

**CEA-Targeted Monoclonal Antibody
Therapy in Colorectal Cancer**

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To Elizabeth, Joshua, Emma & Edward

and to the glory of the Almighty God

who gave them to me.

Abstract

Introduction

The adjuvant treatment of colorectal cancer (CRC) has seen little improvement in terms of mortality of the disease in the last 40 years. There has been a resurgence in research into the use of monoclonal antibodies in the treatment of CRC. Carcinoembryonic antigen (CEA) is a useful target in cancer immunotherapy. The distribution of CEA in CRC differs from that in normal colorectal tissue. In normal colorectal tissue CEA is found only on the luminal surface of the cell which is inaccessible to intravenous antibody, whereas in CRC, CEA is found on all borders of the cell membrane and so becomes accessible to intravenous antibody. However, anti-CEA antibodies are prone to sequestration by circulating CEA. The anti-CEA antibody, PR1A3, binds only membrane-bound CEA and thus is able to overcome this problem. The aim of my research was to assess whether PR1A3 is suitable to be considered as a therapeutic agent in the treatment of CRC and what its mechanism of action might be.

Methods

The level of expression of CEA on a panel of cell lines was determined under different conditions using a solid-phase ELISA and FACS analysis. Humanized PR1A3 (hPR1A3) was assessed in a variety of *in vitro* cytotoxicity assays with colorectal cell lines expressing varying levels of CEA, using peripheral blood mononuclear cells and purified natural killer cells as sources of effector cells. The mechanism of action of PR1A3 was investigated by modifying the Fc fragment of the antibody and using antibodies to block the Fc γ IIIa receptor on the effector cells.

PR1A3 was also investigated in combination with a humanised A33 antibody.

Results

A panel of colorectal cell lines was found to have a range of CEA expression which could be upregulated in certain cell lines by growing the cell line beyond confluence and by treatment with the chemotherapeutic agent, 5-fluorouracil. The *in vitro* assays demonstrated hPR1A3 antibody-dependent and CEA-specific killing of tumour cell lines

by human PBMC. The effect increased with increasing concentration of antibody and was lost by using the parent murine IgG1 PR1A3. Using 50µg/ml hPR1A3, tumour cell lysis was increased by more than 3-fold above spontaneous killing ($p < 0.001$) in a high CEA-expressing cell line. Both antibody-dependent and antibody-independent (spontaneous) killing was blocked by using whole antibody to the Fc-γIIIa receptor, although the spontaneous killing was restored when a F(ab')₂ was used instead of whole antibody. hPR1A3 and the A33 antibody showed potential synergy when used in combination against a high-CEA and a moderate-A33 expressing cell line.

Conclusion

The monoclonal antibody hPR1A3 causes CEA-specific lysis of human colorectal cancer-derived cell lines in the presence of human PBMCs. This lysis is dependent on the dose of the antibody, requires a compatible Fc-receptor and is inhibited by blockade of the FcγIIIa receptor. These findings show that hPR1A3 can kill tumour cells by antibody-mediated cellular cytotoxicity (ADCC) and implicate NK cells as a major contributor to this effect.

The results support the development of hPR1A3 for therapy of colorectal cancer.

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GLOSSARY OF ABBREVIATIONS

5FU	5-fluorouracil
Ab	Antibody
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADCP	Antibody-dependent cell-mediated phagocytosis
ADEPT	Antibody-dependent enzyme prodrug therapy
APC	Adenomatous polyposis coli
APCs	Antigen presenting cells
CDC	Complement-dependent cytotoxicity
CEA	Carcinoembryonic antigen
CK	Cytokeratin
CO ₂	Carbon dioxide
COX-2	Cyclo-oxygenase-2 enzyme
CRC	Colorectal cancer
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
DFS	Disease-free survival
DMSO	Dimethylsulfoxide
EDTA	Ethylene diamine tetra-acetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
EpCAM	Epithelial cell adhesion molecule
Fab	Fragment antibody binding
FACS	Fluorescence activated cell sorting
FAP	Familial adenomatous polyposis
Fc	Crystallisable fragment
FcR	Fc-receptor
FCS	Foetal calf serum
FiTC	Fluorescein isothiocyanate
GPI anchor	Glycosyl phosphatidyl inositol anchor
HACA	Human anti-chimera antibodies
HAMA	Human anti-mouse antibodies
HER	Human epidermal growth factor receptor
HNPCC	Hereditary non-polyposis colorectal cancer
HRC	Human random control
IFN- γ	Interferon-gamma
Ig	Immunoglobulin
IL-2	Interleukin-2
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer Ig-like receptor
LDF	Leucodepletion filter

GLOSSARY OF ABBREVIATIONS (cont)

LoH	Loss of heterozygosity
M	Molar
mAb	Monoclonal antibody
MAC	Membrane attack complex
µg	Microgram
mg	Milligram
MIN	Multiple intestinal neoplasia
µl	Microlitre
ml	Millilitre
mM	Millimolar
MUG	4-methylumbelliferyl-B-D-galactoside
ng	Nanogram
NK cell	Natural killer cell
NSAID	Non-steroidal anti-inflammatory drug
OD	Optical density
OS	Overall survival
PBMC	Peripheral blood mononuclear cells
PBSA	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFS	Progression-free survival
PLL	Poly-L-lysine
RAIT	Radioimmunotherapy
RIGS	Radioimmunoguided surgery
RIS	Radioimmunosciintigraphy
RPMI	Roswell Park Memorial Institute
SEM	Standard error of the mean
TAA	Tumour-associated antigen
T _C cell	Cytotoxic T-cell
TDA	Terpyridine dicarboxylic acid
T _H cell	Helper T-cell
TKI	Tyrosine kinase inhibitor
TP	Thymidine phosphorylase
TS	Thymidylate synthase
UTR	Untranslated region
VEGF	Vascular endothelial growth factor

CHAPTER 1

Introduction

1.1 Colorectal cancer and its treatment

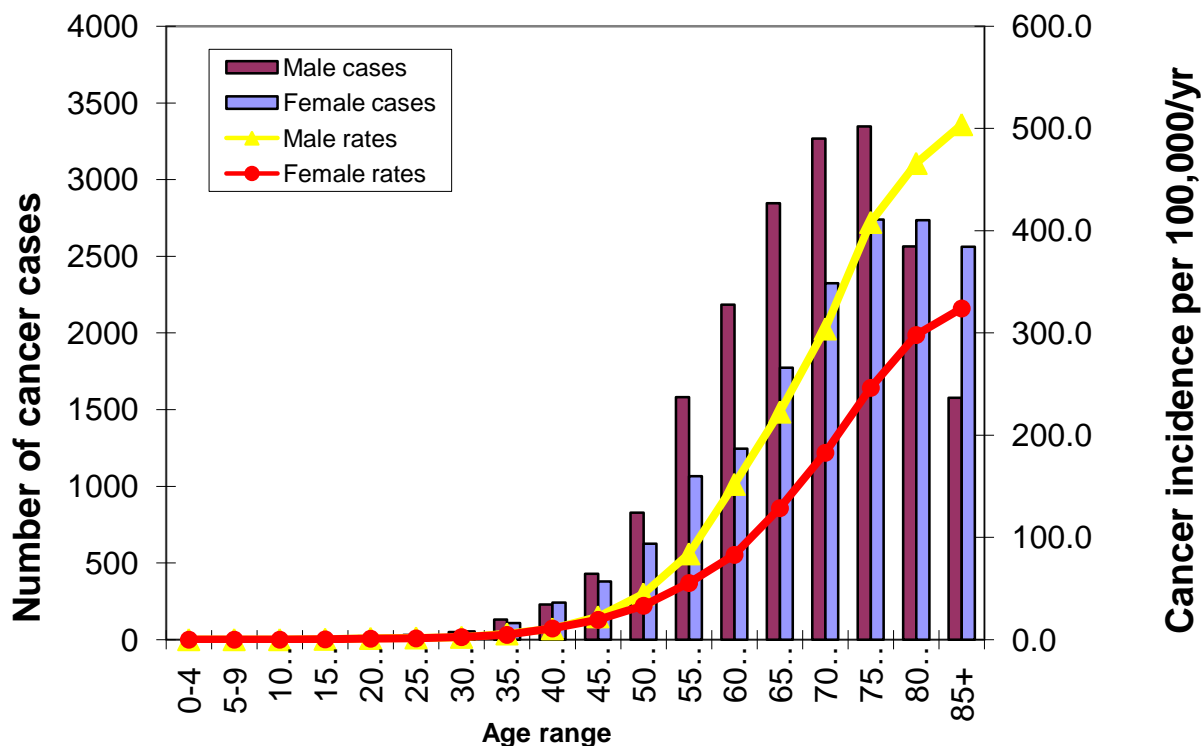
1.1.1 Incidence

Colorectal cancer (CRC) is the second commonest cause of cancer-related mortality in the UK (Cancer-Research-UK, 2007) with approximately 17,000 deaths from 35,000 new cases every year (table 1.i). Worldwide there are about one million new cases every year (Parkin et al., 2005) with a wide variation in incidence. Australia, New Zealand, USA, Western Europe and Japan all have a high incidence of CRC compared with Africa where the incidence is very low. This geographical variation is believed to be mostly due to environmental factors outlined below in chapter 1.1.2. The incidence in the UK is about 60 per 100 000/yr but shows a wide variation with age (figure 1.i). In general the incidence of CRC is rising; in areas with a previously low incidence such as Asia or Africa this rise is rapid, whereas in areas of high CRC incidence the rate is either stabilising or rising only slowly.

Table 1.i: Numbers and incidence of colorectal cancer in the UK

	Cases	Crude rate per 100,000	Age-standardised rate per 100,000
Male	19,096	65.6	54.7
Female	15,910	52.3	34.4
Total	35,006	58.8	43.4

Figure 1.i UK cancer incidence by age: Cancer Research UK Statistics



1.1.2 Aetiology

Both genetic and environmental factors play an important part in the aetiology of CRC. Genetic factors are most clearly seen in the hereditary colorectal cancer syndromes, which account for less than 4% of all CRC (Aaltonen et al., 2007), although inherited susceptibility is believed to be responsible for up to 30% (Lichtenstein et al., 2000). These other low-penetrance susceptibility alleles include variants in the APC gene, Axin1, mismatch repair genes (Fearhead et al., 2004) and the more recently discovered variants in 8q24.21 (Tomlinson et al., 2007) and the CDK8 gene (Firestein et al., 2008) which, singularly do not cause colorectal cancer but together significantly increase the risk of developing the disease.

Hereditary colorectal cancer can be divided into the polyposis syndromes, the most important of which is familial adenomatous polyposis (FAP), and hereditary non-

polyposis colorectal cancer (HNPCC). FAP is an autosomal dominant condition which occurs as a result of germline mutations in the APC gene found on 5q21. It accounts for less than 0.5% of all CRC, but the risk of CRC in affected individuals is 100%. It is characterised by the development of hundreds of colorectal adenomatous polyps in the 2nd to 3rd decades as well as other extra-colonic manifestations. 90% of the mutations occur within 10% of the APC gene (the mutation cluster region) and the mutation can usually be identified in about 80% of probands, allowing screening of the rest of the family. Further polyposis syndromes are listed in table 1.ii.

Table 1.ii: Examples of colorectal polyposis syndromes

	Gene mutation	Inheritance	Major involvement outside colorectum
Familial adenomatous polyposis coli	APC; 5q21	Autosomal dominant	Desmoids, duodenum, retina
Peutz-Jehgers	STK11; 19p13	Autosomal dominant	Small bowel, gastric
Juvenile polyposis	SMAD4; 18q	Autosomal dominant	Other GI
Cowden's disease	PTEN; 10q22	Autosomal dominant	Other GI, skin, thyroid, breast, cervical
MYH polyposis	MYH	Autosomal recessive	Other GI
Li-Fraumeni	p53; 17p	Autosomal dominant	Breast, bone, leukaemia, sarcomas

HNPCC is an autosomal dominant condition which occurs as a result of germline mutations in the mismatch repair (MMR) genes. MMR proteins are responsible for correcting errors in DNA during replication and initiating apoptosis when the DNA is damaged irrevocably. Mutations in these genes result in the accumulation of mutations, predominantly in DNA containing repetitive sequences. HNPCC accounts for about 2% of all CRC, with a lifetime risk in affected individuals of about 80%. Unlike FAP there is no obvious phenotype and so no obvious clinical presentation apart from the development of cancers, particularly colorectal. Other associated cancers include endometrial (40% of women), gastric (15%) and ovarian (12% of women). Clusters of cancers within families

are defined as HNPCC if they conform to the Amsterdam criteria (Vasen et al., 1999) (table 1.iii). Like FAP, a mutation can usually be identified in about 80% of probands.

Table 1.iii: Amsterdam II criteria

- | |
|--|
| <ul style="list-style-type: none">• At least three relatives with a HNPCC-associated cancer, one of whom should be a first degree relative of the other two• At least two successive generations should be affected• At least one CRC should be diagnosed before age 50• FAP should be excluded in the colorectal case(s) if any• Tumours should be verified by pathological examination |
|--|

There is good evidence for adenomatous polyps being the precursor lesion of colorectal cancer. Vogelstein (Vogelstein et al., 1988) showed a stepwise accumulation of genetic mutations from early adenoma through to carcinomas which he concluded was phenotypically expressed by an adenoma to carcinoma sequence. Hence large adenomas show more cellular atypia and have more genetic abnormalities than smaller polyps. Important genetic mutations in this pathway include:

- ***APC (Fearhead et al., 2001)***

Mutations in this large gene found on chromosome 5q21 are found in 60-80% of both adenomas and carcinomas and so are believed to be an early step in the adenoma-carcinoma sequence.

- ***K-ras (Vogelstein et al., 1988)***

This is a proto-oncogene found on chromosome 12p and is mutated in 30-50% of colorectal cancers and polyps. The k-ras protein is a GTP-binding protein with constitutive GTPase activity. Mutations of k-ras cause increased and unregulated cellular proliferation and malignant transformation. As these mutations are more common in larger rather than smaller adenomas, they are felt to be a later event in adenoma-carcinoma sequence

- **18q21 genes (Thiagalingam et al., 1996)**

It is not clear if one or a combination of genes at this locus are involved in colorectal tumourigenesis. 18q mutations are found in more than 60% of sporadic CRCs mostly by loss of heterozygosity (LOH). These mutations are seen more commonly in increasingly dysplastic polyps. Candidate genes include:

- **DCC (Mazelin et al., 2004)**

This is a receptor for netrin-1, a protein which causes chemo-attraction / repulsion of axons and neurons. However it also acts as a survival factor for cells so that the cell dies unless DCC is bound by netrin-1. Hence DCC mutation or deletion could inhibit death signalling in cells thereby predisposing to tumourigenesis

However, actual mutations in DCC are rare and DCC-negative mice do not have a cancer predisposition which would question the involvement of DCC in colorectal carcinogenesis

- **SMAD4** (also known as DPC4/MADH4) (Woodford-Richens et al., 2001)

This gene codes for a downstream regulator in the TGF- β signal transduction pathway. Mutations in SMAD4 occur much more commonly than in DCC in CRC. Constitutional mutations in SMAD4 can cause juvenile polyposis syndrome and inactivation of SMAD4 is commonly seen in metastatic CRC

- **p53**

This is a common mutation in invasive carcinomas (70%) (Baker et al., 1990) but rare in adenomas and thus thought to be a late event in the adenoma-carcinoma sequence. p53 is a tumour-suppressor gene found on chromosome 17p. It is a transcription factor which binds to genes involved in apoptosis during times of DNA damage (genotoxic stress) including p21, BAX and Bcl-2. The loss of p53-mediated pathways of apoptosis is considered to be an important determinant of progression from adenoma to a malignant phenotype.

However, the finding that most cancers harbour a large number of genetic and/or epigenetic changes have led some to suggest that sporadic tumours acquire mutations early-on which lead to an inherent genomic instability (Sieber et al., 2003). Bodmer argues, however, that genetic instability is not a requirement for tumour development and

believes that the power of natural selection, even with its low mutation rates, can readily account for tumour development (Bodmer et al., 2008).

