may be from a sub-clone of NSO used in fusion, which did not appear in the cells sent for DNA fingerprinting.

Table 3.4.2.5: Summary of the Number of Bands found when using various DNA probes on C23/11, HET-37 and NSO.

Probe Used	No. of Bands Produced			
	C23/11 (p = 9-10)	NSO		
multi-locus 33.6 + 33.15 single-locus G3, MS43A, MSA	44 6	43	42	

Table 3.4.2.6: Percentage Multilocus Band Shares between C23/11, HET-37 and NSO Cell Lines.

	C23/11	HET-37	NSO
C23/11	100%	17%	14%
HET-37	17%	100%	87%
NSO	14%	87%	100%

#### 3.4.2.6. Summary of the Characterisation Studies on HET-37.

The cell line characterisation studies undertaken to date show that:

- Chromosome Studies: HET-37 (p = 4) contained a modal number of 60 chromosomes, two of which were bi-armed and may have been from the C23/11 human cell line.
- *Electron Microscopy:* HET-37 (p = 4) retained few myeloma-like ultrastructural inclusions. However, numerous mitochondria and extensive endoplasmic reticulum suggests that the line may secrete antibody and has a fast-doubling time.
- Isoenzyme Analysis: HET-37 (p = 4) contained C23/11-like LDH patterns and did not express nucleoside phosphorylase. However, the murine glucose-6-phosphate dehydrogenase isoenzyme pattern was dominant.
- FACS Analysis: HET-37 (p = 5) expressed cell surface markers contained on either NSO or C23/11 parent cells.
- DNA Fingerprinting: HET35 (p = 10) contained a total of 87% DNA which showed homology with the NSO cell line. The absence of bands of DNA from C23/11 in HET-37 suggests that at this passage number, HET-37 may have lost the human DNA component which was observed in chromosome studies.

### 3.5.1. The Establishment and Characterisation of C23/11.

The aim of this experiment was to establish a human myeloma cell line for use in human monoclonal antibody production. Even though C23/11 was derived from a patient with multiple myeloma, many of the characteristics of this cell line are typically lymphoblastoid.

#### 3.5.1.1. C23/11, A Lymphoblastoid or A Myeloma Cell Line?

C23/11 has a doubling time of between 16 to 23 hours, myeloma-like golgi-apparatus, myeloma-like intracellular inclusions and was shown to express EBVNA and grow in a lymphoblastoid clump-like manner in vitro. These characteristics suggest that C23/11 is an EBV transformed plasma cell.

It is of interest to examine both the source of EBV and the transformation of such a differential cell type. The blood sample was not deliberately transformed by EBV in vitro suggesting that these plasma cells were transformed in vitro by EBV in the patient's own blood sample. A similar natural transformation of blood lymphocytes, co-cultured with EBV infected lymphocytes from the same patient, has been described by Lewin *et al.* (1990). C23/11 is, therefore, more characteristic of a lymphoblastoid rather than a myeloma cell line.

#### 3.5.1.2. Growth and Morphology of C23/11.

Table 3.5.1. summarises many of the characteristics of the lymphoblastoid cell lines (LCL) used in hmab production. Doubling time of between 18 to 30 hours were observed for LCLs, while faster doubling times of between 16 to 23 hours were observed in C23/11. Myeloma cell lines have a much slower growth rate, with average doubling times of between 36 to 37 hours in vitro (Kozbor and Croce, 1985). This slower growth rate leads to long and costly fermentation runs in the large-scale production of hmab from human hybridomas. The morphology of C23/11, observed following Giemsa staining (Figure 3.2.2.), is typical of an immature plasma cell. This cell shows extensive golgi apparatus, which would be useful for the secretion of monoclonal antibodies. The ultrastructural characteristics of C23/11 also show golgi apparatus and extensive endoplasmic reticulum. However, the established myeloma cell lines such as RPMI-8226 have more prominent golgi apparatus and endoplasmic reticulum (Matsuoka *et al.* 1967) than observed in C23/11. Several bi-nucleated and tri-nucleated cells were also recorded by electron microscopy and Giemsa staining, and, these are features of normal plasma cells and myeloma (Karpas *et al.* 1985).

Cell Line	Parent Cell Line	d.t. (Hrs)	EBVNA	Fusion Frequency	Ig	Reference
GM-1500 -GTG	GM-1500	18.25	+	18	IgG₂(k)	Croce et al., 1980
LICR-LON -HMY 2 -8AG	PGLC33H	N.D.	+	N.D.	IgM(k)	Chiorazzi et al., 1982
PGLC33H -8-AG	ARH77	20-30	+	0.1 - 10	IgG1(k)	Edwards <i>et</i> al., 1982
MC/MNS-2 -8AG	MC/CAR	N.D.	+	100	N.S.	Ritts et al., 1983

Table 3.5.1: Characteristics of the main lymphoblastoid cell line used in hmab production.

In this figure, the abbreviations are as follows:

d.t. = doubling time in hours; Ig = antibody secreted; N.D. = not determined; N.S. = non-secretor

#### 3.5.1.3. Antibody Secretion Patterns and Surface Antigen Expression.

The majority of LCL produce intact immunoglobulin, a heavy chain or a light chain, in vitro (Table 3.5.1.). C23/11 appeared to express the human  $\lambda$  light chain (Table 3.2.1.) in early passages but not in later passage numbers. FACS analysis (Table 3.4.2.4.) showed that C23/11 did not produce human Ig in vitro. C23/11, therefore, appears to be a non-secretor. The use of a non-secreting fusion partner is preferable in hmab production as the absence of non-specific antibody would simplify downstream purification procedures. Gregory *et al.* (1987) suggested that a small proportion of myeloma and lymphocytes are 'sterile'. This may result from a gene-rearrangement during cell immortalisation. FACS analysis was also used to investigate the immunophenotype of this cell line. Cluster determinants (CDs) are a nomenclature used to classify antigens expressed on the surface of haemapoetic cell lines. Both CD19 and CD38 are expressed on B lymphocytes at different stages of differentiation while CD19 is expressed on less mature B cells, CD38 is expressed on plasma cells with multiple restricted lineage (Nacheva *et al.* 1990). From Table 3.4.2.4., it can be seen that C23/11 expressed CD38, the plasma cell marker but did not express the CD19 antigen which is associated with lymphoblastoid cells (Tazzari *et al.* 1987). Interestingly, C23/11 expresses the CD23 blast-antigen. The co-expression of CD23 and CD38 is further evidence that C23/11 is an EBV-transformed plasma cell.

#### 3.5.1.4. Isoenzyme Analysis of C23/11.

C23/11 has a type a G-6-P profile (Table 3.4.2.3.), indicating that C23/11 is of human caucasian origin. The five band LDH pattern is also associated with human lineage. Both the presence of 2-MDH bands and the absence of NP can be used as indicator markers of C23/11. Isoenzyme analysis of RPMI-8226 showed a similar G-6-P profile (Matsuoka *et al.* 1967), while Edwards *et al.* (1982) showed that LICR-LON-HMy2 had multiple type bands on analysis.

3.5.1.5. Chromosomal Analysis of C23/11.

C23/11 contained a modal number of 51 chromosomes, similar to the modal chromosome number recorded for LCLS such as LICR-LON-HMy2 (n = 43) (Edwards *et al.*, 1982) and GM-1500 (n = 46) (Croce *et al.*, 1980). However, myelomas have several structural changes to their chromosomes which can affect secretion of antibodies. Myeloma cells have several marker chromosome abnormalities such as translocations involving the long arm of chromosome 15 at the band q32 (Rabbitts *et al.*, 1988) and chromosome 11 at q13 with partners other than chromosome 14, at 1q32.1, 8q24.22 and 13q14.3 (Nacheva *et al.*, 1990). The presence of oncogenes in fusion partners may increase the number of tests required to validate this cell line for use in the production of a mab for human use (Brown, 1990).

Gazdar *et al.* (1986) have reported the over-expression of *c*-myc in the myeloma cell line NC1929. The identification of myeloma-like translocations and the location of oncogenes in C23/11 would be essential before C23/11 could be used to immortalise hmabs for in vivo human use.

#### 3.5.2. Generation of C23/11 with geneticin resistance.

Procedures for the transfection of the plasmid pSV2 *neo* were optimised and mutant cell lines of C23/11 which were resistant to geneticin or G-418 (1mg/ml) were isolated. Wabl *et al.* (1989) reported on the instability of this plasmid mediated drug resistance in B cells. Continuous culture in G-418 without long-term storage in liquid nitrogen (Section 2.3.4.), should maintain the plasmid in C23/11 by selective pressure.

### 3.5.3. Future Work with C23/11.

C23/11 was used to generate the heteromyeloma cell line HET-37, but the fusion of C23/11 G-418 to immunised B cells has not been attempted to date. The success of neuraminidase pre-treatment in increasing the number of heteromyeloma clones produced following fusion, coupled to the favourable ultrastructural characteristics observed in C23/11 implies that further work on hmab production using C23/11 as a fusion partner may be fruitful.

#### 3.5.4. Generation of HET-37, a Heteromyeloma Cell Line.

#### 3.5.4.1. The Effect of Neuraminidase on C23/11-NSO Cell Fusion.

In order to bring C23/11 and NSO cells into closer contact before fusion, several enzyme pre-treatment systems were investigated. As C23/11 cells grow as clumps and these clumps re-form, a successful enzyme pre-treatment method was required to minimise the number of C23/11 to C23/11 fusions which occurred. Igarashi and Bando (1990) proposed that EBV-transformed cells, pre-treated with neuraminidase, should reduce spontaneous blast formation by removing sialic acid from the cell membrane of the human blast cell. This should increase the association between C23/11 and NSO mouse myeloma cells. However, the exact mechanism involved is not clear. Similarly, Rosenberg *et al.* (1972) have suggested that sialic acid residues on the surface of a cell membrane can mask cell surface proteins from detection by cells or antibodies. In addition, electron microscopy studies have shown that there is intimate association of cells after neuraminidase treatment (Bentwich *et al.*, 1973). Igasashi and Bando (1990) recorded a 6.8-fold increase in the number of Ig producing clones when cells were pre-treated with neuraminidase prior to human-mouse cell fusion. In this experiment, a 10.7-fold overall increase in the number of clones formed was recorded in neuraminidase pre-treated cells.

Marginally higher clone formation was observed when equal numbers of newly fused cells were plated in 96 well plates (17 clones) when compared with fusions cultured in 24 well plates (15 clones).

#### 3.5.4.2. Characterisation of HET-37.

HET-37 was established from a parent heteromyeloma clone, HET A1. This parent line was selected from a number of heteromyelomas following fusion of neuraminidase-treated C23/11 cells with NSO cells (Section 3.4.1.). HET A1 grew as a single cell suspension rather than as lymphoblastoid clumps, and, it had a faster growth rate than other hybrids obtained after fusion.

#### (a) Chromosomal Studies:

Chromosomal analysis of HET-37 (p = 3) showed that the cell line contained a modal number of 60 chromosomes. Two of these chromosomes were bi-armed, and may be of human origin (Figure 3.4.2.). No bi-armed chromosomes were observed in the mouse parent line. Attempts to distinguish human from mouse chromosomes by the differential uptake of Hoechst 33258 stain were unsuccessful. Teng et al. (1985) reported that hybridoma clones resulting from fusions using a heteromyeloma with high proportion of human chromosomes (5 - 10) secreted higher levels of hmab in vitro. However, these heteromyelomas were shown to be unstable over longer periods of time. Heterohybridomas (heteromyelomas x human B cells) retain variable amounts of human DNA while clones resulting from the fusion of SHM-D33 (Table 3.5.3.1.) with immunised tonsil lymphocytes, contained between 20% to 50% human DNA. However, loss of antibody production in these heterohybridomas is often accompanied by the loss of structural immunoglobulin genes and generally resulted in the loss of greater than 95% of the human component of hybrid DNA (Koropatnick et al., 1988). HAB-1 (Table 3.5.3.1.) contained a total of 75 - 85 chromosomes (50 mouse and 25 - 35 human chromosomes) after one year in culture. Conversely, F3B6 contained 48 chromosomes (46 mouse and 2 human chromosomes) and also retained both the ability to immortalise human B cells and chromosome stability in vitro. Therefore, the effect of low human chromosome content and the potential of HET-37 as a heteromyeloma fusion partner, cannot be predicted by comparison of retained chromosomes in other heteromyelomas and must be determined by future HET-37 x B cell fusion experiments.

The retention and loss of human chromosomes in human x mouse fusions is not a random process. It is known that human chromosome 14 (heavy chain) and 22 ( $\lambda$  light chain) are preferentially retained (Teng *et al.*, 1985) whereas chromosome 2 ( $\kappa$  chain) is preferentially lost (Croce *et al.*, 1979). Translocations between human chromosomes and mouse chromosomes in heteromyelomas have also been reported (Weiss and Green, 1967) explaining how some heteromyeloma cell lines, with low human chromosome content can still assemble mabs in vitro.

## (b) Growth and Morphology of HET-37.

HET-37 grows as a single cell suspension in vitro, allowing the cell line to be used in both chemical and electrofusion experiments (Zimmermann, 1982) and similarly, HET-37 would be easier to transfect with plasmid DNA than C23/11 or other lymphoblastoid-like cells.

Hetero- myeloma	Mouse Origin	Human Origin	EBVNA	Ig Sec.	Drug Markers	Reference
SHM-D33	X63.Ag8 653x	U226	-	N.S,	6-TH OUA G-418	Teng <i>et al.</i> 1983
3HL3	SHM-D33	B Cell	N.D.	N.S.	6-TG OUA	Östberg et al. 1983
SBC-H2O	Sp2/08A2	B Cell	-	N.S.	6-TG OUA	Foung <i>et al.</i> 1985
MHH-1	X63.Ag8 653	B Cell	N.D.	N.S.	6-TG OUA	Van Meel et al. 1985
K6H6/B5 K6H9/G12	NS-1	Lymphoid	N.D.	N.S.	8Ag 6-TG OUA	Carroll <i>et al.</i> 1986
F3B6	NS-1	B Cell	N.D.	N.S.	6-TG OUA	Pollack et al. 1987
E62 D3-8	X63.Ag8 653	Tonsil B Cell	N.D.	-	8Ag 6-TG OUA	Hirata <i>et al.</i> 1987
SP5/HPT	Sp2/0	PBLxTonsil Cells	-	N.S.	6-TG 8Ag	Martin <i>et al.</i> 1988
CB-F7	X63.Ag8 653	B Cell	N.D.	N.S.	6-TG OUA	Grunow et al. 1988
SPA2-4	Ag8	Tonsil Cell	N.D.	N.S.	6-T <b>G</b>	Goldstein et al. 1990
HAB-1	Ag8	B Cell Lymphoma	-	-	6-TG	Faller <i>et al</i> . 1990
mouse- human	P3U1	Mediastinal Lymphocytes	N.D.	IgG + IgM	N.D.	Uchiyama et al. 1987

Table 3.5.3.1: Summary of Heteromyeloma Cell Lines.
Electron microscopy showed that HET-37 contained a euchromatic nucleus which is indicative of a primative nucleus (P. Dervan, personal communication). The lysosomes appear to be full of polyribosomes and abundant mitochondria can be detected. A large or giant mitochondrion may be observed adjacent to the cell. Several giant mitochondria were seen in the cell line. HET-37 had some endoplasmic reticulum but more golgi apparatus and sheet-like endoplasmic reticulum would be expected in a human-mouse myeloma cell line (Hirata and Sugawara, 1987). The microvilli-like projections seen in HET-37 were also observed in some murine myelomas (X63 - Ag8.653) and some heteromyeloma cell lines (HE62, D3-8). This apparent anomaly between the ultrastructure of a normal murine plasma cell and a murine myeloma <u>in vitro</u>, results from the alteration or adaptation of a cell to tissue culture over a period of time (reviewed by Kozbor, 1985).

#### (c) Isoenzyme Analysis.

Isoenzyme analysis showed that HET-37 retained LDH isoenzymes from both C23/11 and NSO cells (Table 3.4.2.3.). HET-37 has a murine species G-6-P profile, with a migration distance of 1.0 mm, while HET-37 lacks the NP band which is a marker for the NSO cell line. Isoenzyme studies by Uchiyama *et al.* (1987) showed that the mouse-human heteromyeloma retained a murine G-6-P profile following fusion, confirming the dominance of the murine chromosomes.

#### (d) FACS Analysis of Surface Markers on HET-37.

HET-37 lost the expression of the EBVNA antigen following cell fusion. Table 3.5.3. shows that all heteromyelomas lost the EBVNA antigenic phenotype on fusion to murine myeloma cells. The loss of EBVNA expression has favourable implications for the heterohybridomas and hmab products. As these products do not express EBVNA, fewer regulatory and safety processes are required to ensure the safety of these hmabs for human use. The absence of the EBVNA antigen also reflects the loss of the CD23 blast antigen from the HET-37 cell membrane, confirming that HET-37 lost many of the lymphoblastoid characteristics of C23/11 on fusion. However, the expression of both CD38 and CD19 suggests that HET-37 did indeed retain some of the human C23/11 immunophenotype following cell fusion. HET-37 was also shown not to express human or mouse Ig on its cell surface.

That HET-37 (p = 3) is negative for EBVNA and does not produce immunoglobulin suggests that HET-37 may be a useful fusion partner for human lymphocytes.

#### (e) DNA Fingerprinting.

At passage number 10, HET-37 could not be shown to contain any C23/11-derived DNA, when probed

with multilocus probes and human single locus probes. As the previous characterisation studies had shown HET-37 to have altered chromosome and isoenzyme profiles from its parent lines, it was felt that HET-37 may have lost its human chromosomes at this passage. Further cloning of earlier passage number (p = 3) cells and DNA fingerprinting should be undertaken to confirm the presence of human DNA in the HET-37 cell line.

#### 3.5.5. Future Work with the HET-37 Cell Line.

In order to use HET-37 as a fusion partner, the cell line must be made resistant to some drug, by which fusion products may be selected. Drug selection systems which are commonly used in heteromyelomas (Table 3.5.3.1.) include ouabain and thioguanine resistance (Cole and Arlett, 1976), 8-azaguanine resistance (Myhr and DiPaolo, 1975), and G-418 resistance (Section 2.4.7.). Further characterisation of the chromosomes of a stabilised HET-37 clone should reveal the major translocation and chromosomal abnormalities in the cell line.

# 3.6. THE FUTURE OF HUMAN MYELOMA CELL LINES AND hmab PRODUCTION.

#### 3.6.1. Generation of Human Myeloma Cell Lines.

Difficulties in establishing continuous cell lines from samples obtained from patients with myeloma, are well documented (Kozbor *et al*, 1985; James and Bell, 1987). In the experimental work outlined, only one of the myeloma samples isolated from patients was successfully established <u>in vitro</u> even though over 100 samples were processed), and, it appears that this occured by transformation.

Several researchers have tried other culture systems to try to establish myeloma cells in vitro. Millar et al., (1988) used a clonogenic assay system in semi-solid agar to establish granulocyte and granulocyte-macrophage colony forming units. This system used a feeder-layer of irradiated HL-60 promyelocytic leukaemia cells, to support growing cells. To date, only the short-term growth of myeloma cells has been accomplished with this technique (Devereux and Linch, 1989).

Haemapoetic cells are supported in vivo by a system of pluripotent cytokines. The range of cytokines in the blood and their role in blood cell development is currently under investigation. The effect of media supplements containing interleukin-rich culture supernatants and purified interleukins on the growth of myeloma cells has also been investigated (Christie *et al.*, 1987; ; Kishimoto, 1985; Fakoi *et al.*, 1990; Yokoi *et al.*, 1990).

#### 3.6.2. Human Monoclonal Antibody Production

In 1980, Croce and his colleagues produced human monoclonal antibodies to the measles virus. During their investigations, it became apparent that the hybridoma production processes developed for murine monoclonal antibody production did not transfer directly to human B-cells and human myeloma cells. Initial problems involving the source of human B-lymphocytes have largely been solved by in vitro immunisation technology (Section 1.3.2.). Increased numbers of clones have been formed by strategies such as EBV-transformation before fusion to a myeloma or LCL (Kozbor and Croce, 1985) electrofusion (Zimmermann, 1982) and gene cloning technology (Winter and Milstein, 1991).

Many of the LCLs, human and heteromyeloma fusion partners used may not secrete antibody, but continue to synthesise heavy and/or light chains in the cytoplasm. Hybridisation often leads to resecretion of these chains and results in mixed immunoglobulin molecules being produced which are both antigen-specific and non-antigen-specific (Strelkaukas and Taylor, 1986). Many human

hybridomas have very poor secretory capacity and are relatively unstable when compared to murine hybridomas. Low levels of antibody secretion have been related to the low levels of endoplasmic reticulum and golgi apparatus in these cells (Kozbor *et al.*, 1985). Many human hybridomas produce hmabs which build up in the cytoplasm and are not secreted in vitro. Crawford (1985) suggested that adding HLD-Dr-matched-specific helper T cells or antibody to HLA-Dr may help secretion. This approach was less successful than the attempts to demethylate 'inhibitory' genes (Olsson and Brams, 1985) and treatment with phorbol esters (Polke *et al.*, 1986). Alternatively, it may be possible to transfect cells with specific enhancer sequences to promote transcription of the antibody genes (Gillies *et al.*, 1983) and thereby increase hmab secretion rates.

The limited success of human monoclonal antibody technology has resulted in much collaboration between immunologists and molecular biologists in the production of humanised and human recombinant antibodies.

#### 3.6.3. The Impact of Molecular Biology on Human Monoclonal Antibody Production.

Several strategies have been developed for preparing specific antibody-secreting human cell lines. These strategies include the transfection of normal B lymphocytes with tumour cell-derived DNA (Jonak *et al.*, 1984) and conversly, the transfection of permanent human cell lines with DNA from antibodysecreting lines (Neuberger, 1985) and lastly, the introduction of natural or recombinant antibody genes into a suitable cell line to produce chimaeric and humanised monoclonal antibodies (Section 1.4). The use of these recombinant antibodies <u>in vivo</u> will be discussed in more detail in Section 4. Site-directed mutagenesis of existing human monoclonal antibodies has also been proposed as a mechanism to increase the repertoire of available hmabs (Wabl *et al.*, 1989). These products of so-called conventional genetic engineering are currently providing human and human-like antibodies for use <u>in vivo</u>. Newer recombinant antibody techniques such as the expression of antibody fragments, which will make functional human  $F_v$  fragments using phage display libraries (Clackson *et al.*, 1991), rather than naive B-cells, and <u>E. coli</u> rather than human myelomas, will increase both the range of antibodies available for use and the cost-effectiveness of hmab technology. However, these second generation recombinant antibodies are still at the developmental stages. Problems are still encountered with screening, post-translational modification and with purificiation (Coughlan, 1991).

Conventional hmabs have not proved as easy to construct as their murine counterparts. The human antibodies which have been produced to date (reviewed by Borrebaeck *et al.*, 1990) will be useful in optimising techniques for monoclonal antibody-mediated diagnosis and therapy, before more recombinant human monoclonal antibodies and antibody fragments become widely available for human use.

SECTION 4 : IMMUNOSCINTIGRAPHY

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# THE USE OF RADIOLABELLED MONOCLONAL ANTIBODIES IN THE EVALUATION OF OVARIAN CARCINOMA.

Recent advances in human, humanised and chimaeric antibody technology have renewed interest in the detection and treatment of cancer using targeted monoclonal antibodies (mabs). Immunoscintigraphy, or the use of radiolabelled mabs as detection systems in vivo has been shown to be particularly useful in the evaluation of disease in patients with ovarian carcinoma. Conventional radiological examinations such as X-ray are of limited use in these patients, while CAT scan and ultrasound often fail to locate small volume disease or non-tumour masses (Chatal *et al.*, 1985). Metastasis of ovarian carcinoma is found primarily in the peritoneal cavity and may be distributed as large tumour masses matted to the bowel or omentum, as diffuse peritoneal seedlings, or as ascites. Ovarian carcinoma may also be found in the pleural cavity as a pleural effusion. Immunoscintigraphy can therefore be used to locate antigenbearing masses in these areas and to differentiate them from non-antigen bearing masses. These immunoscans may indicate the distribution of the disease within the intraperitoneal and pleural cavities without the need for surgery.

Earlier studies in immunoscintigraphy used isotopes of iodine, ie. <sup>123</sup>I, <sup>125</sup>I and <sup>131</sup>I (reviewed by Lamki *et al.*, 1988). These isotopes were readily available and conjugation techniques such as chloramine T and iodogen were already developed to label proteins with iodine. However, on administration, iodine-mab conjugates were found to be less stable in vivo than in vitro (Finn *et al.*, 1991). High levels of uptake by the thyroid and inefficient imaging by the gamma cameras which are currently available, all pointed to the need for more suitable isotopes for tumour immunoscintigraphy. Attention was then focussed on using <sup>III</sup>In and <sup>99M</sup>Tc which have more suitable imaging properties for gamma imaging.

<sup>In</sup>In has two gamma energies at 173 keV and 247 keV and has a half-life of 72 hours. Mabs are easily labelled with <sup>III</sup>In after conjugation to a variety of chelating agents. Diethylenetriaminepentaacetic acid (DTPA) is the most commonly used bifunctional chelator for antibody labelling and DTPA-mab conjugates are relatively stable <u>in vivo</u>. In recent years, macrocyclic bifunctional chelators such as the nitrobenzyl derivatives of DTPA, EDTA and tetraazacyclododecane-N-N',N'',N'''-tetraacetic acid (DOTA) have been shown to have improved stability over DTPA <u>in vivo</u> (Meares *et al.*, 1990). Despite favourable imaging properties, it has been shown that patients who were administered with <sup>III</sup>In experienced problems with increased accretion and residence of conjugates in the liver and spleen. The uptake by these organs leads to a waiting period of a number of days, during which non-tumour bound conjugate is removed before images can be obtained. Unlike <sup>99M</sup>Tc, <sup>III</sup>In is not routinely available in Nuclear Medicine Departments and this increases both the expense and the waiting time before

immuniscintigraphy can take place. In terms of availability, cost, gamma ray energy (140 keV) and user familiarity, <sup>99M</sup>Tc is the most desirable of all isotopes currently available for imaging. As <sup>99M</sup>Tc has a six hour half-life, it is essential to use F(ab'), fragments of mab (Figure 1.2) to expedite the clearance of unbound conjugate from the blood stream and non-tumour regions. Blood pool clearance is also affected by the choice of antibody (Section 1.8).  $F(ab')_2$  fragments clear more quickly from the circulating blood pool and have less non-specific interactions with the liver, due to the absence of the Fc receptor (Buraggi et al., 1987). <sup>99M</sup>Tc-F(ab')<sub>2</sub> fragments have been used to image colorectal and liver metastases (Granowska et al., 1990; Hertel et al., 1990). Improvements in the quality of 99MTcmab images result from the availability of improved labelling methods, especially in newly reported reduction procedures. The preparation of most <sup>99M</sup>Tc-F(ab')<sub>2</sub> conjugates involves the reduction of <sup>99M</sup>Tcpertechnate and the subsequent non-specific binding of the reduced technetium to donor groups present in the protein (such as thiol, amide, amino and carboxylate groups). Most of the 99MTcimmunoscintigraphy studies to date have used stannous chloride as the reducing agent (Rhodes and Burchel, 1983). Increasing numbers of reductants have been used for conjugation procedures with the aim of increasing the stability of <sup>99M</sup>Tc-mab conjugates in vivo. These reductants include dithiothrietol (DTT) (Pak et al., 1989), dithioerythritol (DTE) (Del Resallo et al., 1989), ascorbic acid, (Thakur and DeFulvio, 1991), dithionate (Fitzberg et al., 1988) and recently, 2-mercaptoethanol (Mather and Ellison, 1990).