Other evidence suggesting that colorectal adenomas are the precursors of carcinomas includes:

- The prevalence of adenomas correlates with that of carcinomas, the average age of adenoma patients being around five years younger than patients with cancer (Muto et al., 1975)
- Adenomatous tissue often accompanies cancer, and it is unusual to find small cancers with no contiguous adenomatous tissue (Morson, 1966)
- Sporadic adenomas are identical histologically to the adenomas of FAP, and this condition is unequivocally premalignant
- The distribution of adenomas throughout the colon is similar to that for carcinomas (Granqvist, 1981)
- Adenomas are found in up to one-third of all surgical specimens resected for CRC (Chu et al., 1986)
- The incidence of CRC has been shown to fall with a long-term screening programme involving polypectomy (Mandel et al., 2000).

Environmental and dietary factors play a clear role in development of CRC. This is demonstrated well in migrants moving from areas of low-incidence of CRC to an area of high CRC incidence where rates of CRC can be seen to increase towards that of the host nation within one generation (McMichael et al., 1980). Studies looking at individual dietary risk factors have suggested that meat (Armstrong and Doll, 1975; Willett et al., 1990) and animal fat (Willett et al., 1990) may increase the risk of CRC. In contrast dietary fibre has been shown in two large studies to reduce the risk of colonic adenomas (Peters et al., 2003) and cancer (Bingham et al., 2003). In a case-controlled study, Peters et al (Peters et al., 2003) showed a lower incidence of colonic (but not rectal) adenomas in patients taking high levels of fibre in their diet. For those taking the highest levels of fibre (highest quintile) there was a 27% reduction in the numbers of colonic adenomas compared with those in the lowest quintile for fibre intake. To add to this evidence,

Bingham et al (Bingham et al., 2003) carried out a prospective study looking at the effect of dietary fibre intake on colorectal cancer. They found that intake of dietary fibre was inversely proportional to the incidence of CRC, an effect which was greatest in the left colon and least for the rectum. More circumstantial evidence points to cruciferous vegetables (eg broccoli), which contain isothiocyanates, having a protective effect in bowel cancer.

Bile acids, especially following breakdown in the GI tract by bacteria to form secondary bile acids, have been implicated in the development of CRC (Imray et al., 1992) and calcium supplementation has been shown to reduce that risk in a randomised trial, possibly by binding to these secondary bile acids (Grau et al., 2007). There is some evidence that cholecystectomy may increase the risk of CRC by increasing the production of secondary bile salts (Schottenfeld and Winawer, 1983).

Certain other conditions predispose patients to CRC. The best known of these is inflammatory bowel disease (ulcerative colitis and Crohn's colitis) where the risk is proportional to the length of colon involved and to the length of time that the patient has had inflammatory bowel disease.

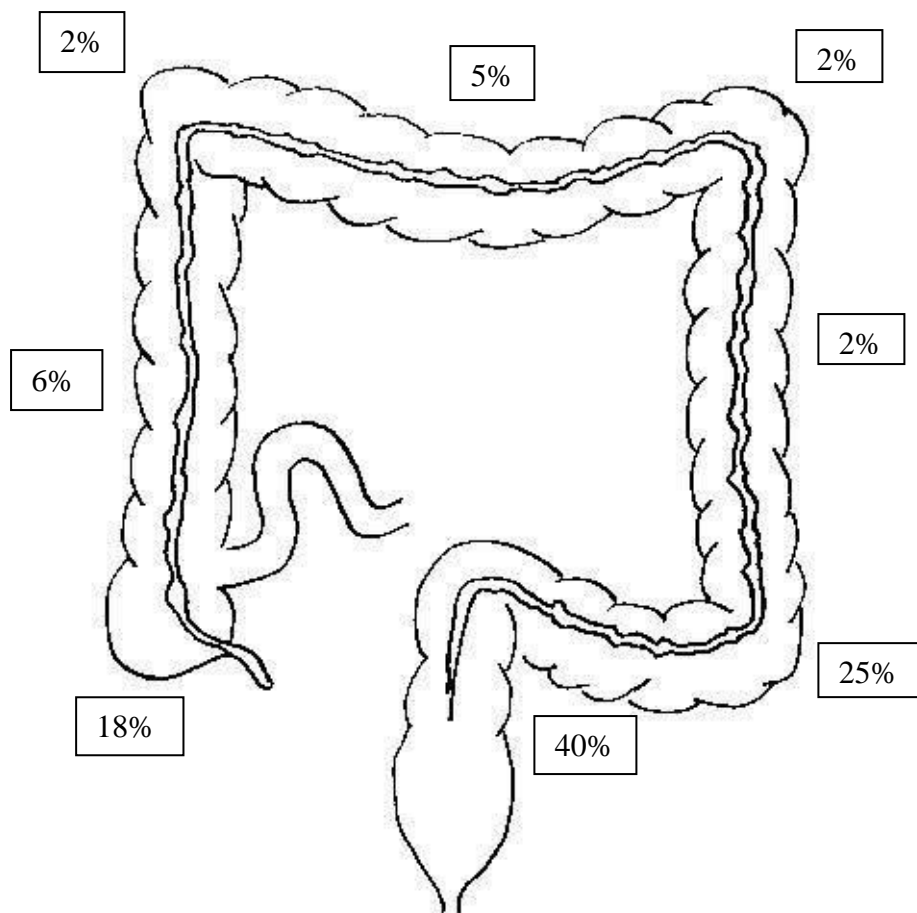
1.1.3 Natural history of CRC

The majority of CRCs are found in the rectum (40%) with sigmoid and caecum the other two major sites. A detailed breakdown can be found in figure 1.ii. 4-5% of patients with CRC will have a 2nd tumour at the time of presentation (synchronous tumour) (Mella et al., 1997) and about 25% will have a synchronous polyp (Kronborg et al., 1986), although the increasing recognition of "flat adenomas" (Rembacken et al., 2000) may mean the rate of synchronous polyps is even higher.

CRC can spread locally, or to distant sites by lymphatic and haematogenous spread. Local spread is commonly radial rather than longitudinal. Radial spread involves extending out into the pericolonic or perirectal tissues and tumour extent in this plane is the most important factor in determining local recurrence and indeed in deciding whether the

tumour is resectable by surgery with curative intent. Spread in the longitudinal plane is usually very limited but increases in tumours which are poorly differentiated. This is important when considering the resection of rectal tumours as it determines the length of bowel which must be left below the tumour to consider the operation curative. When the tumour is in the low rectum this could be the difference between either making an anastomosis (join between the two ends of bowel) and restoring continence to the patient, or leaving the patient with a permanent colostomy. There is increasing evidence that as long as the distal margin itself does not have microscopic involvement (ie >1mm) the risk of local recurrence is not increased (Karanjia et al., 1990).

Figure 1.ii: Distribution of colorectal cancer (Cancer Research UK figures, 2006)
Percentage distribution of all colorectal cancer cases by site within the large bowel.



Lymphatic spread is common in CRC and the likelihood of lymph node involvement increases with tumour size. However, even small tumours which don't even involve the muscle coat of the bowel wall (T1 tumours (Sobin and Wittekind, 2002)) have a 14% risk of nodal disease, with other factors such as degree of differentiation contributing to this risk (Kikuchi et al., 1995; Nascimbeni et al., 2002). The lymphatics tend to follow the arterial blood vessels back eventually to the para-aortic lymph nodes.

Haematogenous spread is again common and accounts for spread through the portal venous system to the liver. Liver metastases are the commonest site of distant spread in CRC with 30% of patients having distant disease at the time of presentation (chapter 1.1.7).

Other routes of spread such as transperitoneal or direct implantation are unusual in CRC.

1.1.4 Presentation

The presentation of bowel cancer is extremely variable. Many symptoms are reported but, given the high incidence of CRC, some of these may simply be triggers for investigation, during which a bowel cancer is discovered, and not a symptom of the cancer itself. Right-sided cancers in particular are frequently asymptomatic and often present in an occult fashion with symptoms of anaemia (eg shortness of breath, lethargy, occult vascular insufficiency such as angina or intermittent claudication). The more liquid contents of the right colon, together with its wide capacity, make any obstructive symptoms less likely in right sided disease.

Left sided cancers are more likely to be symptomatic and common presentations include:

- Change in bowel habit, particularly if towards looser stools and when the change is persistent
- Rectal bleeding, especially darker red and mixed in with the stool.
- Abdominal pain and distension, which are obstructive symptoms
- Colovaginal or colovesical fistulation where the cancer has invaded locally into the vagina or bladder respectively

Rectal cancers are more likely to cause rectal bleeding and can produce more local symptoms such as tenesmus (the sensation of needing to defecate in the absence of stool)

and rectal irritability causing urgency, frequency, explosive diarrhoea and even incontinence if the cancer abuts or invades the anal sphincters.

20% of all CRCs present as emergencies with 80% of these being due to obstruction. Bleeding and perforation are the other common emergency presentations of CRC.

All colorectal cancers can cause anorexia and weight loss although these are often signs of disseminated disease, a finding in up to 30% of patients with CRC at presentation (Cancer-Research-UK, 2007). Rarely CRC can present with symptoms referable directly to the metastases, such as jaundice from widespread liver metastases.

Clinical examination in patients with CRC is most commonly normal. About 60% of rectal cancers are digitally palpable during rectal examination. Caecal tumours may present with a mass in the right lower abdomen.

The differential diagnosis of a patient presenting with the above symptoms and a normal examination is obviously wide and includes most pathological conditions of the colorectum. Hence investigation of a targeted group with new onset symptoms such as those described warrant investigation, even though the detection rate of CRC as a result is small. Common differentials include complications of diverticular disease, irritable bowel syndrome, coeliac disease and inflammatory bowel disease. Differentials of the rectal symptoms described include:

- Proctitis
 - IBD
 - Irradiation
 - Ischaemia
- Pelvic floor disorders
 - Rectal prolapse
 - Solitary rectal ulcer syndrome / intussusception
- Prostate cancer
- Haemorrhoids

1.1.5 Investigation

Direct investigation of the colorectum tends to be either radiological or endoscopic or a combination. A rigid sigmoidoscopy to examine the rectum is often the first simple investigation to rule out an obvious rectal cancer. A double-contrast barium enema has been the most popular radiological procedure for the investigation of CRC for many years. It is not a good investigation for the rectum and it is difficult to exclude small cancers in areas of significant diverticular disease. Hence it is sometimes combined with a flexible sigmoidoscopy which is an endoscopic examination of the left colon and rectum. A colonoscopy remains the gold standard for the investigation of potential CRC as it not only visualises all areas of the large bowel well but allows the detection and endoscopic treatment of early cancer and adenomatous polyps which are known to be the precursors of colorectal cancer. However, colonoscopy is more invasive and carries a greater risk of complications.

Long oral preparation CT scans of the colon are used as an investigation for patients deemed not suitable for barium enema or colonoscopy on the grounds of fitness. It avoids both mechanical bowel preparation and the need to retain air or barium introduced rectally, and is non invasive. However these advantages are at the cost of accuracy, being able only to accurately detect sizeable colorectal tumours. CT colonography and MR colonography are developing areas of investigation which aim to retain the accuracy of colonoscopy, being able to detect polyps of 6mm, but without the need for invasive investigation and perhaps in the future, without the need for bowel preparation.

1.1.6 Pre-operative staging

A tissue diagnosis is required for all rectal cancers and hence biopsy of the lesion is required. Although this would be ideal for all colonic tumours also, pre-operative staging and subsequent treatment can be instigated on the basis of appearances on a barium enema or CT scan without the need for colonoscopy and biopsy (ACPGBI, 2007). The necessity for histological proof in the case of rectal cancer relates partly to the ease of obtaining such tissue but also due to the use of neo-adjuvant (ie pre-operative)

radiotherapy or chemoradiotherapy in the treatment of rectal cancer, which requires a definitive diagnosis of cancer before treatment is started.

Pre-operative staging involves assessing both the extent of local spread of the tumour and whether there is any distant spread, to ensure that the tumour is operable with curative intent before surgery is undertaken.

A CT scan of chest, abdomen and pelvis is often used to assess distant spread for all CRCs. Occasionally a plain chest radiograph and an abdominal ultrasound scan are used as substitutes. In rectal cancers an MRI has now become standard in assessing the relationship of the tumour to the mesorectal envelope and the chance of lymph node involvement. This aids in the planning of pre-operative (neo-adjuvant) treatment.

Endoanal ultrasound scanning can be useful in experienced hands to assess the depth of penetration of early rectal cancers if a local transanal resection is being considered instead of a formal bowel resection.

1.1.7 Pathology and post-operative staging

Over 95% of cancers found in the colorectum are adenocarcinomas. They are normally graded according to the degree of differentiation that the tumour shows on histological section, mainly based on the architectural features of the tubules. If the tubules are regular and look like adenomatous epithelium, these are termed well differentiated (20% of all cancers) but if the tubules are completely unformed or highly irregular these are termed poorly differentiated (20%). The majority are between these extremes and are termed moderately differentiated. However these are subjective assessments and the interobserver variability is large.

Prognosis of CRC is directly dependent on the stage of the tumour as assessed by the pathologist post-operatively. Staging not only enables post-operative predictions on survival to be made but it also guides the use of post-operative treatment. On this basis the factors of greatest importance are:

- Depth of tumour penetration
- Involvement of adjacent structures

- Involvement of associated lymph nodes
- Involvement of distant organs (ie presence of metastases)
- Distance of tumour from the nearest circumferential resection margin
- Distance of tumour from the nearest longitudinal resection margin
- Presence of lymphovascular invasion
- Presence of bowel perforation at site of the tumour

Many of these factors have been incorporated in the two well-used staging systems for colorectal cancer: Dukes' classification (originally devised for rectal cancers (Dukes, 1949) and the UICC TNM (Tumour / Nodal / Metastasis) classification. These are summarised in table 1.iv.

In broad terms, within the group of patients who have been treated with curative intent and have recovered well from surgery, post-operative chemotherapy is offered to all who have nodal disease, and only a small proportion of those with node-negative disease. This will be discussed further below. The survival for patients by Dukes' stage and the proportion of cancers falling into each of the Dukes' categories are given in table 1.v

1.1.8 Screening for bowel cancer

For some years the UK has had a colorectal cancer screening programme for high risk groups and these guidelines are published and updated by the British Society of Gastroenterology (Cairns and Scholefield, 2002). These programmes are disease-specific and cover patients with inheritable conditions, inflammatory bowel disease and a strong family history of bowel cancer as well as patients with previously removed cancers and polyps. More recently, in April 2006, the UK cancer screening programme for colorectal cancer began in parts of the UK following a pilot study in 5 UK districts screening people between the ages of 50 and 69 years old (NHS-Screening-Pilot, 2004). The screening programme uses a guaiac-based test for detecting occult blood in faeces to identify a higher-risk group of the population. A positive result is obtained from the peroxidase-like activity of haematin from blood in the stool. The peroxidase-like activity reduces as blood

Table 1.iv: Staging of colorectal cancer

Tumour (T) –stage		Nodal (N) –stage	
T _X	Primary tumour can't be assessed	N _X	Nodal status can't be assessed
T ₀	No primary tumour found	N ₀	No regional lymph nodes involved
T ₁	Tumour invades submucosa	N ₁	1-3 pericolic lymph nodes involved
T ₂	Tumour invades muscularis propria	N ₂	>4 pericolic lymph nodes involved
T ₃	Invades through muscularis propria into subserosa or non-peritonealised pericolic / perirectal tissue	N ₃	Involved node along a named vessel
		Metastasis (M) –stage	
		M _X	Metastases can't be assessed
T ₄	Invades adjacent structures and / or breaches visceral peritoneum	M ₀	No metastatic disease
		M ₁	Metastatic disease present

TNM grouping	AICC stage	Dukes' stage
T ₁₋₂ N ₀ M ₀	Stage I	A
T ₃₋₄ N ₀ M ₀	Stage II	B
Any T N ₁₋₃ M ₀	Stage III	C
Any T Any N M ₁	Stage IV	D

Table 1.v: Approximate frequency and 5 year relative survival (%) by Dukes' stage

Mod Dukes' stage	Freq at diagnosis (%)	5-yr survival (%)
A	11	83
B	35	64
C	26	38
D	29	3

travels through the gut and thus is less sensitive from more proximal causes of bleeding, for example within the oral cavity. A positive test led to the patients being invited for colonoscopy. This technique has been used in previous randomised controlled trials in the UK (Hardcastle et al., 1996), USA (Mandel et al., 1999) and Denmark (Kronborg et al., 1996) with reductions in colorectal cancer-specific mortality of 15%, 21% and 18% respectively, using biannual testing. However in all three studies there was no change in overall mortality, as CRC was too small a percentage of overall mortality to influence this, and the incidence of CRC was unchanged except in the 18-year follow-up from the Minnesota group where cumulative incidence fell from 39 per 1000 in the control group to 33 per 1000 in the screened group (Mandel et al., 2000).