In this section, the preparation of <sup>III</sup>In-mab conjugates by the DTPA anhydride method and of <sup>99M</sup>Tc-mab conjugates by 2-ME reduction are described. These conjugates were administered to patients with ovarian cancer and the results of the immunoscans will be discussed in detail.

A total of three IgG mabs which react with ovarian carcinoma cells were conjugated to DTPA. The antibodies used were HMFG-1, AUA-1 and H17E2; these antibodies are described in more detail in Section 2.5.1. Twenty one patients with ovarian carcinoma were administered with <sup>III</sup>In-DTPA-mab conjugates.  $F(ab')_2$  fragments of HMFG-1,  $(F(ab')_2)$ , were conjugated with <sup>99M</sup>Tc following reduction with 2-ME. One patient with ovarian carcinoma had residiual tumour and was imaged with <sup>99M</sup>Tc- $F(ab')_2$ . Extensive testing of both <sup>III</sup>In-mab and <sup>99M</sup>Tc- $F(ab')_2$  was undertaken to ensure that conjugates used were able to bind to tumours and antigen-bearing cells before administration to patients. In order to ensure the safety of the immunoconjugated antibody for administration to patients, the sterility and absence of pyrogenicity were determined. The results of the immunoscans were compared with suspected ovarian carcinoma and the advantages and disadvantages of <sup>III</sup>In-mab and <sup>99M</sup>Tc are discussed.

# 4.2. PREPARATION AND CHARACTERISATION OF "In-MAB AND ""Tc-F(ab')<sub>2</sub> CONJUGATES

#### 4.2.1. Preparation of "In-mab Conjugates.

HMFG-1, AUA-1 and H17E2 were labelled with <sup>III</sup>In by the DTPA anhydride method, which is outlined in Section 2.5.3. Hnatowich *et al.* (1983) showed that the conjugation of between 1 and 5 DTPA molecules to a range of IgG antibodies had no effect on antibody binding capacities of the resulting DTPA-mab conjugates. A molar ratio of 3.5:1 DTPA : antibody was used with all antibodies. As DTPA has a long shelf-life, it was possible to prepare DTPA-mab conjugates in batch form and to store in aliquots of 2 mg at 4°C. All conjugates were tested for their ability to chelate <sup>III</sup>In, to bind to target cells <u>in vitro</u> as well as for sterility and pyrogenicity before administration to patients. Since DTPA forms strong chelates with a large number of metals, it was important to minimise trace metal contamination prior to <sup>III</sup>In-chelation. Sterile plastics, sterile water for injection ( which was free of trace metals ) and acid-treated glassware were used to minimise the competition between trace metals in the mab preparation and <sup>III</sup>In.

#### 4.2.2. Preparation of <sup>99M</sup>Tc-F(ab')<sub>2</sub> Conjugates.

Though some investigators prepared <sup>99M</sup>Tc-mab conjugates using the DTPA anhydride method, the stability of these <sup>99M</sup>Tc-DTPA-mab conjugates in vivo was low (Childs and Hnatowich, 1985). Mather and Ellison (1990) showed that by reducing the di-sulphite bond in the  $F(ab')_2$  with 2-mercaptoethanol (2-ME) and reacting 2-ME-F(ab')<sub>2</sub> with the weakly competitive reductant methylene diphosphonate (MDP), stable <sup>99M</sup>Tc-F(ab')<sub>2</sub> conjugates could be formed.  $F(ab')_2$  fragments of HMFG-1 (referred to as  $F(ab')_2$ ) were reacted with 2-ME at a molar ratio of 1:1000  $F(ab')_2$ : 2-ME. Details of this procedure are given in Section 2.5.4. The effect of reduction on the  $F(ab')_2$  structure and antibody binding capacity will be reported in detail.

# 4.2.3. SDS-PAGE Analysis of <sup>III</sup>In-mab and <sup>99M</sup>Tc-F(ab')<sub>2</sub> Conjugates.

SDS-PAGE was used to determine the effect of DTPA conjugation and 2-ME reduction on mabs and  $F(ab')_2$ . While unconjugated HMFG-1 (Figure 4.2.1., Lane 2) appeared to be a homogenous intact IgG, conjugation of DTPA to HMFG-1 resulted in the formation of both higher and lower molecular weight complexes. As DTPA is a bifunctional chelator, more than one antibody molecule can bind to each DTPA molecule allowing for protein dimer and polymer formation. These aggregates would account for the higher molecular weight species seen in Lane 3. A significant proportion of DTPA-HMFG-1



Figure 4.2.1: SDS-PAGE Polyacrylamide Gel Electrophoresis (SDS-Page) of HMFG-1, DTPA-HMFG-1, F(ab')<sub>2</sub> and 2 ME - F(ab')<sub>2</sub> conducted under nonreducing conditions.

conjugated also formed a low molecular weight protein product. The extremes of pH used in the conjugation of DTPA to HMFG-1, and to a lesser extent, physical disruption during the manipulation, may have caused this fractionation of HMFG-1. These fragments may be light chains. The sterility and endotoxin tests showed there were no bacteria present in DTPA-HMFG-1 and therefore bacterial proteases were not responsible for this fractionation. Similar results were obtained for both DTPA-H17E2 and DTPA-AUA-1 conjugates. The reduction of  $F(ab')_2$  by 2-ME did not appear to result in F(ab) fragments or other light and heavy chain fragmentation (Lanes 4 and 5).

### 4.2.4. Immunoreactivity of DTPA-mab and 2-ME reduced <sup>99M</sup>Tc

DTPA conjugation or 2-ME reduction may affect the ability of a monoclonal antibody to bind to target and non-target antigens. Four techniques were used to assess DTPA-mab and 2-ME-reduced-F(ab')<sub>2</sub> (referred to as 2-ME-F(ab')<sub>2</sub>) conjugates. The non-radioactive methods used were FACS, ELISA and immunofluorescence and the Lindmo assay, which assesses the ability of <sup>10</sup>In-mab and <sup>99M</sup>Tc-F(ab')<sub>2</sub> to bind to antigen-expressing and non-antigen expressing cells <u>in vitro</u>. The results of each method are considered in turn.

#### 4.2.4.1. FACS Analysis

FACS was used to determine the number of cells which bound the mab as a percentage of the total cells which were incubated with antibody. Where the percentage of cells which bound DTPA-mab or 2-ME- $F(ab')_2$  differed from the percentage of cells which bound unconjugated antibodies, the immunoreactivity was said to be altered. Figure 4.2.2.(a) shows that HMFG-1 bound to 88.2% of MCF-7 cells, while 93.4% of MCF-7 cells bound DTPA-HMFG-1 on their surface membrane. Differences in the concentration of DTPA-HMFG-1 and HMFG-1 may explain the apparent increase in immunoreactivity of the DTPA-HMFG-1 conjugate. Plots 4.2.2. (b)-(d) show that there was no alteration in DTPA-HMFG-1 binding to EJ-138 cells, LOVO cells or RT-112 cells, when compared with unconjugated HMFG-1. While HMFG-1 did not react with EJ-138 or RT-112 bladder cell lines, the LOVO adenocarcinoma cell appears to express HMFG-1-like antigens. This cross-reactivity with colon adenocarcinoma was also observed by Taylor-Papadimitriou *et al.* (1981). The results of FACS analysis of DTPA-H17E2, DTPA-AUA-1, and 2-ME-F(ab')<sub>2</sub> using cell lines known to express these antigens, are shown in Figure 4.2.3 (a)-(c). No alteration in immunoreactivity can be seen between these chemically modified antibodies and their unconjugated equivalents.

ELISA a very useful initial test to examine the effects of conjugation or reduction on antibodies, but these results were not as reproducible as the results obtained by FACS analysis. Indirect



Figure 4.2.2: Assessment of the immunoreactivity of HMFG-1 and DTPA-HMFG-1 on cells lines using FACS analysis with (a) MCF-7 cells [which express the HMFG antigen], (b) EJ-138 cells, (c) LOVO cells and (d) RT-112 cells.



**Figure 4.2.3:** Comparison of the immunoreactivity of H17E2, AUA-1 and F(ab')<sub>2</sub> before and after chemical modification using FACS Analysis.

immunofluorescence analysis was also carried out to assess whether the antibodies bound to various cell types, and like ELISA, was used as a preliminary indicator of the immunoreactivity of the modified antibodies.

#### 4.2.4.2. ELISA and Immunofluorescence

A solid phase ELISA capture assay was used to determine immunoreactivity in vitro; details of the experimental technique used are given in Section 2.5.9. These results confirmed that all batches of conjugated antibody bound to target antigens. The ease of access to all necessary equipment made both ELISA and immunofluorescence convenient assays for the peliminary assessment of immunoreactivity.

#### 4.2.4.3. Lindmo Assay

The Lindmo assay assesses the immunoreactivity of a radiolabelled antibody by measuring the amount of radioactivity bound to varying dilutions of cells in <u>vitro</u>. Cells were incubated with excess radiolabelled antibody. The amount of radiolabelled antibody bound to the cells [B] is related to the total amount of radioactivity added to the cells [T] by the following equation:



The ratio [T]/[B] is related to the reciprocal of cell concentration by a constant 1/r, where r is defined as the immunoreactivity fraction of that conjugation. C defines the concentration of free antigen and  $K_{\star}$  is the association constant. The immunoreactivity fraction is independent of the cell numbers and the excess of antibody used is also a valuable indicator of batch-to-batch variations in different preparations of radiolabelled monoclonal antibodies (Lindmo *et al.*, 1984). Figure 4.2.4. illustrates the Lindmo plot of <sup>III</sup>In-HMFG-1 (Batch 3) when bound to MCF-7 cells in vitro. The intercept with the ordinate axis shows at 1/r = 0.51, r = 1.967. Three different batches of DTPA-labelled HMFG-1 were prepared and found to have immunoreactivity fractions of n = 2.5, 1.75 and 2.0, respectively. Single batches of DTPA-AUA-1 (r = 0.666), DTPA-H17E2 (r = 1.176) and <sup>99M</sup>Tc-F(ab')<sub>2</sub> (r = 1.81) were also prepared.



Figure 4.2.4: Determination of the immunoreactivity fraction, r, of <sup>111</sup>In-HMFG, assessed by the Lindmo Assay.

#### 4.2.5. Thin Layer Chromatography

Thin layer chromatography was used to determine the number of DTPA molecules which bound to each antibody molecule. It was also used as a quality control procedure to ensure radiolabelling of antibody prior to administration to the patient. TLC was carried out behind lead shielding and the plates were visualised using a GE type 400 AC Gamma Camera system. After the samples were resolved, images of the radiolabelled components were obtained using the General Electrics Software accompanying the camera system. Protein (mab) which had taken up the radiolabel was unable to migrate with the solvent front through the silica matrix and could be resolved from both the unbound radiolabel and DTPAradiolabel which migrated to the top of the plate. Therefore, the number of radioactive counts at the bottom of the TLC plate represented radiolabel bound to antibody while the counts at the top of the TLC plate represented a mixture of both free and DTPA-bound radiolabel, respectively. Figure 4.2.5.(a) illustrates the image of the TLC plate obtained after scanning with the gamma camera. The three lanes in the centre of the picture indicate the three TLC samples which were resolved. Lane 1 contained a sample of DTPA-HMFG-1 taken before free DTPA was separated. The gamma camera used to acquire the TLC plate image was unable to resolve <sup>10</sup>In from <sup>10</sup>In-conjugated to DTPA. The densitometry scan shown above the TLC plate shows that 18.2% of the total <sup>III</sup>In in this lane migrated to the top of the TLC plate. This implies that 18.2% of the DTPA did not bind to HMFG-1, and the actual molar ratio of DTPA : mab was 3.07:1, or 87.8% efficiency, in conjugating 3.5 moles of DTPA. Lane 2 represents a sample of <sup>III</sup>In-mab immediately before injection to the patient. Further analysis of this sample showed that there was no free <sup>m</sup>In present in the sample. Lane 3 contained free <sup>m</sup>In, and, was included as an experimental control.

Figure 4.2.5.(b) illustrates the images obtained on TLC analysis of various <sup>99M</sup>Tc samples. Lane 1 contains free <sup>99M</sup>Tc. The scan above the TLC plates relates to Lane 1 only and shows that the majority of the <sup>99M</sup>Tc migrated to the top of the plate. Lane 2 contained <sup>99M</sup>Tc mixed with 2-ME-F(ab')<sub>2</sub> immediately after mixing with the MDP kit, while Lane 3 shows <sup>99M</sup>Tc-F(ab')<sub>2</sub> before administration to the patient. This final <sup>99M</sup>Tc-mab mixture contained 19.72% unconjugated <sup>99M</sup>Tc. As the <sup>99M</sup>Tc-F(ab')<sub>2</sub> was eluted in the same fractions as unconjugated <sup>99M</sup>Tc, further purification using gel filtration (Section 2.4. .) was not possible. Granowska *et al.* (1990) achieved between 85-95% labelling efficiency by this method. However, Mather and Ellison (1990) commented that efficiency obtained when HMFG-1 was labelled with <sup>99M</sup>Tc was lower than the efficientcy of H17E2-F(ab')<sub>2</sub> fragment labelling.

#### 4.2.6. Pyrogen Detection

Bacterial lipopolysaccarides when administered to patients are known to cause a pyrogenic or fever reaction. All procedures were carried out under sterile and pyrogen-free conditions to avoid bacterial contamination. Each stage of the DTPA-mab and 2-ME-F(ab')<sub>2</sub> procedures was monitored for the presence of pyrogens using a KabiVitrum CoATest kit (Section 2.5.12.). All antibodies for administration had pyrogen levels below the sensitivity level of this kit (0.1 EU/ml = 12 pg/ml) and no

inhibition of the enzyme reaction was observed. All final batches of antibody for injection were also tested by the less quantitative Rabbit Pyrogen Test at Biological Laboratories (Ballina) Ltd., Carrentrila, Ballina, Co. Mayo, Ireland.

#### 4.2.7. Sterility Assessment

All antibodies were tested according to the protocols outlined in Section 2.5.11. None of the samples for injection in vivo had detectable microbial contamination (Biological Laboratories (Ballina) Ltd., Ballina, Co. Mayo, Ireland).



**Figure 4.2.5:** Images obtained following gamma camera scanning of <sup>III</sup> labeled antibody (A) Lane 1 contained <sup>III</sup>In, <sup>III</sup>In-DTPA and <sup>III</sup>In-DTPA-HMFG-1; Lane 2 contained <sup>III</sup>In-HMFG-1 before administration and Lane 3 contained free <sup>III</sup>In, and (B) <sup>99M</sup>Tc-F(ab')<sub>2</sub> samples where Lane 1 contained unbound <sup>99M</sup>Tc, Lane 2 contained <sup>99M</sup>Tc-MDP-F(ab')<sub>2</sub> and Lane 3 contained <sup>99M</sup>Tc F(ab')<sub>2</sub>. A densitometry scan of one lane of TLC each plate (Lane 1) is also shown in these photographs. Indicators showing radioactive intensity are also shown in each photograph.

#### 4.3.1. Patient Selection

<sup>10</sup>In-mab immunoscintigraphy was carried out on 21 patients with ovarian carcinoma at the end of their treatment with chemotherapy. <sup>III</sup>In-mab immunoscintigraphy was carried out in parallel with a number of routine non-surgical investigations, including measurment of serum CA-125 levels, CAT scan, X-ray and, in some cases, ultrasound (in 6 of 21 patients included in this study). Surgical evaluation by either laparotomy or laparoscopy was carried out where possible (in 17/21 patients). The histological findings following surgery were taken as the 'gold standard' result for the detection of ovarian carcinoma. In a separate study, one patient who also had an elevated serum CA-125 level, was evaluated using <sup>99M</sup>Tc- $F(ab')_2$  immunoscintigraphy soon after surgery to remove ovarian tumour.

#### 4.3.2. Choice of Monoclonal Antibody.

The tumour to background ratio is the term used to describe the ratio of antibody which was taken up by the tumour versus the amount of non-specific uptake of radioactivity in an area without tumour (in this case the pelvis or sacro-ileal crest) there days after the administration of <sup>III</sup>In-mab. Fifteen patients were administered <sup>III</sup>In-HMFG-1, three patients received <sup>III</sup>In-AUA-1 and three patients received <sup>III</sup>In-H17E2. Preliminary studies showed that <sup>III</sup>In-AUA-1 had a tumour to background ratio of 1.9 : 1, while <sup>III</sup>In-H17E2 had a tumour to background ratio of 1.44:1, indicating that these antibodies were not as efficient in distinguishing between areas of specific uptake by neoplastic tissue and non-specific uptake or the highest tumour to background (2.4:1) ratio of the three antibodies examined and was therefore used for the majority of the patients in the study.

#### 4.3.3. Interpretation of <sup>III</sup>In-mab Immunoscans.

All patients had planar (2-dimensional) scans of the chest and abdominal areas one hour after <sup>III</sup>In-mab injection (Day = 0). Follow-up scans were performed after 24 hours (Day = 1), Day 3 and, in some cases, on Day = 6. Tomographic (3-dimensional) scans were taken on Day 3 where the planar images showed abnormal uptake of <sup>III</sup>In.

Free <sup>III</sup>In is normally taken up by the liver, and, to a lesser extent, by vascular areas such as the bone marrow, spleen and kidney. In the absence of tumour, radioactivity recorded in these areas after the injection of <sup>III</sup>In-mab was reported as normal uptake or a negative immunoscan. Figure 4.3.1. shows



Figure 4.3.1:Gamma camera image of the chest area. Image 1 shows abnormal uptake in the<br/>lung, while Image 2 shows normal uptake in the liver, spleen and sternum.

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Figure 4.3.2:

Gamma camera images of the abdominal areas of patients administered <sup>111</sup>Inmabs. Image 3 shows uptake in the umbilical node, Image 5 shows diffuse uptake indicating peritoneal seedlings, while, Images 4 and 6 show background or normal uptake. <sup>In</sup>In uptake patterns in a positive (Image No. 1) and a negative (Image No. 2) immunoscan of the chest area. Image No. 2 shows <sup>III</sup>In-mab uptake in the top of the liver and in the sternum area. This was reported by the physician as background or normal uptake. Image No. 1 shows a diffuse uptake on the right hand side of the sternum. This represents uptake by a metastatic ovarian carcinoma in the right lung. The presence of metastatic tumour was confirmed by histology. Figure 4.3.2. represents planar immunoscans of the abdomen. The <sup>111</sup>In-mab uptake in the liver, spleen, bone marrow and pelvis (sacro-ileal crest) is normal uptake (Figure 4.3.2., Image No. 4). However, in Image No. 4 (Figure 4.3.2.), there was a small tumour nodule below the liver, which, on tomography was seen to be at the anterior of the patient. Surgery soon after the scan showed that this umbilical node was malignant. The distribution of ovarian cancer in the peritoneal cavity may affect the type of treatment that the patient will receive. Image No. 5 shows how a patient with peritoneal seedings was differentiated from a patient with a solid tumour using abdominal immunoscintigraphy. The diffuse uptake in the entire peritoneum was confirmed by laparotomy. Image No. 6 is a copy of Image No. 3 and was included for comparison purposes only. These scans show how <sup>III</sup>In-mab scans can be used to locate tumours. Their importance as part of a panel of investigations must also be evaluated, in order to determine if <sup>III</sup>In-mabs are a useful method of detecting disease routinely.

#### 4.3.4. <sup>III</sup>In-mab Immunoscans as a Non-Invasive Diagnostic Technique.

The immunoscans shown in Figures 4.3.1. and 4.3.2. are among the clearest images obtained in the <sup>fu</sup>Inmab study and these patients were known to have extensive disease. It is of much more relevance to be able to detect disease in patients with low levels of tumour, that is, to determine the lower limits of <sup>III</sup>In-mab sensitivity. The results of <sup>III</sup>In immunoscans were compared with the results obtained from other non-surgical evaluations such as serum CA-125 levels, CAT scan and chest X-ray. Where available, all of these non-surgical techniques were then related to the 'gold standard' result obtained from the histological evaluation of tumour tissue taken from patients during surgery (laparotomy or laparoscopy) or to disease progression. The results of all these evaluations are summarised in Table 4.3.1. and the relationship between <sup>III</sup>In-mab immunoscintigraphy and each technique is discussed.

#### 4.3.4.1. Chest X-ray in Ovarian Cancer

Chest X-ray is used to detect abnormal fluid in a patient's pleural cavity. This may indicate metastatic ovarian carcinoma in the form of a pleural effusion. Although patient no. 1 had a positive pleural effusion (Figure 4.3.1. [Image No. 1]), the results of the chest X-ray did not clearly indicate this. This patient also suffered from pulmonary sarcoidosis which would also have led to an abnormal X-ray result. Immunoscans were particularly useful in differentiating antigen-bearing tumour fluid from non-antigen bearing sarcoid tissue in this patient.

Patient	FIGO Stage	Serum CA-125 (IU/ml)	CAT Scan	Chest X-Ray	"In-mab	Histology
1	IV	1558	+/-	+/-	+	+
2	III <sub>R2</sub>	400	+/-	+/-	+	+
3	IV	192.1	-	-	+	+
4	III	77	+/-	-	-	+
5	п	49.9	-	-	-	+
6	ш	76.4	-	-	+	+
7	III	74.6	-	-	-	ND ( )
8	III <sub>R2</sub>	15.0	-	-	-	+
9	I	59.8	-	-	+	-
10	III	18.4	-	-	-	-
11	III	18.5	-	-	-	ND (-)
12	111	8.9	-	-	-	+
13	III <sub>R2</sub>	101.1	+	-	+	+
14	II <sub>A</sub>	9.1	-	-	-	-
15	IIB	26.0	-	-	-	-
16	III	62.5	+/-	-	+	+
17	Ι <sub>c</sub>	21.4	-	-	-	ND (-)
18	Ш	26	-	-	-	22
19	III	252.5	+	-	+	+
20	III	67.8	+	-	+	+
21	IV	26.8	1 (đ.)	16	*	÷.

Table 4.3.1: Results of the evaluation of 21 patients with ovarian carcinoma post-chemotherapy.

#### 4.3.4.2. CAT Scan and Ovarian Cancer

Computerised Axial Tomography (CAT) scan is used clinically to determine the density of tissue. Abnormal density changes in tissues may indicate the presence of a tumour mass. CAT scan is used routinely in the follow up of patients with ovarian carcinoma. The first line of treatment for these patients is usually surgical removal of the tumour and, in many cases, hysterectomy, bi-lateral salpingo-oophorectomy (BSOH), and further debulking surgery are also undertaken. As a result of surgery, many of these patients have scar tissue and fibroid masses which have abnormal tissue densities. Such masses can not be distinguished from residual or recurrent tumour in the peritoneal cavity using present CAT scan techniques. Table 4.3.1. shows that while 8/13 patients had true positive immunoscans, 3/13 patients had true positive CAT scans. An <sup>III</sup>In immunoscan would, therefore, appear to be more sensitive than a CAT scan in the evaluation of ovarian carcinoma in this patient group.

#### 4.3.4.3. Serum CA-125 and Ovarian Cancer

The OC-125 monoclonal antibody was first reported by Bast et al. (1981) and it reacts with CA-125 . CA-125 is an ovarian cancer-associated antigen (200,000 kD molecular weight) that is expressed in 80% of non-mucinous epithelial ovarian carcinomas. This antigen is detected in the sera of patients with intra-peritoneal disease and it has been shown to be a sensitive, but non-localising indicator of persistent and recurrent disease and has also been used to monitor a patient's response to treatment (Muto et al., 1989). A normal CA-125 is given as less than 35 IU/ml. Levels of serum CA-125 greater than 35 IU/ml are considered a sensitive indicator of disseminated disease in ovarian carcinoma patients (Tholander et al., 1990). From Table 4.3.2. it can be seen that 10/13 patients with elevated CA-125 levels had detectable disease, while 9/13 of these patients had positive immunoscans. All patients with positive immunoscans had elevated serum CA-125 levels. The correlation between serum CA-125 levels and <sup>III</sup>In-mab scanning is examined in more detail in Table 4.3.2. Three distinct groups can be observed on the basis of serum CA-125 levels. The first group contained five patients with serum CA-125 levels greater than 100 IU/ml. All of these patients had positive immunoscans regardless of which antibody was used. The second group contained seven patients with serum CA-125 levels between 35-100 IU/ml. No direct correlation between the results of immunoscintigraphy, serum CA-125 and histology could be determined in this patient group. While three patients had positive immunoscans and histology, another group of three patients had false negative immunoscans. One patient (No. 9) had false-positive serum CA-125 and immunoscans. This gives an overall accuracy of 42% in this patient group, while CAT scan had a 14% accuracy level in this patient group. The third group contained nine patients with normal serum CA-125 levels (less than 35 IU/ml). All patients with negative serum CA-125 levels had negative immunoscans. However, two patients (Nos. 8 and 12) had false negative immunoscans and serum CA-125 levels when compared to the histology results.

Patient (No. from Table)	<sup>111</sup> In-immunosc (Antibody, [Res	an ult])	Serum CA-125 (IU/ml)	Histology
1	HMFG-1	+	1558	+
2	HMFG-1	+	400	+
19	H17E2	+	252.5	+
3	HMFG-1	+	192.1	+
13	HMFG-1	+ 101.1		+
6	HMFG-1	+	76.4	+
20	H17E2	+	67.8	+
16	AUA-1	+	62.5	+
9	HMFG-1	+	59.8	-
4	HMFG-1	- 77		+
7	HMFG-1	-	74.6	N.D. (+)
5	HMFG-1	-	49.9	+
21	H17E2	-	26.8	-
15	HMFG-1	-	26.0	N.D. (-)
18	AUA-1	-	26	-
17	AUA-1	- 21.4		N.D. (-)
11	HMFG-1	- 18.5		N.D. (-)
10	10 HMFG-1		18.4	-
8 HMFG-1		-	15	+
14	HMFG-1	-	9.1	-
12	HMFG-1	-	8.9	+

Table 4.3.2. Relationship between serum CA-125 levels and <sup>111</sup>In immunoscans.

Where: (+) = positive result, (-) = negative result, N.D. () = result of clinically observed turnour progression and not at histological evaluation.

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In summary, serum CA-125 appears to be a more sensitive indicator of the presence of ovarian carcinoma than <sup>III</sup>In-mabs. <sup>III</sup>In-mabs are particularly useful in determining the distribution of tumour in patients with elevated serum CA-125 levels greater than 100 IU/ml.

#### 4.3.5. Sensitivity and Specificity of <sup>III</sup>In-mabs Conjugates.

The sensitivity and specificity of radiolabelled mabs are terms which are used to assess the overall success of tumour localisation and for comparison purposes between studies. Sensitivity is calculated by expressing the number of true positive (TP) tumours which were visualised as a percentage of the number of TP plus false negative (FN) scans, i.e.  $(TP/[TP + FN]) \times 100\%$ . The specificity is defined as the number of true negative patients (TN) expressed as a percentage of TN and false positive (FP) results, i.e.  $(TN/[TN + FP]) \times 100\%$ .

Table 4.3.3. Sensitivity and Specificity of <sup>III</sup>In-mabs.

Number of Patients	ТР	FN	TN	FP	Sensitivity	Specificity
21	8	3	9	1	72.7%	90%
Where $TP = true positive$ , $TN = true negative$ , $FN = false negative$ , $FP = false positive$						

The results contained in Table 4.3.3. relate to the overall study, and, will be compared to the results obtained by other groups.

#### 4.3.6. <sup>99M</sup>Tc-F(ab')<sub>2</sub> Immunoscintigraphy.

<sup>99M</sup>Tc-F(ab')<sub>2</sub> was administered to one patient with residual ovarian carcinoma. This patient had had extensive surgery a number of weeks before immunoscintigraphy and also had a negative CAT scan at that time. Her serum CA-125 level at the time of immunoscintigraphy was 294.2 IU/ml, indicating significant residual disease. Non-specific or non-tumour uptake of <sup>99M</sup>Tc-F(ab')<sub>2</sub> differs from the uptake already seen with <sup>III</sup>In-mab conjugates. <sup>99M</sup>Tc uptake was observed in the kidney, stomach and bladder. Free <sup>99M</sup>Tc would normally be seen in the stomach, but, <sup>99M</sup>Tc-F(ab')<sub>2</sub> appears to bind to the colon area. Four planar scans of the peritoneal and pleural cavities were recorded at 10 minutes, 2 hours, 6 hours and 26 hours after <sup>99M</sup>Tc-F(ab')<sub>2</sub> administration. These scans are shown in Figure 4.3.3.(a). After 10 minutes, the blood pool was observed in the kidneys, liver and bladder areas. The diffuse area below the left kidney could not be explained by blood pool. The scan taken at 2 hours again showed blood pool levels in the kidney and bladder. By 6 hours, some colon uptake was apparent. The high level of radioactive uptake seen in the right kidney was attributed to overlap with the liver and colon areas. The area of abnormal uptake in the right lower abdomen may be neoplastic and was reported as a positive scan (Figure 4.3.3.(b)). The image obtained after 26 hours showed  $^{99M}$ Tc-F(ab')<sub>2</sub> uptake in the colon. This uptake was considered to be normal uptake. As this patient had undergone recent surgery, a follow-up laparotomy was not performed. However, significant residual ovarian cancer in the peritonealical method of immunoscintigraphy than <sup>III</sup>In-mab immunoscanning and its application to othertumour will be discussed in Section 4.4.4.



**Figure 4.3.3:** Gamma camera immunoscans of a patient who was administered <sup>99M</sup>Tc-F(ab')<sub>2</sub> conjugate. (A) shows images after 10 minutes (Image No. 5), 2 hours (No. 6) 6 hours (No. 7) and after 26 hours (No. 8). (B) is an enlargement of the image obtained after 6 hours (No. 7). 4.4.

#### 4.4.1. Preparation of <sup>III</sup>In-mab and <sup>99M</sup>Tc-F(ab')<sub>2</sub> Fragments.

The aim of any protocol by which antibodies are labelled for use in clinical studies is to produce a conjugate with high stability in vivo using a procedure with minimal effects on the immunoreactivity of the antibody. The physical stability of DTPA-mab and of 2-ME-F(ab')<sub>2</sub> was monitored by SDS-Figure 4.2.1. (Lane 3) shows that higher molecular weight complex and some mab PAGE. fragmentation could be seen in DTPA-HMFG-1 conjugates; these were also seen in DTPA-H17E2 and DTPA-AUA1 conjugates. The lower molecular weight fragments accounted for a large proportion of the total antibody injected. They may have resulted from denaturation of antibody due to the pH or other physical conditions arising during the labelling procedure. Dimer and polymer formation were found to increase with the molar ratio of DTPA : mab. Hnatowich et al. (1983) estimated that approximately 15.5 - 18.7% of the DTPA : mab conjugate would be in the form of higher molecular weight conjugates. The presence of larger DTPA-mab polymers has significant clinical implications. Firstly, larger complexes are more immunogenic and may lead to a HAMA reaction (Section 1.2.1.). However, no patient in this study developed a detectable HAMA reaction following <sup>111</sup>In-mab immunoscintigraphy. As no patient received more than one dose of antibody, it must be noted that few patients were expected to develop HAMA reactions. Secondly, larger molecular complexes may have altered immunoreactivity with the target antigens. FACS analysis showed that the overall immunoreactivity of DTPA-HMFG-1 remained virtually unchanged when compared with unconjugated HMFG-1. Finally, larger molecular weight complexes may also accumulate in the liver, increasing the non-specific background in the scan and reducing the target to background ratio. The precise effect of <sup>In</sup>In-mab accumulation in the liver in this study could not be determined. However, Peltier *et al.* (1987) reported that the target to background ratio of <sup>III</sup>In-F(ab')<sub>2</sub> fragments was far superior to that of <sup>III</sup>Inmabs. The absence of Fc receptors and a molar ratio of 2 DTPA : 1 F(ab')<sub>2</sub> significantly reduced the non-specific uptake of these conjugates. New macrocylic chelators are more stable in vivo and can bind sufficient radiolabel when using lower molar ratios of chelator. Meares et al. (1990) showed that chelation of <sup>III</sup>In to nitrobenzyl-DOTA resulted in no fragmentation or complexation. Despite the relatively severe reduction step, no bands corresponding to possible antibody fragments were seen in the F(ab')<sub>2</sub> fragments reduced by two 2-ME. Other <sup>99M</sup>Tc-labelling techniques have been shown to lead to colloid complex formation and polymer formation when analysed by high pressure liquid chromatography (HPLC) (Thakur and DeFulvio, 1991).

Neither DTPA conjugation nor 2-ME reduction affected the immunoreactivity of the antibody conjugates. Each of the four methods used to assess immunoreactivity had its own specific merits. The

cell based ELISA used here provided a very rapid assay system requiring the use of very small amounts of antibody. However, some problems were encountered with the reproducibility of the assay due to the growth pattern of the LOVO cells used in measure immunoreactivity of DTPA-H17E2 and unconjugated H17E2.

This cell line grows in multi-layers rather than as a sheet of single cells. This leads to uneven sloughing of cells from the 96 wells during the washing procedures and therefore to inaccurate results. The high cost of purified antigens precluded the use of antigen capture ELISA system in this study.

Immunofluorescence was found to be a useful method for assessing the immunoreactivities of antibodies on a wide range of cells and tissues. All of the cell-lines used for immunofluorescence analysis were tumour derived. The inclusion of normal colon tissue in the immunoreactivity assessment might have indicated the high levels of uptake of <sup>III</sup>In-AUA-1 and <sup>III</sup>In-H17E2 by colon cells before immunoscans were performed. Immunofluorescence analysis on primary tumour samples from Patients 8 and 12 may also have shown that these tumours did not express HMFG-1 before <sup>III</sup>In-mab immunoscintigraphy was carried out. Facilities for performing immunofluorescence and ELISA were available locally. Both techniques gave qualitative assessments of alterations in immunoreactivities of DTPA-mab and 2-ME- $F(ab')_2$  and were useful for preliminary investigations into immunoreactivity without the use of radioisotopes.

FACS analysis provided a quantitative measure of the immunoreactivity of each antibody with various tumour cells. The patterns of antibody binding (Figures 4.2.2. and 4.2.3.) were useful in comparing variabilities between the three preparations of DTPA-HMFG-1. No variability between preparations was observed. FACS analysis showed that HMFG-1 and DTPA-HMFG-1 reacted with a subpopulation of LOVO cells. The results of the <sup>III</sup>In-HMFG-1 immunoscans did not show significant uptake by normal colon. The uptake observed in the 26 hour <sup>99M</sup>Tc-F(ab')<sub>2</sub> immunoscan by normal colon or non-tumour tissue (Figure 4.3.3.(a)) was thought to be due to the vascularity of the colon rather than the presence of antigen-bearing cells. Immunofluorescence analysis of these conjugates on normal colon tissue may have indicated this uptake before in vivo administration.

ELISA, immunofluorescence and FACS were used to assess immunoreactivity. All these techniques compared mab to DTPA-mab and 2-ME-F(ab')<sub>2</sub>. However, the Lindmo assay assessed the immunoreactivity of radiolabelled <sup>III</sup>In-mab and <sup>99M</sup>Tc-F(ab')<sub>2</sub> conjugates. The determination of numerical values for immunoreactivity which could be compared from batch-to-batch and from laboratory-to-laboratory was a further control which ensured the safety of conjugates for human administration. The Lindmo assay showed that both intact HMFG-1 (r = 2.08 ± 0.3) and F(ab')<sub>2</sub> fragments of HMFG-1, (r = 1.81) had similar immunoreactivity fractions. Thus, pepsin digestion of

the intact mab to produce F(ab')<sub>2</sub> fragments had little effect on the immunoreactivity fraction in vitro.

The safety of the DTPA-mab and 2-ME-F(ab')<sub>2</sub> conjugates for human administration was assessed by pyrogen and sterility testing. The rabbit pyrogen test is still used by the FDA as the definitive method for determining the pyrogenicity of a product. This test is particularly unsuitable for the testing of murine antibody products as the rabbit may have anti-mouse antibodies, (RAMA). This pyrogen test result would falsely indicate the toxicity of the immunoconjugate (Brown, 1990). However, the LAL assay provides a quantitative and reproducible method for the determination of bacterial endotoxin levels during conjugate preparation. Endotoxins were not detected within the assay range (0.1 - 1.2 EU/ml). The maximum permitted endotoxin level for immunoglobulin products for human administration is 350 EU/ml (or per 70 kg body weight) (Brown, 1990). As less than 1 ml (2mg) of antibody was administered to each patient, all antibody preparations were considered pyrogen-free.

#### 4.4.2. <sup>III</sup>In-mab Immunoscintigraphy.

<sup>III</sup>In-labelled monoclonal antibody was successfully used to determine the distribution of tumours in ovarian carcinoma patients. The results of <sup>III</sup>In-mab immunoscintigraphy which was performed on these patients will be discussed. The usefulness and potential of <sup>III</sup>In-mabs in routine evaluation of ovarian cancer will be determined and compared with the current non-surgical techniques used, such as X-ray, CAT scan and serum CA-125.

#### 4.4.2.1. Choice of Antibody.

<sup>III</sup>In-HMFG-1, <sup>III</sup>In-H17E2 and <sup>III</sup>In-AUA-1 conjugates were all used to localise ovarian cancers. The <sup>III</sup>In-HMFG-1 antibody was found to have a tumour to background ratio of 2.4:1 (in organs except the liver), while <sup>III</sup>In-AUA-1 and <sup>III</sup>H17E2 had slightly lower tumour : background ratios of 1.9:1 and 1.44:1, respectively. As a result of this ratio, two further batches of HMFG-1 were prepared. The high tumour to background ratio seen with <sup>III</sup>In-HMFG-1 was also reported by Hird *et al.* (1990), (10:1) and by Epenetos *et al.* (1982), who recorded a ratio of 8.5:1, and is thought to result from the high levels of expression of HMFG on breast and ovarian tissues (Verhoeyn *et al.*, 1990). Epenetos *et al.* (1985), reported the use of <sup>III</sup>In-H17E2 for the detection of neoplasms of the testes, ovary and cervix. Patients (n = 6) with cystadenocarcinoma of the ovary, who had pelvic masses were successfully imaged, but patients with tumour deposits of 1 - 2 cm diameter were not successfully imaged. The reduced sensitivity of H17E2 was thought to be due to the presence of trace levels of PLAP, the target antigen of H17E2, on many healthy tissues (McLaughlin *et al.*, 1984). As PLAP is not ovarian tumour-specific, it was used by Pectasides *et al.* (1990) to image patients with germ cell tumours of the testes. They found that the accuracy of <sup>131</sup>I-H17E2 imaging was related to the degree of differentiation of the primary tumour. In their study, heterogeneity of H17E2 was observed and higher levels of PLAP were found on active germ cell tumours than on more differentiated tumours.

A similar heterogeneity of tumour expression was found in studies using the AUA-1 antibody. Löhde *et al.* (1990) reported a higher target to background ratio for well differentiated colon tumours (5.88:1) when compared with less differentiated tumour (0.81:1) when using <sup>131</sup>I-AUA-1. Immunohistochemical analysis of bladder carcinomas confirmed this heterogeneous expression of the AUA-1 antigen (Anagnostaki *et al.*, 1990). Therefore, the higher tumour to background ratio observed with <sup>III</sup>In-HMFG-1 appears to be related to the expression of the human milk fat globule antigens on 90% of both undifferentiated and well differentiated ovarian tumours (Ward *et al.*, 1987). The high tumour to background ratio observed with HMFG-1 has led to novel applications for this mab. Y<sup>90</sup>-HMFG-1 has been used in the immunotherapy of patients with minimal residual ovarian carcinoma (Hird *et al.*, 1990) and studies to produce humanised HMFG-1 by CDR grafting (Section 1.4.2.) are at an advanced stage (Verhoeyen *et al.*, 1990) for both imaging and therapy. Snook *et al.* (1991) reported the use of a reshaped or humanised monoclonal HuZ PLAP (reshaped H17E2) labelled with DOTA-<sup>III</sup>In on six patients with colon adenocarcinoma. However, 3/6 patients developed anti-DOTA responses, and tumours were not imaged.

## 4.4.2.2. Sensitivity and Selectivity of <sup>III</sup>In-mab Immunoscintigraphy.

In order to determine the usefulness of <sup>III</sup>In-mab immunoscintigraphy in the evaluation of ovarian carcinoma, the results of this study were compared with other studies using similar mabs to locate ovarian cancers. The sensitivities and selectivities (as defined in Section 4.3.5.) obtained in these studies are summarised in Table 4.6.

In the research described in this thesis, an overall sensitivity of 73% and a selectivity of 90% was obtained. These results appear to be comparable to those obtained in other studies (Table 4.6.1.) which use a variety of radioisotopes and antibody types. The sensitivity observed in studies using  $F(ab')_2$  fragments (Chatal *et al.*, 1987) does not appear to be significantly greater than that observed with intact antibodies, despite faster blood clearance and lower liver accumulation.

# 4.4.2.3. Comparison of the Results of Chest X-ray and CAT Scan with <sup>III</sup>In-mab.

Chest X-ray and CAT scan are used routinely in the follow-up of patients with ovarian carcinoma (Goldhirsh *et al.*, 1983). In this study, both chest X-ray and CAT scan were of limited value in the detection of residual disease. CAT scan detected tumours in 3/13 patients having histologically positive tumour at second-look laparotomy (or by disease progression) while 9/13 patients had positive

Antibody	Туре	Isotope	No. of	%	%	Reference
			Patients	Sensit.	Select.	
HMFG-1/HMFG-2	IgG	<sup>131</sup> I	15	62	-	Epenetos et
						al. (1982)
HMFG-2	IgG	<sup>123</sup> I	1.4	91	· •	Granowska et
						al. (1984)
HMFG-2	IgG	<sup>131</sup> I	17	90	÷	Paleisky et al.
						(1985)
OC-125	F(ab') <sub>2</sub>	<sup>111</sup> In	15	67	-	Chatal et al.
						(1987)
OC-125	F(ab') <sub>2</sub>	<sup>111</sup> In	20	72	86	Chatal et al.
						(1987)
CEA/OC-125	F(ab') <sub>2</sub>	<sup>111</sup> In	23	88	50	Hunter et al.
		111-		0		(1987)
CEA/OC-125	F(ab') <sub>2</sub>	i'''In	12	91.6	-	Liehn et al.
		131-		100		(1987)
79IT/36	lgG2D		2	100	-	Liehn et al.
	LOT(-b)	1111- 4311	70	07.5	79.0	(1987)
0C-125/HMFG-2	$1gG/F(ab)_2$		/8	91.5	/6.9	McPowell <i>et</i>
		1117_	405	07	67	al. (1987
		1317	403	0/	07	Foon <i>et al</i> .
		123 T				(1990)
	F(ab') <sub>2</sub>	1				
OVTL-3		<sup>111</sup> In	42	86	-	Kanamana at
UVIL-5	F(ab') <sub>2</sub>		12			d (1000)
OC-125		<sup>111</sup> In	47	94	67	Dulin at al
						(1991)
						(1))1)
HMFG-1	IgGla	<sup>IN</sup> In	21	72.7	90	This study
AUA-1						
H17E2						

<u>Table 4.4</u>: Summary of the Sensitivities and Selectivity obtained in studies using immunoscintigraphy of ovarian cancer patients.

Where: Sens. = sensitivity = (IP/[IP + FN]) x 100%

Select. = selectivity =  $(TN/[TN + FP]) \times 100\%$  as in Section 4.3.5.).

immunoscans. The increased sensitivity of <sup>111</sup>In immunoscintigraphy over CAT scan has also been reported by Chatal *et al.* (1985), and with <sup>131</sup>I-mabs by Granowska *et al.* (1984), Benner *et al.* (1990), Barzon *et al.* (1991) and Dulin *et al.* (1991) stressed the *complementary roles* that may be played in tumour diagnosis by each of these techniques, rather than the advantage of immunoscans over CAT scans in the evaluation of ovarian cancer.

#### 4.4.2.4. Relationship Between Serum CA-125 Levels and Immunoscintigraphy.

<sup>III</sup>In-mab immunoscintigraphy located neoplastic tissue in 9 tumours out of a total of 13 patients with ovarian carcinoma while abnormal serum CA-125 levels were observed in 11 out of 13 patients. Tholander *et al.* (1990) observed that serum CA-125 levels can be related to the *extent* of disease. Their study found that patients with limited ovarian epithelial caricnoma had a mean serum CA-125 level of 151 IU/ml, while a mean of 805 IU/ml was determined for patients with extensive disease. Many immunoscintigraphy studies have shown that intact antibodies in particular are unable to specifically localise tumour in patients with limited disease (Thomas *et al.*, 1990). In this study, the accuracy of <sup>III</sup>In-mab immunoscintigraphy in patients with limited disease (CA-125 levels between 35-100 IU/ml) was compared with the accuracy of immunoscintigraphy observed in patients with more extensive disease (serum CA-125 levels greater than 100 IU/ml). From Table 4.3.2., it can be seen that all patients with serum CA-125 levels greater than 100 IU/ml had positive immunoscans, while the accuracy (3/6) of immunoscintigraphy in patients with serum CA-125 levels between 35 - 100 IU/ml was much lower. This suggests that serum CA-125 is a sensitive indicator of the presence of limited disease, while immunoscintigraphy is a less sensitive technique for the localisation of tumours in patients with limited disease.

Many immunoscintigraphy studies using <sup>III</sup>In-OC125 and <sup>131</sup>I-OC125 (Table 4.4.) have also reported on serum CA-125 levels of patients used in these studies. This antigen is present in the blood stream as serum CA-125 and on the surface of the tumour as tumour associated (t) CA-125. <sup>III</sup>In-OC125 bound to serum CA-125 increases the level of label in the blood stream and reduces the target to background ratio. The production of human anti-murine (OC-125) antibodies following immunoscintigraphy has been shown to interfere with serum CA-125 assays conducted following immunoscintigraphy (Muto *et al.*, 1989). Problems with interference by anti-OC-125 antibodies in immunoscintigraphy were not reported (Chatal *et al.*, 1987; Kenemans *et al.*, 1990) although some interference by HAMA antibodies was found in subsequent serum CA-125 assays (Muto *et al.*, 1989). In studies by Peltier *et al.* (1987) and Chatal *et al.* (1987) few patients with serum CA-125 levels less than 100 IU/ml were included. Chatal *et al.* (1987) found that 18/24 (75%) of patients with proven ovarian carcinoma and CA-125 levels levels greater than 100 IU/ml had positive immunoscans, while 2/4 (50%) with serum CA-125 levels

in the range 35 - 100 IU/ml had positive scans. Patients in the study by Hunter *et al.* (1987) were preselected for immunoscintigraphy on the basis of the results of pelvic examination and CAT scan, and not their serum CA-125 level. A similar pre-selection of patients on the basis of a positive ultrasound scan was seen by Peltier *et al.* (1987) and Barzon *et al.* (1991). The increased sensitivity and selectivity of <sup>10</sup>In-OC125 immunoscintigraphy observed by Dulin *et al.* (1991) may reflect this pre-selection of patients with highly elevated CA-125 levels (94%) for immunoscintigraphy.

#### 4.4.2.5. Usefulness of <sup>III</sup>In-mab Immunoscintigraphy in Ovarian Carcinoma.

The results of this study suggest that <sup>III</sup>In-HMFG-1 immunoscintigraphy is a useful method for determining the distribution of tumour in patients with ovarian carcinoma. The technique proved to be most sensitive in patients with more extensive disease and serum CA-125 levels greater than 100 IU/ml. Though chest X-ray and CAT scan are less sensitive techniques, they complement the results of CA-125 assays and <sup>III</sup>In-mab immunoscans. Finally, <sup>III</sup>In-mabs may be an effective, if costly method to assess ovarian carcinoma when used as part of a panel of non-invasive techniques.

#### 4.4.3. Limitations of <sup>III</sup>In-mab Immunoscintigraphy.

A realistic assessment of <sup>III</sup>In-mabs immunoscintigraphy must take into account several limitations which exist with <sup>III</sup>In. Firstly, <sup>III</sup>In is not readily available in most Nuclear Medicine Departments and it is costly to purchase. The delay incurred in the delivery of the isotope increases the time taken to process immunoscan request. Aside from these logistic problems, <sup>III</sup>In is not an ideal isotope. <sup>III</sup>Inimmunoscintigraphy may still take up to 72 hours to complete (Britton et al., 1989) even using F(ab'), and F(ab) fragments. Advances in the digital subtraction of non-specific uptake of <sup>III</sup>In in the liver have reduced the interference of <sup>III</sup>In blood pooling to minimal levels (Vaughan et al., 1987), but further models would be needed to reduce this blood pool to the levels seen with <sup>99M</sup>Tc (Thomas et al., 1990). The energy emissions of <sup>m</sup>In are not optimal for the gamma camera equipment available in most Nuclear Medicine Departments. <sup>III</sup>In has two gamma ray energies; the higher 247 keV energy requires that the gamma camera is used with a heavier collimator which reduces the sensitivity and resolution which may be achieved. <sup>99M</sup>Tc has a gamma ray energy of 140 keV which is optimal for the standard equipment in Nuclear Medicine Departments. An example of the improved sensitivity of 99MTc-F(ab'), over <sup>III</sup>In-mabs can be seen in the TLC analysis of these conjugates. The spots recorded for <sup>III</sup>In samples (Figure 4.2.5.(a)) are significantly less clear than those seen for <sup>99M</sup>Tc (Figure 4.2.5.(b)), even though equal levels of radioactivity were recorded in each scan. As <sup>III</sup>In has a 72 hour half-life, the radiation dose absorbed by the patient is higher than that absorbed by patients administered with 99MTc- $F(ab')_2$  which has a half-life of six hours. When <sup>III</sup>In-labelled antibodies are metabolised in the reticuloendothelial system, the <sup>III</sup>In is deposited in tissues giving high liver and bone marrow background and, in some cases, high activity in the large bowel. Ovarian tumours located in these areas are often more dificult to locate with <sup>III</sup>In-mabs. Buy *et al.* (1988), have reported that CAT scanning is particularly sensitive in locating tumours in the perihepatic and subdiaphramal regions which <sup>III</sup>In-mabs that second look laparotomy often fails to detect. This strengthens the proposal that <sup>III</sup>In-mab is most useful as part of a panel of non-invasive and invasive techniques for tumour localisation.

#### 4.4.4. <sup>99M</sup>Tc-mab Immunoscintigraphy in Ovarian Cancer.

Stable <sup>99M</sup>Tc-labelled HMFG-1-F(ab')<sub>2</sub> fragments were successfully used to locate ovarian carcinoma in a patient with known residual ovarian carcinoma in the peritoneal cavity. Figure 4.3.(a) shows that the images obtained after 6 hours were of diagnostic quality. The absence of free <sup>99M</sup>Tc in the stomach demonstrated the stability of the <sup>99M</sup>Tc-conjugate <u>in vivo</u>. The clearance of free <sup>99M</sup>Tc-conjugate from the blood pool by 6 hours was also reported by Granowska *et al.* (1990; 1991), using <sup>99M</sup>Tc-PRIA3 in colorectal cancer. In these studies, target to background ratios of up to 63:1 were obtained. The stability of these <sup>99M</sup>Tc-labelled antibodies <u>in vivo</u> has been attributed to the 2-ME reduction procedure used. Intact mabs labelled with <sup>99M</sup>Tc by the 2-ME method were also used to image granulocytes associated with inflammatory processes (Lind *et al.*, 1990). An overall sensitivity of 95% was observed in this study, but some residual uptake in the colon was also found. Eary *et al.* (1990) used <sup>99M</sup>Tc-F(ab')<sub>2</sub> and F(ab) fragments to image patients with melanoma. These fragments were labelled with the bifunctional chelating agent dithionite (Fritzberg *et al.*, 1988). F(ab) fragments had faster clearance rates than F(ab')<sub>2</sub>. No details were given referring to the stability of the antibody conjugate <u>in vivo</u>, but the presence of intestinal activity and images of stomach activity indicated free isotope.

<sup>99M</sup>Tc is continuously available in all Nuclear Medicine Departments. It is cheap and gives a low radiation absorbed dose and it is ideally suited to the modern gamma camera collimator. The stability of <sup>99M</sup>Tc-F(ab')<sub>2</sub> conjugates produced by the 2-ME/MDP reduction method has overcome the problem of free <sup>99M</sup>Tc interference in imaging. The major advantages of <sup>99M</sup>Tc-F(ab')<sub>2</sub> over <sup>III</sup>In-mab scanning are the efficiency in terms of both cost and convenience. A request for immunoscintigraphy received in the morning can be undertaken and a provisional result given at the end of the same afternoon. This result may be confirmed by imaging after 26 hours. This shows that the immunoscintigraphy of cancer can be a routine, rather than a specialised technique.

#### 4.4.5. Future Role of Immunoscintigraphy in Cancer Diagnosis.

The ability to produce <sup>99M</sup>Tc-labelled mabs shows that the chemistry associated with antibody labelling has advanced significantly in recent years. Further development in bifunctional chelators and methods for the protection of the antibody binding site during conjugation (Kröhn and Eary, 1991) will further increase the efficiency of mab conjugations. Parallel developments in the identification of newer tumour antigens, the production of second generation mabs and the application of new software to analyse the images obtained, and improved gamma cameras, will all increase the sensitivity and selectivity of immunoscintigraphy.