The UK pilot study demonstrated a positive predictive value for detecting polyps of 35% (and 11% for detecting cancer) and needed to screen 500 patients to detect one cancer.

1.1.9 Current treatment of colorectal cancer

1.1.9.1 Early rectal cancer

Surgery is the only well-established method of curative treatment for colorectal cancer. In early rectal cancer with a complete pathological response to pre-operative CRT, there is now an argument for a non-surgical approach followed by salvage if recurrence occurs (Habr-Gama et al., 2005; Habr-Gama et al., 2004). In early rectal cancer there is the possibility of local trans-anal resection precluding the need for major surgery. However this is only suitable for the earliest T-stage of cancer (T1 sm1; tumour invading less than 0.3mm into the submucosa (Kikuchi et al., 1995)) and is done with the knowledge that the draining lymph nodes are left behind, as the likelihood of involvement is extremely small.

1.1.9.2 Surgery with curative intent for colorectal cancer

Surgical treatment of colorectal cancer with curative intent involves, for the large majority of patients, a segmental resection of the diseased bowel along with its draining lymph nodes. Surgery for curative intent is indicated in the absence of known metastatic disease or in the presence of metastatic disease that is also deemed resectable. The precise nature of each operation is dependent on the site of the tumour. In the majority of cases

the two ends of bowel left following resection can be rejoined (anastomosed) although occasionally this is not possible, especially in certain patients with a low rectal cancer and an end colostomy is formed.

There is increasing interest in laparoscopic colorectal resections for cancer as well as other colorectal conditions. Two major trials have shown that, in the colon, this is feasible technically and with no compromise to oncological principles of cancer surgery (Anonymous, 2004; Guillou et al., 2005). The follow-up period is relatively short and so data are interpreted with some caution. Only one of these trials, the UK CLASICC trial (Guillou et al., 2005) looked at rectal cancers and they found an increase in the rate of positive resection margins in the laparoscopic group which, although not statistically significant, gave the authors cause to conclude that, on current evidence at least, laparoscopic resection of the rectum was not justified. This did not translate to a higher local recurrence rate at 3 years however (Jayne et al., 2007). Other groups have shown good outcomes for rectal cancer resection with no oncological compromise and a recent systematic review supports this (Breukink et al., 2006).

1.1.9.3 Neoadjuvant treatment for rectal cancer

The treatment of rectal cancer again differs from that of colon cancer in that the large majority of patients are now offered treatment prior to surgery with the aim of reducing the risk of local recurrence of their disease within the pelvis, and with the long-term hope of increasing their chances of survival. Pre-operative (neoadjuvant) treatment is given in two circumstances. Patients with locally advanced rectal cancer in whom there is a risk of leaving residual disease with surgery alone are given a combination of chemotherapy with radiotherapy. This consists of a long course of radiotherapy (45-50Gray (Gy) over 5 weeks using 1.8-2 Gy per fraction) with 5-fluorouracil (5-FU) or its prodrug, capecitabine, given in 2 week long doses during the first and last weeks of radiotherapy. Surgery is then undertaken 6-10 weeks post-chemoradiotherapy. In all other patients with rectal cancer, recent data from the MRC CR07 trial (Sebag-Montefiore et al., 2009) and from a large Dutch randomised controlled study (Kapiteijn et al., 2001), suggested that “short-course” radiotherapy, which consists of 5 days of 5Gy, followed by surgery within 5 days of finishing the radiotherapy improves local control of rectal cancer and disease-

free survival compared with surgery alone. However this reduced local recurrence rate is at the expense of an increased risk of long-term bowel dysfunction (Peeters et al., 2005) and an increased risk of other GI complications (Birgisson et al., 2008).

1.1.9.4 Adjuvant treatment for colorectal cancer

Although about 80% of CRC is macroscopically resectable, 50% subsequently develop metastases. This is due to micrometastatic disease at the time of surgery. The aim of all adjuvant treatments is to eradicate these micrometastases. Adjuvant treatment can consist of either chemotherapy or radiotherapy or both dependent upon the site of the tumour, the pre-operative treatment given and the pathological stage of the tumour. For rectal cancer if pre-operative radiotherapy has been given, as is increasingly the case in the UK, then no post-operative radiotherapy is prescribed. If however pre-operative radiotherapy has not been given and a tumour-involved resection margin is noted on pathological analysis, then post-operative radiotherapy is an option.

Chemotherapy following colorectal resection is largely dependent on the nodal status of the tumour and the fitness of the patient. In general, tumour which has spread to lymph nodes on pathological analysis (stage III; Dukes' C) is treated post-operatively with chemotherapy in an otherwise fit patient (O'Connell et al., 1998), whereas for disease which is node negative, post-operative treatment is only used in a small subset of patients who are deemed to be at higher risk of local or distant recurrence. These high risk Dukes' B features include:

- T₄ tumours
- Tumour perforation
- Obstruction caused by the tumour
- Extramural vascular invasion
- Poorly differentiated tumours

The problem with the Dukes' B subset of tumours is its wide variation in tumour penetration ranging from those just breaching the muscular coat of the bowel to those which have invaded other organs. The benefit of treating higher risk Dukes' B patients with chemotherapy is small (IMPACT-B2-Investigators, 1999) and as a result, selection of a subset more likely to respond would be invaluable. A recent scoring system, the

Petersen index, based on independent prognostic factors in a multivariate analysis has been proposed (Morris et al., 2007; Petersen et al., 2002). This is shown in table 1.vi. Molecular analysis will hopefully provide further stratification of stage II and III disease which will give better information on which patients should receive chemotherapy and possibly also which type of chemotherapy.

Table 1.vi: Petersen prognostic index for Dukes' B colorectal cancer

	Score
Peritoneal involvement	1
Vascular invasion	1
Margin involvement	1
Tumour perforation	2

	Risk	5-yr survival
Score 0-1	Low risk	70-86%
Score 2-5	High risk	43-50%

The overall benefit of giving post-operative chemotherapy to patients who have Dukes' C tumours, however, is now well established for an overall benefit of 5-10% in absolute 5-year survival (QUASAR-Collab-Gp, 2000). The standard chemotherapy given is 5-FU / capecitabine with folinic acid. 5-FU acts by inhibiting the enzyme thymidylate synthase, inhibiting the synthesis of thymidine and hence DNA. The oral prodrug of 5-FU capecitabine, has several advantages over the intravenous 5-FU. As well as the convenience of oral over intravenous medication, another advantage is that capecitabine itself is not toxic and only acts on thymidylate synthase after conversion within cells to 5-FU by the enzyme thymidine phosphorylase (TP). The activity of TP is much higher in colorectal cancer cells than in normal epithelial cells, hence the toxicity is targeted in some part to the tumour cells (Schuller et al., 2000).

The recent MOSAIC trial (Andre et al., 2004) suggested that the addition of oxaliplatin to this current regime improves the rate of disease-free survival at 3 years in both stage II and stage III patients but at the cost of increased toxicity.

The ongoing adjuvant trials have recently looked at the addition of bevacizumab, an antibody against vascular endothelial growth factor (VEGF), to standard adjuvant chemotherapy regimens. The AVANT trial, which has only recently closed, is looking at oxaliplatin / 5FU treatment with or without bevacizumab but the results have not yet been published. QUASAR 2 is an ongoing trial looking at capecitabine with or without bevacizumab. There is also interest in using cancer ‘vaccines’ which will be discussed further below.

1.1.9.5 Metastatic disease

Although the majority of patients with metastatic (stage IV) disease will be incurable and treated with palliative therapy (see below), about 20-30% who have disease confined to the liver will be deemed suitable for resection of both primary and secondary tumours with curative intent (Stangl et al., 1994). The liver is by far the most common site of metastatic disease and approximately 25% of patients will have detectable liver metastases at presentation with a further 40% developing metastases after resection of the primary (Scheele et al., 1995). Hepatic resections for colorectal metastases in published series leave 20% of patients disease-free at five years with an overall five-year survival figure of about 30% (Simmonds et al., 2006).

1.1.9.6 Palliative treatment

Current first line options in the UK are a fluoropyrimidine alone (usually capecitabine) or combination chemotherapy, usually oxaliplatin plus a fluoropyrimidine (capecitabine or infusional 5-FU). Second line treatment is often with irinotecan (Douillard et al., 2000; Saltz et al., 2000). In other countries bevacizumab is sometimes offered in combination with first line chemotherapy (Kabbinavar et al., 2005a; Kabbinavar et al., 2005b). Similarly, in other countries, third line treatment is sometimes offered with irinotecan or, after irinotecan failure, cetuximab, which is an antibody against the epidermal growth factor receptor (EGFR), as this too has a proven, although small, survival benefit (Jonker

et al., 2007). Various combinations of fluoropyrimidine, oxaliplatin, irinotecan, bevacizumab and cetuximab have been published and there is a wide variety in clinical practice between countries, mainly due to the lack of direct comparative efficacy trials between regimens used in first and second line treatments. A meta-analysis of various combinations compared to standard 5-FU / folinic acid has been published (Golfopoulos et al., 2007), although this analysis looks at survival parameters, and this always has to be balanced against quality of life.

There are two major UK trials currently looking at the optimal treatment in palliative disease. The COIN trial is studying first line chemotherapy for metastatic disease, essentially looking at oxaliplatin and 5-FU with or without cetuximab as this has not previously been tested in the first line setting. This study is likely to close soon, having recruited over 2000 patients in a short space of time. The PICCOLO trial is examining irinotecan with or without panitumumab, a fully human anti-EGFR antibody, as second line chemotherapy for metastatic disease.

Colonic stenting has its major role in the palliative treatment of obstructing colorectal tumours, usually in the presence of metastatic disease. This involves placing a self-expanding metal stent (SEMS) by a combined endoscopic and fluoroscopic approach. This enables the colon to remain patent and avoids the need for surgery, and the high risk of requiring a stoma, in patients who have incurable disease. Pooled data from heterogeneous studies would suggest that the success at relieving the obstruction is about 91% with a risk of perforating the bowel of 4% and a 12% risk of the stent migrating, resulting in recurrence of the obstruction (Sebastian et al., 2004). However, despite these results, a recent randomised trial comparing SEMS with surgery for obstructing tumours in incurable disease was closed early due to the high rate of complications, in particular perforation, in the SEMS group (van Hooft et al., 2008).

1.1.10 Novel treatments

1.1.10.1 Tailoring treatment based on genetic profiles

The increasingly in-depth analysis of the development of colorectal cancer has led to increasing interest in tailoring adjuvant treatments to the individual cancer, dependent not simply on the histopathological findings, but on the genetic analysis of the resected or biopsied cancer. Preclinical work has suggested numerous candidates both for targeting particular treatments and also to identify subgroups of tumours with a better or worse prognosis than their Dukes' staging would suggest. Such candidates include k-ras, p53, MMR genes, 18q and 20q (Andreyev et al., 2001; Conlin et al., 2005; Diep et al., 2003; Forslund et al., 2001; Hawkins et al., 2001; Smith et al., 2002).

K-ras in particular has been shown to be a useful marker in predicting the response to treatment with cetuximab. Karapetis et al (Karapetis et al., 2008) reviewed specimens from patients previously randomised to cetuximab or best supportive care in a clinical trial. They found that the presence of *k-ras* mutations was strongly associated with a poor response to cetuximab, whereas those with wild type *k-ras* had an improvement in overall survival and progression-free survival compared to the control group.

Given that k-ras is seen as a marker of a poor prognosis, it has encouraged the development of targeted chemotherapy in the form of farnesyl transferase inhibitors. The k-ras protein is a GTPase which requires transfer of a farnesyl group for its activation and inhibition of this transfer leads to disruption of k-ras activity. However in a phase III trial in refractory advanced CRC, a farnesyl transferase inhibitor did not improve progression-free or overall survival (Rao et al., 2004). Thoughts of targeting the key wnt-signalling pathway to disrupt colorectal carcinogenesis have led to the development of high throughput assays to screen compounds for ability to bind to different members of this pathway (Lepourcelet et al., 2004), but this research has not been translated to the clinical arena.

1.1.10.2 Targeting the cyclooxygenase (COX) enzymes

The COX enzymes are involved in, amongst other reactions, the synthesis of prostaglandins which take part in a wide range of physiological processes. Two major isoforms are described; COX-1 which is constitutively expressed in many tissues and is

important for the homeostatic functions of prostaglandins and COX-2 which is inducible in several cell types in response to proinflammatory cytokines and other inflammatory mediators, and has a role in the inflammatory and proliferative activities of prostaglandins. Although the latter isoform is believed to have the biggest influence in the development of CRC, COX-1 may also have a role, perhaps through the synthesis of prostaglandin E2. Inhibition of COX-1 synthesis in a mouse model of CRC (*Apc*^{min/+}) showed a reduced burden of gastrointestinal adenomas compared to control mice (Chulada et al., 2000). However the majority of the evidence points to the role of COX-2. Eberhart and colleagues showed that there was upregulation of COX-2 in colorectal carcinomas and some adenomas, whereas levels of the enzyme are largely undetectable in normal colonic mucosa (Eberhart et al., 1994). COX-1 levels however are comparable in neoplastic and normal tissue. There is also genetic polymorphism in both *COX-1* and *COX-2* genes which influence both the risk of developing colorectal adenomas but also the response to treatment (Ulrich et al., 2005) and this may influence in future those who are treated with COX inhibitors – the class of drug known as non-steroidal anti-inflammatory drugs (NSAIDs).

The use of aspirin, a NSAID, in chemoprevention of colorectal adenomas and carcinomas has been supported by a number of epidemiological studies and more latterly, randomised controlled trials. Baron et al (Baron et al., 2003) randomised patients with previous adenomas to take aspirin or placebo for a median of 33 months. At three years following beginning of treatment, they found a reduction in the number of patients with adenomas in the group taking low-dose (81mg) aspirin (47% vs 38%) with a relative risk of 0.81 for all polyps and 0.59 for advanced lesions (which included high risk polyps and invasive cancer). Sandler et al (Sandler et al., 2003) randomised patients with previous curative resections for colorectal cancer to 325mg aspirin or placebo. The trial was terminated early due to a significant reduction in the number of adenomas found in the treatment group. At colonoscopy, a median of 13 months from the beginning of treatment, 17% of patients in the aspirin group had at least one adenoma compared to 27% in the placebo group with a relative risk of 0.65. In addition, a prospective study looking at the incidence of CRC in 83,000 women enrolled in the Nurses' Health Study over 20 years who had their aspirin intake closely documented, found that there was a reduction in CRC risk in

those women taking more than two 325mg tablets daily, although the effect was not seen before 10 years of follow-up (relative risk=0.77) (Chan et al., 2005).

The advantages of using a specific COX-2 inhibitor are obvious especially given their lower risk of gastric side-effects. However, two randomised trials of the COX-2 inhibitor, celecoxib versus placebo in patients who had recent previous adenomas, were stopped early due to the increased cardiovascular risk in patients taking the COX-2 inhibitor. A similar trial using rofecoxib was also stopped for identical reasons with an excess of cardiovascular morbidity in the active group. In all three trials, however, the use of COX-2 inhibitors was associated with a significant reduction in adenoma detection at 3 years following treatment commencing. The magnitude of the effect ranged from a relative risk of 0.76 with rofecoxib (Baron et al., 2006), to 0.43 in the Adenoma Prevention with Celecoxib (APC) trial (Bertagnolli et al., 2006) and 0.49 in the Prevention of Colorectal Sporadic Adenomatous Polyps (PreSAP) trial (Arber et al., 2006). This is a greater effect than that seen in the aspirin trials but the morbidity associated with taking COX-2 inhibitors would seem to place aspirin as a better prospect for chemoprevention of colorectal adenomas.

1.1.10.3 Small molecule tyrosine-kinase inhibitors (TKIs)

The EGFR is a tyrosine kinase receptor and a target for small molecule inhibitors in a number of cancers, given that it is over-expressed in a number of cancers, including in 60-80% of colorectal cancers. The antibody-targeting of the extracellular domains of the EGFR will be discussed below but more recently attention has also been given to the intracellular tyrosine kinase portion of the receptor which initiates an intracellular cascade following receptor dimerisation. Inhibition of this kinase can prevent down-stream signalling. Two TKIs have already been used in other EGFR positive cancers, gefitinib and erlotinib, and both these as well as a third TKI, lapatinib, have been used in metastatic colorectal cancer. The results, alone or in combination with other chemotherapeutic agents have been largely disappointing, adding little to the current treatment in terms of response, although the occasional trials have suggested some promise (Kuo et al., 2005).

1.2 Antibody-based cancer therapy

1.2.1 Introduction

Until recently the use of monoclonal antibodies (mAb) in cancer therapy had been very disappointing. The initial prediction of Erlich in 1908 that “magic bullets” would be developed to target specific diseases following his work on diphtheria toxin, and the complementary work by Metchnikov that these “bullets” would work by allowing white cells to target microbes, was followed by the realisation that the specificity of antibodies for their target could be harnessed for therapeutic purposes. The ability to produce and develop antibodies for therapeutic purposes, however, was not realistic until the Nobel-prize winning work of Köhler and Millstein (Köhler and Milstein, 1975) in 1975 who developed a technique for producing immortalised plasma cells producing Abs of a single specificity as detailed later. Within ten years, murine antibodies were used to protect against acute rejection of transplanted organs using a monoclonal against CD3 (Anonymous, 1985), and later many patients received mAbs to treat metastatic cancer, but enthusiasm for these early experiments in cancer-based therapy in the 1980s waned following trial data showing no significant clinical effect. Problems with immune reactivity against murine protein (human anti-mouse antibodies; HAMA) and the resulting short antibody half-lives are largely responsible for these failures. In addition, much of the early effort in developing monoclonal antibodies as therapeutic agents concentrated on using them as vehicles for delivery of radioactivity. As discussed in chapter 1.2.6, this failed to improve the treatment of solid tumours despite often excellent preclinical results, mainly because of a failure to deliver an adequate dose to the tumour without a significant bystander effect (Sharkey and Goldenberg, 2005).

The development of firstly chimerisation and later humanisation of these mAbs, as well as a better appreciation of how antibodies may exert their effect *in vivo*, has led to a resurgence of interest and the development of numerous antibodies against a variety of targets. Over 200 mAb have been studied so far in clinical trials for the treatment of cancer. There are currently about nineteen antibodies licensed for clinical use, eleven of which are for the treatment of cancer (Reichert and Valge-Archer, 2007), with other areas

such as chronic inflammatory conditions (eg rheumatoid arthritis, Crohn's disease), transplantation, infectious diseases and cardiovascular medicine looking at antibody therapy as part of the treatment armamentarium.

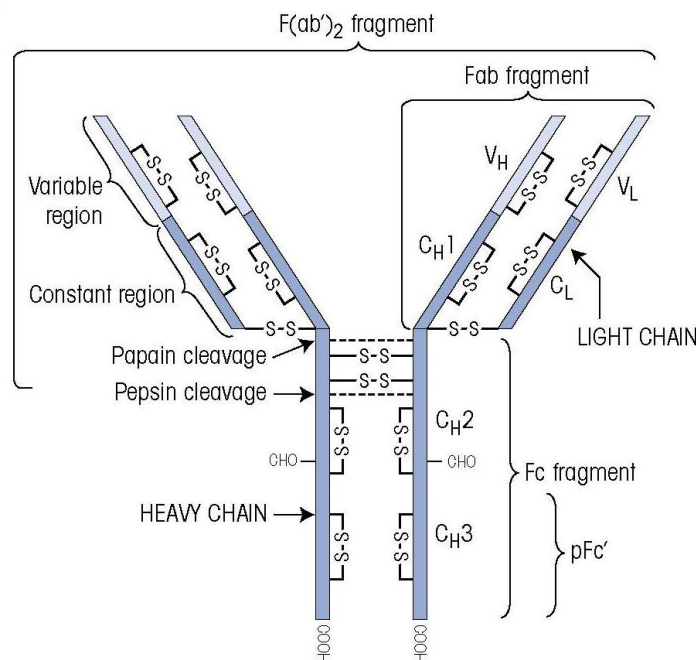
1.2.2 Antibody structure and function

All antibodies share a basic structure consisting of two identical light and two identical heavy chains joined together by disulphide bridges (figure 1.iii). There are 2 isotypes of the light chain – kappa and lambda – and five isotypes of the heavy chain – G, A, M, D and E. Each chain has a constant region and a variable region, the latter being responsible for creating the unique antigen-binding area within the antibody. Antibodies are encoded by the immunoglobulin gene family situated on chromosomes 14, 2 and 22, for the heavy chain, kappa light chain and lambda light chain respectively. Protein cleavage of whole antibody leads to the identification of two important basic subunits of the antibody: the Fab (fragment antibody binding) subunit, and the Fc (crystallisable fragment) subunit. The $F(ab')_2$ subunit describes the construct where the two Fab fragments remain joined maintaining a divalent molecule but without the Fc-portion of the antibody. The Fab subunit contains the variable regions of the light and heavy chains. At the tips of the variable regions are the six hypervariable loops which make up the diverse binding sites for antigen, an area known as the complementarity determining region. Variability in this area of the Ab is the result of recombination of variable, diversity and junctional gene segments during B-cell differentiation (VDJ-recombination) and secondarily from somatic hypermutation during the antigen-dependent germinal centre response which enables antibody of higher affinity for a given antigen to be produced.

The Fc subunit of the antibody is responsible for antibody interaction with other parts of the immune system such as effector cells and complement. Different heavy chain isotypes have different half-lives, distribution throughout the body, times of production, and ability to bind complement and interact with effector cells. Further variability is found even within each isotype (eg amongst the four IgG subclasses). IgG is the commonest isotype in the secondary response and the only isotype used currently in successful

antibody therapy. It diffuses more readily than other immunoglobulins into extravascular spaces and apart from mucosal tissues, where IgA predominates, it is the most important isotype outside the vascular space. It will be the isotype discussed here. There are three domains (C_{H1-3}) to the constant part of the heavy chain (figure 1.iii), two of which, C_{H2} and C_{H3} , make up the Fc portion of the antibody which interacts with other components of the immune system. This will be discussed further in chapter 1.3. Sites for glycosylation are discussed later in chapter 1.6.1.3.

Figure 1.iii: Structure of IgG antibody (Roitt, 2001)



Antibody function *in vivo* includes:

- Antibody-dependent cell-mediated cytotoxicity (ADCC) (Steplewski et al., 1983). This is described in detail in chapter 1.2.4.1. In brief, it involves antibody, bound to antigen on target cells, being engaged by the FcγRIIIa (CD16a) receptor on effector cells, predominantly NK cells. This leads to the activation of NK cells and the release of perforins and granzymes, killing the target cell.
- Antibody-dependent cell-mediated phagocytosis (ADCP) (Huber et al., 2001)

In a similar process to ADCC, antibodies coating micro-organisms or cells are engaged by Fc γ receptors found, for example on macrophages and neutrophils. This binding facilitates phagocytosis by the effector cells, in particular macrophages via Fc γ RI.

- Neutralisation of toxins

Bacterial toxins (eg. tetanus toxin) are neutralised when they are bound by specific antibody, preventing the toxins from carrying out their action. IgG is particularly important for this function.

- Immobilisation of bacteria

Antibodies specific for bacterial cilia or flagellae hinder their movement and reduce their ability to evade phagocytic cells.

- Complement activation

The classical pathway of the complement cascade is initiated by C1q binding to the C_H2 subunit of the Fc region of antibodies, particularly IgM and IgG. This leads to destruction of the target cell either by the eventual construction of the membrane attack complex, which causes cell lysis by forming a transmembrane channel fully permeable to electrolytes and water, or by enhancing the ability of complement to induce phagocytosis in capable cells possessing a receptor for C3b (eg neutrophils). It is discussed in more detail in chapter 1.2.4.2.

- Mucosal protection

This is a function mainly of IgA and, to a lesser degree, IgE. IgA acts by preventing pathogens from gaining attachment to mucosal surfaces. IgE attached to mast cells can bind foreign antigen precipitating the release of mediators from the mast cell.

- Conferring immunity to the foetus by the transplacental passage of IgG, and to the newborn by secretion of IgG in breast milk.

- Autoimmune activation of cell surface receptors: eg. Graves' disease in which anti-thyroid-stimulating hormone (TSH) receptor antibodies bind to the receptor, mimicking TSH and causing the unregulated release of thyroid hormones.

The function of naked antibodies used as anti-cancer drugs is discussed below in chapter 1.2.5.

1.2.3 Monoclonal antibodies

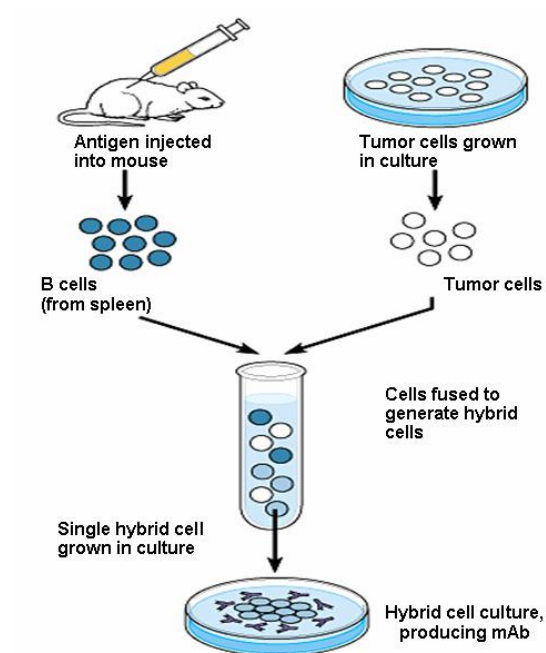
Monoclonal antibodies are those produced by cultures derived from a single B-cell, producing antibodies of identical specificity for a single epitope with the same affinity and immunoglobulin class. The use of monoclonal antibodies in the clinical and research arenas was made possible by the work of Kohler and Milstein (Kohler and Milstein, 1975) who produced immortalised clones from murine B-cells by fusing them with myeloma cell lines. Mice were immunised to a particular antigen and the splenocytes harvested and fused in polyethylene glycol with a myeloma line. The resulting hybridomas were selected out by growing in medium which did not support either of the parent cells beyond two weeks and the surviving hybridomas were isolated to single-cell cultures by serial dilution. Wells containing clones could then be grown up in culture and the purified antibody tested against the original antigen for specificity and affinity (figure 1.iv).

The clinical application of monoclonal antibodies is diverse including uses in diagnosis of malignancy, immunoassays, serotyping of micro-organisms, typing of haematological malignancies, immunohistochemistry, and as drugs. The latter application includes uses as anti-rejection drugs (eg muromonab, basiliximab), anti-inflammatory drugs (infliximab, adalimumab) and anti-cancer therapy which will form the main part of this thesis and will be discussed presently.

There are a number of advantages to using antibodies as therapy in cancer treatment. Modern antibody production techniques allow different antigen-binding areas to be incorporated into a successful antibody “frame”. Hence by changing the target of the antibody, a new drug is created. The Fc subunit of the antibody can also be modified to alter the characteristics of the generated immune response and clearance from the body. Antibodies are generally well tolerated with few side-effects, in contrast to most

chemotherapeutic agents. They are occasionally associated with first-dose reactions which are mild-moderate allergic type responses which are abrogated by slowing the infusion. In addition certain antibodies bind to a sufficient quantity of normal tissue expressing the target antigen and this can lead to cytotoxicity against this normal tissue (eg skin rash with cetuximab). Rarely, however, they can cause serious side-effects when used to target immune activation molecules, as was demonstrated by the anti-CD28 antibody TGN1412 which led to the aborted phase 1 healthy volunteers trial in 2006 (Suntharalingam et al., 2006). The risk of such antibodies should be distinguished from antibodies eliciting ADCC or blocking function. Antibodies conjugated with radioactive or cytotoxic ligands have additional problems of systemic exposure to these conjugates.

Figure 1.iv: The production of monoclonal antibodies

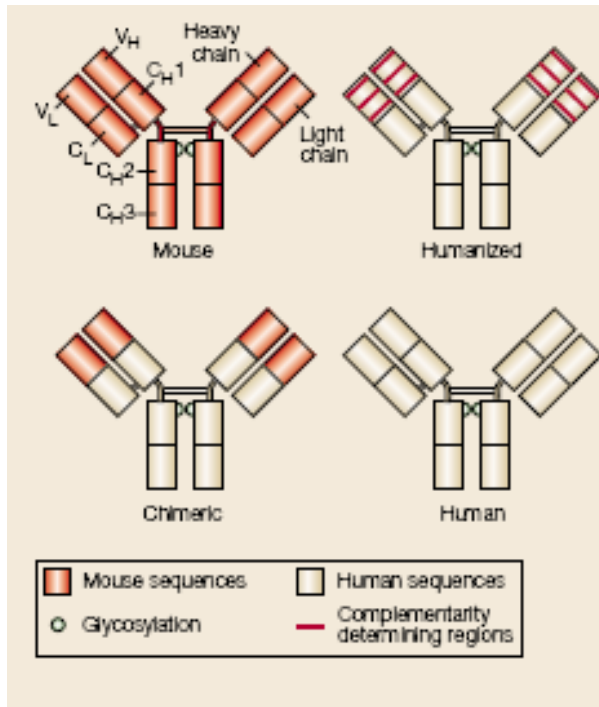


A further problem with antibody-based therapeutics is that they are dependent on cell surface antigen expression and the rate of internalisation of that antigen. Thus there is the

theoretical risk of antigen-loss variants being selected for following treatment if the antigen targeted is not vital for tumourigenesis.

The original murine monoclonal antibodies used in early clinical therapy were not as efficacious as initially hoped. The immunogenicity of murine protein led to a human antibody response to the foreign protein, with the development of human anti-mouse antibodies (HAMA). These HAMA not only reduced the half-life of the therapeutic antibody by precipitating its clearance from the body, they also neutralised the antibody, diminishing its ability to reach its target. In addition murine antibodies had weaker interactions with human Fc γ III receptors and human complement, leading to inefficient effector function (Kipps et al., 1985). A similar inability to bind with good affinity to the human neonatal Fc receptor (FcRn) further shortens the half-life of murine antibodies. The problem of immunogenicity of these murine antibodies has led to their modification to include progressively less murine protein, being replaced by more human protein (figure 1.v). The first development was that of chimeric antibodies such as rituximab and cetuximab. This was achieved by substituting the constant subunits of both heavy and light chains (C_H , C_L) with human sequences (Morrison et al., 1984). However these antibodies still had 33% murine protein and remained a target of antibody against the murine component of the antibody (known as human anti-chimera antibody; HACA) (Albert et al., 2008). Further refinements of this model led to the development of humanised antibodies in which the complementarity-determining regions (CDRs) were grafted onto a human antibody frame (Jones et al., 1986). This includes antibodies such as bevacizumab, trastuzumab and the major antibody of this thesis, hPR1A3. The CDR sequence is introduced by site-directed mutagenesis of the areas of human V_H and V_L which make up the CDR. A higher binding affinity can be achieved by the additional transfer of one or more framework-region residues from the mouse parent. This framework region makes up the structural element of the variable regions forming a scaffold for the CDRs to attach to. It remains 10% murine and very occasionally antibodies to the foreign component still arises (Welt et al., 2003). More recently fully human antibodies have been developed and used in the clinic (eg panitumumab, adalimumab). This has been achieved by two main techniques. One such technique

Figure 1.v: Progressive humanisation of monoclonal antibodies (after Carter, 2001(Carter, 2001))



involves the use of transgenic mice and was first described by Lonberg in 1994 (Lonberg et al., 1994). Large portions of the human immunoglobulin genome are inserted into that of the mouse and the murine immunoglobulin sequences are disabled by insertional mutagenesis. Hence when the mice are subsequently stimulated by foreign antigen, their B-cells produce human antibodies. These B-cells can be harvested and immortalised in the same way as before to produce antibody-producing hybridomas. The repertoire of antibodies produced is limited by the number of variable gene sequences introduced into the mouse although the more recent transfer of chromosome fragments which contain the entire human heavy- and light-chain loci, into mouse embryonic stem cells has largely overcome this problem (Tomizuka et al., 2000). In addition to producing human antibodies, these hybridomas often produce high-affinity antibodies, the B-cells having undergone the affinity maturation that would occur in a wild-type mouse.