The ideal tumour antigen for use in immunoscintigraphy should be present on tumour cells but not on normal tissue. This ideal antigen should be abundant on the surface of the cell (greater than 10<sup>6</sup> antigen sites per cell), and should be present at all stages of tumour differentiation (Larson, 1985). The antigen should be present on the surface of the cell but not shed into the blood stream to neutralise injected antibody and delay rates of clearance from the blood pool. Studies have shown that antigen sites inside the cell may also be available for imaging (Ronay *et al.*, 1990). Although the HMFG and CA-125 antigens have been useful in a large proportion of ovarian cancers, and CEA, PLAP, AUA-1 and hCG have been useful markers in a wide range of other tumour types, new antigens are constantly being found. Tumour associated glycoprotein (TAG72) (Correale *et al.*, 1991), the TAC antigen (Brown *et al.*, 1991) and certain oncogene-related antigens such as p97 (Rose *et al.*, 1986), <u>neu-oncogene</u> (Drebin *et al.*, 1988) and c-<u>myc</u> (Chan and Sikora, 1987) have all been proposed as potential targets for immunoscintigraphy. However, the low levels of expression of many of the oncogene markers and their homology with normal antigens on the surfaces of cells has reduced their potential as imaging antigens (Hellström and Hellström, 1989).

Developments in murine antibodies, human mabs (hmabs) and recombinant or second generation antibodies, are constantly being applied to immunoscintigraphy. The difficulties encountered in the production of stable hmab have limited their applications in tumour imaging to date. Studies by Masuho *et al.* (1990) showed that the half-life of human mab <u>in vivo</u> is between 22 - 26 days and they also found a six-fold increase in stability <u>in vivo</u> over murine antibodies. Although these properties are of greater significance for mab-therapy than imaging, their reduced immunogenicity <u>in vivo</u> would allow repeated application of antibody for both imaging and therapy. Ryan *et al.* (1988) used hmab <sup>III</sup>In-IgM conjugate to image patients with ovarian carcinoma. The slow clearance of IgM (i.e., increased circulation time required to clear blood pool) and poor resolution of images appears to suggest that IgM hmabs have few advantages over cheaper, more easily produced murine mabs. However, cardiac imaging with a human IgG anti-myosin monoclonal antibody is now commercially available in kit form. Centocor, the immunopharmaceutical company, have launched a product called 'Myoscinct', for imaging
myosin-associated myocardial necrosis (Strauss *et al.* 1991; Van Dam Mieras *et al.*, 1991). Reulander *et al.* (1991) have also shown that these mabs may be of use in imaging neonatal and infantile tumours with a higher myosin content than normal tissue. The production of 'Myoscinct' represents a landmark in mab technology. The commercialisation of one mab product provides a test case for future mab products (Spalding, 1991).

Recombinant mabs are currently being produced (Section 1.4.) and evaluated for use in imaging and therapy. Chimaeric antibodies which consist of the entire variable region of the mouse antibody with a human constant antibody fragment have been used in vivo. Chimeric antibodies are easier to produce in large quantities than human mabs but are, in theory, less immunogenic. However, when the chimaeric anti-OKT3 antibody was used in patients, much of the antibody response was directed against the human variable region rather than the mouse constant region (Jaffers et al., 1986) a human antihuman (HAHA) reaction. Chimaeric antibodies appear to be more immunogenic if their light or heavy chain V region is not homologous to any human V region (Co and Queen, 1991(b)). However, studies by Bischof-Delalyoe et al. (1989) showed that the <sup>99M</sup>Tc-chimaeric CEA antibodies were less immunogenic than <sup>99M</sup>Tc-murine CEA antibodies when used to image patients with colorectal carcinoma. With the general trend towards using F(ab')<sub>2</sub> murine antibody fragments in immunoscintigraphy, the advantages of chimeric antibodies over murine monoclonal antibodies appear to be minimal. Theoretically, the immunogenicity of humanised or re-shaped hmabs which consist of only six murine complementary-determining regions (CDRs) embedded in a framework region should be lower than murine or chimeric antibodies. To date, few studies have used humanised mabs for imaging, but they have been used to treat patients with non-Hodgkins lymphoma (Hale et al., 1988). Both of the patients in this study experienced allergic responses after repeated therapy. These reactions were not attributed to HAMA production. The fast rate of clearance from the blood stream seen with humanised anti-TAC antibody suggests that these antibodies have favourable clearance properties. Hu-mabs have improved half-lives, stabilities and are less immunogenic when compared to murine antibodies in vivo (Coughlan, 1991). These properties will allow multiple therapy and imaging protocols to be established using humanised mabs. Humanised HMFG-1 and H17E2 are currently being evaluated for use in clinical trials on ovarian carcinoma (Verhoeyn et al., 1990; Snook et al., 1991).

Chemically produced bifunctional antibodies (BFAs) (Section 1.5) have been used to image patients with colorectal carcinoma (Stickney *et al.*, 1988). One arm of the antibody bound to the CEA antigen while the second arm of the antibody bound <sup>III</sup>In-benzyl-EDTA. Approximately 95% of the known lesions were detected by this system with efficient blood clearance. There are some limitations to the use of BFAs in vivo. When the two arms of an antibody bind to antigens, the binding is said to be co-operative. By binding only one arm of an antibody to the cell, the immunoconjugate bound less tightly than a monoclonal antibody and is more sensitive to shearing factors from blood. One approach used

to improve, or to exploit, this concept was proposed by Wong *et al.* (1987). BFAs which bound to CD3 and CD4, and a second BFA which bound to CD3 and CD8 antigens were produced. Cell binding and cell toxicity were increased by 25 - 31 times by this stimulated co-operative binding. As there are two antigens available to BFA binding, there is an increased in the antigen density or number of binding sites for antibody on tumours. By targeting combinations of antigens which react with both well-differentiated and undifferentiated cells, increase in the tumour to background ratio is achievable. This approach may prove more effective than the cocktails of murine, human or humanised mabs proposed by Glassy and Dillman (1988) and Fanger *et al.* (1991).

Attempts to reduce non-specific uptake of isotopes and antibody in the liver by the administration of a BFA reacting with a breast carcinoma antigen and a hapten 3-(3'-iodo-, 4'-hydroxy)-phenyl propionic acid have also been proposed (Bator and Reading, 1990). This hapten forms the labelling group of iodinated Bolton-Hunter reagent (IBH). The administration of the non-radiolabelled antibody before the radiolabelled IBH, allows minimal non-specific absorption of the radiolabelled BFA. Similar two stage immunotherapy strategies such as the delayed administration of the radiolabel using streptavidin-labelled antibodies and biotinylated radioisotopes have also been suggested with the aim of reducing non-specific background levels of radiolabel using the BFA concept (Paganelli *et al.*, 1991). The use of BFAs comprised of hmabs or other recombinant mabs will be of clinical importance in the application of second generation mabs <u>in vivo</u> (Nolan and O'Kennedy, 1990; Fanger *et al.*, 1991).

Recombinant and bifunctional mabs may allow more stable conjugates to be administered with a greater frequency. This is particularly important when both immunoscintigraphy and immunotherapy are conducted on the same patient. Further developments in the use of alternative routes of administration, such as immunolymphoscintigraphy (Lehtovirta *et al.*, 1990) and improvements in the time-course of antibody and isotope administration (using the avidin/biotin systems) may increase the attainable target to background ratio. The use of imaging during surgery, as proposed by Jäger *et al.*, (1990), may also help identify tumour sites *during* second-look surgery.

Progress in imaging technology will also affect the future role of monoclonal antibodies in the evaluation of tumours. Position emission tomography, (PET), allows the detection of smaller lesions and will permit quantitative imaging (Larson, 1991). By combining the information obtained from magnetic resonance imaging, (MRI), PET, CAT scan with images obtained from immunoscintigraphy, a more complete panel of non-invasive techniques will be available. Fusion of these images by computer graphics techniques (Davis, 1990; Kramer *et al.*, 1991) will enhance the potential of non-invasive tumour diagnosis by such techniques rather than by repeated surgery.

SECTION 5 : IMMUNOPHOTODYNAMIC THERAPY

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#### 5.1 INTRODUCTION

When a monoclonal antibody (mab) which reacts with a tumour associated antigen (Taa), is administered to a patient, many non-tumour cells such as Kupffer cells and macrophages bind the Fc portion of the mab non-specifically. In Section 4 it was shown that the use of  $F(ab')_2$  fragments and digital subtraction techniques can minimise the effect of this non-specific uptake when tumours are localised by immunoscintigraphy. However, when mabs are conjugated to toxic agents such as drugs, toxins and istopes, this non-specific uptake leads to the killing of non-tumour cells.

In Section 1.10, various two-stage immunotherapy strategies such as the use of prodrugs, biotin-avidin and photodynamic therapy (PDT) with mab-photosensitiser immunoconjugates were outlined. PDT relies on the interaction of light with a photosensitiser to produce a localised toxic effect in a cell or organ. Photosensitisers are only partially selective for tumour tissue and light can only be delivered to an area, not a tissue type. This leads to the destruction of healthy tissue in the irradiated area. By increasing the ability of photosensitisers to selectively accumulate in tumour cells rather than healthy cells, non-specific phototoxicity to normal cells could be minimised. Such targeting of these photosensitisers to tumour sites in vivo may be achieved by coupling the photoactive agent to a mab with specificity for antigens on the surface of tumour cells. The use of mab-directed photosensitisers is termed immunophotodynamic therapy, (IPDT).

The major objectives of this experimental section were:

- (a) To compare two known photosensitising agents, hematoporphyrin (Hp) and its more photoactive derivative, HpD, with acridine orange, coumarin, 7-hydroxycoumarin, 4hydroxycoumarin and amino-methylcoumarin acetic acid, (AMCA) using an <u>in vitro</u> assay system. All of these compounds have known fluorescent and photochemical properties, and, some have been used clinically as laser dyes. However, their potential as photosensitisers has not been fully explored to date.
- (b) The second experimental objective was to produce an immunophotosensitiser for use in IPDT. Although HpD is an effective photosensitiser, the chemical composition of the photoactive components of HpD is still disputed (Byrne *et al.*, 1990). This chemical complexity makes the stoichiometry of reactions between HpD and proteins difficult to predict and monitor. As Hp is a single compound, the reaction chemistry with a mab is more defined and accurate

molar ratios for conjugation can be predicted. Hp was conjugated to the mab HMFG-1 which reacts with antigens on the surface of breast and ovarian tumours. HMFG-1 has been used for both immunoscintigraphy (Section 4) and immunotherapy (Epenetos *et al.*, 1985). The techniques which were used to assess the immunorectivity of mabs following conjugation to DTPA (Section 4.2) were also used to determine if chemical conjugation of Hp to HMFG-1 altered the immunoreactivity of the mab. Preliminary experiments to determine the ability of HMFG-1-Hp to selectively kill MCF-7 breast adenocarcinoma cells following irradiation were undertaken using an <u>in vitro</u> assay system.



Figure 5.1.:Schematic view of the system used to irradiate cells in vitro. Broadband light<br/>(200 mW) from a Xenon Arc Lamp was reflected by a mirror onto the cells to<br/>be irradiated. Cells were irradiated for a total of 20 minutes.

### THE EVALUATION OF HEMATOPORPHYRINS, ACRIDINE ORANGE AND COUMARIN COMPOUNDS AS PHOTOSENSITISERS

The effectiveness or efficacy of Hp, HpD and other test compounds as photosensitisers was determined by their ability to kill Landschütz ascites tumours (LAT cells) in vitro following irradiation. An in vitro assay system was designed which used LAT cells in 24 well and 6 well tissue culture plates and broadband Xenon Arc Lamp light. The method used is outlined in Section 2.2.5. The experimental irradiation system which was used is shown schematically in Figure 5.1. All work was undertaken in darkened conditions to minimise non-specific activation of photosensitisers. A large number of control experiments were necessary to eliminate the effects of light and drug toxicity. These control experiments included cells which were treated with drug but which were not irradiated. This control was included in order to assess drug toxicity in the absence of light, i.e. its drug toxicity. Similarly, cells irradiated in the absence of drugs, were included to determine the effect of the irradiation protocol (200 mW irradiation for 20 minutes) on the viability of cells in culture, i.e. light toxicity.

From Figure 5.2.1.(B), it can be seen that at a concentration of 0.1 mg/ml, Hp exhibits greater phototoxicity than drug toxicity. The  $IC_{50}$  is the term used to describe the concentration of drug at which 50% of the cells are growth inhibited. The  $IC_{50}$  for Hp is 0.048 mg/ml following irradiation but is greater than 0.1 mg/ml for drug when not irradiated. Similarly, Figures 5.2.1.(A) and 5.2.3. give  $IC_{50}$  values of 0.02 mg/ml and 0.042 mg/ml for HpD and acridine orange respectively, following irradiation. However, when higher doses of HpD (0.1 mg/ml) and acridine orange (1.0 mg/ml) were added to the cells, some cells were killed in the absence of light. These figures also show that  $IC_{50}$  drug toxicity values for both HpD and acridine orange in the absence of irradiation were greater than 0.1 mg/ml and 1.0 mg/ml, respectively. Table 5.1.1. summarises the  $IC_{50}$  values obtained for hematoporphyrin and other test photosensitisers.

Viability was assessed in these experiments by dye exclusion methods (Section 2.3.3.). However, it has been suggested that such staining techniques detect membrane damage and do not directly measure cell viability (Yuhus *et al.*, 1974). As Hp and HpD are known to result in membrane lipid oxidation, cell viability following irradiation was also assessed by LAT cell transplantability studies (Section 2.5.7.). Table 5.2.2. shows that the average dry weight of the untreated control cells was 0.44  $g \pm 0.16$  g (mean weight of tumour  $\pm$  standard deviation) after 7 days growth in vivo. The average weight of tumour obtained from mice (n = 5) whose cells had been treated with HpD and irradiated was 0.05  $g \pm 0.02$  g. This value represents a significant reduction in cell dry weight (p < 0.002, Student's t-test) confirming the results shown in Figure 5.2.2. Therefore, the toxicities of Hp, HpD and acridine orange are considerably increased following irradiation.

5.2.



**Figure 5.2.1.:** The effect of HpD (A) and Hp (B) on the viability of Landschütz ascites tumour (LAT) cells in the presence (**●**) and absence (**○**) of 200 mW Xenon Arc Lamp broadband light (Bars indicate standard deviation).



Figure 5.2.2.: The effect of acridine orange on the viability of LAT cells in the presence (•) and absence (O) of 200 mW Xenon Arc Lamp broadband light (Bars indicate standard deviation).

Table 5.2.1. shows that incubation of LAT cells with varying concentrations of coumarin,

7-hydroxycoumarin, 4-hydroxycoumarin and AMCA for 18 hours, in the presence or absence of irradiation, had no effect on tumour viability in <u>vitro</u>. None of these compounds showed any potential as photosensitisers under the conditions used.

Several researchers have shown that coumarin affects the growth of cells in culture (reviewed by Egan *et al.*, 1990). LAT cells were incubated with coumarin and AMCA for two days before washing and transplantation into Schofield mice. Table 5.2.3. showed that incubating LAT cells with coumarin for two days (short-term) before tansplanting the cells had no effect on the mean weight of that was tumour harvested when compared to the transplanted cells which were not pre-incubated with coumarin.

The effect on LAT cell viability following incubation with coumarin for 9 days in vitro before transplantation are shown on Table 5.2.3. and in Figure 5.2.3. ( $IC_{50} = 0.375$  mM). From these results, 7-hydroxycoumarin appears to have cytostatic rather than cytotoxic effects on LAT cells in vitro. Figure 5.2.3. shows that the number of LAT cells fails to increase following treatment with 7-hydroxycoumarin even though the viability of the cells was unaltered (Figure 5.2.4.).

Compound	IC <sub>50</sub> (irradiated)	IC <sub>50</sub> (not irradiated)
Нр	0.048 mg/ml	> 0.1 mg/ml
HpD	0.02 mg/ml	> 0.1 mg/ml
Acridine orange	0.042 mg/ml	> 0.1 mg/ml
Coumarin	> 1 mM	> 1 mM
7-hydroxycoumarin	> 1 mM	> 1 mM
4-hydroxycoumarin	> 1 mM	> 1 mM
AMCA	> 1 mM	> 1 mM

<u>Table 5.2.1</u>: Summary of the  $IC_{50}$  values recorded for compounds in the presence and absence of light.

 Table 5.2.2. The Results of a Transplantability Study to Determine the Effect of Photodynamic

 Irradiation on the Growth of Landschütz Acites Tumour Cells In Vivo

Treatment	Concentration of HpD (per ml <sup>-1</sup> )	Irradiation	Tumour wt.* (g)
None	0.00	+	$\begin{array}{c} 0.44 \pm 0.16 \ (5) \\ 0.47 \pm 0.1 \ (4) \end{array}$
НрD	0.025 0.025	+	$0.05 \pm 0.02 (5)^{**}$ 0.41 \pm 0.11 (4)

\* Mean dry weight of tumour in  $g \pm S.D.$ ; number of determinations in parenthesis

\*\* Significantly different from control (P < 0.002)

Treatment	Concentration of Coumarin of Derivatives (mM)	Drug Incubation Period, <u>in vitro</u> (days)	Tumour wt.* (g)
None	0.00	2	0.30 ± 0.04 (5)
Coumarin	1.00	2	0.30 ± 0.08 (5)**
АМСА	1.00	2	0.30 ± 0.01 (3)**
None	0.00	9	0.43 ± 0.10 (6)
Coumarin	0.20	9	0.25 ± 0.06 (6)
Coumarin	1.00	9	$0.05 \pm 0.04 \ (6)^+$

# Table 5.2.3. The Results of a Transplantability Study on LAT Cells Treated with Coumarin Compounds

AMCA = Amino-methyl-coumarin acetic acid

\* Mean dry weight of tumour in  $g \pm S.D.$ ; number of determinations in parentheses

\*\* Not significantly different from control



Figure 5.2.3.: The effect of coumarin (△) 7-hydroxycoumarin (●) and control medium (O) on the growth of LAT cells during a 9-day incubation period in vitro. Drugs were used at a concentration of 0.5 mM. (Bars indicate standard deviation of mean).



**Figure 5.2.4.:** The effect of coumarin (O) 7-hydroxycoumarin (●) and AMCA (△) on the growth of LAT cells during a 9-day incubation period. (Bars indicate standard deviation of mean).

The aim of attaching a mab to a photosensitiser is to increase the selectivity of the drug for target tissues expressing antigens for that mab. The conjugation procedure used should also ensure that the monoclonal antibody retains its specificity for the target tissue following chemical modification, i.e. that the antibody retains its specific immunoreactivity (Lindmo *et al.*, 1984). Moreover, chemical conjugation may also alter the drug toxicity of a compound (reviewed by Koppel, 1990). Therefore, control experiments were undertaken to monitor the effect of conjugation on the selectivity of the antibody and the efficacy of Hp.

#### 5.3.1. Conjugation of Monoclonal Antibody to Hp

HMFG-1 was linked to hematoporphyrin dichloride by reaction with the water soluble carbodiimide, 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide (EDC) as outlined in Method 3.5.2. EDC was used to activate the carboxyl groups on the Hp molecule forming an 0-acyl-isourea intermediate compound. This intermediate then reacted with amino groups on the protein forming an amide linkage and releasing N-acetyl urea. The amino groups were provided by the lysine residues in HMFG-1. A molar ratio of 300:1 (Hp : HMFG-1) was selected for this conjugation following preliminary experimentation with a range of mabs including H17E2 and AUA-1. The results obtained following SDS-polyacrylamide gel electrophoresis (SDS-PAGE) are shown in Figure 5.3.1. Lane 4 indicates that the covalent conjugation of Hp to HMFG-1 resulted in the formation of larger molecular weight compounds which were not present in unconjugated HMFG-1 (Lane 2). These complexes may be due to cross-linking reactions between HMFG-1 molecules resulting in HMFG-1-HMFG-1 conjugates. As the antibody contains both free carboxyl groups and amino groups, this cross-linking can be mediated by EDC. Non-covalent attachment of Hp to HMFG-1 had no effect on the molecular weight observed (Lane 3).

The SDS-PAGE gels were run under non-reducing conditions, and, as a result of this, the exact molecular weights of the HMFG-1-Hp conjugate and higher molecular weight species were not determined. BSA-Hp conjugates were also prepared and were used as control conjugates in IPDT experiments in vitro to monitor toxicity which was not caused by HMFG-1-Hp. Such toxicity may have been caused by the presence of undialysed EDC and hydrolysed 0-acyl-isourea intermediate compounds in the BSA-Hp or HMFG-1-Hp preparations.



Figure 5.3.1.: Determination of the effect of EDC conjugation and non-covalent attachment of Hp to the monoclonal antibody HMFG-1, using SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

#### 5.3.2. Determination of the Hp content of HMFG-1-Hp

Protein molecules adsorb porphyrin molecules in a non-specific manner by non-covalent association (Mew et al., 1983). Thin layer chromatography was used to determine the proportion of Hp which was non-covalently attached to HMFG-1-Hp and the amount of Hp which was chemically conjugated to HMFG-1. Though dialysis removed most of the unconjugated Hp, the HMFG-1-Hp conjugate contained approximately 19% Hp which was non-covalently attached to HMFG-1. Spectrophotometric analysis of the conjugate at 383 nm, showed that an average 0.270 mg Hp were bound to each mg HMFG-1, representing a molar ratio of 87.1:1 (Hp: HMFG-1). However, only 60 of the total of 90 amino sites present in the antibody molecule are available for carbodiimide conjugation (Goodfriend et al., 1964). The excess of Hp molecules in the HMFG-1-Hp was attributed to non-specific absorption. Mew et al. (1983; 1985), used gel filtration to purify the HMFG-1-Hp conjugate after dialysis. Attempts to purify HMFG-1-Hp by gel filtration were unsuccessful due to the absorption of HMFG-1-Hp onto the G-25 column. The limited availability of HMFG-1-Hp and the high cost of HMFG-1 prevented further experimentation to optimise the conditions used for gel filtration. Ultrafiltration using a 30,000 m.w. cut-off membrane (Millipore) replaced gel filtration and reduced the unconjugated Hp content in HMFG-1-Hp from approximately 30% to approximately 19%, while recording a 90% recovery of protein.

#### 5.3.3. FACS Analysis of HMFG-1-Hp

In Section 4.2.4., methods for assessing the effect of chemical modification on the specific binding of mab-conjugates to various cells (i.e. the immunoreactivity of the conjugates) were outlined. FACS analysis may be used to establish if conjugation has altered the percentage of cells which bound unconjugated HMFG-1. Figure 5.4.1. shows that HMFG-1-Hp conjugate bound to a higher proportion of MCF-7 cells (97.4%) than an equal concentration of free HMFG-1 (86.6%). The MCF-7 cell line is estimated to express  $2 - 5 \times 10^5$  HMFG-antigens on the surface of each cell ( M. Verhoeyn, Unipath Ltd., Colworth, UK, personal communication), and is, therefore, considered highly reactive with the HMFG-1 antibody. Fluorescence by Hp molecules (or autofluorescence) was not detected at the scanning wavelengths used. Both HMFG-1 (55%) and HMFG-1-Hp (76.6%) bound to a portion of LOVO colon adenocarcinoma cells suggesting the LOVO cells may express a HMFG-1 like antigen at some stages of the cell cycle. Similar binding patterns were observed when FACS analysis was carried out on HMFG-1, DTPA-HMFG-1 and HMFG-1-Hp conjugates (Section 4.2.4.). Free HMFG-1 did not bind to the RT-112 bladder carcinoma cell line while HMFG-1-Hp bound to the cells under identical conditions. Neither HMFG-1-Hp nor HMFG-1 bound to the EJ-138 bladder carcinoma cells. These results were confirmed by cell-based ELISA (data not shown).



Figure 5.3.2: Assessment of the immunoreactivity of the monoclonal antibody HMFG-1 and the immunophotosensitiser HMFG-1-Hp using FACS analysis, on (a) MCF-7 cells; (b) EJ-138 cells; (c) LOVO cells and (d) RT-112 cells.

#### 5.4.1. <u>In Vitro</u> Assay

An <u>in vitro</u> assay system was developed to test whether Hp which was bound to HMFG-1 retained its phototoxic potential. Details of the assay system are given in Section 2.4.6. The control experiments conducted to monitor non-specific irradiation, cytotoxic effects of the monoclonal antibody and drug effects are described in Section 5.4.2.

MCF-7 and EJ-138 cells were incubated with varying concentrations of HMFG-1-Hp and were irradiated with 200 mW Xenon Arc Lamp light for 20 minutes. Graph 5.4.1. shows that HMFG-1-Hp had significant effects on MCF-7 cell viability following irradiation, but had no effect on viability in the absence of light. The IC<sub>50</sub> (with irradiation) for HMFG-1-Hp was found to be 2.75 x  $10^{-2}$  mg/ml HMFG-1-Hp. As it was previously shown that HMFG-1-Hp contains 87.1 Hp molecules per HMFG-1 molecules, this suggests that this concentration of HMFG-1-Hp contains a maximum of 7.6 x  $10^{-3}$  mg/ml of Hp (both conjugated and non-specifically bound). This also suggests that HMFG-1-Hp is significantly more toxic than equivalent concentrations of free Hp in vitro (Figure 5.4.1.(A)). Incubation of HMFG-1-Hp with EJ-138 cells, in the presence or absence of irradiation, had no significant effects on overall cell viability (Figure 5.4.1.(B)). These results indicate that HMFG-1-Hp can be used as an immunophotosensitiser to selectively kill cells which express the HMFG-1 antigen.

#### 5.4.2. Non-specific Controls in Immunophotodynamic Therapy.

A large number of control experiments were conducted in order to ensure that the MCF-7 cell killing seen in Figure 5.4.1. was not non-specific toxicity. As in Figure 5.4.1., EJ-138 cells were included as non-antigen bearing control cells. Two types of controls were conducted. The first group of controls monitored the effects of light and various compounds on the viability of MCF-7 and EJ-138 cells, where the compound was *removed* after an incubation period of 30 minutes. These controls monitored antibody binding and the uptake of unconjugated Hp during the 30 minute HMFG-1-Hp incubation period. In the second group of controls, the test compound were *not removed* before irradiation. These were included to determine if HMFG-1-Hp had sufficient phototoxicity to kill MCF-7 and EJ-138 cells *irrespective* of binding to antigens on the tumour cell surface. All controls were irradiated. The results of these controls are shown in Figure 5.4.2.

Control 1 was included as a standard, and showed maximal EJ-138 and MCF-7 cell growth. Unconjugated Hp is only weakly soluble in DMEM- $S_{10}$ . Therefore, Hp was dissolved in a minimal

volume of ethanol, before being diluted to the appropriate drug concentration. Control 2 showed that this minimal volume of ethanol had no significant effect on MCF-7 or EJ-138 viability. Controls 3 and 4 showed that a small amount of unconjugated Hp was taken up by both EJ-138 and MCF-7 cells. The uptake appeared to be proportional to the concentration of free drug in the medium. Hp concentrations greatly exceeded the total amount of Hp (bound to HMFG-1) that was added to MCF-7 and EJ-138 cells which was reported in Figure 5.4.1. Therefore, non-specific uptake of Hp during the 30 minute incubation period was not responsible for the MCF-7 toxicity shown in Figure 5.4.1. Control 5 was included to see if non-covalent attachment of Hp to HMFG-1 resulted in cell toxicity. A reduction in the viability of both cell types EJ-138 (82.5%) MCF (86%) was observed. This toxicity was due to non-specific uptake of Hp and not due to HP which was non-covalently attached to HMFG-1. This effect was evident in both antigen-bearing and non-antigen bearing cells.

In Control 7, Hp was not removed. Both the Hp which had been taken up by the cell, and Hp in DMEM- $S_{10}$  could produce singlet oxygen following light activation, leading to cell death. Similarly in Control 6, the HMFG-1-Hp which had bound to the antigen and unbound HMFG-1-Hp were available for light activation and singlet-oxygen production. However, the HMFG-1-Hp conjugate (0.1 mg/ml) contained a maximum of 0.027 mg/ml Hp. That HMFG-1-Hp killed similar numbers of EJ-138 and MCF-7 cells shows that binding to the MCF-7 cells was not necessary to cause cell damage using HMFG-1-Hp.

The BSA-Hp control (8) contains total of 0.023 mg/ml of Hp (bound and free) which did not have a significant effect on cell viability. This suggests that undialysed EDC and O-acyliso-urea intermediates did not cause cell death. Control 9 was included to examine whether or not HMFG-1-Hp bound to the same antigen as free HMFG-1. Pre-incubation of MCF-7 cells with HMFG-1 (0.1 mg/ml), should block all antigens to which HMFG-1-Hp could bind. The failure of HMFG-1-Hp to kill cells following pre-incubation with HMFG-1 confirms that pre-binding of HMFG-1 to MCF-7 cells blocked subsequent binding by HMFG-1-Hp and that antigen recognition by HMFG-1 was not adversely affected by coupling to Hp. A similar control was included in the Lindmo Assay in order to detect altered binding of mabs which had been conjugated to DTPA (Section 4.2.4.3.). Further controls which were not irradiated did not effect MCF-7 or EJ-138 cell viability. All these control experiments confirm that the selective killing of MCF-7 cells shown in Figure 5.4.1. was due to the immunophotosensitiser HMFG-1-Hp and not the non-specific absorption of Hp by the cells.



Figure 5.4.1:The effect of the immunophotosensitiser HMFG-1-Hp on (A) MCF-7 cells and<br/>(B) EJ-138 cells, in the presence of (•) and absence (O) of 200 mW Xenon Arc<br/>Lamp light.



Figure 5.4.2:Determination of non-specific antibody and drug effects associated with the use<br/>in immunophotodynamic therapy (IPDT) in vitro. HMFG-1-Hp was used as the<br/>immunophotosensitiser and 200 mW Xenon Arc Lamp light was used to irradiate<br/>the cells.

immunophotosensitiser and 200 mW Xenon Arc Lamp light was used to irradiate the cells.

#### 5.5. DISCUSSION

The hematoporphyrins, acridine orange and coumarins and the immunophotosensitiser HMFG-1-Hp were shown to affect cancer cell growth in vitro. Each of the following topics will be discussed in more detail.

- (1) The toxicity and phototoxicity of acridine orange and hematoporphyrins.
- (2) The cytotoxic and cytostatic effects of coumarin compounds.
- (3) The potential of HMFG-1-Hp as an immunophotosensitiser.
- (4) The evaluation of the potential of IPRT, ADEPT and other two-stage immunotherapy systems and to look at other delivery systems such as liposomes and polymer systems in cancer therapy.

#### 5.5.1. Hematoporphyrin Compounds and Acridine Orange as Photosensitisers in PDT.

Dougherty and his colleagues (1976) showed that incubating TA-3 mouse carcinoma cells with between 0.36 - 0.54 mg/ml Hp resulted in the death of 90% of the cells ( $IC_{90}$ ) in vitro. In another set of experiments by Gomer *et al.* (1983; 1985), using a different incubation time and different irradiation system, concentrations of Hp (0.060 mg/ml) and HpD (0.025 mg/ml) resulted in 50% growth inhibition of of Chinese Hamster Ovary (CHO) cells. Figure 5.2.1. shows Hp (0.048 mg/ml) and HpD (0.020 mg/ml) resulted in the death of 50% of LAT cells in vitro. As these results agree with the studies by Gomer and his colleagues. Therefore, the LAT cell assay appear to be a useful system to determine the potential of various compounds as photosensitisers.

The IC<sub>50</sub> of acridine orange (0.042 mg/ml) was determined using this assay system. This confirms the results of <u>in vitro</u> studies using acridine orange which were undertaken by Tomson *et al.* (1974), and Alvarez (1975). However, acridine orange is a known mutagen that intercalates DNA. Although many of the chemotherapeutic drugs which are currently in use are extremely mutagenic, the large doses of acridine orange which would be needed to cause phototoxicity would limit the potential of acridine orange in PDT.

#### 5.5.2. Toxicity of Coumarin Compounds.

None of the coumarin compounds investigated showed any phototoxicity in the presence or absence of irradiation in the LAT assay system previously described. Photodynamic activity had been previously reported for some coumarin compounds including the furocoumarins (Rodighiero et al., 1984), and benzyl-coumarin derivatives (Kaidbey Klingman, 1981; Opdyke, 1981). However, both coumarin and 7-hydroxycoumarin inhibited the growth of LAT cells, when incubated with the cells for 9 days. The IC<sub>50</sub> for coumarin (9 days) was found to be 375 µM, while 7-hydroxycoumarin appeared to have cytostatic effects. Cox et al. (1989) found that both coumarin and 7-hydroxycoumarin inhibited Sp2/0 myeloma cells. In a separate study, Moran (1990) showed that 7-hydroxycoumarin (500 µM) had a cytostatic rather than a cytotoxic effect on G-CCM and G-UVM human brain cell lines while Conely and Marhsall, (1987) also showed evidence of the cytostatic action of 7-hydroxycoumarin on ACHN and Caki-2 renal cells and on the K562 erythroleukemic cell line. Conely and his colleagues reported that coumarin acted by arresting cells in the  $G_1/G_1$  stage of the cell cycle, preventing entry into the S phase. The 18 hour incubation period which was used in the LAT cell PRT assay system was too short to observe the cytotoxic and cytostatic effects of coumarin. Variations in the metabolism of coumarin by different animal species (Raunio et al., 1988; Dominguez et al., 1990) and metabolic activation of the cytochrome p450 multienzyme complex (reviewed by Egan et al, 1990) may account for some of the conflicting reports on the cytotoxic/cytostatic actions of coumarin compounds.

#### 5.5.3. Evaluation of the Immunophotosensitiser HMFG-1-Hp.

Photosensitisers have been conjugated directly to mabs or have been attached via carrier molecules and spacer molecules (including dextran and polyvinyl alcohol) and they have also been encapsulated in microspheres and liposomes (summarised in Table 5.5.1.).

The HMFG-1-Hp immunophotosensitiser used in this experimental section, was prepared by labelling HMFG-1 directly with Hp. HMFG-1-Hp was shown to contain 87.1 Hp molecules per HMFG-1 molecules with approximately 19% of the Hp non-covalently attached to the mab. Mew *et al.* (1983; 1985) reported conjugation ratios in excess of 60 Hp/mab, while Steele *et al.* (1988) reported that ratios of up to 100 Hp/mab could be obtained by direct conjugation using EDC. They also reported that at these higher conjugation ratios, the antigen-binding capacity of the immunophotosensitiser was severely limited. The results of FACS analysis (Figure 5.3.2.) and ELISA showed that steric hindrance by Hp did not reduce the specificity of the HMFG-1-Hp.

The results of SDS-PAGE (Figure 5.3.1.) showed that there were large molecular weight complexes in the HMFG-1-Hp preparation. Excess EDC may have led to the conjugation of HMFG-1 to HMFG-1,

resulting in complex formation. A HAMA response may be produced when large molecular weight complexes are injected into patients. Steele *et al.* (1988) also reported significant antibody-antibody complexing when the anti-T suppressor factor B16G was conjugated to Hp by the EDC method outlined above. They also reported a significant decrease in the solubility of the mab after conjugation to Hp in aqueous solutions.

Oseroff *et al.* (1985) conjugated Hp dihydrazine to a number of anti-leukaemic mabs via the carbohydrate residues on the Fc portion of the mab. Conjugation ratios of 0.85 - 3 Hp/mab were recorded with no alteration in immunospecificity. In a later series of experiments, Oseroff and his colleagues (1986) conjugated a larger number of chlorin  $e_6$  molecules to a carrier (dextran) and subsequently conjugated the carrier to the carbohydrate residues on the Fc portion of antibody. Conjugation ratios of up to 24 - 36 chlorin  $e_6$ /mab were achieved using this method. Similarly, Rakestaw *et al.*, (1990) reported conjugating up to 18.9 SnCe<sub>6</sub> molecules per mab using a dextran carrier. Jiang *et al.* (1990) used the spacer polyvinyl alcohol to conjugate benzoporphyrin to an anti-T-cell mab. This resulted in a conjugation ratio of 3/4 residues per mab molecule. Therefore, the carrier systems used to date do not appear to increase the number of photosensitisers delivered to each antigen when compared to HMFG-1-Hp.

Figure 5.4.1.(a), shows that at a concentration of 2.7 x  $10^{-2}$  mg/ml, HMFG-1-Hp killed 50% of the MCF-7 cells (IC<sub>50</sub>). This concentration of HMFG-1-Hp contains a total of 7.4 x  $10^{-3}$  mg Hp/ml. This dose is significantly lower than the dose (2.3 x  $10^{-2}$  mg/ml) used in toxicity studies by Bellinier and Dougherty (1982). Determination of the exact concentration of intracellular and membrane-associated HMFG-1-Hp and Hp was not possible and may be investigated at a later stage.

Both Mew *et al.* (1983; 1985) and Oseroff *et al.* (1987) have commented on the increased toxicity of photosensitisers when they are conjugated to monoclonal antibodies. Oseroff *et al.* reported that 200 to 1,000 times more unconjugated photosensitiser was required to kill antigen-bearing cells under similar conditions. They went on to argue that this affects results from the *localisation* of porphyrin conjugates at the cell membrane. Singlet oxygen produced by Hp reacts with local membrane lipoproteins to cause oxidation and cell death. This theory has been supported by recent studies by Maziere *et al.* (1990) and Ricchelli *et al.* (1990). Using liposomes as a cell model, they showed that photosensitisers directly associated with the lipid layer gave higher levels of lipid oxidation than photosensitiser which was encapsulated within the liposome or in solution around the liposomes.

A. Direct Labelling	References	
Hp - anti-myosarcoma	Mew et al., 1983; 1985	
Hp dihydrazine - anti-Leu1	Oseroff et al., 1985	
anti-Leu2a		
anti-Leu3		
Chlorin e <sub>6</sub> - anti-Leu1	Oseroff et al., 1986	
anti-Leu2a		
anti-Leu3		
Hp - anti-T cell	Steele et al., 1988	
Chlorin e <sub>6</sub> (CMA) - anti-leukaemia	Hasan, 1988	
bladder		
anti-ovarian carcinoma		
Chlorin e <sub>6</sub> - anti-ovarian carcinoma	Goff et al., 1991	
Pthalocyanine - anti-EMA	Dawson et al., 1991	
Iodofluorescine - anti- <u>E</u> . <u>coli</u>	Devanatan et al., 1991	
TCPP - anti-lym-1	Biddle et al., 1991	
B. Carrier Systems	References	
Alspc - liposome-anti-CEA	Rakestraw et al., 1990	
Benzoporphyrin - polyvinyl alcohol-anti-T cell	Oseroff et al., 1990	
chlorin e <sub>6</sub> - Dextran anti-Leul	Jiang et al., 1990	
Sn Ce <sub>6</sub> - Dextran anti-melanoma	Yemul et al., 1990	
Pyrene - liposome-anti-OKT4	Morgan et al., 1989	
C. Non-Antibody Carriers	References	
Photosensitiser - low density lipoproteins	Reviewed by Richelli et al., 1990	
	Cuomo et al., 1990	
Photosensitiser - liposome	Mosley et al., 1984	

## Table 5.5.1: Summary of the methods used to prepare immunophotosensitisers.

The proximity of HMFG-1-Hp to the cell membrane when bound to the HMFG-1-Hp antigen allows singlet oxygen to diffuse more rapidly to membrane lipid components. Extracellular and intracellular Hp are diffusion-limited and this results in lower levels of membrane oxidation and therefore, less cell killing. Other drug delivery systems such as liposomes (Cuomo *et al.*, 1990) and microspheres (Bachor *et al.*, 1991) have also shown increased levels of cell kill <u>in vitro</u>. These carrier systems are thought to enter the cell by phagocytosis and Bachor and his colleagues have surmised that the high level of cell kill results from damage to lysosomal membranes and subsequent proteolytic breakdown, rather than through cell membrane damage. A more detailed study of both the chemistry and pharmacology of HMFG-1-Hp is required before the mechanism of action is fully understood.

#### 5.5.4. Two-Stage Immunotherapy and Drug Delivery Systems.

IPDT with Hp-mab immunophotosensitisers appears to be an effective treatment in vitro and it has shown some promising results in animal studies (Mew *et al.*, 1986; Steele *et al.*, 1988). However, immunophotosensitisers have not been used in clinical studies to date.

Unconjugated photosensitisers such as HpD and phthalocyanines have been used to treat patients with several types of tumour. In recent reviews by Ash and Brown (1989), Dougherty (1989) and Brown (1990), PDT was found to be particularly useful in treating smaller tumours of the colon, trachea and bladder. All of these tumours can be irradiated by the use of endoscopes fitted with fibre optic cables carrying laser light. The main side-effect associated with PDT is the patient's sensitivity to sunlight after treatment. Patients treated with photosensitisers have to remain in a darkened room for 6 - 8 weeks to avoid skin ulceration and discomfort. By using immunophotosensitisers, the selectivity of the photosensitiser for tumour cells should increase. A more selectively delivered drug requires a lower dose to achieve the same result and this would reduce the time the patient would need to spend in the absence of sunlight.

Immunophotosensitisers would also bind to the liver and bone marrow cells. As these tissues would not be irradiated (either by laser or sunlight) IPDT also minimises the side-effects of immunotherapy on non-tumour cells. IPDT will not be useful in treating all stages and grade of tumour. The main challenge is to identify *which* tumour types can be treated by IPDT and to concentrate on developing effective treatment schedules which minimise the side-effects of both the photosensitiser (i.e. skin ulceration) and the mab (i.e. HAMA). Some of the more novel strategies which have been proposed include Suppressor Deletion Therapy, intravessicle treatment of bladder carcinoma and the intrathecal administration of immunoconjugates to treat parenchymal brain tumours. Each of these strategies will be looked at in more detail. Steele and his co-workers (1988), argued that elevated levels of T-suppressor cells (Ts) stops a patient producing large numbers of anti-Taa antibodies. Suppressor Deletion Therapy is based on the principle of removing Ts cells, and as a result, the patient should increase their production of anti-Taa antibodies. They proposed that Hp-anti-Ts immunoconjugates could be used to purge Ts cells from a patient's bone marrow. The bone marrow cells (without Ts cells) could then be returned to the body. Similarly, Gulliay and Mathews (1988), proposed the use of ex-vivo PDT to kill leukaemia cells in bone marrow. Administration of the immunophotosensitiser a number of days before irradiation allows the conjugates to bind to the target cells and allows clearance of the conjugate from non-target cells. The volume of bone marrow which could be safely removed from a patient would limit the success of this technique. However, Löhde and his colleagues (1991) showed that patients could be treated with immunoconjugates after ex-vivo perfusion using an ex-vivo blood oxygenation system. This technique would require surgery to insert the external blood oxygenation system.

IPDT may also be used to treat superficial bladder cancers. Fitzpatrick (1989), has proposed that immunophotosensitisers which bind to superficial bladder cancers, may be irradiated by laser light, delivered through an adapted endoscope and may be particularly useful in treating minimal residual disease of drug resistant tumours.

Bamias *et al.* (1991) have also shown that immunophotosensitisers can be administered intravesically (directly to the bladder). With intravesical mab administration the antibody does not enter the blood stream directly. This allows the repeated use of IPDT *without* adverse HAMA reactions.

Work by Colombatti and his colleagues (1988), indicates that immunotherapy is of use in treating parenchymal brain tumours. The tumours of the central nervous system are "immunologically privileged" which means that immune reactions (such as HAMA reactions) rarely take place under normal conditions. They proposed that imunoconjugates could be administered intrathecally or by other regional administration systems. The tumours could then be irradiated after a number of days by light delivered through a fibre optic cable which could be inserted through a biopsy needle. All of these novel treatment strategies try to work around the problems of non-specific localisation and killing of non-tumour cells. A number of other two-stage immunotherapy strategies were outlined in Section 1.10., but many of these strategies have only been used in vitro.

(a) ADEPT:

Antibody-directed enzyme prodrug therapy, ADEPT, has not been used in clinical studies but had reasonable success in animal models. These results were discussed at a recent conference held in Halkidiki, Greece. Haisma and his colleagues (1991), showed that anti-CEA conjugated to alkaline phosphatase successfully cleaved the prodrug phosphorylated etoposide (EP) into its active form in mice. Pre-treatment of the mice with unconjugated anti-CEA reduced the absorption of anti-CEA-AP by the liver and an overall 10-fold increase in cytotoxicity was reported. Similarly, Rowlinson-Busza and his colleagues (1991), showed that HMFG-1 and H17E2 antibodies linked to beta-glucosidase successfully cleaved glycoside amygdalin into free cyanide in vitro. They have called this type of prodrug therapy antibody - guided enzyme nitrite therapy or AGENT. Senter (1991), remarked that a cocktail of mabenzyme-fusion proteins directed against a range of Taas will increase the effectiveness of ADEPT in tumours which express a variety of Taas.

#### (b) Avidin-Biotin Therapy:

The specificity of avidin and streptavidin for biotin has led to the use of biotin-avidin immunoconjugates to localise and treat cancers (Sections 1.10. and 4.4.5.). Several researchers have used avidin-biotin techniques to localise tumours (reviewed by Paganelli *et al.*, 1991) and to treat cancer (reviewed by Britton *et al.*, 1991). Britton and his colleagues also proposed using a three-stage radioimmunotherapy strategy:

Stage 1: Administration of mab-biotin on Day 0.

- <u>Stage 2</u>: Administration of free avidin to clear unbound mab-avidin from the blood stream on Day 1.
- <u>Stage 3</u>: Administration of radiolabelled (or photosensitiser drug, toxin or cytokine)labelled avidin on Day 3.

They also proposed the use of two and three stage therapy systems which would simultaneously image and treat tumour cells.

- <u>Stage 1</u>: Inject a <sup>99M</sup>Tc-bifunctional antibody which reacts with tumour and a hapten (i.e. biotin or IBH).
- <u>Stage 2</u>: Image the tumour by <sup>99M</sup>Tc-scanning to record when the conjugate is in the correct location in the tumour.
- Stage 3: A P-32 linked ligand (i.e. avidin) which is a  $\beta$ -emitter binds to the BFA and is released and incorporated into tumour DNA after internalisation of the immunoconjugate.

Clinical studies using these two and three stage immunotherapy systems are currently underway in St. Bartholomew's Hospital, London (Britton, 1991b).

#### 5.5.5. Mabs and Other Drug Delivery Systems.

Mab-conjugates are one of a range of strategies to deliver drugs to target areas. Liposomes, reconstituted viral envelopes, microsponges, microspheres and "signalling targets" are just some of these strategies.

Liposomes are phospholipid vesicles which can be used to carry drugs and other substances within the blood stream (reviewed by Alving, 1991). Liposomes have been quite successful as artificial surfactants in the treatment of respiratory distress syndrome when administered as a nasal aerosols. However, they have had very limited success in clinical studies. Mab-liposomes or immunoliposomes have also been prepared (reviewed by Peeters *et al.*, 1988). However, like liposomes, most immunoliposomes are taken up by the liver after administration. The use of immunoliposomes incorporating photosensitisers such as Si(IV)-naphthalocyanine to successfully treat mice bearing MS-2 fibrosarcomas (Cuomo *et al.*, 1990), has overcome this problem. Immunoliposomes have also been used to carry pyrene and AlsPC as shown in Table 5.5.1. Immunoliposomes delivered to the liver are not irradiated and therefore, were not toxic to the mice. A further injection of immunophotosensitiser at the tumour site increase the amount of immunoliposome at the tumour site. Following irradiation, the tumour micro-vasculature was destroyed. This led to a reduction in the oxygen delivered to the tumour and resulted in tumour anoxia.

Reconsistuted viral envelopes (RVEs) are similar to liposomes. REVs are formed by solubilising intact virus in detergent and reassembling the envelope on removal of detergent (reviewed by Blumenthal and Loyster, 1991; Kingsman *et al.*, 1991). Like liposomes, RVEs have been used to deliver biological therapeutics, and they have been of particular use in delivering genes, antisense nucleic acids, ribosomes and proteins. However, the presence of viral proteins on the surface of REVs means that a patient may raise an immune-response to REVs which were injected. REVs may be used as "Trojan Horses" to deliver drugs and genes. This may be particularly useful in delivering chemoimmunoconjugates and immunotoxins which need to be internalised in order to kill cells. However, the amount of drug, DNA or protein which can be encapsulated in a REV is quite low and this has slowed clinical studies on REV targeting.

Microspheres and microsponges are both made of synthetic polymers and are not immunogenic (reviewed by Van Brunt, 1989). Morgan *et al.*, 1986, have used Chlorin  $e_6$ -conjugated to microspheres to deliver photosensitiser in mice. Unlike immunophotosensitisers, the Chlorin  $e_6$ -conjugates were delivered to tumours which had increased blood supply.

"Smart drugs", or drugs that act on particular cell functions, are also used to target specific cell types. Several drugs are already available which can act on protein serine/threonine kinases, protein tyrosine kinases and phospholipase C as well as inhibitors of *myo*-inositol signalling. These enzymes are involved in growth control and are particularly important in the search for more selective drugs for treating cancer (reviewed by Powis, 1991). To combine this technology (i.e. identify drugs which selectively interact with cancer cell-specific signalling targets) with ADEPT and other two-stage monoclonal antibody therapies may help to selectively kill cancer cells.

Finally, conventional mabs, mab-conjugates and the new genetically engineered mabs will not provide the cure to all cancers. The challenge is to involve chemists, biochemists, geneticists, physicists and oncologists in the design of more effective conjugates. These conjugates should then be used to treat specific types of tumours or minimal residual disease which are amenable to immunotherapy. It is highly unlikely that all cancers will be suitable candidates for immunotherapy.

# SECTION 6 : CONCLUSION

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In conclusion, three separate aspects of monoclonal antibody technology were investigated in this thesis.

In Section 3, the use of myeloma-derived cell lines to produce human mabs was explored. Two new cell lines were established. C23/11 was shown to have many of the characteristics of a lymhphoblastoid cell line, including the expression of the CD23 antigen, the presence of EBVNA and a clump-like growth pattern in vivo. The C23/11 cell line was used as a fusion partner with the NSO mouse myeloma cell line to produce the heteromyeloma cell line, HET-37. HET-37 grows as a single cell suspension. Both C23/11 and HET-37 have ulstrastructural characteristics such as endoplasmic reticulum and golgi apparatus which are associated with the secretion of antibodies. The advances in recombinant mab technology allows the production of human mabs by transfection of human B-cell DNA into human, murine and microbial hosts provides the addition strategy for producing human mabs for use in the diagnosis and treatment of cancers.

<sup>In</sup>In-labelled and <sup>99M</sup>Tc-labelled fragments were used to detect the distribution of tumour in patients with ovarian carcinoma. <sup>III</sup>In-HMFG-1 conjugates were found to have the highest tumour to background ratio and were therefore used on the majority of patients in the study. Tumours were also localised using <sup>III</sup>In-AUA-1 and <sup>III</sup>-H17E2 conjugates. <sup>III</sup>In-mab immunoscintigraphy was found to be more sensitive than techniques such as CAT scanning, ultrasound and X-ray for detecting tumours in patients with suspected ovarian carcinoma. The presence of the tumour associated antigen serum CA-125 in elevated levels was found to be more sensitive than immunoscintigraphy for indicating the presence of tumour. However, this marker gives no information on the location of the tumour or the distribution of the tumour <u>in vivo</u>. Preliminary results using <sup>99M</sup>Tc-F(ab')<sub>2</sub> fragments on one patient with residual carcinoma suggests that this technique is a more cost-efficient and convenient alternative to <sup>III</sup>In-mab immunoscintigraphy.

In Section 5, a two-step strategy for killing tumour cells in vitro was investigated. Hematoporphyrin (Hp), and its more toxic derivative HpD, were shown to be potent photosensitizers which killed Landschütz ascites tumours (LAT) cells when activated by light. While acridine orange was shown to be an effective photosensitizer in vitro, coumarin, 7-hydroxycoumarin, 4-hydroxycoumarin and AMCA did not show any phototoxic effects on LAT cells in vitro. Tumouristatic effects were detected with some of these coumarin compounds. The mab HMFG-1 was chemically linked to Hp and this conjugate killed MCF-7 cells which expressed the HMFG antigen, following irradiation with light from a Xenon Arc Lamp. HMFG-1-Hp did not kill EJ-138 cells which do not express the HMFG antigen.

Thus, the work described clearly indicates that radiolabelled mabs can successfully be applied to image tumours and that they may be used as a non-surgical technique to localise tumours, particularly when used in combination with serum markers. It is also evident that antibody targeted drugs can specifically kill tumours in vitro. However, for this system to be applied in vivo problems associated with non-specific uptake and immunogenicity associated with the administration of repeated doses of murine mab must be overcome. It may be possible to use C23/11 and HET-37 fusion partners and/or genetic approaches to make less immunogenic human mab with the required specificity for clinical use. Realistic antibody therapies must be devised and used in combination with conventional drugs and radiotherapy to improve the scope of treatment available to cancer patients.

## REFERENCES

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V.

Alley, C. D. and McDermott, R. P. (1980).

'Separation and characterisation of human bone marrow mononuclear cells from aspirates and ribs'. J. Immunol. Methods, <u>32</u>: 223-237.

Altshuler, G.L., Dziewulski, D.M., Sowek, J.A. and Belfort, G. (1986).

'Continuous hybridoma growth and monoclonal antibody production in hollow-fibre reactors - separators'. *Biotechnol. Bioeng.*, <u>28</u>: 646-656.

Alvarez, M.R. (1975).

'Microfluorometric comparisons of heat-induced nuclear acridine orange metachromasia between normal cells and neoplastic cells from primary tumours of diverse origin'. *Cancer Res.*, <u>53</u>: 93-98.

Alving, V.(1991).

'Liposomes as carriers of antigens and adjuvants'. J. Immunol. Methods, 140: 1-13.

Anagnostaki, E., Skarlos, D., Tamvakis, N., Psaropoulou, P., Blana, E., Bamias, A., Legaki, S., Aravantinos, G., Deliveliotis, Ch. and Dimopoulous, K. (1990).

'Immunohistochemical and immunocytochemical study of bladder carcinoma using the epitheliumspecific, tumour-associated monoclonal antibodies HMFG-1 and AUA-1'. Br. J. Cancer: <u>62</u> (Suppl. X): 52-56.

Arklie, J., Taylor-Papadimitriou, J., Bodimer, W.F., Egan, M. and Mellis, M. (1980).
'Differentiation antigens expressed by epithelial cells in the lactating breast are also detectable in breast cancer'. *Int. J. Cancer*, <u>28</u>: 17-21.

Ash, D. and Brown, S.B. (1989). 'Photodynamic therapy - achievements and prospects'. Br. J. Cancer, <u>60</u>: 151-152.

Bachor, R., Shea, C.R., Gilles, R. and Hasan, T. (1991).