The second technique for producing human antibodies is by using phage-display libraries (McCafferty et al., 1990). The V_H and V_L genes are cloned into a bacteriophage and the resultant antibody protein fragments (known as scFvs – see chapter 1.2.8) are then expressed on the phage surface and used to screen for those that bind the relevant antigen. Those that do are amplified and then further selected for affinity. The advantages of this technique are that it allows large libraries of antibodies to be produced against an antigen although there is obviously a need to screen further for antibodies with appropriate affinity. Other techniques that have been proposed include ribosome mRNA libraries, yeast-display libraries, human hybridomas from patients and antibody-cDNA cloning from single lymphocytes selected on the tumour-associated antigen.

1.2.4 Suggested mechanisms of naked antibody based cancer therapy

There is considerable debate about the mechanism of antibody action *in vivo* when used as anti-cancer therapy. Most agree that there are likely to be multiple mechanisms to the action of most therapeutic antibodies although clearly certain mechanisms predominate. It is in better understanding the mechanism of antibody action *in vivo* that we will be able to design antibodies for clinical therapeutics, since different mechanisms of action rely on different aspects of the antibody molecule and these can be modified to enhance the mechanism in question. For many of the antibodies in clinical use, there has been much discussion about the mechanism of antibody action and especially on the subject of receptor blockade. However, much of the experimental data would be more in keeping with ADCC being the primary mechanism of action.

1.2.4.1 Antibody dependent cellular cytotoxicity (ADCC)

The *in vitro* evidence for ADCC as a mechanism for antibody-based cancer therapy forms part of the work of this thesis. The evidence for *in vivo* action will be presented here. ADCC occurs when effector cells such as natural killer (NK) cells and macrophages, bind the Fc-subunit of the antibody, which in turn is bound to the target cell via the antigen-binding sites in the Fab fragment of the antibody (figure 1.vi). Cross-linking of the antibody Fc-receptor on the effector cell leads to the release of preformed cytoplasmic

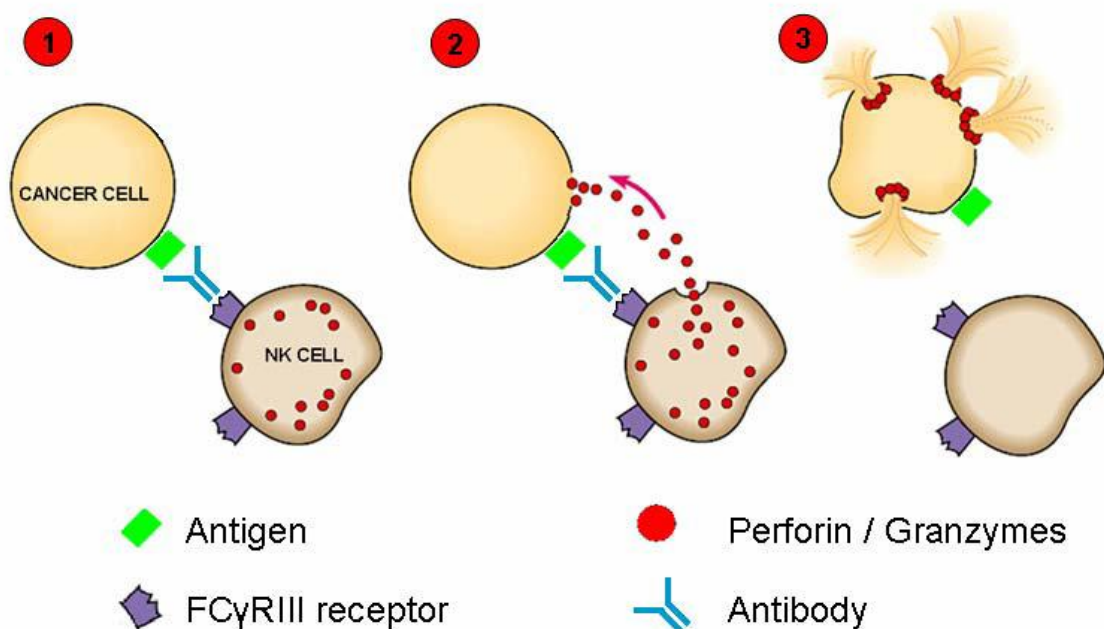
granules. In the case of NK cells these include perforin and granzymes. Perforin inserts into the target cell membrane and possibly also in the membrane of granzyme-containing vesicles, forming a channel through which granzymes reach the target cell cytoplasm. Granzymes activate caspases, especially caspase-3, leading to destruction of the target cell by apoptosis (figure 1.vi). In mice the interaction is predominantly between IgG2a and the Fc γ IV receptor (Nimmerjahn and Ravetch, 2005) and in humans between IgG1 and the Fc γ IIIa (CD16a) receptor.

Different IgG subclasses are known to have differing abilities to initiate ADCC, mostly on the strength of their binding to the human CD16a receptor. This was initially noted with the differing ability of murine antibodies to cause ADCC with human effector cells, where murine IgG2a was superior to both IgG2b and IgG1 in initiating ADCC (Kipps et al., 1985). Early antibody-based cancer therapy used murine antibodies and found IgG2a to be the most efficacious, supporting the view that ADCC was a central mechanism to their action. Edrecolomab, an IgG2a antibody against epithelial cell adhesion molecule (EpCAM), was one of the earliest antibodies used in the treatment of a solid tumour (Riethmuller et al., 1998). It's ability to induce ADCC had previously been noted (Steplewski et al., 1983). Other murine antibodies brought to trial again tended to rely on IgG2a as the isotype of choice (Welt et al., 1990) although the technology of chimerisation soon led to progressive humanisation as the focus of attention. Recent work using switch variants of the anti-melanoma antibody TA99 (anti-gp75 antigen) demonstrated not only the importance of IgG2a in the immunotherapy of mice but also the reason why, showing that it was the differing affinities of the various antibody isotypes for the murine Fc receptors that determined the ability of these different isotypes to inhibit tumour cell growth (Nimmerjahn and Ravetch, 2005). In particular, Nimmerjahn and Ravetch demonstrated that the affinity of IgG2a for the Fc γ IV receptor in mice (closest homolog to the human Fc γ IIIa receptor), enabling ADCC by the effector cells, coupled with the poor affinity of this antibody for the inhibitory Fc γ RIIB receptor, led to it being the most successful isotype *in vivo*, highlighting ADCC as the major mechanism of action. Thus the ability of each antibody isotype to initiate ADCC was determined by the balance of its affinity for the various activating and inhibitory

receptors, a balance described as the activating / inhibitory (A:I) ratio. Table 1.vii summarises the affinities of murine and human IgGs for their Fc γ receptors.

Figure 1.vi: Antibody-dependent cellular cytotoxicity:

(1) Antibody bound to antigen on the target cell is engaged by the Fc γ IIIa receptor on NK cells. (2) This results in activation of the NK cell and release of their granzyme vesicles, causing target cell lysis (3).



Key evidence emphasising the importance of ADCC in antibody therapy was demonstrated by Clynes in two papers using Fc γ receptor knockout mice. These mice contain genetic deletions of the gamma chain (part of the activating complex of most Fc receptors), leading to the failure to express the activating receptors Fc γ RI and Fc γ RIII and consequently a failure to initiate ADCC (Clynes et al., 1998; Clynes et al., 2000). The TA99 anti-melanoma antibody reduced the number of lung metastases in wild-type mice which had been injected with a melanoma cell line. However no such reduction in metastases occurred in the knockout mice suggesting a key role for Fc-Fc γ R interaction in

Table 1.vii: Relative binding affinities of murine (A) and human (B) IgG subclasses for their respective Fcγ receptors; derived from Nimmerjahn & Ravetch (Nimmerjahn and Ravetch, 2005) and Clynes (Clynes, 2006)

A: Murine IgG-FcR interaction

	FcγRI	FcγRIIb	FcγRIII	FcγIV
IgG1	-	++	+	-
IgG2a	+++	+/-	-	+++
IgG2b	-	+	-	+++

B: Human IgG-FcR interaction

	FcγRI	FcγRIIA	FcγRIIB-1	FcγIIB-2	FcγRIIIA	FcγIIIB
IgG1	++	++	++	++	+++	+++
IgG2	+/-	+	+/-	+/-	+	+
IgG3	++	++	++	++	+++	+++
IgG4	+	+/-	+	+	+	+

the mechanism of antibody action. Further work by Clynes subsequently showed a similar result using xenografts of breast cancer and B-cell lymphoma and using the therapeutic antibodies trastuzumab (anti-HER2) and rituximab (anti-CD20) respectively. Anti-tumour activity was reduced in the knockout mice. It was also reduced in the wild-type mouse if a modified version of the respective antibodies was used in which the Fc portion was mutated to prevent effective interaction with FcγIII receptor. A similar loss of antibody activity was shown in xenografts using a F(ab')₂ of cetuximab, which lacks the Fc-subunit, instead of the whole antibody, hence preventing interaction between antibody and effector cell. This resulted in a loss of 50% of the antibody's activity against the squamous carcinoma cell line A431 in a mouse xenograft model (Fan et al., 1993).

Cumulatively, these data provide compelling evidence that interactions between antibody and effector cells via the Fc γ -receptors are essential to the antibody mechanism of action. Clynes and colleagues further looked at mice deficient in the inhibitory Fc γ IIB receptor. These mice had increased spontaneous tumour protection against a melanoma cell line and a potentiated antibody response, even at subclinical doses of anti-gp75. The interaction between activating Fc-receptors and the inhibitory Fc γ IIB receptor is known to be important in regulating ADCC (Nimmerjahn and Ravetch, 2005).

Following progressive humanisation of murine antibodies, affinity for the human Fc γ IIIa receptor has still remained an important consideration, with human IgG1 Fc-subunits forming the framework for the huge majority of antibodies in clinical use for cancer (table 1.viii). For example, cetuximab (see chapter 1.2.5.3), a chimeric anti-epidermal growth factor (EGF) receptor mAb used in the treatments of colorectal and lung cancers, has an IgG1 Fc-subunit and, although it is commonly stated to work by blocking the function of the EGF receptor on the surface of the cell, there is increasing evidence that ADCC forms a large part of its action. Work from our laboratory and by others have shown that cetuximab and trastuzumab both have the potential to work by ADCC at very low, and thus clinically achievable, concentrations (figure 1.vii). Bleeker et al (Bleeker et al., 2004) showed using a fully human anti-EGF receptor (EGF-R) antibody, that ADCC was the likeliest mechanism of action both *in vitro* and *in vivo*. They achieved cell lysis *in vitro* using doses which would equate to less than 5% receptor occupancy, suggesting that receptor blockade was not the cause, a finding supported by other groups (Kurai et al., 2007). They also demonstrated that EGF-R blockade only occurred when there was complete receptor saturation, something which was only achievable *in vivo* with supra-normal doses (100mg/kg body weight; >600 μ g/ml concentration) whereas the normal cetuximab plasma concentration in humans is only 50-100 μ g/ml. Therefore given that in a tumour xenograft model they showed that their human IgG1 antibody inhibited tumour growth at low concentrations, where receptor occupation would be limited, they concluded that ADCC was the likely mechanism of action. It would also explain why, in contrast, a human IgG2 anti-EGF-R antibody, which would not cause ADCC, required a

total dose of at least 60mg/kg to achieve downsizing of the same xenograft (Yang et al., 1999) compared to the 5mg/kg of IgG1 human antibody used in the Bleeker study.

The suggestion that ADCC is the dominant mechanism in anti-EGF-R antibodies has also been suggested by experiments demonstrating good tumour downsizing in EGF-R-expressing xenografts with the chimeric IgG1 cetuximab (c225), but not so with the murine IgG1 m225 parent (Goldstein et al., 1995). It would also help explain the reason why EGF-R status does not correlate with clinical outcome in cetuximab treatment of metastatic colorectal cancer (Cunningham et al., 2004; Saltz et al., 2004).

Table 1.viii: Anti-cancer antibodies approved for clinical use

Antibody	Construct	Isotype	Target	Indication
Rituximab	Chimeric	IgG1	CD20	Non-Hodgkins lymphoma
Trastuzumab	Humanised	IgG1	HER2	Breast cancer
Gemtuzumab ozogamicin	Humanised / calicheamicin conjugate	IgG4	CD33	Acute myeloid leukaemia
Alemtuzumab	Humanised	IgG1	CD52	Chronic lymphocytic leukaemia
Ibritumomab tiuxetan	Murine / ⁹⁰ Y-radioconjugate	IgG1	CD20	Non-Hodgkins lymphoma
Tositumomab	Murine / ¹³¹ Y-radioconjugate	IgG2a	CD20	Non-Hodgkins lymphoma
Cetuximab	Chimeric	IgG1	EGFR	Colorectal cancer / head and neck SCC
Bevacizumab	Humanised	IgG1	VEGF-A	Colorectal / breast / NSC lung cancer
Panitumumab	Human	IgG2	EGFR	Colorectal

SCC = squamous cell cancer

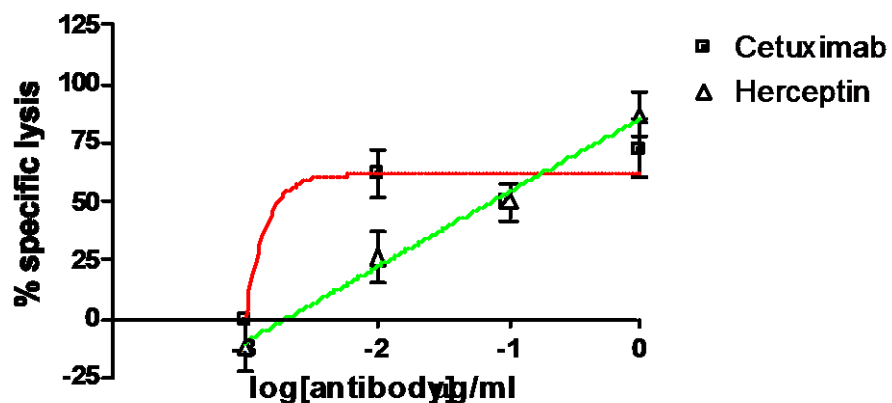
NSC = non-small cell

There is evidence to suggest that the interaction between the Fc fragment of the IgG antibody and the FcγIIIa receptor on effector cells, in particular NK cells, is influenced by polymorphisms in the gene encoding this receptor and this translates to differential activation in response to binding antibody (Koene et al., 1997; Wu et al., 1997).

Prominent amongst these polymorphisms is the T to G substitution at nucleotide 559 (exon 4) of the FcγRIIIa gene which changes the amino acid sequence of the receptor from phenylalanine to valine in an area of the receptor responsible for antibody binding (Tamm and Schmidt, 1996). This has been tested in the clinical arena using rituximab in the treatment of non-Hodgkin's lymphoma and trastuzumab in the treatment of metastatic breast cancer. Patients with valine/valine (V/V) at amino acid 158 had better outcomes with rituximab and trastuzumab than patients with the valine/phenylalanine (V/F) or phenylalanine/phenylalanine (F/F) polymorphism (Cartron et al., 2002; Musolino et al., 2008). This can be explained by the higher affinity of the Fc segment of an antibody to FcγRIIIa with valine at position 158. That this interaction leads to an improved clinical response would suggest that ADCC is an important mechanism in the action of rituximab. A similar finding has now been shown for breast cancer patients with the higher binding polymorphism being treated with trastuzumab.