'Photosensitized destruction of human bladder carcinoma cells treated with chlorin e<sub>e</sub>-conjugated microspheres'. *Proc. Natl. Acad. Sci. USA*, <u>88</u>: 1580-1584.

Bagshawe, K.D. (1989).

'Towards generating cytotoxic agents at cancer sites'. Br. J. Cancer, 60: 275-281.
Bamias, A., Keane, P., Krausz, T., Williams, G. and Epenetos, A.A. (1991).

'Intravesical administration of monoclonal antibodies in patients with superficial bladder carcinoma.' [Abst.], in Proceedings of the Eight International Hammersmith Meeting of the Advances in the Applications of Monoclonal Antibodies in Clinical Oncology, *Halkidiki*, *Greece*, 8-13th May, pp 45.

Barzon, G., Hierholzer, J., Cordes, M., Friedmann, W. and Felix, R. (1991).

"Primary diagnosis and follow-up of ovarian cancer : radioimmunoscintigraphy with Iodine-131 labelled OC 125 monoclonal antibodies versus computed tomography and "second look". *Nucl. Med. Biol.*, <u>18</u>: 69-76.

Bassan, H., Gutmann, H. and Djaldetti, M. (1981).

'Ultrastructural characteristics of the plasma cells of a patient with nonsecretory myeloma and plasma cell leukaemia'. Acta. Haemat., <u>66</u>: 129-133.

Bast, R.C., Feeney, M., Lazarus, H., Nadler, L.M., Colvin, R.B. and Knapp, R.C. (1981). 'Reactivity of a monoclonal antibody with human ovarian carcinoma'. J. Clin. Invest., <u>68</u>: 1331-1337.

Bator, J.M. and Reading, C.L. (1990).

'Antibody-mediated cancer diagnosis and therapy', in Therapeutic Monoclonal Antibodies (eds. Borrebaeck, C.A.K. and Larrick, J.W.). Stockton Press, New York: pp 35-56.

Benner, D.E., Schoeppel, S. and Roberts, J.A. (1990).
'Non-invasive technology assessment: How should treatment of ovarian cancer be assessed?' *Cancer Invest.*, <u>8</u>: 563-564.

Bentwich, B.Z., Douglas, S.D., Skuteksky, E. and Kunkel, H.G. (1973). 'Sheep red cell binding to human lymphocytes treated with neuraminidase: Enhancement of T cell binding and identification of a subpopulation of B-cells'. J. Exp. Med., <u>137</u>: 1532-1540.

Biddle, W.C., Pancook, J.D., Musser, D.A. and Oseroff, A. (1991). <u>'In vitro</u> photoablation of human B lymphoma cells by a monoclonal anti-porphyrin conjugate'.[Abst.], *Proc. Am. Assoc. Cancer Res.*, <u>32</u>: 265.

Birch, J.R., Boraston, R. and Wood, L. (1985).'Bulk production of monoclonal antibodies in fermenters'. *Trends. Biotechnol.*, <u>3</u>: 162.

Bischof-Delaloye, A., Dalaloye, B., Buchegger, F., Mach, J.P., Heusser, Ch. and Hardman, N. (1989). 'Chimaeric mouse-human anti-CEA monoclonal antibody of IgG4 isotope used in a pilot immunoscintigraphy study of patients with colorectal carcinomas'. J. Nucl. Med., <u>30</u>: 809-819.

Bjorn, M.J. and Villemez, C.L. (1988).

'Immunotoxins : Selection of cell-surface antigens and their corresponding monoclonal antibodies'. Cancer Treat. Res., <u>37</u>: 255-277.

Blumenthal, R. and Loyter, A. (1991). 'Reconstituted viral envelopes - "Trojan Horses" for drug delivery and gene therapy?' *Tibtech.*, <u>9</u>: 41-44.

Bonnet, R., Ridge, R.J., Scourides, P.A. and Berenbaum, M.C. (1981).
'On the nature of haematoporphyrin derivative'. J. Chem. Soc. Perkins, <u>1</u>: 3135-3140.

Borrebaeck, C.A.K. (1986).

"In vitro immunisation for the production of murine and human monoclonal antibodies : Present status". Trends Biotechnol., <u>4</u>: 147-153.

Borrebaeck, C.A.K. (1988(a)). 'Human MAbs produced by primary <u>in vitro</u> immunization'. *Immunol. Today*, <u>9</u>: 355-359.

Borrebaeck, C.A.K., Danielson, L. and Möller, S. (1988(b)). 'Human monoclonal antibodies produced by primary in vitro immunization of peripheral blood lymphocytes'. *Proc. Natl. Acad. Sci. (USA)*, <u>85</u>: 3995-3999.

Borrebaeck, C.A.K., Danielsson, L., Ohlin, M., Carisson, J. and Carlsson, R. (1990). 'The use of <u>in vitro</u> immunization, cloning of variable regions and SCID mice for the production of human monoclonal antibodies' in Therapeutic Monoclonal Antibodies (eds, Borrebaeck, C.A.K. and Larrick, J.W.), *Stockton Press, New York, pp 1-16.* 

Bosslet, K., Steinstraesser, A., Hermentin, P., Kuhlmann, L., Bruynck, A., Magerstaedt, M., Seemann, G., Schwartz, A. and Sedlaeck, H.H. (1991(a)).

'Generation of bispecific monoclonal antibodies for two phase radioimmunotherapy'. Br. J. Cancer, 63: 681-686.

Bosslet, K., Czech, J., Lorenz, P., Schuermann, M., Seemann, G., Kolar, C., Hoffman, D. and Sealaeck, H.H. (1991(b)).

'Molecular and Functional characterisation of a fusion protein suited for tumour specific pro-drug activation'. [Abst.], in Proceedings 8th International Hammersmith Meeting of the Advances in the Applications of Monoclonal Antibodies in Clinical Oncology, *Halkidiki*, *Greece*, 8-13th May, 1991.

Boulianne, G.L., Hozumi, N. and Shulman, M.J. (1984).

'Production of functional chimaeric mouse/human antibody'. Nature, 312: 643-651.

Bown, S.G. (1990).

'Photodynamic therapy to scientists and clinicians - one world or two?'. J. Photochem. Photobiol. B, <u>6</u>: 1-12.

Bradford, M.M. (1976).

'A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding'. Anal. Biochem. 72: 248-254.

Brennan, M., Davison, P.F. and Palus, H. (1985).
'Preparation of bispecific antibodies by chemical recombination of monoclonal IgG1 fragments'. Science, <u>229</u>: 81-83.

Britton, K.E., Granowska, M. and Nimmon, C.C. (1989).
'Monitoring the <u>in vivo</u> behaviour of immunoglobulins with radiotracers: Imaging approaches and data processing techniques'. *Nucl. Med. Biol.*, <u>16</u>: 105-111.

Britton, K.E., Mather, S.J. and Granowska. (1991 (a)).
'Radiolabelled monoclonal antibodies in oncology III: Radioimmunotherapy'. Nuclear Med. Comms., 12: 333-347.

Britton, K.E. (1991 (b)).

'Radionuclide therapy of cancer - past, present and future. An overview'. [Abst.]', in Proceedings of the Eight International Hammersmith Meeting of the Advances in the Applications of Monoclonal Antibodies in Clinical Oncology, *Halkidiki*, *Greece*, 8-13th May, pp 50.

Brown, J. (1990).

'Therapeutic monoclonal antibodies: European regulatory issues', in Therapeutic Monoclonal Antibodies (eds. Borrebaeck, C. and Larrick, J.), *Stockton Press, New York, pp 321-361.* 

Brown, P., Parenteau, G.L, Dirbas, F.M., Garsia, R.J., Goldman, C.K., Bukowski, M,A., Junghans, R.P.,
Queen, C., Hakimi, J., Benjamin, W.R., Clark, R.E. and Waldmann, T.A. (1991).
'Anti-Tac-H, a humanized antibody to the interleukin 2 receptor, prolongs primate cardiac allograft survival ', *Proc. Natl. Acad. Sci. USA*, <u>88</u>: 2663-2667.

Buraggi, G.L., Callegaro, L., Turrin, A., Gennari, L., Bombardieri, E., Mariani, G., Deleide, G., Davis,
M., Gasparini, M., Doci, R., Regalia, E., Seregni, E. and Scassellati, G.A. (1987).
'Immunoscintigraphy of colorectal carcinoma with F(ab')<sub>2</sub> fragments of anti-CEA monoclonal antibody'. *Cancer Detect. Prevent.*, <u>10</u>: 335-345.

Burton, D.R. (1991).

'Human and mouse monoclonal antibodies by repertoire cloning'. Tibtech., 2: 169-175.

Buy, J.N., Moss, A.A., Ghossain, M.A., Sciot, C., Malbet, L., Vadrot, D., Paniel, D.J. and Decroix, Y. (1988).

'Peritoneal implants from ovarian tumours: CT findings'. Radiology, 169: 691-694.

Byrne, C.J., Marshallsay, L.V. and Ward, A.D. (1990). 'The chemical composition of photofrin II'. J. Photochem. Photobiol. B., <u>6</u>: 13-28.

Campbell, A.M., Whitford, P. and Leake, R.E. (1987). 'Human monoclonal antibodies and monoclonal multispecificity'. Br. J. Cancer, <u>56</u>: 709-713.

Carroll, K. and O'Kennedy, R. (1988).

'The elimination of mycoplasma from infected hybridomas by passaging in BALB/c mice'. J. Immunol. Method, <u>108</u>: 189-193.

Carroll, K., Prosser, E. and O'Kennedy, R. (1990).

'Parameters affecting the <u>In vitro</u> immunization of human tonsilar lymphocytes : Effects of rIL-2 and muramyl dipeptide'. *Hybridoma*, <u>9</u>: 81-89.

Carroll, K., Prosser, E. and O'Kennedy, R. (1991).

'In vitro immunization of human tonsilar lymphocytes : Effects of PWM and rIL-6. Hybridoma, <u>10</u>: 229-239.

Carroll, W.L., Threlemans, K., Dilley, R. and Levy, R. (1986).

'Human-mouse heterohybridomas as fusion partners with human B cell tumours'. J. Immunol. Methods, 89: 61-70.

Castro, D.J., Saxton, R.E., Fetterman, H.R. and Ward, P.H. (1988). 'The effects of argon lasers on human melanoma cells sensitized with rhodamine-123 in vitro'. Am. J. Otolar, <u>9</u>: 18-29.

Cavagnaro, J. (1987).

'Immunotoxicology and the new biotechnology'. Immunol. Today, 8: 102-104.

Cerny, T., Kupfer, A., Zeugin, T. and Brunner, K.W. (1990). 'Bioavailability of subcutaneous ifosfamide and feasibility of continuous output application in cancer patients'. Ann. Oncol., <u>1</u>: 365-368.

Chan, V.T. and Sikora, K. (1987). 'The potential of oncogene products as tumour markers'. *Cancer Surv.*, <u>6</u>: 185-207.

Chaney, S.G., Gibbons, G.K., Wyrick, S.D. and Podhasky, P. (1991).
'An unexpected biotransformation pathway for tetrachloro-(d,1-trans)-1,2-daiminocyto-hexaneplatinum (IV) (tetraplatin) in the L1210 cell line'. *Cancer Res.* <u>51</u>: 969-973.

Chatal, J.F., Fumoleau, P. and Kremer, M. (1985). 'Complementary roles of immunoscintigraphy and ultrasonography'. *Eur. J. Nucl. Med.*, <u>10</u>: 464-466.

Chatal, J.F., Fumoleu, P., Saccavini, J.C., Thedrez, P., Curtet, C., Bianco-Arco, A., Chetanneau, A., Peltier, P., Dremer, M. and Guillard, Y. (1987).
'Immunoscintigraphy of recurrences of gynaecologic carcinomas'. J. Nucl. Med., 28: 1807-1819.

Chovnick, A., Schneider, W.P., Tso, J.Y., Queen, C. and Chang, C.N. (1991). 'A recombinant, membrane-acting immunotoxin'. *Cancer Res.*, <u>51</u>: 465-467.

Childs, R.L. and Hnatowich, D.J. (1985).

'Optimum conditions for labeling of DTPA-coupled antibodies with technetium-99M'. J. Nucl. Med., <u>26</u>: 293-299.

Chiorazzi, N., Wasserman, R.L. and Kunkel, H.G. (1982).

'Use of Epstein-Barr virus-transformed B cell lines for the generation of immunoglobulin-producing human B cell hybridomas'. J. Exp. Med., <u>156</u>: 930-935.

Christie, J.F., Gallagher, G. and Stimson, W.H. (1987). 'The human bladder carcinoma cell line T-24 secretes a human B-cell differentiation factor'. *Immunology*, <u>60</u>: 467-473.

Clackson, T., Hoogenboom, H.R., Griffith, A.D. and Winter, G. (1991). 'Making antibody fragments using phage display libraries'. *Nature*, <u>352</u>: 624-628.

Co, M.S., Deschamps, M., Whitley, R.J. and Queen, C. (1991(a)). 'Humanized antibodies for anti-viral therapy'. *Proc. Natl. Acad. Sci. USA*, <u>88</u>: 2869-2873.

Co, M.S. and Queen, C. (1991(b)). 'Humanized antibodies for therapy'. *Nature*, <u>351</u>: 501-502.

Cole, J. and Arlett, C.F. (1976).
'Ethyl Methanesulphonate Mutagenesis with L5278Y mouse lymphoma cells: A comparison of oubain, thioguanine and excess thymidine resistance'. *Mutation Res.*, <u>24</u>: 507-526.

Colombalti, M., Bisconti, M., Dell'Arcipete, L., Gerosa, M.A. and Tridente, G. (1988). 'Sensitivity of human glioma cells to cytoxic heteroconjugates'. Int. J. Cancer, <u>42</u>: 441-448.

Conley, D. and Marshall, M.E. (1987).

'Effects of coumarin on human tumour cell growth and cell cycle analysis in vitro.' [Abst.], Proc. Am. Assoc. Cancer Res., 28: 63.

Coppel, R.L. (1986). 'Prospects for a malaria vaccine'. *Microbiol. Sci.*, <u>3</u>: 292-295.

Correale, M., Abbate, I., Gargano, G., Garrubba, M., Muncipinto, A., Addabbo, L. and Colangelo, D. (1991).

'Serum TAG-72 levels in different carcinomas'. Nucl. Med. Biol., 18: 101-105.

Coughlan, A. (1991).

'A second chance for antibodies'. New Scientist, 2: 34-39.

Courtenay-Luck, N.S., Epenetos, A.A., Sirolapenko, G.B., Larche, M., Barkans, J.R. and Ritter, M.A. (1988).

'Development of anti-idiotypic antibodies against tumour antigens and autoantigens in ovarian cancer patients treated intraperitoneally with mouse monoclonal antibodies'. *Lancet*, *ii*: 894-897.

Cox, D., O'Kennedy, R. and Thornes, R.D. (1989).

'The rarity of liver toxicity in patients treated with coumarin (1,2-benzopyrone)'. Human Toxicol., <u>8</u>: 501-506.

Crawford, D.H. (1985).

'Production of human monoclonal antibodies using the Epstein-Barr virus', in Human Hybridomas and Monoclonal Antibodies (eds, Engleman, E.G., Foung, S.K.H., Larrick, J. and Raubitschek, A.), *Plenum Press, New York, pp 37-50.* 

Croce, C.M., Shander, M., Martinis, J., Circurel, L., D'Ancona, G.G., Dolby, T.W. and Koprowski, H. (1979).

'Chromosomal locations of the genes for human immunoglobulin heavy chains'. Eur. J. Immunol., <u>10</u>: 486-488.

Croce, C.M., Linnenbach, A., Hall, W., Steplewski, Z. and Koprowski, H. (1980). 'Production of human hybridomas secreting antibodies to measles virus'. *Nature*, <u>228</u>: 488-489.

Davis, M. (1990). 'The current and future use of tumour-localizing agents'. J. Nucl. Med., <u>31</u>: 1658-1661.

Davis, G.T., Bedzyk, W.D., Voss, E.W. and Jacobs, T.W. (1991).
'Single chain antibody (SCA) encoding genes: One-step construction and expression in eukaryotic cells'. *Biotechnology*, <u>9</u>: 165-169.

Dawson, J.W., Morgan, J., Dean, C., MacRobert, A., Savage, F. and Boulos, P. (1991). 'Epithelial membrane antigen: A suitable target for an antibody-photosensitiser conjugate in the treatment of sub-clinical deposits of cancer.' [Abst], in Proceedings of Eight International Hammersmith Meeting of the Advances in the Applications of Monoclonal Antibodies in Clinical Oncology, *Halkidiki*, *Greece*, 8-13th May, p 45. Del Resario, R.B. and Wahl, R.L. (1989).

'Site-specific radiolabeling of monoclonal antibodies with biotin/streptavidin'. Nucl. Med. Biol., <u>6</u>: 525-531.

Devereux, S. and Linch, D.C. (1989). 'Clinical significance of haemopoietic growth factors'. Br. J. Cancer, <u>59</u>: 2-5.

Dillman, R.O. (1987).

'Antibody Therapy', in Principles of Cancer Biotherapy (eds. Oldham, R.K.), Raven Press, New York, pp 291-318.

Di Maggio, J.J., Scheinberg, D.A. and Houghton, A.N. (1990). 'Monoclonal antibody therapy of cancer'. Cancer Chemother. Biol. Resp. Modif., <u>11</u>: 177-203.

Dominguez, K., Fentem, J., Garle, M. and Fry, J. (1990).

'Comparison of mongolian gerbil and rat hepatic microsomal monooxygenase activities: High coumarin in 7-hydroxylase activity in the gerbil'. *Biochem. Pharmacol.*, <u>39</u>: 1629-1631.

Dougherty, T.J., Gomer, C.J. and Weishaupt, K.R. (1976). 'Energetics and efficiency of photoinactivation of murine tumour cells containing hematoporphyrin'. *Cancer Res.*, <u>36</u>: 2330-2333.

Dougherty, T.J. (1989). 'Photodynamic therapy - new approaches'. Sem. Surg. Oncol., <u>5</u>: 6-16.

Drebin, J.A., Link, V.C. and Greene, M.I. (1988).
'Monoclonal antibodies specific for the <u>neu</u> oncogene directly mediate antitumour effects in <u>vivo</u>'.
Oncogene, <u>2</u>: 387-394.

Dulin, J.D., Peltier, P. and Chatal, J.F. (1991).
'Bayesian analysis of the utility of In-III OC 125 immunoscintigraphy in the diagnosis of ovarian cancer'. [Abst.] J. Nucl. Med., <u>32</u>: 941.

Duncan, A.R. and Winter, G. (1988). 'The binding site of Clq on IgG'. Nature, <u>332</u>: 738-740. Eary, J.F., Press, O.W., Badger, C.C., Durack, L.D., Richter, K.P., Addison, S.J., Krohn, K.A., Fisher, D.R., Porter, B.A., Williams, D.L., Martin, P.J., Applebaum, F.K., Brown, S., Miller, R.A., Nelp, W.B. and Bernstein, I.D. (1990).

'Imaging and treatment of B-cell lymphoma'. J. Nucl. Med., 31: 1257-1258.

Edwards, P.A.W., Smith, C.M., Neville, A.M. and O'Hare, M.J. (1982). 'A human-human hybridoma system based on a fast-growing mutant of the ARH-77 plasma cell leukaemia-derived line'. *Eur. J. Immunol.*, <u>12</u>: 641-648.

Egan, D., O'Kennedy, R., Moran, E., Cox, D., Prosser, E. and Thornes, R.D. (1990). 'The pharmacology, metabolism, analysis and applications of coumarin and coumarin-related compounds'. *Drug Metab. Rev.*, <u>22</u>: 503-529.

Epenetos, A.A., Britton, K.E., Mather, S., Shepherd, J., Granowska, M., Taylor-Papadimetriou, J., Nimmon, D., Durbin, H., Hawkins, L.R., Malpas, J.S. and Bodimer, W.F. (1982). 'Targeting of <sup>123</sup>Iodine-labelled tumour-associated monoclonal antibodies to ovarian, breast and gastrointestinal tumours'. *Lancet*, <u>ii</u>: 99-1004.

Epenetos, A.A., Hooker, G., Durbin, H., Bodimer, W.F., Snook, D., Begent, R., Oliver, R.T.D. and Lavender, J.P. (1985).

'Indium III labelled monoclonal to placental alkaline phosphatase in the detection of neoplasms of testis, ovary and cervix'. *Lancet*, <u>ii</u>: 350-353.

Epstein, A.I., Chan, D. and Ansari, A. (1990). 'Radioimmunodetection of necrotic lesions in human tumours using I-131 labelled TNF-1 F(ab')<sub>2</sub> monoclonal antibody'. *Antibody Immunoconj. Radiopharm.*, <u>3</u>: 38-46.

Evans, L., Marago, C. and May, J.T. (1984).

'Human lymphoblastoid cell lines established from peripheral blood lymphocytes secreting immunoglobulins directed against Herpes simplex virus'. *Immunol. Letts.*, <u>8</u>, 39-49.

Faller, G., Vollmers, H.P., Weiglein, I., Marx, A., Zink, C., Pfaff, M. and Müller-Hermelink (1990). 'HAB-1, a new heteromyeloma for continuous production of human monoclonal antibodies'. Br. J. Cancer, <u>62</u> (Suppl. X): 595-598.

Fanger, M.W., Segal, D.M. and Romet-Lemonne, J.L. (1991).'Bispecific antibodies and targeted cellular cytotoxicity'. *Immunol. Today*, <u>12</u>: 51-54.

Ferrini, S., Melioli, G. and Moretta, L. (1990).

'Immunotherapy and immunity to cancer : Cellular mechanisms'. Curr. Opin. Immunol., 2: 683-688.

Figlin, K.A. (1989). 'Biotherapy in clinical practice'. Semin. Hemtol., <u>26</u> (Suppl. 3): 15-25.

Finn, R., Cheung, N.K.V., Dirgi, C., St Germain, J., Graham, M., Pentlow, K. and Larson, S.M. (1991). 'Technical challanges associated with the radiolabeling of monoclonal antibodies utilizing short-lvied, positron emitting radionuclides'. *Nucl. Med. Biol.*, <u>18</u>: 9-13.

Fitzpatrick, J.M. (1989). 'Superficial bladder cancer: Evaluation and management'. *Cancer Topics*, <u>7</u>: 82-83.

Foon, K.A. (1989).

'Biological response modifiers: The new immunotherapy'. Cancer Res., 49: 1621-1639.

Foon, K.A. and Fanning, J. (1990). 'Immunotherapy of gynecological malignancies'. Semin. Surg. Oncol., <u>6</u>: 364-368.

Foung, S.K., Perkins, S., Raubitschek, A., Larrick, J., Lizak, G., Fishwild, D., Engleman, E.G. and Grumet, F.C. (1984).

'Rescue of human monoclonal antibody production from an EBV-transformed B cell line by fusion to a human-mouse hybridoma'. J. Immunol. Methods, <u>70</u>: 83-90.

Foung, S.K.H., Perkins, S., Arvin, A., Lifson, J., Mohagheghpour, N., Fishwild, D., Grumet, F.C. and Erngleman, E.G. (1985).

'Production of human monoclonal antibodies using a mouse fusion partner', in Human Hybridomas and Monoclonal Antibodies (eds, Engleman, E.G., Foung, S.K.H., Larrick, J. and Raubitschek, A.), *Plenum Press, New York, pp 135-148.* 

Fritzberg, A.R., Abrams, P.G., Beaumier, P.L., Kasina, S., Morgan, A.C., Rao, T.N., Reno, J.M., Sanderson, J.A. and Srinivasan, A. (1988).

'Specific and stable labeling of antibodies with technetium-99M with a diamide dithiolate chelating agent'. *Proc. Natl. Acad. Sci. USA*, <u>85</u>: 4025-4029.

Gallatin, M., St. John, T.P., Siegelman, M., Reichert, R., Butcher, E.C. and Weissman, I.L. (1986). 'Lymphocyte homing receptors'. Cell, <u>44</u>: 673-680. Garzelli, C., Taub, F.E., Scharff, J.E., Prabhakar, B.S., Ginsberg-Fellner, F. and Notkins, A.L. (1984). 'Epstein-Barr transformed lymphocytes produce monoclonal antibodies that react with antigens in multiple organs'. J. Virol., <u>52</u>: 722.

Gazdar, A.F., Ore, H.K., Kirsh, I.R. and Hollis, G.F. (1986).

'Establishment and characterization of human plasma cell myeloma culture having a rearranged cellular *myc* proto-oncogene'. *Blood*, <u>67</u>: 1542-1549.

Geisow, M.J. (1991).

'Binding contracts : Antibody engineering'. Tibtech., 9: 113-115.

Ghosh, S. and Campbell, A.M. (1986). 'Multispecific monoclonal antibodies'. *Immunol. Today*, <u>7</u>: 217-221.

Gillies, S.D., Morrison, S.L., Oi, V.T. and Tonegawa, S. (1983).
'A tissue-specific transcription enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene'. *Cell*, <u>33</u>: 717-722.

Glad, C. and Nilsson, I. (1990).

'Large-scale production of monoclonal antibodies', in Therapeutic Monoclonal Antibodies (eds., Borrebaeck, C.A.K. and Larrick, J.W.), *Stockton Press, New York, pp 303-320.* 

Glassy, M.C., Handley, H.H., Hagiwara, H. and Royston, I. (1983).
'UC 729-6, a human lymphoblastoid B-cell line useful for generating antibody-secreting human-human hybridomas'. *Proc. Natl. Acad. Sci. USA*, <u>80</u>: 6327-6331.

Glassy, M.C. and Dillman, R.O. (1988). 'Molecular biotherapy with human monoclonal antibodies'. *Mol. Biother.*, <u>1</u>: 7-13.

Glennie, M.J., McBride, N.M., Worth, A.J. and Stevenson, O.T. (1987). 'Preparation and performance of bispecific F(ab'y) fragments. J. Immunol., <u>139</u>: 2367-2375.

Goff, B., Bamberg, M., Hasan, T. (1991).

'Photoimmuno-targeting of carcinoma cells from human ovarian cancer patients'. [Abst.], Proc. Am. Soc. Cancer Res., <u>32</u>: 426.

Goldhirsh, A., Triller, J.K., Greiner, R., Dreher, E. and Davis, B.W. (1983).
'Computed tomography prior to second-look operation in advanced ovarian cancer'. J. Obstet.
Gynecol., 62: 630-634.

Goldstein, N.I., Nagle, R., Villar, H., Hersh, E. and Fisher, P.B. (1990). 'Isolation and characterization of a human monoclonal antibody which reacts with breast and colorectal carcinoma'. *Anticancer Res.*, <u>10</u>: 1491-1500.

Gomer, C.J., Rucker, N., Banerjee, A. and Benedict, W.F. (1983). 'Comparison of mutagenicity and sister chromatid exchange in Chinese hamster cells exposed to hematoporphyrin derivative photodynamic therapy'. *Cancer Res.*, <u>43</u>: 2622-2627.

Gomer, C.J., Rucker, N., Razum, N.J., Lin, I. and Murphree, A. (1985). <u>'In vitro and in vivo</u> light dose rate effects related to hematoporphyrin derivative photodynamic therapy'. *Cancer Res.*, <u>45</u>: 1973-1977.