Figure 1.vii: Cetuximab and trastuzumab cause ADCC at low concentrations *in vitro* against colorectal cell lines

COLO678 cell line (high expressor of EGF-R and HER-2 receptors) was pre-incubated with cetuximab and trastuzumab in 2 separate experiments using the same donor effector cells at a ratio of 40:1 effector:target cells, and analysed in an ADCC assay (unpublished results; SQ Ashraf, Cancer and Immunogenetics laboratory, Weatherall Institute of Molecular Medicine, Oxford).



Finally new technologies, which involve altering the glycosylation pattern or amino acid sequence in the Fc segment, have resulted in the production of antibodies with enhanced binding affinities to FcγIIIa receptor. When translated to *in vivo* models, amino acid modifications which enhance binding to FcγRIIIa and reduce binding to FcγRIIb result in enhanced CD20+ B-cell depletion in primates treated with rituximab by about 50-fold.

Despite the increasing evidence for ADCC being the primary mechanism in naked antibody-based cancer therapy, only two (rituximab and alemtuzumab) of five naked IgG1 mAb approved for clinical use are advertised as being ADCC-based. Many however have been noted to have ADCC activity. For example the anti-HER2 antibody, trastuzumab, is advertised as working by a number of immune mechanisms, particularly by receptor modulation - enhancement of HER2 degradation and inhibition of cell cycle progression by blockade of HER2 signalling - although these effects are not always apparent *in vivo* (Mohsin et al., 2005). Arnould et al (Arnould et al., 2006) demonstrated that neoadjuvant treatment of breast cancer patients with trastuzumab led to increased numbers of NK cells in the tumour and increased expression of granzyme B and TiA1 (markers of cells with cytolytic activity). This was not true in patients treated with chemotherapy alone (controls). In addition they also demonstrated the persistence of HER2 over-expression in residual tumour following treatment with trastuzumab, again favouring ADCC as being the primary action *in vivo* and not receptor down-regulation and blockade. Gennari et al found that patients induced to complete or partial remission by trastuzumab alone in the neoadjuvant setting were found to have a higher intensity of ADCC and higher in-situ infiltration of cytotoxic lymphocytes, including NK cells (Gennari et al., 2004). Most recently the combination of lapatinib, a tyrosine kinase inhibitor, with trastuzumab showed synergistic activity (Scaltriti et al., 2009). Lapatinib prevented internalisation of the HER2 receptor thus leaving an increased number of inactive receptors on the cell surface, potentiating ADCC *in vitro* in HER2 expressing breast cancer cell lines

Thus, in summary, the evidence for ADCC being of primary importance in the mechanism of action of antibody-based cancer therapy is:

- Importance of IgG2a and IgG1 isotypes for effective mAb action *in vivo* – these are isotypes able to elicit ADCC (Nimmerjahn and Ravetch, 2005). They include the major naked antibodies edrecolomab, cetuximab, trastuzumab, bevacizumab and others (table 1.viii)
- Knockout mice unable to express FcγIIIa or FcγI receptors failed to mount a response against melanoma cells following the injection of an anti-gp75 antibody (TA99) and failed to develop immunity against the same antigen when immunised prior to tumour injection (Clynes et al., 1998). These receptors are critical for ADCC.
- Mice deficient in FcγIIB receptor had an enhanced spontaneous immune response against tumour, even in the absence of TA99, and a potentiated response with TA99, allowing treatment with subclinical doses (Clynes et al., 2000).
- Mutation of the Fc region of rituximab and trastuzumab in order to weaken their binding to the FcRs, led to a weakened antibody response in mouse xenografts against CD20 positive lymphoma and HER2 positive breast cancer respectively (Clynes et al., 2000).
- F(ab')₂ of cetuximab lost 50% of its activity against a xenograft of squamous cell carcinoma expressing EGF-R (Fan et al., 1993).
- The action of cetuximab and trastuzumab *in vivo* is at low concentrations which would favour ADCC being the mode of action rather than the reported block of function, which would require saturation of the receptors and hence supra-therapeutic doses (Bleeker et al., 2004; Goldenberg, 1999).
- A human IgG2 version of an anti-EGF-R antibody requires supra-therapeutic doses for its effect (Yang et al., 1999) (more than 10 times that of a human IgG1 anti EGF-R antibody).
- Many therapeutic antibodies believed to work by a block of function do not work at therapeutic levels *in vitro* in the absence of effector cells (Bleeker et al., 2004), suggesting the latter are critical for antibody action.

- Murine IgG1 m225 (parent of cetuximab) compares badly in murine xenograft models with cetuximab (Goldstein et al., 1995) (chimeric; has a human IgG1 Fc-subunit). Mouse Fc γ IV receptor has a considerably better affinity for human IgG1 than murine IgG1.
- A polymorphism in the Fc γ IIIa receptor influences its affinity for the Fc-subunit of IgG1. The presence of the higher-binding polymorphism leads to an improved clinical response compared to the lower-binding polymorphisms (Cartron et al., 2002).
- Rituximab modified to produce a higher affinity for the Fc γ IIIa receptor and a lower affinity for the Fc γ IIb receptor, has an increased potency *in vivo* using primates.

1.2.4.2 Complement-dependent cytotoxicity

Complement is a group of proteins within the blood and on cell membranes which carry out three major functions:

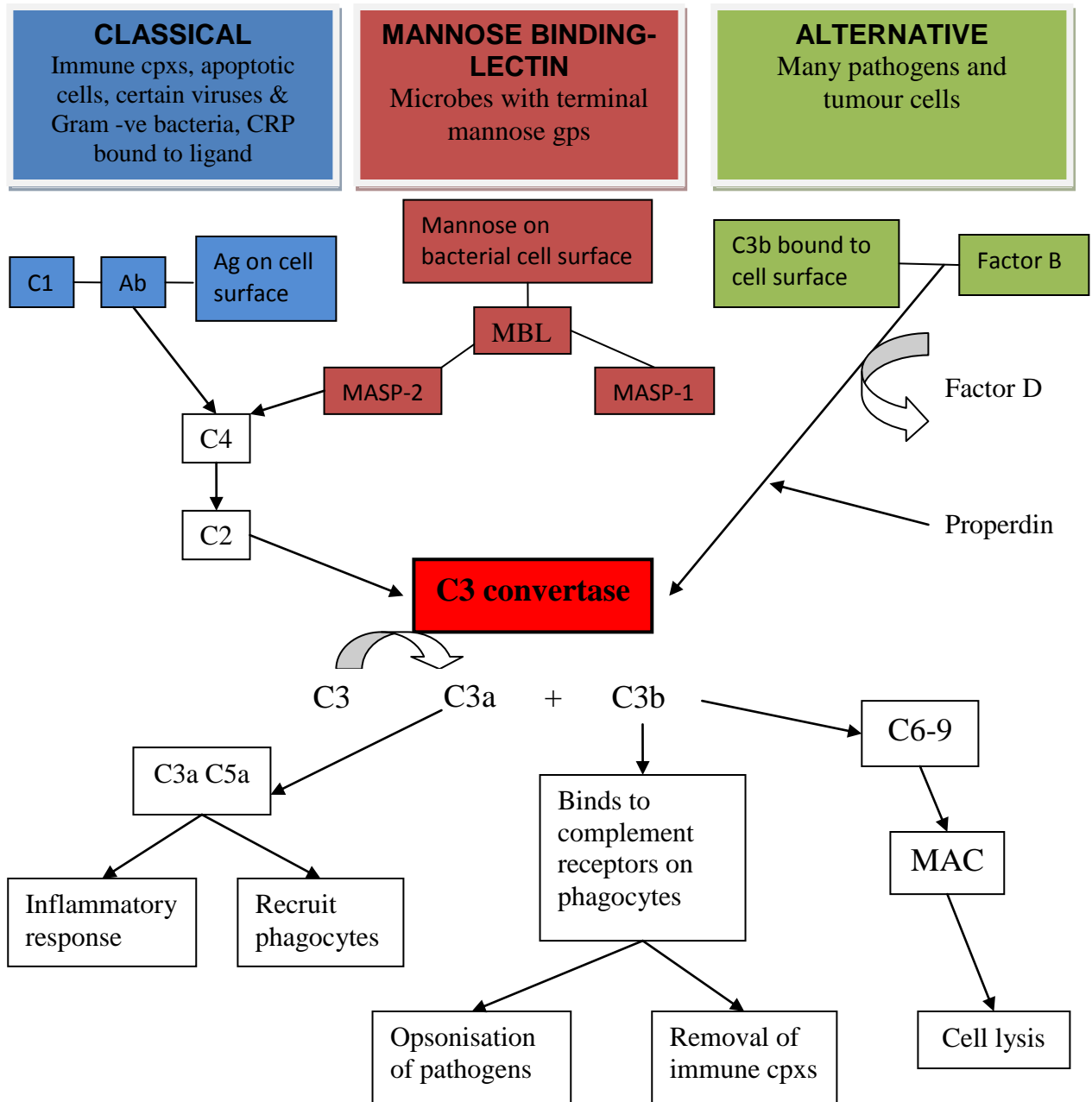
- Host protection against infection
- Link between innate and adaptive immune response
- Removal of immune complexes and other inflammatory debris

The various proteins making up complement can be activated in three ways; the classical pathway, alternative pathway and mannose-binding lectin pathway (figure 1.viii). The classical pathway carries the most relevance to this discussion. The inactive complement proteins are activated by being cleaved in an enzymatic cascade. The end result of all 3 pathways is often the cleavage of the C3 protein. This C3 protein has a multi-functional role, the best known of which leads to the formation of a membrane attack complex (MAC) on the surface of the target cell, leading to cell lysis (Walport, 2001).

The interaction between complement and antibodies is predominantly through binding sites for C1q on C_H2 of the Fc-subunit of IgG1 and IgG3 antibodies, leading to the activation of the classical pathway. Binding of antibody to antigen leads to the unclenching of multiple C1q binding sites (Adams and Weiner, 2005). In addition, C1q can act as a ligand for receptors such as C1qR, CR1 (CD35; see below) and CR3. These receptors are found on neutrophils, macrophages and NK cells. The interaction between C1q and its

Figure 1.viii: The three activation pathways of complement

The 3 pathways converge at the cleavage of the C3 protein to its active forms C3a and C3b. These C3 subunits initiate a wide range of immunological and inflammatory responses some of which are important in the anti-tumour response



MBL: mannose-binding lectin; MASP: mannose-binding lectin associated serine protease

receptor triggers cell-mediated tumour cell lysis or phagocytosis (Carter, 2001). A further interaction between complement and antibody is on the surface of effector cells. Many tumour cells can prevent the action of complement by complement-inhibitory proteins on their cell surface such as CD59, which inhibits formation of the MAC, and CD46, CD35 (complement receptor-1) and CD55, which inactivate the central complement component C3b on the cell surface, forming C3bi (Gorter and Meri, 1999). However, complement proteins such as the inactive fragment C3bi can still act as an opsonin by binding to C3 receptors such as Mac-1 (CD11b) on effector cells. There is a synergistic interaction between Mac-1 and Fc γ R-mediated signaling, whereby Mac-1 engagement enhances Fc γ R-mediated phagocytosis, cellular migration, and ADCC (van Spruiel et al., 2001). Hence although the antibody is not directly involved in CDC in this instance, complement mechanisms enhance ADCC. A further indirect effect of complement activation on antibody-based cytotoxic mechanisms is mediated by the complement protein fragment C5a which can cause upregulation of activating Fc γ Rs and downregulation of inhibitory Fc γ Rs (Schmidt and Gessner, 2005), hence enhancing ADCC in any given system.

Hence, complement can enhance ADCC by:

- C1q binding to Fc-portion of antibody and the resulting MAC
- C1q acting as a ligand for receptors on neutrophils, macrophages and NK cells resulting in phagocytosis or cell lysis
- C3bi acting as an opsonin by binding to C3 receptors such as Mac-1 (CD11b) on effector cells. There is a synergistic interaction between Mac-1 engagement and Fc γ R-mediated signaling, which enhances Fc γ R-mediated phagocytosis, cellular migration, and ADCC
- C5a causing upregulation of activating Fc γ Rs and downregulation of inhibitory Fc γ Rs, hence enhancing ADCC.

Evidence put forward to support the role of complement in antibody-based cancer therapy comes from *in vivo* work. Knockout mice deficient for C1q reduced the ability of rituximab to clear a CD20-transfected thymoma cell line (Di Gaetano et al., 2003). However this could simply be the result of reduced levels of C5a at the site of the tumour

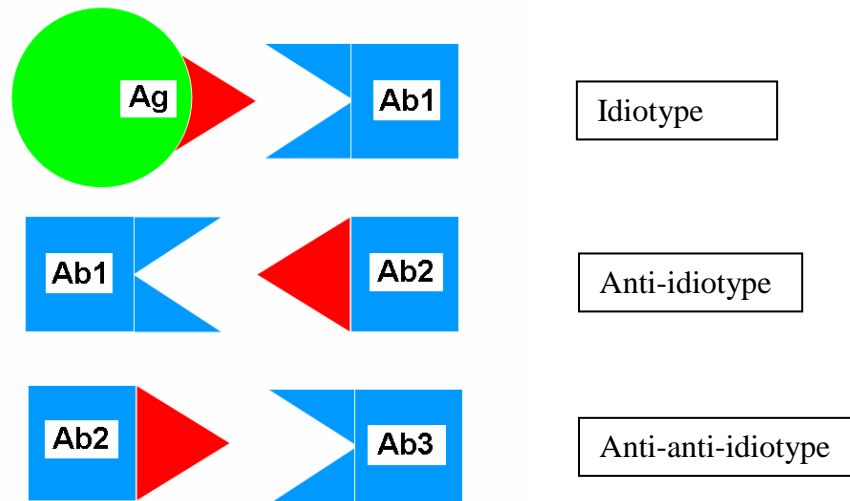
and the enhancement that this brings on ADCC potency as mentioned above, rather than evidence for complement having a direct role in antibody-based therapy. Further evidence for this comes from studies looking at the association between response to antibody therapy and the presence of complement inhibitory proteins on tumours. These proteins, CD59, CD44, CD35, prevent MAC formation on the cell surface and so would be expected to inhibit antibody action if CDC played a significant direct role. However in patients treated with rituximab for non-Hodgkins lymphoma, there is no correlation between the presence of these three proteins on the tumour cell surface and the response to rituximab, or indeed to the susceptibility of primary cultures grown from these samples to *in vitro* CDC (Weng and Levy, 2001). Hence although many antibodies used in cancer therapy can activate complement *in vitro* and demonstrate CDC, it is unlikely that this contributes significantly to their *in vivo* action.

1.2.4.3 Anti-idiotypic networks

This forms the basis of the mechanism behind using antibodies as “cancer vaccines”. It involves using antibodies to perpetuate an active immune response against a tumour antigen, in contrast to a passive immune response seen when simply giving antibody as a drug to elicit, for example, ADCC or CDC as described above. Foreign protein in non-human antibodies have the potential to stimulate production of antibodies to the therapeutic antibody (eg HAMA, HACA), including antibodies against areas in the Ag-binding site area of the antibody. The original antibody is described as Ab1 and the antibody created against it is Ab2 (anti-idiotypic). It is then possible for antibodies to develop against the configuration of the complementarity-determining region of Ab2 creating an antibody, referred to as Ab3 (anti-anti-idiotypic), which recognises a similar epitope to Ab1 and can bind to the tumour (figure 1.ix). Hence in this way the original Ab1 antibody has stimulated the humoral immune system and can overcome a patients’ unresponsiveness to the original tumour antigen which can have low or no immunogenicity on account of being ‘self’. The adaptive system continues to mount a response as long as the tumour antigen persists. In turn, this then involves the cell-mediated arm of the immune response (see later: chapter 1.2.4.5). This has most commonly been studied for the haematological malignancies where antibodies against the

Figure 1.ix: The development of an anti-idiotypic antibody network

Ab1 is the initial antibody administered to a tumour-associated antigen. Ab2 is an anti-idiotypic of Ab1 which contains within the CDR a configuration which mimics the original antigen (Ag). Consequently Ab3, the anti-idiotypic against Ab2, is very similar in structure to Ab1 but is now part of a sustained humoral response.



B-cell receptor from the malignant B-cell clone have been studied with some limited success in phase II trials, where both humoral and cell-mediated responses can be measured (Inoges et al., 2006; Redfern et al., 2006). However trials with vaccines against solid tumours have been less successful. One of the best studied is CeaVac, an anti-idiotypic mAb against the CEA molecule. A recent phase II trial failed to show any benefit of vaccination following potentially curative liver resection for colorectal cancer metastases, compared to a historical control of liver resection alone (Posner et al., 2008). One postulated reason for the failure of anti-idiotypic vaccinations is that they rely on an active immune response both during the induction of immunity and in the effector phase of the response, but both are inhibited by an immunosuppressive tumour microenvironment. Attempts to overcome this suppression have included targeting CTLA-4, a protein found on activated T-cells which serves to inhibit the activated

phenotype (Phan et al., 2003), and depleting regulatory T-cells (T-reg) by targeting the IL2-receptor (CD25) found in abundance on T-reg (Mahnke et al., 2007).

However at the current time, although detectable anti-idiotypes can be found *in vivo*, there is no evidence it plays any significant role in the action of Ab-based cancer therapy.

1.2.4.4 Receptor modulation

This is the most commonly stated method of action of the antibodies against solid tumours in clinical practice. Of the four mentioned in table 1.viii, three - trastuzumab, cetuximab and panitumumab - are said to work by receptor modulation. The targets in these situations are often growth factor receptors which are commonly over-expressed in solid malignant tumours. Their activation in the normal cell environment serves to promote cell division and cell survival, and hence their over-expression could lead to an abnormal expansion in cell numbers and cell survival. The three mentioned above are against members of the epidermal growth factor family of receptors; trastuzumab against the HER2 receptor and cetuximab and panitumumab against the EGF receptor (HER1). The methods proposed by which these antibodies are believed to block signal transduction include

- Blockade of ligand-receptor interaction (Sunada et al., 1986)
- Blockade of receptor dimerisation, thus preventing activation (Franklin et al., 2004)
- Steric inhibition of the receptor preventing the receptor from adopting the conformation required for dimerisation (Li et al., 2005).
- Promotes internalisation and degradation of the receptor, hence reducing the number of receptors on the cell surface which are available for interaction with ligand (Klapper et al., 2000).

Early studies demonstrated the ability of anti-EGFR antibodies to inhibit EGF-induced proliferation *in vitro* (Sato et al., 1983) and this was later repeated with cetuximab, showing that the antibody had a higher affinity for the receptor than the natural ligands (eg. EGF, TGF- α) and as such could prevent EGFR phosphorylation (Goldstein et al., 1995). Internalisation of the receptor was also noted leading to the conclusion that this

was another mechanism of cetuximab action (Sunada et al., 1986). The effect of the resulting blockade, by whichever mechanism, has been shown to cause numerous intracellular effects which serve to inhibit unregulated cell division and abnormal cell survival. These include halting cell cycle progression (Huang et al., 1999), inhibiting the release of paracrine angiogenic factors (Petit et al., 1997) and stimulating apoptosis (Liu et al., 2000). This evidence is obtained mostly from *in vitro* work on cell lines where saturation of receptors is possible at high doses of antibody, although it remains debatable whether such profound receptor blockade is possible *in vivo* (Bleeker et al., 2004).

1.2.4.5 Activation of the adaptive immune response (figure 1.x)

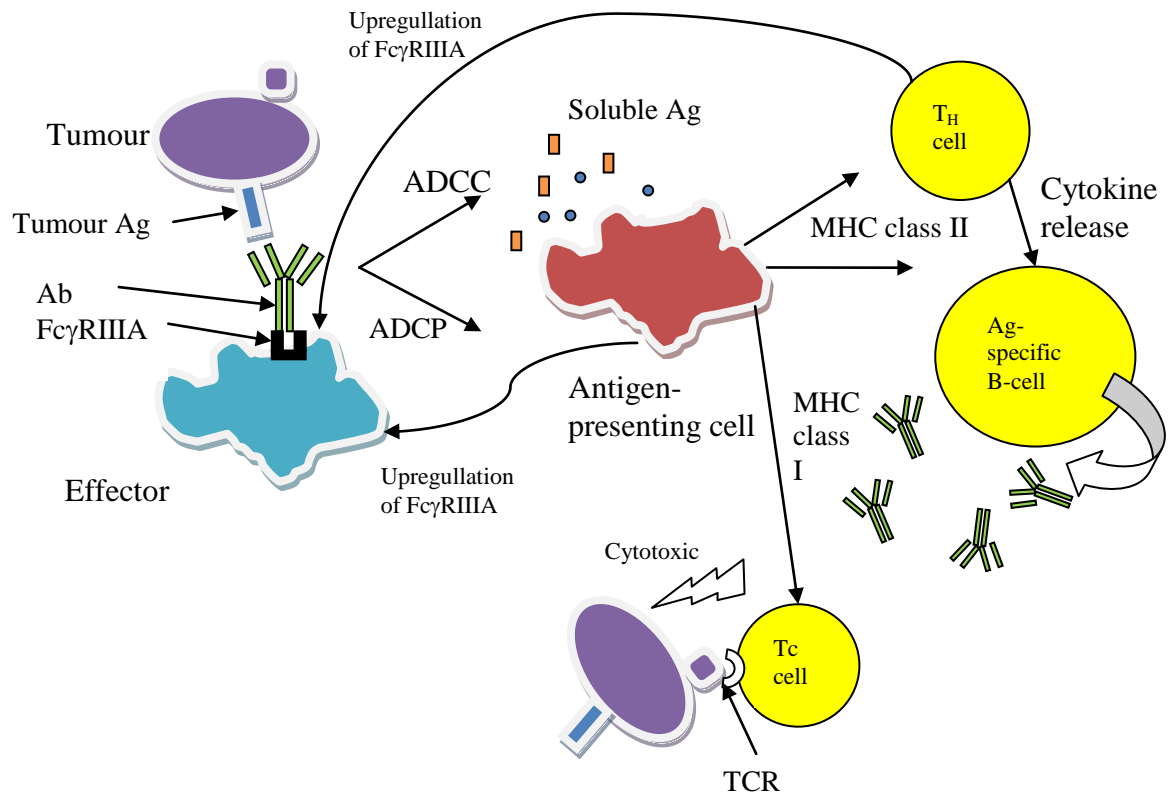
Passive immunotherapy with antitumor antibodies has the potential to induce active tumour immunity by interaction of the antibody with antigen-presenting cells (APCs). Firstly, lysed tumour cells, for example by ADCC, can release novel soluble antigen allowing uptake by APCs such as dendritic cells, macrophages and B-cells. These can then present foreign antigen to CD4⁺ helper T-cells (T_H-cells) and B-cells via MHC-class II, and CD8⁺ cytotoxic T-cells (T_C) via MHC-class I, leading to T-cell activation, cytotoxic activity, cytokine release and activation of B-cells and other effector cells and with this, the development of further anti-tumour pathways. Certain cytokines released in this response (eg. IFN- γ & GM-CSF) can lead to upregulation of the expression of activating Fc γ Rs (Schiff et al., 1997) thus providing further opportunity for antibody-mediated toxicity to the tumour and the presentation of further novel peptides.

This cascade of the immune response can also be initiated directly by the antibody bound to the tumour cell or soluble immune complexes. Fc γ Rs on the surface of APCs such as dendritic cells allow uptake of the antibody-antigen complex and, through the antigen-presenting capabilities of dendritic cells thereby stimulate the adaptive immune response, leading to a specific T_C cell response (Dhodapkar and Dhodapkar, 2005; Vasovic et al., 1997). The antibody again could thus be seen to be acting as a vaccine – allowing delivery of antigen to dendritic cells and the subsequent development of memory to the tumour antigen through the adaptive immune response. In fact, mice immunised with DCs which had previously been exposed to a tumour antigen, developed both a T_C- and T_H-mediated tumour immunity (Akiyama et al., 2003). This has been further shown in

Figure 1.x: ADCC-mediated adaptive immunity switch

Unconjugated monoclonal antibodies have the potential through ADCC to induce an adaptive tumour-specific immune response through the processing of peptides from lysed cells by dendritic cells and their subsequent presentation in the context of MHC class I or II to T_C cells, T_H cells and B cells.

Ab: antibody; Ag: antigen; TCR: T-cell receptor



patients with breast cancer treated with the anti-HER2 antibody trastuzumab and chemotherapy. Clynes' group demonstrated a significant increase in the presence of endogenous anti-HER2 antibodies in the serum of these patients, which were not anti-idiotypes, and an augmented HER2-specific T_H response (Taylor et al., 2007). In addition they noted that of those who had metastatic disease, a clinical response was more likely in those with more frequent and larger treatment-associated anti-HER2 humoral responses.

Finally, humoral immunity can be stimulated through the anti-idiotypic pathway as discussed above in chapter 1.2.4.3.

1.2.4.6 Effect on angiogenesis

There has been interest in targeting angiogenic factors to reduce or modify the blood supply to tumours. Most interest currently has been on vascular endothelial growth factor (VEGF)-A and its receptor. It is known that tumours frequently over-produce both VEGF and VEGF-receptors creating an autocrine loop which encourages tumour growth. One method of targeting the angiogenic machinery has been to use antibodies to VEGF, and one antibody in particular, bevacizumab, has already been used in clinical trials and is licensed for use in colorectal cancer. The effect of bevacizumab on the tumour vasculature, however, is unclear with a number of potential effects being considered. These include

- Inhibition of neovascularisation within the tumour.

Preventing stimulation of the VEGF receptor can reduce tumour vascularisation, inducing hypoxia and subsequent cell death. There is direct evidence that bevacizumab can inhibit neovascularisation. Willett et al (Willett et al., 2004) looked at the vascularity of tumours in patients with locally advanced primary rectal cancers after a single dose of bevacizumab. They found a reduction in tumour perfusion, vascular volume and microvascular density as well as a reduction in the number of viable circulating endothelial cells suggesting a direct effect on the ability of the tumour to stimulate neovascularisation, consistent with previous *in vitro* work.

- ‘Normalisation’ of tumour vasculature

It has been noted that the above theory alone is not consistent with evidence from clinical trials showing synergy of bevacizumab with chemotherapy (Hurwitz et al., 2004) and radiotherapy (Winkler et al., 2004). If bevacizumab only reduces blood flow to the tumour, it would be unlikely to aid the effect of chemotherapeutic agents which are delivered to the tumour via the bloodstream. The same would apply to the oxygen-dependent effects of radiotherapy and again these are enhanced rather than limited by prior treatment with anti-angiogenic treatment. In light of this it has been hypothesised that these anti-angiogenic antibodies may cause a temporary

normalisation of tumour vasculature from the more immature vessels normally encountered in tumour tissue. Such an effect would serve to increase the blood flow to tumour tissue during a short time-frame following bevacizumab treatment and allow increased delivery of chemotherapy via the bloodstream and improve tumour oxygenation to enhance the effect of radiotherapy. There is some evidence that such a process may occur. The tumour vasculature of a glioblastoma xenograft in a murine model was examined following treatment with an anti-VEGF receptor antibody (Winkler et al., 2004). Two to five days following treatment, tissue hypoxia reduced significantly before increasing again by day eight. During this window there was an increase in pericyte coverage of brain tumour vessels and a thinning of their pathologically thick basement membrane.

A further effect in tumour tissue seen following treatment with bevacizumab *in vivo* is a reduction in tumour interstitial pressure (Willett et al., 2004). Elevated interstitial fluid pressure is universal in solid tumours and is believed to be the result of abnormalities in the tumour vessels. Hence the reduction in interstitial pressure noted has again been attributed to a normalisation of the tumour vasculature. The result would again be a temporary enhancement of tumour blood flow.

An anti-angiogenic mechanism has also been proposed for antibodies acting against the human EGF receptor family. Both cetuximab and trastuzumab have been shown to down-regulate VEGF production by tumour cells via the blockade of the EGFR and HER2 receptors respectively (Perrotte et al., 1999; Petit et al., 1997). However such results relied on saturation of the receptors *in vitro* and in the well-vascularised model of the xenograft in nude mice. Whether such blockade can occur in the clinical situation is debatable as discussed above.

1.2.5 Naked antibodies in clinical practice

A brief overview of the literature concerning naked antibodies in current clinical practice is now given.

1.2.5.1 Rituximab

Rituximab is a chimeric IgG1 antibody targeting CD20, a transmembrane cell surface protein of uncertain function found on all normal B-cells and 95% of B-cell lymphomas but not on plasma cells. Its predominant use in cancer is in the treatment of non-Hodgkins lymphoma (table 1.viii). The mechanism of its action is likely through ADCC (Cartron et al., 2002; Clynes et al., 2000; Maloney, 2005) although contributions from CDC (Di Gaetano et al., 2003; van Meerten et al., 2006) and a direct action by apoptosis (Shan et al., 1998) have been suggested.

Several clinical trials have shown the efficacy of rituximab in a variety of clinical settings. Responses to single agent rituximab have ranged from 72% in previously untreated patients with follicular lymphoma (Witzig et al., 2005), to 48% in patients with relapsed indolent lymphoma (McLaughlin et al., 1998), to 40% in patients with relapsed lymphoma who had previously received rituximab (Davis et al., 2000).

However the best results have been in combination with standard chemotherapy consisting of cyclophosphamide, doxorubicin, vincristine and prednisolone (CHOP). Czuczman and colleagues demonstrated a median time to progression of 82 months in a long term follow-up of a phase II trial of rituximab and CHOP for non-Hodgkins lymphoma (Czuczman et al., 2004) and randomised phase III trials of rituximab and chemotherapy versus chemotherapy alone have also demonstrated a survival advantage with 97.3% alive at 3 years in combination treatment compared with 91.7% in the CHOP alone group (Hiddemann et al., 2005). A recent Cochrane meta-analysis of almost 2000 patients concurs with these results, demonstrating that the addition of rituximab to chemotherapy led to an improvement in overall survival from non-Hodgkins lymphoma when compared to chemotherapy alone (hazard ratio for mortality 0.65) (Schulz et al., 2007).

Rituximab has more recently been used in maintenance therapy after the induction of remission. A number of phase III randomised trials have looked at rituximab at a variety of dosing schedules and compared this with observation alone. These have all shown that prolonged therapy with rituximab is able to increase overall response and prolong remissions in patients with non-aggressive lymphomas. A Dutch trial looked at patients who had shown a complete or partial response to either CHOP alone or CHOP in

combination with rituximab (van Oers et al., 2006). They demonstrated an improved progression-free survival (PFS) with rituximab maintenance of 51.5 months compared with 15 months for observation alone. Overall survival (OS) was also improved from 77% at 3 years with observation to 85% with rituximab maintenance (once every 3 months for a maximum of 2 years).

Toxicity with rituximab is minimal. However, given that CD20 is expressed on normal B-cells, these too are targets for rituximab and consequently profound B-cell depletion post-treatment is common and can last for many months (Maloney et al., 1997). In rare circumstances this can lead to overwhelming sepsis. Another problem is the development of transfusion related reactions which are the result of the burden of murine protein in chimeric antibodies.

Given its chimeric nature, humanised and human versions of anti-CD20 antibodies are now being evaluated. Ofatumumab is a human anti-CD20 antibody and is currently under evaluation in clinical trials (Coiffier et al., 2008). This is in addition to the conjugated anti-CD20 antibodies which will be discussed below (chapter 1.2.6).

1.2.5.2 Trastuzumab

Over-expression of the HER2 (human EGF receptor 2) receptor occurs in about 20-30% of breast cancers and also in other cancers such as non-small cell lung cancer, ovarian cancer and prostate cancer, and is associated with a poor prognosis (Slamon et al., 1987). The HER2 receptor is a 185KDa transmembrane tyrosine-kinase receptor without a known ligand and is able to form homodimers with itself or heterodimers with other members of the human EGF receptor family. The mechanism of action is, however, contentious and has been discussed above (chapter 1.2.4).