Goodfriend, T.L., Levine, L. and Fasman, G. (1964). 'Antibodies to bradykin and angiotensin: A use of carbodiimides in immunology'. *Science*, <u>144</u>: 1344-1346.

Goodwin, D.A., Mears, C.F., McTigue, M. and David, G.S. (1986). 'Monoclonal antibody hapten radiopharmaceutical delivery'. *Nucl. Med. Commun.*, <u>7</u>: 569-580.

Granowska, M., Britton, K.E. and Shepherd, J. (1984). 'The detection of ovarian cancer using I-123 monoclonal antibody'. *Radiobiol. Radiother.*, <u>25</u>: 153-160.

Granowska, M., Britton, K.E., Shephard, J.H., Nimmon, C.C., Mather, S., Ward, B., Osborne, R.J. and Slevin, M.L. (1986).

'A prospective study of I<sup>123</sup> labelled monoclonal antibody imaging in ovarian cancer'. J. Clin. Oncol., <u>4</u>: 730-736.

Granowska, M., Mather, S.J., Britton, K.E., Bentley, S., Richman, P., Phillips, R.K.S. and Northover, J.M.A. (1990).

<sup>(99M</sup>Tc radioimmunoscintigraphy of colorectal cancer'. Br. J. Cancer, <u>62</u> (Suppl. X): 30-32.

γ.

Granowska, M., Morris, G., Britton, K.E., Mather, S.J., Ellison, D. and Phillips, R.K.S. (1991). 'Routine Application of Tc<sup>-99M</sup> PRIA3 Monoclonal Antibody in colorectal cancer'. [Abst.], J. Nucl. Med., <u>32</u>: 940.

Green, B.S. and Tawfik, D.S. (1989).

'Catalytic monoclonal antibodies : Tailor-made, enzyme-like catalysts for chemical reactions'. *Tibtech.*, <u>7</u>: 304-310.

Gregory, C.D., Kirchgens, C., Edwards, C.F., Young, L.S., Rowe, M., Forster, A., Rabbitts, T.H. and Rickinson, A.B. (1987).

'Epstein-Barr virus transformed human precursor B cell lines; altered growth phenotypes of lines with germ-line or rearranged but nonexpressed heavy chain genes'. Eur. J. Immunol., <u>17</u>: 1199-1207.

Grunow, R., Jahn, S., Portsmann, T., Kiessig, S.S., Steinkellner, H., Steindl, F., Mattanovich, D., Gurtler, L., Deinhart, F., Katinger, H. and Von Baehr, R. (1988).

'The highly efficient, human B cell immortalizing heteromyeloma CB-F7 used in the production of human monoclonal antibodies to human immunodeficiency virus. J. Immunol. Methods, 60: 257-265.

Gulliya, K.S. and Mathews, J.L. (1988).

'Photosensitizations of leukaemic cells and normal bone marrow cells by 514nm laser light and effects of laser light on migration inhibition and lymphokine response'. Cell Biol. Int. Reports, <u>12</u>: 302-312.

Hagan, P., Halpern, S.E., Dillman, R.O., Shawler, D.L., Johnson, D.E., Chen, A., Krishnan, L., Frincke, J., Bartholomew, R.M., David, G.S. and Carlo, D. (1986).

'Tumour size : Effect of monoclonal antibody uptake in tumour models'. J. Nucl. Med., 27: 422-427.

Haisma, H.J., Moseley, K.R., Battaille, A.I., Griffiths, T.C., Zurawski, V.R. and Knapp, R.C. (1988).
'Biodistribution pharmacokinetics and imaging of <sup>131</sup>I-labelled OC125 in ovarian cancer'. *In. J. Cancer*, <u>2</u>: 109-113.

Hale, G., Dyer, M.J.S., Clark, M.R., Phillips, J.M., Marcus, R., Riechmann, L., Winter, G. and Waldmann, H. (1988).

'Remission induction in non-Hodgkin lymphoma with reshaped human monoclonal antibody CAMPATH-1H'. Lancet, <u>ii</u>: 1394-1399.

Haisma, H.J., Boven, E., van Muijen, M., DeVries, R., Lankelma, J. and Pinedo, H.M. (1991). 'Monoclonal antibody-enzyme conjugates and prodrugs for selective cancer therapy' [abst], in Proceedings of Eight International Hammersmith Meeting of the Advances in the Applications of Monoclonal Antibodies in Clinical Oncology, *Halkidiki*, *Greece*, 8-13th May, p 66.

Halton, D.M., Peterson, W.D. and Bharati, H. (1983). 'Cell culture quality control by rapid enzymatic characterisation'. In Vitro, <u>19</u>: 16-24.

Harris, T.J.R. (1991). 'Catalytic antibodies - something for everyone'. *Tibiech.*, <u>9</u>: 39-41.

Hasan, T. (1988). 'Selective phototoxicity using monoclonal antibody-chromophore conjugates'. SPIE Proc., <u>997</u>, 42-47.

Hasan, T., Lin, C.W. and Lin, A. (1989).

'Laser-induced selective cytotoxicity using monoclonal antibody-chromophore conjugates'. Immun. Cancer, <u>2</u>, 471-477.

Hellström, K.E. and Hellström, I. (1989).'Oncogene-associated tumour antigens as targets for immunotherapy'. Oncogene, <u>3</u>: 1715-1722.

Herlyn, D., Ross, A.H. and Koprowski, H. (1986).'Anti-idiotypic antibodies bear the internal image of the human tumour antigen'. *Science*, 232: 100-106.

Hertel, A., Baum, R.D., Lorenz, M., Baew-Christow, T., Encke, A. and Hör, G. (1990). 'Immunoscintigraphy using a technetium-99M labelled monoclonal anti-CEA antibody in the follow-up of colorectal cancer and other tumours producing CEA'. Br. J. Cancer, <u>62</u> (Suppl. X): 34-36.

Hirata, Y. and Sugawara, I. (1987).

÷

'Characterisation of mouse-human hybridoma as a useful fusion partner for the establishment of mousehuman-human hybridoma secreting anti-tetanus toxoid human monoclonal antibody of IgM or IgG class'. *Microbiol. Immunol.*, <u>31</u>: 231-245. Hird, V., Stewart, J.S.W., Snook, D., Dhokia, B., Coulter, C., Lambert, H.E., Mason, W.P., Soulter, W.P. and Eppenetos, A.A. (1990).

'Intraperitoneal administered <sup>99</sup>Y-labelled monoclonal antibodies as a third line treatment in ovarian cancer. A phase 1-2 trial: problems encountered and possible solutions'. Br. J. Cancer, <u>62</u> (Suppl. X): 48-52.

Hirohashi, S., Shimosato, Y. and Ino, Y. (1982).

'Antibodies from EB-virus-transformed lymphocytes of lymph nodes adjoining lung cancer'. Br. J. Cancer, <u>46</u>: 802-812.

Hnatowich, D.J., Childs, R.L., Lanteigne, D. and Najafi, A. (1983).
'The preparation of DTPA-Coupled Antibodies Radiolabelled with Metallic Radionuclides: An Improved Method'. J. Immunol. Methods, 65: 147-157.

Ho, M.K., Rand, N., Murray, J., Kato, K. and Rabin, H. (1985).
<u>'In vitro</u> immunization of human lymphocytes. I. Production of human monoclonal antibodies against bombesin and tetanus toxoid'. J. Immunol., <u>135</u>: 3831-3838.

Ho, M.K. (1988).

'Human monoclonal antibodies produced by <u>in vitro</u> immunization', in Progress in Biotechnology 5, In Vitro Immunization in Hybridoma Technology, (eds. Borrebaeck, C.A.K.), *Elsevier*, *Amsterdam*, *pp* 247-266.

Hoffman, M.K. and Hirst, J.A. (1985).

"In vitro stimulation of human B lymphocytes', in Human Hybridomas and Monoclonal Antibodies, (eds. Englemann, E.G., Foung, S.K.H., Larrick, J. and Raubitschek, A.), *Plenum Press, New York, pp* 466-471.

Horsfall, A.C., Hay, F.C., Soltys, A.J. and Jones, M.C. (1991). 'Epitope mapping'. *Immunol. Today*, <u>12</u>: 211-213.

Hsu, T.C., Markvong, A. and Marshall, J.T. (1978).
'G-band patterns of six species of mice belonging to subgenus Mus'. Cytogenet. Cell Genet., <u>20</u>: 304-307.

Hunter, R.E., Doherty, P., Griffin, T.W., Gionet, M., Hnatowich, D.J., Bianco, J.A. and Dillon, M.B. (1987).

'Use of Indium-III-labelled OC-125 monoclonal antibody in the detection of ovarian cancer'. *Gynecol.* Oncol., <u>27</u>: 325-337.

Igarashi, M. and Bando, Y. (1990).

'Enhanced efficiency of cell hybridisation by neuraminidase treatment'. J. Immunol. Methods, <u>135</u>: 91-93.

Jäger, W., Feistel, H., Paterock, E.M., Ronay, G., Tulusan, A.H., Wolf, F. and Lang, N. (1990). 'Resection guided by antibodies (REGAJ) a diagnostic procedure during second-look operation in ovarian cancer patients'. Br. J. Cancer, <u>62</u> (Suppl. X): 18-21.

Jahn, S., Grunow, R., Kiessig, S.T., Bogachera, G.T. Arsenjeva, E.L., Hlinak, A., Rocklin, O.V. and Von Baehr, R. (1987).

'Cell biology of human IgM-producing hybridomas derived from a fusion of human spleen lymphocytes with mouse myeloma cells'. *Hybridoma*, <u>6</u>: 679-687.

James, K. and Bell, G.T. (1987). 'Human monoclonal antibody production - current status and future prospects'. J. Immunol. Methods, <u>100</u>: 5-40.

Jeffreys, A.J., Wilson, V. and Thein, S.L. (1985). 'Individual-specific 'fingerprints' of human DNA'. *Nature*, <u>316</u>: 76-79.

Jiang, F.N., Jiang, S., Liu, D., Richter, A. and Levy, J. (1990).
'Development of technology for linking photosensitizers to a model monoclonal antibody'. J. Immunol. Methods, <u>134</u>: 139-149.

Johnson, I.S., Spearman, M.E., Todd, G.C., Zimmermann, J.L. and Bumol, T.F. (1987). 'Monoclonal antibody drug conjugates for site-directed cancer chemotherapy: Preclinical pharmacology and toxicology studies'. *Cancer Treat. Rev.*, <u>14</u>: 193-196. Jonak, Z.L., Braman, V. and Kennett, R.H. (1984).

'Production of continuous mouse plasma cell lines by transfection with human leukemia DNA'. *Hybridoma*,  $\underline{3}$ : 107-118.

Kaidbey, K.H. and Klingman, A.M. (1981).

'Photosensitization by coumarin derivatives. Structure-activity relationships'. Arch. Dermatol., <u>117</u>: 258-263.

Karpas, A., Fischer, P. and Swirsky, D. (1982).'Human myeloma cell line carrying a Philadelphia chromosome'. Science, <u>216</u>: 997-999.

Kenemans, P., Buist, M., Massuger, L., Roos, J., Classen, R., Verhoeyn, K., Vermorken, J., Burger, C. and Corstens, F. (1990).

'OV-TL3 F(ab')<sub>2</sub> <sup>111</sup>In imaging of ovarian cancer'. [Abst.] in Proceedings 7th International Meeting of the Advances in the Applications of Monoclonal Antibodies in Clinical Oncology. RCOG, Regent's Park, London, 14th - 16th May, pp 26.

Kingsman, A.J., Adams, S.E., Burns, N.R. and Kingsman, S.M. (1991). 'Retroelement particles as purification, presentation and target vehicles'. *Tibtech.*, *2: 304-309*.

Kishimoto, T. (1985).

'Factors affecting the growth and differentiation of B-lymphocytes'. Ann. Rev. Immunol., 3: 241-252.

Kitano, K., Shintani, Y., Ichimori, Y., Tsukamoto, K., Sasal, S. and Kida, M. (1986). 'Production of human monoclonal antibodies by heterohybridomas'. *Appl. Micro. Biotech.*, <u>24</u>: 282-293.

Klein, B., Lewinski, U., Shabtai, F., Freidin, N. and Djaldetti, M. (1977). 'Transmission and scanning electron microscopy study on plasma cell leukaemia'. *Blut*, <u>35</u>: 11-19.

Klibanov, A.L., Martynov, A.V., Slinkin, M.A., Sakharov, I.Y.U., Smirnov, M.D., Muzykantov, V.R., Danilov, S.M. and Torchilin, V.P. (1988).

'Blood clearance of radiolabeled antibody: Enhancement by lactrosamination and treatment with biotinavidin or anti-mouse IgG antibodies'. J. Nucl. Med., 29: 1951-1956.

Köhler, G. and Milstein, C. (1975).

'Continuous cultures of fused cells secreting antibody of predefined specificity'. Nature, 256: 495-497.

Koppel, G.A. (1990).

'Recent advances in monoclonal antibody drug targeting for the treatment of human cancer'. Bioconjugate Chem., <u>1</u>: 13-23.

Koropatnick, J., Pearson, J. and Harris, J.F. (1988).

'Extensive loss of human DNA accompanies loss of antibody production in heteromyeloma hybridoma cells'. *Mol. Biol. Med.*, <u>5</u>: 69-83.

Kosinski, S. and Hämmerling, U. (1986).

'A new cloning method for antibody-forming lymphoblastoid cells'. J. Immunol. Methods, 94: 201-209.

Kozak, C.A., Lawrence, J.B. and Ruddle, F.H. (1977).

'A sequential staining technique for the chromosomal analysis of interspecific mouse/hamster and mouse/human somatic cell hybrids'. *Exp. Cell Res.*, 105: 109-117.

Kozbor, D., Abramow-Newerly, W., Tripputi, P., Cole, S.P.C., Weibel, J., Roder, J.C. and Croce, C.M. (1985).

'Specific immunoglobulin production and enhanced tumourigenicity following ascites growth of human hybridomas'. J. Immunol. Methods, <u>81</u>: 31-43.

Kozbor, D. and Croce, C. (1985).

'Fusion partners for production of human monoclonal antibodies', in Human Hybridomas and Monoclonal Antibodies (eds., Engleman, E.G., Foung, S.K.H., Larrick, J. and Raubit-Schek, A.), *Plenum Press, New York, pp 21-36.* 

Kramer, E.L. and Noz, M.E. (1991). 'CT-SPECT fusion for analysis of radiolabelled antibodies: applications in gastrointestinal and lung carcinoma'. *Nucl. Med. Biol.*, 18: 27-43.

Kröhn, K.A. and Eary, J.F. (1991). 'Protecting the Antigen-Binding Site'. J. Nucl. Med., <u>32</u>: 122-123.

Laemmli, U.K. (1970).

'Cleavage of structural proteins during the assembly of the head of bacteriophage  $T_4$ '. *Nature*, <u>227</u>: 680-685.

Lagace, J. and Brodeur, B.R. (1985).

'Parameter affecting the in vitro immunisation of human lymphocytes'. J. Immunol. Methods, <u>85</u>: 127-136.

Laguzza, B.C., Nichols, C.L., Briggs, S.L., Cullinam, G.J., Johnson, D.A., Starling, J.J., Baker, A.L., Bumol, T.F. and Corralan, J.R. (1989).

'New anti-tumour monoclonal antibody-vinca conjugates LY203625 and related compounds: Design, preparation and representative in vitro activity'. J. Med. Chem., <u>32</u>: 548-555.

Lamki, L.M., Kasi, L.P., Kim, E.E., Podoloff, D.A. and Haynie, T.P. (1988). 'Radioimmunoscintigraphy of cancer'. *Cancer Bulletin*, <u>40</u>: 143-150.

Larrick, J.W. and Bourla, J.M. (1986).

'Prospects for the therapeutic use of human monoclonal antibodies'. J. Biol. Response Mod., <u>5</u>: 379-393.

Larrick, J.W., Gavilondo, J.V., Coloma, M.J. and Fry, K.E. (1990). 'Construction of recombinant therapeutic monoclonal antibodies', in Therapeutic Monoclonal Antibodies, (eds. Borrebaeck, C.A.K. and Larrick, J.W.), *Stockton Press, New York, pp 17-34.* 

Larson, S.M. (1985).

'Radiolabelled monoclonal anti-tumour antibodies in diagnosis and therapy'. J. Nucl. Med., <u>26</u>: 538-545.

Larson, S.M. (1991). 'Radioimmunology: Imaging and Therapy'. *Cancer*, 67 (Suppl. 4): 1253-1260.

Lehtovirta, P., Kairem, K.J., Liewendahl, K. and Seppalia, M. (1990). 'Immunolymphoscintigraphy and immunoscintigraphy of ovarian and fallopian tube cancer using F(ab')<sub>2</sub> fragments of monoclonal antibody OC125'. *Cancer Res.*, <u>50</u> (Suppl. 3): 9375-9405.

Lerner, R.A. and Tramantano, A. (1988). 'Catalytic antibodies'. Sci. Am., <u>258</u>(3): 58-70.

Lerner, R.A., Benkovic, S.J. and Schultz, P.G. (1991). 'At the crossroads of chemistry and immunology: Catalytic antibodies'. *Science*, 252: 659-667. Levy, R. and Miller, R.A. (1991).

'Radiolabelled antibodies : Another form of cytotoxic treatment'. J. Clin. Oncol., 9: 537-538.

Lewin, N., Aman, P., Akerlund, B., Gustavsson, E., Carenfelt, C., Lejdeborn, L., Klein, G. and Klein, E. (1990).

'Epstein-Barr virus-carrying B cells in the blood during acute infectious mononucleosis give rise to lymphoblastoid lines in vitro by release of transforming virus and by proliferation'. *Immunology Letts.*, 26: 59-65.

Liehn, J.C., Hannequin, P., Nasca, S., Lebrun, D., Cattan, D. and Valeyre, J. (1987). 'A new approach to image subtraction in immunoscintigraphy: Preliminary Results.' *Eur. J. Nucl. Med.*, <u>13</u>: 391-396.

Lind, P., Langsteger, W., Költringer, P., Dimai, H.P., Passl, K. and Eber, O. (1990). 'Immunoscintigraphy of inflammatory processes with technetium-99M-labelled monoclonal antigranulocyte antibody (MAb BW 250/183).' J. Nucl. Med., <u>31</u>: 417-423.

Lindmo, T., Boven, E., Cuttitta, F., Fedorko, J. and Bunn, P.A. (1984). 'Determination of the immunoreactive fraction of radiolabelled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess'. J. Immunol. Methods, <u>72</u>: 77-89.

Ling, N.R. and Lowe, J.A. (1989).

'Changes in the phenotype and immunoglobulin secretion of human B cells following co-culture with cells of an EBV<sup>+</sup> lymphoblastoid line or fusion with mouse plasmacytoma cells. Studies in short-term and long-term culture'. *Clin. Exp. Immunol.*, <u>75</u>: 311-316.

Löhde, E., Schwarzendahl, P., Schlicker, H., Abri, O., Kalthoff, H., Matzku, S., Epenetos, A.A. and Krass, E. (1990).

<sup>(</sup>Accumulation characteristics of human colon carcinomas after monoclonal antibody ex-perfusion'. Br. J. Cancer, <u>62</u> (Suppl. X): 12-15.

Lowe, D., Handley, H., Schmidt, J., Royston, I. and Glassy, M. (1984).'A human monoclonal antibody reactive with human prostrate'. J. Urol., <u>132</u>: 780-785.

Mafane, N., Barth, R.F. and Steplewski, Z. (1987).

'Purification and characterisation of boron-10 linked monoclonal antibody conjugates for neutron capture therapy'. *Proc. Amer. Assoc. Cancer Res.*, <u>28</u>: 391.

.....

Martin, D., Larose, Y., Hamel, J., Lagace, J. and Brodeur, B.R. (1988).
'Heterohybridomas secreting human monoclonal antibodies against <u>Haemophilus influenza</u> type b'. *Eur. J. Immunol.*, <u>18</u>: 601.

Martinis, J., Kull, J.F., Franz, G. and Bartholomew, R.M. (1982). 'Monoclonal antibodies with dual antigen specificity', in Proceedings of the 13th Colloquium on Protides of the Biological Fluids, (eds. Peeters, H.), *Pergamon Press, New York, pp 311*.

Masucci, G., Wersäll, P., Ragnhammer, P. and Mellstedt, H. (1989). 'Granulocyte-monocyte-colony-stimulating factor augments the cytotoxic capacity of lymphocytes and monocytes in antibody-dependent cellular cytotoxicity'. *Cancer Immunol. Immunother.*, <u>29</u>, 288-292.

Masuho, Y., Matsumoto, Y., Sugano, T., Tomliyama, T., Sasaki, S. and Tamotsu, K. (1990). 'Development of a Human Monoclonal Antibody against cytomegalovirus with the aim of passive immunotherapy', in Therapeutic Monoclonal Antibodies (eds, Borrebaeck, C.A.K. and Larrick, J.W.) *Stockton Press, New York, pp 187-207.* 

Mather, S.J. and Ellison, D. (1990). 'Reduction mediated technetium-99M labeling of monoclonal antibodies'. J. Nucl. Med., <u>31</u>: 692-697.

Matsumoto, Y.I., Sugano, T., Miyamoto, C. and Masuho, Y. (1986). 'Generation of hybridomas producing human monoclonal antibodies against human cytomegalovinis'. Biochem. Biophys. Res. Commun., <u>137</u>: 273-283.

Matsuoka, Y., Moore, G.E., Yagi, Y. and Pressman, D. (1967). 'Production of free light chains of immunoglobulin by a hematopoietic cell line derived from a patient with multiple myeloma'. *Proc. Soc. Exp. Biol.*, <u>125</u>: 1246-1250.

McDicken, I.W., Stamp, G.H., McLoughlin, P.J. and Johnson, P.M. (1983). 'Expression of human placental-type alkaline phosphatase in primary breast cancer'. *Int. J. Cancer*, <u>32</u>: 205-209.

McDowell, E. M. and Trump, B. F. (1976). 'Histologic fixatives suitable for diagnostic light and electron microscopy'. Arch. Pathol. Lab. Med., 100: 405-414. McHale, A.P. and McHale, L. (1988).

'The effect of haematoporphyrin derivative and human erythrocyte ghost-encapsulated hematoporphyrin on a mouse myeloma cell line'. *Cancer Biochem. Biophys.*, <u>20</u>: 157-164.

McLaughlin, P.J., Travers, P., McDicken, I.W. and Johnson, P.M. (1984). 'Demonstration of placental and placental-like alkaline phosphatase in non-malignant tumour tissue extracts using an enzyme immunoassay'. *Clin. Chim. Acta*, <u>137</u>: 341-348.

Meares, C.F., Moi, M.K., Diril, H., Kukis, D.L., McCall, M.J., Deshpande, S.V., DeNardo, S.J., Snook, D. and Epenetos, A.A. (1990).

'Macrocyclic chelates of radiometals for diagnosis and therapy'. Br. J. Cancer, 62 (Suppl. X): 21-22.

Mellstedt, H. (1990).

'Monoclonal antibodies in cancer therapy'. Curr. Opin. Immunol., 2: 708-713.

Mendelsohn, J. (1990).

'Anti-epidermal growth factor receptor monoclonal antibodies as potential anti-cancer agents'. J. Steroid. Biochem. Mol. Biol., <u>37</u>: 889-892.

Mew, D., Wat, C.K., Towers, G.H.N. and Levy, J.G. (1983). 'Photoimmunotherapy: Treatment of animal tumours with tumour specific monoclonal antibodyhematoporphyrin conjugates'. J. Immunol., <u>130</u>: 1473-1477.

Mew, D., Wat, C.K., Towers, G.H.N., Sun, C.H.C., Walters, R.J., Wright, W., Berns, M.W. and Levy, J.T. (1985).

'Ability of specific monoclonal antibodies and conventional antisera conjugated to hematoporphyrin to label and kill selected cell lines subsequent to light activation'. *Cancer Res.*, <u>45</u>: 4380-4386.

Millar, B.C., Bell, J.B.G., Lakhani, A., Ayliffe, M.J., Selby, P.J. and McElwain, J.J. (1988). 'A simple method for culturing myeloma cells from bone marrow aspirates and peripheral blood in vitro'. Br. J. Haematol., <u>69</u>: 197-203.

Milstein, C. and Cuello, A.C. (1984).

'Hybrid hybridomas and the production of bi-specific monoclonal antibodies'. *Immunol. Today*, <u>5</u>: 299-304.. Mishell, B.B., Shiigi, S.M., Henry, C., Chan, E.L., North, J., Gallily, R., Slomich, M., Miller, K., Marbrook, J., Parks, D. and Good, A.H. (1980).

'Preparation of mouse cell suspensions', in Selected Method in Cellular Immunology (eds, Mishell, B.B. and Shiigi, S.M.). W.H. Freeman and Company, San Francisco, pp 3-28.

Moore, G.E., Gerner, R.E. and Franklin, H.A. (1967). 'Culture of normal human leukocytes'. J. Amer. Med. Assoc., <u>199</u>: 519-524.

Moran, E. (1990).

'Studies on the antigenic nature and effects of drugs on astrocytoma cells'. Ph.D. Thesis, University of Dublin, Trinity College.

Morrison, S.L. and Oi, V.T. (1989). 'Genetically engineered antibody molecules'. Adv. Immunol., <u>44</u>: 65-92.

Muto, M.C., Lepisto, E.M., Van den Abeele, A., Knapp, R.C. and Kassis, A.I. (1989). 'Influence of human antimurine antibody on CA-125 levels in patients with ovarian cancer undergoing radioimmunotherapy or immunoscintigraphy with murine monoclonal antibody OC-125'. Am. J. Obstet. Gynecol., <u>161</u>: 1206-1212.

Myhr, B.C. and DiPaolo, J.A. (1975).

'Requirements for cell dispersion prior to selection of induced azaguanine-resistant colonies of chinese hamster cells'. *Genetics*, <u>80</u>: 157-169.

Nacheva, E., Fischer, P.A., Sherrington, P.P., Labastide, W., Lawlor, E., Conneally, E., Blaney, C., Hayhoe, F.G.J. and Karpas, A. (1990).

'A new human plasma cell line, Karpas-620 with translocations involving chromosomes 1, 11 and 14'. Br. J. Haematol., <u>74</u>: 70-76.

Nelson, J.S., Liaw, L-H., Orenstein, A., Roberts, W.G. and Berns, M.W. (1988).
'Mechanism of tumour destruction following photodynamic therapy with hematoporphyrin derivative, chlorin and phthalocyanine'. J. Natl. Cancer Inst., <u>80</u>: 1599-1605.

Neuberger, M.S., Williams, G.T. and Fox, R.O. (1984). 'Recombinant antibodies possessing novel effector functions'. *Nature*, <u>312</u>: 604-608. Neuberger, M.S. (1985).

'Making novel antibodies by expressing transfected immunoglobulin genes'. Trends Biotechnol., <u>3</u>: 347-355.

Nolan, O. and O'Kennedy, R. (1990). 'Bifunctional antibodies: Concepts, production and applications'. *Biochim. Biophys. Acta.*, <u>1040</u>: 1-11.

O'Brien, S.J., Kleiner, G., Olson, R. and Shannon, J.E. (1977). 'Enzyme polymorphisms as genetic signatures in human cell cultures'. *Science*, <u>195</u>: 1345-1348.

O'Kennedy, R. and Roben, P. (1991). 'Antibody engineering: An overview'. Essays in Biochemistry, <u>26(6)</u>: 59-75.

Olsson, L. and Kaplan, H.S. (1980).
'Human-human hybridomas producing monoclonal antibodies of predefined antigenic specificity'. *Proc.* Natl. Acad. Sci. USA, <u>77</u>: 5429-5431.

Olsson, L. and Kaplan, H.S. (1984). 'Human-human antibody producing hybridomas: Technical aspects'. Methods in Enzymol., <u>92</u>: 3-16.

Ollson, L. and Brams, P. (1985).

'Human-Human Hybridoma Technology. Five years of technical improvements and its application in cancer biology', in Human Hybridomas and Monoclonal Antibodies (eds, Engleman, E.G., Foung, S.K.H., Larrick, J. and Raubitschek, A.), *Plenum Press, New York: pp 37-50.* 

Opdyke, D.L. (1981). 'The structure-activity relationships of some substituted coumarins with respect to skin reactions'. Dragoco Report, <u>2</u>: 42-48.

Oseroff, A.R., Wimberly, J., Lee, C., Alvarez, V. and Parrish, J.A. (1985). 'Photosensitized destruction of normal and leukaemic T cells using monoclonal antibody (mab) directed hematoporphyrin '. [Abst.], J. Invest. Dermatol., <u>84</u>: 335.

Oseroff, A.R., Ohuoha, D., Hasan, T., Bommer, C. and Yarmush, M.L. (1986). 'Antibody-targeted photolysis: Selective photodestruction of human T cell leukaemia cells using monoclonal antibody - chlorin e<sub>6</sub> conjugates. *Proc. Natl. Acad. Sci. USA*, <u>183</u>: 8744-8748. Oseroff, A.R., Ara, G., Ohuoha, D., Aprille, J., Bommer, J.C., Yarmush, M.L., Foley, J. and Cincotta, L. (1987).

'Strategies for selective cancer photochemotherapy: Antibody-targeted and selective carcinoma cell photolysis'. *Photochem. Photobiol.*, <u>46</u>: 83-96.

Oseroff, A.R., Wan, W., Wanda, K.S., Barrie, K.A., Park, Y., and Whitaker, J.E. (1990) 'Cationic dyes permit selective fluorescence and detection of subcutaneous tumours'. [Abst.], *Proc.Am.Assoc.Cancer Res.*, <u>31</u>: 444.

Östberg, L. and Pursche, E. (1983). 'Human x (mouse x human) hybridomas stably producing human antibodies'. *Hybridoma*, <u>2</u>: 361-367.

Paganelli, G., Malcovati, M. and Fazio, F. (1991).
'Monoclonal antibody pretargetting technique for tumour localisation: The avidin-biotin system'. Nucl. Med. Commun., <u>12</u>: 211-234.

Pak, K.Y., Nedelman, M.A. and Stewart, R. (1989).
'A rapid and efficient method of labelling IgG antibodies with Tc-99m and comparison with Tc-99m
Fab' antibody fragments'. J. Nucl. Med., <u>30</u>: 793-801.

Pateisky, N., Phillip, K. and Skolder, W.D. (1985).
'Radioimmunodetection in patients with suspected ovarian cancer'. J. Nucl. Med., <u>26</u>: 1369-1376.

Paulie, S., Lundblad, M.L., Hansson, Y., Koho, H., Ben-Aissa, H. and Perlmann, P. (1984).
'Production of antibodies to cellular antigens by EBV-transformed lymphocytes from patients with urinary bladder carcinoma'. Scand. J. Immunol., <u>20</u>: 461-469.

Pectasides, D., Vonorta, P., Tsialta-Salihou, A., Pateniotis, K., Barbounis, V., Kayianni, H., Arapantoni, P., Taylor-Papadimitriou, J., Epenetos, A., Doutsioubq, P. and Athanassion, A. (1990). 'Immunoscintigraphy with <sup>131</sup>I-labelled H17E2 monoclonal antibody compared with conventional lymphangiography and computed tomography in the detection of metastases in patients with testicular germ cell tumours'. *Br. J. Cancer*, <u>62</u> (*Suppl. X*): 74-77.

Peeters, P.A.M., Storm, G. and Crommelin, D.J.A. (1987). 'Immunoliposomes in vivo: State of the art'. Adv. Drug Del. Rev., <u>1</u>: 249-266. Peltier, P., Fumoleau, P., Kremer, M., Tournemaine, N., Le Mável, B., Tédrez, P. and Curlet, C. (1987). 'Immunoscintigraphie a l'OC 125-DTPA-In<sup>III</sup>'. J. de Biophysique Biomécanique, <u>11</u>: 91-93.

Perkins, A.C., Pimm, M.V., Gie, C., Marksman, R.A., Symonds, E.M. and Baldwin, R.W. (1989). 'Intraperitoneal <sup>131</sup>I- and <sup>10</sup>In-791T/36 monoclonal antibody in recurrent ovarian cancer: imaging and biodistribution'. *Nuclear Med. Comm.*, <u>10</u>: 577-584.

Pietersz, G.A., Kanellos, J., Smyth, M.J., Zaleberg, J. and McKenzie, I.F.C. (1987).
'The use of monoclonal antibody conjugates for the diagnosis and treatment of cancer'. *Immunol. Cell Biol.*, 65: 111-125.

Pietersz, G.A. (1990).

'The linkage of cytotoxic drugs to monoclonal antibodies for the treatment of cancer'. *Bioconjugate* Chem.,  $\underline{1}$ : 90-95.

Pollack, M., Raubitschek, A.A. and Larrick, J.W. (1987).
'Human monoclonal antibodies that recognize conserved epitopes in the core-lipid A region of lipopolysaccharides'. J. Clin. Invest., 79: 1421-1428.

Polke, C.R., Lowenthal, J.W., Roth, S.A., Rohwer, P., Macdonald, H. and Kalden, J.R. (1986). 'Phorbol ester enhances both interleukin receptor expression and immunoglobulin secretion in human Epstein-Barr virus-immortalized B cells'. *Eur. J. Immunol.*, <u>16</u>: 146-152.

Powis, (1991). 'Signalling targets for anti-cancer drug development'. TIPS., <u>12</u>: 188-194.

Price, P.J. (1985).

'A simple rapid method for freezing hybridomas'. J. Tissue Culture Methods, 2: 167-170.

Rabbitts, P.H., Douglas, J., Fisher, P., Nacheva, E., Karpas, A., Catovsky, D., Melo, J.V., Baer, R.,
Stinton, M.A. and Rabbitts, T.H. (1988).
'Chromosome abnormalities at 11q13 in B cell tumours'. Oncogene, 3: 99-103.

Rakestraw, S.L., Tompkins, R.G. and Yarmush, M.L. (1990). 'Preparation and characterization of immunoconjugates for antibody targeted photolysis'. *Bioconjugate Chem.*, <u>1</u>: 212-221. Reid, Y.A. and Cour, M.I. (1985).

'Cryopreservation of hybridomas'. J. Tissue Culture Methods, 9: 163-166.

Reichmann, L., Clark, M., Waldmann, H. and Winter, G. (1988). 'Reshaping human antibodies for therapies'. *Nature*, <u>332</u>: 323-327.

Reisfeld, R.A. and Schrappe, M. (1990).

'Current status and future perspectives of chemoimmunoconjugates for the treatment of cancer', in Therapeutic Monoclonal Antibodies, (eds. Borrebaeck, C.A.K. and Larrick, J.W.), Stockton Press, New York: pp 57-73.

Reuland, P., Kosceiniak, E., Ruck, P., Treuner, J. and Feine, U. (1991).
'Application of anti-myosin antibody for scintigraphic differential diagnosis of infantile tumours'. Nucl. Med. Biol., <u>18</u>: 89-95.

Rhodes, B.A. and Burchel, S.W. (1983).

'Radiolabelling of antibodies with techetium-99M', in Radioimmunoimaging (eds, Burchiel, S.W. and Rhodes, B.A.) *Elsevier Science, New York:* 207-222.

Ricchelli, F., Jori, G., Moreno, G., Vinzens, F. and Salet, C. (1990).
'Factors influencing the distribution pattern of porphyrins in cell membranes'. J. Photochem. Photobiol.
B:, <u>6</u>: 69-77.

Ritts, R.E., Ruiz-Arguelles, A., Weyl, K.G., Bradley, A.L., Weihmeir, B., Jacobsen, D.J. and Strehlo, B.L. (1983).

'Establishment and characterisation of a human non-secretory plasmacytoid cell line and its hybridization with human B cells'. Int. J. Cancer, <u>31</u>: 133-141.

Rodighiero, G., Dall'Acqua, F. and Pathak, M.A. (1984).
'Photobiological properties of monofunctional furocoumarin derivatives', in Topics in Photomedicine, (ed. Smith, K.C.), *Plenum Press, New York, pp 319-398.*

Ronay, G., Jäger, W., Weiss, K., Feistel, H., Wolf, F., Tulusan, A.H. and Lang, N. (1990). 'Immunohistochemical identification of CA125 and the  $F(ab')_2$  fragments of the murine monoclonal antibody OC125 in ovarian cancer tissue'. Br. J. Cancer, <u>62</u> (Suppl. X): 67-70. Rose, T.M., Plowman, G.D., Teplow, D.B., Dreyer, W.J., Hellstöm, K.E. and Brown, J.P. (1986). 'Primary structure of the human melanoma antigen p97 (melanotransferin) deducted from the MRNA sequence'. *Proc. Natl. Acad. Sci. USA*, <u>83</u>: 1261-1265.

Rosén, A., Noma, T., Wendel-Hansen, V., Zeuthen, J., Lin, C.Y. and Honjo, T. (1988). 'A new progression factor for stimulation of human B cells <u>in vitro</u>', in Progress in Biotechnology 5, <u>In vitro</u> Immunization in Hybridoma Technology, (ed. Borrebaeck, C.A.K.), *Elsevier, Amsterdam, pp* 311-335.

Rosenberg, S.A., Plocinik, B.A. and Nicholas, G. (1972).

"Unmasking'of human lymphoid cell heteroantigens by neuraminidase treatment'. J. Natl. Cancer Inst., <u>48</u>: 1271.

Rousset, F., Garcia, E. and Banchereau, J. (1991). 'Cytokine-induced proliferation and immunoglobulin production of human B lymphocytes triggered through their CD40 antigen'. J. Exper. Med., <u>173</u>: 705-710.

Ryan, K.P., Dillman, R.O., DeNardo, S.J., DeNardo, G.L., Beuregard, J., Hagan, P.L., Amox, P.G., Clutter, M.L., Burnett, K.G. and Rubt, G.M. (1988). 'Imaging of breast cancer metastases using an III-indium radiolabelled human IgM monoclonal

antibody'. Radiology, <u>167</u>: 71-75.

Sany, J. (1990). 'Immunological treatment of rheumatoid arthritis'. Clin. Exp. Rheumatol., <u>8</u>: (Suppl. 5), 81-88.

Sasaki, M., Peterson, J.A. and Ceriani, R.L. (1981).
'Quantitation of human mammary epithelial antigens in cells cultured from normal and cancerous breast tissues'. *In Vitro*, <u>17</u>: 150-158.

Scheirer, W., Nilsson, K., Merten, D.W., Katinger, H.W.D. and Mosbach, K. (1984).
'Entrapment of animal cells for the production of biomolecules such as monoclonal antibodies'. *Dev. Biol. Stand.*, <u>55</u>: 155-167.

Schroder, J. (1985).

'Identification of human chromosomes in mouse-human lymphocyte hybrids', in Human Hybridomas and Monoclonal Antibodies, (eds, Engleman, E.G., Foung, S.K.H., Larrick, J. and Raubitschek, A.). *Plenum Press, New York:* 449-453.

Schroff, R.W., Foon, K.A., Beatty, S.M., Oldham, R.K. and Morgan, A.G. (1985).

'Human anti-murine immunoglobulin responses in patients receiving monoclonal antibody therapy'. Cancer Res., <u>45</u>: 879-885.

Searle, F., Bier, C., Buckley, R.G. (1986).

'The potential of carboxypeptidase G2 antibody conjugates as anti-tumour agents. 1. Preparation of anti-human chorionic gonadotrophin carboxypeptidase G2 and cytotoxicity of the conjugate against JAR choriocarcinoma cells in vitro. Br. J. Cancer, <u>53</u>: 377-385.

Seigneurin, J.M., Desgranges, C., Seigneuvin, D., Paire, J., Renversez, J.C., Jacquemont, B. and Micouin, C. (1983).

'Herpes simplex virus glycoprotein D : Human monoclonal antibody produced by bone marrow cell line'. Science, <u>221</u>: 173-175.

Senter, P. (1991).

'Activation of prodrugs by antibody-enzyme conjugates'. [Abst.], in Proceedings of the Eight International Hammersmith Meeting of the Advances in the Applications of Monoclonal Antibodies in Clinical Oncology, Halkidiki, Greece, 8-13th May: p 67.

Shawler, D.L., Bartholomew, R.M., Smith, L.M. and Dillman, R.O. (1985).
'Human immune response to multiple injections of murine monoclonal IgG'. J. Immunol., <u>135</u>: 1530-1535.

Sherwood, R. (1991). 'Protein fusions: Bioseparation and application'. *Tibtech.*, <u>9</u>: 1-3.

Shokat, K.M. and Schultz, P.G. (1990). 'Catalyic antibodies'. Ann. Rev. Immunol., <u>8</u>: 335-363.

Shulman, M., Wilde, C.D. and Köhler, G. (1978).'A better cell line for making hybridomas secreting specific antibodies'. *Nature*, <u>276</u>: 269-272.

Sikora, K., Alderson, T., Phillips, J. and Watson, J.V. (1982). 'Human hybridomas from malignant gliomas'. *Lancet*, *i*: 11-14.

Sikora, K., Alderson, T., Ellis, Y., Phillips, J. and Watson, J. (1983). 'Human hybridomas from patients with malignant disease'. Br. J. Cancer, <u>47</u>: 135-145. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fugiomoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985).

'Measurement of protein using biocinchoninic acid'. Anal. Biochem. 150: 76-85.

Smyth, H., Farrell, D.J., O'Kennedy, R. and Corrigan, A. (1977). 'Transplantability of neuraminidase-treated ascites tumour cells'. *Eur. J. Cancer*, <u>13</u>: 1313-1320.

Snook, D., Verhoeyen, M., Kosmas, C., Badley, R.A., Hird, V., Meares, C., Lavender, J.P. and Epenetos, A.A. (1991).

'A preliminary comparison of a tumour-associated reshaped human monoclonal antibody (HU2PLAP) with its murine equivalent (H17E2)' [Abst.] in Proceedings Eight International Hammersmith Meeting of the Advances in the Applications of Monoclonal Antibodies in Clinical Oncology, *Halkidiki*, *Greece*, *8-13th May: pp 51*.

Spalding, B.J. (1991).

'Cancer immunoconjugates: Will clinical success lead to commercial success'? Biotechnol., 2: 701-704.

Spooner, R.A. and Lord, J.M. (1990). 'Immunotoxins: Status and prospects'. *Tibtech.*, <u>8</u>: 189-193.

Steele, J.K., Liu, D., Stammers, A.T., Deal, H., Whitney, S. and Levy, J.G. (1988). 'Supressor deletion therapy: Selective elimination of T suppressor cells using a hematoporphyrinconjugated monoclonal antibody', in Antibody-Mediated Delivery Systems, (ed. Rodwell, J.D.), *Marcel Dekker, Inc., New York: pp 157-189.* 

Steintz, M., Sepälä, I., Eichmann, K. and Klein, G. (1979).
'Establishment of a human lymphoblastoid cell line with specific antibody production against group A streptococcal carbohydrate'. *Immunobiology*, 156: 41-47.

Stickney, D.R., Frincke, J.M., Slater, J.B., Ahlem, C.N., Merchant, B. and Slater, J.M. (1988). 'Bifunctional antibody technology: Clinical applications for CEA expressing tumours'. [Abst.] in Proceedings of the Third International Conference on Monoclonal Antibody Conjugates for Cancer, San Diego, CA, USA, 4th - 6th February: pp 74.

Strauss, H.W., Fischerman, A.J., Khaw, B.A. and Rubin, R.H. (1991). 'Non-tumour applications of radioimmune imaging', *Nucl. Med. Biol.*, <u>18</u>: 127-134.

÷

Strelkauskas, A.J. and Taylor, C.L. (1986).

'Human monoclonal antibody: Construction of stable clones reactive with human breast cancer'. Cancer Immunol. Immunother., 23: 31-38.

Sun, L.K., Curtin, P., Rakowicz-Szulcznska, E., Ghrayeb, J., Chang, N., Morrison, S.L. and Koprowski, H. (1987).

'Chimaeric antibody with human constant regions and mouse variable regions directed against carcinoma-associated antigen 17-1A. Proc. Natl. Acad. Sci. USA, <u>84</u>: 214-222.

Swain, W.F. (1991). 'Antibodies in plants'. *Tibtech.*, <u>9</u>: 107-109.

Taylor-Papadimitriou, J., Bodimer, W.F., Egan, M. and Mellis, M. (1981).
'Monoclonal antibodies to epithelium-specific components of the human milk fat globule membrane:
Production and reaction with cells in culture', *Int. J. Cancer*, <u>28</u>: 23-29.

Tazzari, P.L., Gobbi, M., Dinota, A., Bontadini, A., Grassi, G., Cerato, C., Cavo, M., Pileri, S., Caligaris-Cappio, F. and Tura, S. (1987).

'Normal and neoplastic plasma cell membrane phenotype: Studies with new monoclonal antibodies'. Clin. Exp. Immunol., <u>70</u>: 192-200.

Teng, N.N., Lam, K.S., Riera, F.C. and Kaplan, H.S. (1983).
'Constructing and testing of mouse-human heteromyelomas for human monoclonal antibody production'. *Proc. Natl. Acad. Sci. USA*, <u>80</u>: 7308-7312.

Teng, N.N.H., Reyes, G.R., Bieber, M., Fry, K.E., Lam, K.S. and Herbert, J.M. (1985). 'Strategies for stable human monoclonal antibody production: Construction of heteromyelomas in vitro sensitization and molecular cloning of human immunoglobulin genes', in Human Hybridomas and Monoclonal Antibodies, (eds. Engleman, E.G., Foung, S.K.H., Larrick, J. and Raubitschek, A.), *Plenum Press, New York: pp 71-91.* 

Thakur, M.L. and DeFulvio, J.D. (1991).

'Technetium-99M-labeled monoclonal antibodies for immunoscintigraphy', J. Immunol. Methods, <u>137</u>: 217-224.

Thomas, G.D., Chappell, M.J., Dykes, P.W., Ramsden, D.B., Godfrey, K.R., Ellis, J.R.M. and Bradwell, A.K. (1990).

'Theoretical considerations for improved tumour targeting', Br. J. Cancer, 62: 70-73.

Tholander, B., Taube, A., Lindgree, A., Sjöberg, O., Stendahl, U. and Tamsen, L. (1990). 'Pretreatment serum levels of CA-125, carcinoembryonic antigen, tissue polypeptide antigen and placental alkaline phosphatase in patients with ovarian carcinoma: Influence of histological type, grade of differentiation and clinical stage of disease', *Gynecol. Oncol.*, <u>39</u>: 26-33.

Thurin, J. (1990).

'Characterisation and molecular biology of tumour-associated antigens'. Curr. Opin. Immunol., <u>2</u>: 702-707.

Tomson, S., Emmet, E.A. and Fox, S. (1974).

'Photodestruction of mouse epithelial tumours after oral acridine orange and argon laser'. Cancer Res., <u>34</u>: 3124-3127.

Tong, A.W., Lee, J.C., Wang, R.M., Ordonez, G. and Stone, M.J. (1989).

'Augmentation of lymphokine-activated killer cell cytotoxicity by monoclonal antibodies against human small cell lung carcinoma'. *Cancer Res.*, <u>49</u>: 4103-4108.

Traunecker, A., Oliveri, F. and Karjalainen, K. (1991).

'Myeloma based expression systems for the production of large mammalian proteins'. *Tibtech.*, 2: 109-113.

Trauth, B.C., Klas, C., Peters, A.M.J., Matzuku, S., Möller, P., Falk, W., Debatin, K.M. and Krammer, P.H. (1989).

'Monoclonal antibody-mediated tumour regression by induction of apoptosis'. Science, 245: 301-305.

Trouet, A., Masquelier, M., Baurain, R. and Deprez-de Campeneere, D. (1982).

'A covalent linkage between daunorubicin and proteins that is stable in serum and reversible by lysosomal hydrolases, as required for a lysosomotropic drug-carrier conjugate; <u>In vitro</u> and <u>in vivo</u> studies'. *Proc. Natl. Acad. Sci. USA*, <u>79</u>: 626-629.

Tsuruo, T. and Hamada, H. (1990).

'Therapeutic implications of monoclonal antibodies against multi-drug resistant tumour cells', in Therapeutic Monoclonal Antibodies, (eds. Borrebaeck, C.A.K. and Larrick, J.W.), Stockton Press, New York: pp 109-126.

Uchiyama, K., Saito, H., Tokuhisa, T., Imai, K. and Taniguchi, M. (1987). 'High Frequency of loss of human kappa light chain expression in mouse-human heterohybridomas'. *Hybridoma*, 6: 645-654.

Vadham-Raj, S., Cordon-Cardo, C. and Carswell, E. (1988).
'Phase I trial of a mouse monoclonal antibody against GD3 ganglioside in patients with melanoma: Induction of inflammatory responses at tumour sites'. J. Clin. Oncol., <u>6</u>: 1636-1648.

Van Brunt, J. (1989). 'Novel drug delivery systems'. *Biotechnology*, <u>7</u>: 127-130.

Van Dam-Mieras, M.C.E., de Jeu, W.H., DeVries, J., Currell, B.R., James, J.W., Leech, C.K. and Patmore, R.A. (eds). (1991).

'Case study: Myoscint - a monoclonal antibody preparation used for cardiac imaging', in Biotechnological Innovations in Health Care, Butterworth-Heinemann, Oxford: p 225-249.

Van Meel, F.C.M., Steenbakkers, P.G.A. and Oomen, J.E.H. (1985). 'Human and chimpanzee monoclonal antibodies'. J. Immunol. Methods, <u>80</u>: 267-277.

Vaughan, A.T.M., Anderson, P., Dykes, P.W., Chapman, C.E. and Bradwell, A.R. (1987). 'Limitations to the killing of tumours using radiolabelled antibodies', *Br. J. Radiol.*, <u>60</u>: 567-578.

Verhoeyn, M., Saunders, J., Broderick, L., Eida, S. and Bradley, A. (1990).
'Production of reshaped human antibodies for cancer diagnosis and therapy', in Monoclonal Antibodies:
Applications in Clinical Oncology (ed. Epenetos, A.A.), *Chapman Hall, UK: pp 37-43*.

Vitetta, E.S., Fulton, R.J., May, R.D., Till, M. and Uhr, J.W. (1987). 'Redesigning nature's poisons to create anti-tumour reagents'. *Science*, <u>238</u>: 1098-1104.

Wabl, M.R., Jäck, H-M., Von Borstel, R.C. and Steinberg, C.M. (1989).'Scope of action of the immunoglobulin mutator system'. *Genome*, <u>31</u>: 118-121.

Waldmann, T.A., Goldman, C.K., Bongiovanni, K.F., Sharrow, S.O., Davey, M.P., Cease, K.B., Greenberg, S.J. and Longo, D.L. (1989).

'Therapy of patients with human T-cell lymphotrophic virus I-induced adult T-cell leukemia with anti-Tac, a monoclonal antibody to the receptor for interleukin-2'. *Blood*, <u>72</u>: 1805-1816.

Wang, F., Gregory, C., Sample, C., Rowe, M., Liebowitz, D., Murray, R., Rickinson, A. and Kieff, E. (1990).

'Epstein-Barr virus latent membrane protein (LMPI) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMPI cooperatively induce CD23'. J. Virology, 64: 2309-2318.

Ward, B.G., Lowe, D.G. and Shepherd, J.H. (1987).

'Patterns of expression of a tumour-associated antigen, defined by the monoclonal antibody HMFG2 in human epithelial ovarian carcinoma. Comparison with expression of the HMFG-1, AUA-1 and F36/22 antigens'. *Cancer*, <u>60</u>: 787-792.

Watson, J. and Whitlock, C. (1978).

'Effect of a synthetic adjuvant on the induction of primary immune responses in T-cell depleted spleen cultures'. J. Immunol., <u>121</u>: 383-389.

Weiner, L.M., Moldofsky, P.J., Gatenby, R.A., O'Dwyer, J., O'Brien, J., Litwin, S. and Comis, R.L. (1988).

'Antibody delivery and effector cell activation in a phase II trial of recombinant  $\gamma$ -interferon-murine monoclonal antibody CO17-A in advanced colorectal carcinoma'. *Cancer Res.*, <u>48</u>: 2568-2573.

Weiss, M.C. and Green, H. (1967).

'Human-mouse hybrid cell lines containing partial complements of human chromosomes and functional human genes'. *Proc. Natl. Acad. Sci. USA*, <u>58</u>: 1104-1111.

Wile, A.G., Coffey, J., Nahabedian, M.Y., Baghdassarian, R., Mason, G.R. and Berns, M.W. (1984). 'Laser photoradiation therapy of cancer: An update of the experience at the University of California, Irvine'. Lasers Surg. Med., <u>4</u>: 5-12.

Winter, G. and Milstein, C. (1991). 'Man-made antibodies'. *Nature*, <u>394</u>: 293-299. Winterbourne, D.J., Thomas, S., Herman-Taylor, J., Hussain, I. and Johnstone, A.P. (1988). 'Electric shock-mediated transfections of cells: Characterisation and optimisation of electrical parameters'. *Biochem. J.*, <u>251</u>: 427-434.

Wong, C.H. (1989). 'Enzymatic catalysts in organic synthesis'. Science, <u>244</u>: 1145-1152.

Wong, J.T. and Colvin, R.B. (1987).

'Bi-specific monoclonal antibodies: Selective binding and complement fixation to cells that express two different antigens '. J. Immunol., <u>139</u>: 1369.

Yakoi, T., Miyawaki, T., Yachie, A., Kato, K., Kashara, Y. and Taniguchi, N. (1990). 'Epstein-Barr virus-immortalized B cells produce IL-6 as an autocrine growth factor'. *Immunology*, <u>70</u>: 100-105.

Yamaura, N., Makino, M., Walsh, L.J., Bruce, A.W. and Choe, B.K. (1985).
'Production of monoclonal antibodies against prostatic acid phosphatase by <u>in vitro</u> immunisation of human spleen cells'. J. Immunol. Methods, <u>84</u>: 105-116.

Yu, D.S., Chang, S.Y. and Ma, P.S. (1990).

'Photoactivation of bladder tumour cells by methylene blue: Study of a variety of tumour and normal cells'. J. Urology, <u>144</u>: 164-168.

Yuhus, J.M., Toya, R.E. and Sehon, A.H. (1974). 'Neuraminidase-induced enhancement of tumour growth in mice'. J. Natl. Cancer Inst., 52: 1905-1911.

Zimmermann, U. (1982).

'Electric field mediated fusion and related electrical phenomena'. Biochim. Biophys. Acta, <u>694</u>: 227-277.