Trastuzumab is a humanised IgG1 mAb against HER2 and was the first mAb against a solid tumour to be licensed in the UK. In a randomised controlled trial of trastuzumab with standard chemotherapy compared with chemotherapy alone in patients with HER2⁺ untreated metastatic breast cancer, the group receiving trastuzumab had a statistically significant improvement in median survival (25.4 months vs 20.3 months) (Slamon et al., 2001). Although it is normal for new antibodies to be used in metastatic incurable disease, it does not make optimal use of the naked antibody given that these whole antibodies are

large 150KDa molecules which penetrate only slowly through the interstitium of a poorly vascularised tumour, which is the nature of metastatic disease. Obviously the setting of potential micrometastatic disease would be a better testing ground for the efficacy of such antibodies. This would require the antibody to be used as adjuvant treatment in those at risk of metastatic disease as a result of nodal involvement or poor prognostic features of the tumour. Trastuzumab was tested in such an environment in two randomised controlled trials (Piccart-Gebhart et al., 2005; Romond et al., 2005). Both compared trastuzumab in the adjuvant setting following locoregional control (surgery with or without radiotherapy). In the American trial, trastuzumab treatment following initial post-operative chemotherapy was associated with a 33% reduction in mortality risk compared to chemotherapy alone with an advantage in disease free survival (DFS) at 3 years of 85.3% vs 67.1%. In the European study analysis at one year showed no difference in overall survival as might be expected in such a time-frame but it did show an improved DFS in the group receiving trastuzumab following chemotherapy (or having had neoadjuvant chemotherapy) compared to the group having standard chemotherapy alone; 13 vs 7.5%. Hence it would seem that trastuzumab is beneficial in the adjuvant setting and not just in the context of metastatic disease.

Toxicity has been a concern with trastuzumab especially cardiotoxicity which has been reported in up to 27% of women, this higher incidence being seen in those receiving anthracycline chemotherapy during trastuzumab treatment. Pre-existing cardiac disease is a relative contraindication to treatment with trastuzumab.

1.2.5.3 Cetuximab

Like trastuzumab, cetuximab targets one of the human EGF receptor family, HER1 - more commonly known as the EGF receptor (EGFR). This is a 170KDa transmembrane tyrosine-kinase receptor which is over-expressed in a variety of solid tumours including CRC, lung, squamous cell carcinoma of the head and neck, breast, ovary and oesophagus. It has two major ligands, EGF and transforming growth factor alpha (TGF α) and when the receptor is bound by ligand it undergoes a conformational change which allows dimerisation and activation through the tyrosine kinase domains. The EGFR is important

in mediating cell growth, differentiation, angiogenesis and cell survival and thus it is apparent why dysregulation can lead to tumorigenic processes within the cell.

Cetuximab is a chimeric IgG1 mAb which binds to the EGFR with a higher affinity than the natural ligands but does not activate the receptor. The controversy over the *in vivo* mode of action is detailed above (chapter 1.2.4). Cetuximab has been shown to be cytotoxic *in vitro* and shows synergy when combined with chemotherapy (Inoue et al., 2000) or radiation (Huang et al., 1999; Huang and Harari, 2000) both in *in vitro* and in murine xenograft models of EGFR-overexpressing tumours.

In the clinical arena cetuximab has for the most part only been used in the metastatic setting. The synergy with chemotherapy has proven useful where tumours have not responded to first- or second-line chemotherapy but have responded when combined with cetuximab. Cunningham et al (Cunningham et al., 2004) demonstrated this in a randomised controlled trial in patients with metastatic CRC refractory to irinotecan, a drug commonly used in the UK for second-line palliative treatment. They examined the benefit of cetuximab alone or in combination with irinotecan and found an improved response rate (23% vs 11%) and time to progression (4.1 months vs 1.5 months) with the combination therapy despite the patients having previously been resistant to irinotecan. Later studies have demonstrated that cetuximab monotherapy also produces a survival advantage in patients who have progressed on or are intolerant of a fluoropyrimidine, oxaliplatin or irinotecan (Jonker et al., 2007). However the improvement in overall survival was small albeit statistically significant. Compared to best supportive care, cetuximab improved overall survival by only 1.5 months (6.1 months vs 4.6 months for best supportive care).

Recent work has highlighted the importance of genetic analysis in tailoring treatments in CRC. A review of the pathological specimens from the Jonker trial has found that the presence of *k-ras* mutations in the tumour was strongly associated with a poor response to cetuximab, whereas those patients with wild type *k-ras* had an improvement in overall survival and progression-free survival compared to the control group (Karapetis et al., 2008). This and other data supporting the association between wild-type *k-ras* and response to anti-EGFR therapy in general (Linardou et al., 2008) have emphasised the importance of *k-ras* analysis of tumours in trial design of EGFR therapy.

Results from a Swiss phase II randomised trial showed promising results when randomising patients to capecitabine / oxaliplatin alone or with cetuximab (Borner et al., 2008). They demonstrated a response rate of 41% in the cetuximab group compared with 14% in chemotherapy alone. This didn't translate to a statistically significant survival advantage, likely due to underpowering. However given the impressive increase in response rates there may be an advantage in using cetuximab to create resectable metastatic disease from an unresectable position.

Current trials involving cetuximab include the COIN trial which is looking at the inclusion of cetuximab as first line chemotherapy for metastatic disease in combination with oxaliplatin and 5-FU. In addition the PETACC-8 trial is currently recruiting for enrolment in an adjuvant phase III trial for patients with resected stage III CRC. This trial will randomise patients to FOLFOX-4 regimen (5-fluorouracil, folinic acid and oxaliplatin) with or without the addition of cetuximab.

Cetuximab has been generally well tolerated with minimal morbidity. In addition to the side-effects of transfusion of a chimeric antibody, much of the toxicity reported is due to an acneiform rash which is seen in about 80% of patients but severe in less than 10%. This rash is believed to be due to antibody-mediated caused by binding of the antibody to the EGFR-bearing cells of the skin. However, the presence and severity of the rash correlates with response to cetuximab and to survival (Cunningham et al., 2004).

1.2.5.4 Bevacizumab

Vascular endothelial growth factor A (VEGF-A) is a proangiogenic growth factor that regulates vascular proliferation and permeability and inhibits apoptosis. It acts on two transmembrane receptors VEGF-1 and 2 which are expressed on vascular endothelial cells. VEGFR expression is upregulated in response to hypoxia and various cytokines, and by certain oncogenes. High levels of expression in tumour tissue are associated with a poor prognosis (Rosen, 2002).

Bevacizumab is a humanised IgG1 mAb which binds to all isoforms of VEGF-A and has been used in clinical trials in a number of cancers but predominantly colorectal. Its potential mechanisms of action are discussed above (chapter 1.2.4.6). Bevacizumab shows synergy with chemotherapeutic agents as demonstrated by Hurwitz et al in 2004.

They randomised patients to receive chemotherapy (irinotecan / fluorouracil / folinic acid) alone or in combination with bevacizumab. They found that bevacizumab increased the response rate (45% vs 35%), increased progression-free survival (10.6 months vs 6.2 months) and prolonged median survival (20.3 months vs 15.6 months) compared to chemotherapy alone in patients with previously untreated metastatic colorectal cancer (Hurwitz et al., 2004). A similar trial randomising patients to oxaliplatin-based chemotherapy with bevacizumab or placebo possibly showed a small improvement in progression free survival with bevacizumab (21.3 months vs 19.9 months; $p=0.077$) but no significant increase in overall survival despite randomising 1400 patients (Saltz et al., 2008).

However, the performance of bevacizumab in colorectal cancer is inconsistent with only about 12% of colorectal cancers responding to treatment (Jubb et al., 2006). Varey et al (Varey et al., 2008) put forward the hypothesis that anti-angiogenic treatments would be more successful in tumours with low levels of VEGF-b expression. The b-isoform, a splice variant of the angiogenic VEGF, acts in an anti-angiogenic manner. They demonstrated that bevacizumab indeed bound to the b-isoform with equal affinity to VEGF and that although bevacizumab could inhibit the growth of cell lines predominantly expressing angiogenic VEGF, it failed to inhibit the growth of a transfected cell line predominantly expressing VEGF-b. They postulated that the variability in response to bevacizumab could be explained by the variation in VEGF-b expression amongst colorectal cancers.

Following the results in the cetuximab trials, some researchers have tried bevacizumab and cetuximab together in the hope of further enhancing the effect of chemotherapy in metastatic CRC. The BOND-2 trial (Saltz et al., 2007) looked at this combination in patients with irinotecan-refractory metastatic CRC, much the same group that Cunningham et al had examined for the original cetuximab trial. They randomised patients to the two antibodies alone or in combination with continued irinotecan therapy. They found the greatest advantage when the antibodies were combined with continued irinotecan therapy (response rate of 37% vs 20% for the antibodies alone).

Currently, there is no evidence to support the use of bevacizumab in the adjuvant setting, although trials to assess this question are in progress. A large European trial and a large

North American trial will randomize patients to oxaliplatin / fluoropyrimidine based chemotherapy with or without bevacizumab.

Phase I trials of neoadjuvant bevacizumab prior to and then in combination with standard chemoradiotherapy are already underway in locally advanced rectal cancer (Czito et al., 2007). Despite small numbers there was no significant additional toxicity above that expected for the agents alone. Of 11 patients, 2 had a pathologically complete response, 3 had only microscopic residual disease and a further one had a partial response. The remaining 5 patients had stable disease with no-one progressing on treatment. However 2 of the resected specimen had involved margins.

There are significant concerns regarding toxicity with bevacizumab. There is an increased risk of both bowel perforation and arterial thrombotic events which, although occur rarely, are of great concern given their seriousness. There were 6 (1.5%) gastrointestinal perforations in the bevacizumab arm of the Hurwitz trial (Hurwitz et al., 2004) which included a perforated gastric ulcer, small bowel perforations, and free air under the diaphragm without an identified source. There were no such perforations in the control arm.

1.2.5.5 Panitumumab

Like cetuximab, panitumumab targets the EGF receptor. Its major differences are that it is a fully human antibody, the first to be licensed for clinical use and secondly it is an IgG2 isotype compared to the IgG1 platform of all other antibodies in use for solid tumours. This latter point is significant given that IgG2 antibodies bind poorly to the Fc γ IIIa receptor which would effectively reduce the potential of ADCC and CDC *in vivo*, although the weaker affinity of IgG2 for the inhibitory Fc γ IIb receptor may compensate for this. For the same reasons antibody-dependent cellular phagocytosis (ADCP) may also occur despite its poor binding to the Fc γ IIa receptor.

Panitumumab was made in a transgenic mouse expressing human immunoglobulin genes (see chapter 1.2.3) and binds with good affinity to the EGF receptor. FDA approval was achieved on marginal evidence from a phase II trial which was an open label randomised trial of third-line treatment of metastatic CRC in patients who had previously failed two different chemotherapy regimens (Giusti et al., 2008). The trial randomised 463 patients

to best supportive care alone or with panitumumab. The response rate to the antibody was only 8% and median progression-free survival (PFS) was no different than controls, although mean PFS was improved from 60 to 96 days. There was no difference in overall survival.

A recent multicenter randomized phase III trial was conducted in patients with metastatic CRC who had failed at least one chemotherapy regimen (Van Cutsem et al., 2007). They randomised 463 patients with 1% or more EGFR expression in the tumour, to panitumumab and best supportive care (BSC) or BSC alone ($n = 232$). The primary end point was PFS. Median PFS was 8 weeks in the antibody group compared with 7.3 weeks in the BSC alone group and mean PFS was 13.8 weeks and 8.5 weeks respectively, both figures statistically significant. The objective response rate was 10% and a further 28% of patients in the panitumumab arm experienced stable disease compared with 10% in the BSC alone group. No difference in overall survival was seen between the two arms with 19% of patients alive at 1 year in the panitumumab group and 16% in the BSC group. The toxicity profile of the drug was mild which is important in the palliative setting.

However the large ($n=1054$) phase III PACCE trial comparing a regimen of bevacizumab and FOLFOX with or without the addition of panitumumab was stopped after a pre-planned interim analysis revealed a statistically significant advantage in PFS in the control group (not receiving panitumumab) as well as an excess of complications including pulmonary emboli and infectious complications.

Trials comparing irinotecan with or without panitumumab are underway to assess any synergy or additive effect of the two treatments.

It remains to be seen how much of the effect seen with cetuximab is lost in panitumumab by the use of the IgG2 isotype.

1.2.6 Radioimmunotherapy

The early concepts of antibody-based cancer therapy concentrated on antibodies as delivery vehicles for toxic payloads, in particular radiotherapy (Epenetos et al., 1987). In addition, the ability of antibodies to localise a target made them an attractive proposition for radiological detection of tumours and recurrence even before monoclonal antibody

technology was developed. Hence, the technology to attach a radioisotope to an antibody has therefore been developed over more than 30 years (Mach et al., 1974a; Mach et al., 1974b). However the development of radioimmunotherapy (RAIT), where antibodies target tumour tissue with radioactive isotopes allowing localised high-dose radiotherapy without significant systemic toxicity, has not progressed in any significant way for solid tumours, although there are some clear advantages in haematological malignancies. Hence, of the 9 antibodies that are currently licensed in the US or Europe for use in oncology, only 3 are conjugated to a radioactive ligand or toxin, and none are for use in solid tumours (see table 1.viii). Despite often excellent results in preclinical trials, the translation to the clinical arena often failed (Tempero et al., 2000). The reason for this failure was multifactorial but the major problem was a failure to deliver an adequate toxic dose to the tumour without a significant bystander effect (Sharkey and Goldenberg, 2005), resulting in either failure to irradiate the tumour or radiation injury to both surrounding tissues and organs involved in clearing the antibody, in particular the bone marrow and kidney. In addition, the complex chemistry required to conjugate the radioisotopes to the antibody makes the construct technically more difficult to make, store and administer. The problem is highlighted by a radio-iodine conjugated version of the A33 antibody which targets a 43KDa cell-surface glycoprotein of unknown function whose expression is restricted to colorectal epithelium and continues to be expressed homogeneously in more than 95% of colorectal cancers. In a phase I trial they demonstrated selective targeting of the antigen with the A33 antibody but, as has been shown previously with other radiolabelled antibodies, only a tiny proportion of the antibody was retained by the tumour (0.002% per gram of tumour) (Scott et al., 2005; Weiden et al., 1993). Although the toxicity level was still acceptable in their study there was no improvement in any of the fifteen patients treated (Chong et al., 2005).

However there has been some encouragement in haematological malignancies. Building on the success of rituximab, further anti-CD20 antibodies were developed, some of which were conjugated to radioisotopes. Those licensed for use in B-cell tumours are ibritumomab tiuxetan, which emits ^{90}Y trium, and tositumomab, which emits ^{131}I odide. Both are potent β -emitting isotopes and both have had success in trials treating B-cell lymphomas. Comparison trials with rituximab have favoured RAIT in terms of response

rates and progression-free survival. A phase III randomised trial comparing ibritumomab tiuxetan with rituximab in non-Hodgkins lymphoma, found that the RAIT achieved a greater level of response (80% vs 56%) including complete response (34% vs 20%), although it was underpowered to detect differences in parameters of survival (Gordon et al., 2004).

A list of antibodies involved in recent RAIT trials is given in table 1.ix.

Table 1.ix: Selection of radiolabelled mAb involved in recent clinical trials; after Milenic et al (Milenic et al., 2004)

Antibody	Isotype/species	Isotope	Antigen	Cancer	Status
Tositumomab	mu IgG2a	¹³¹ I	CD20	NHL	FDA
Ibritumomab	mu IgG1	⁹⁰ Y	CD20	NHL	FDA
Epratuzumab	hu IgG1	⁹⁰ Y	CD22	NHL	Phase III
MN-14	hu IgG1	⁹⁰ Y	CEA	Carcinoma – varied	Phase II
TNT-1/B	ch IgG1	¹³¹ I	DNA	Glioblastoma	Phase III
¹³¹ I-Lym-1	hu IgG1	¹³¹ I	HLA-DR10	NHL, CLL	Phase II
Pentumomab	mu IgG1	⁹⁰ Y	PEM	Ovarian, gastric	Phase III

Abbreviations: mu – murine; ch – chimeric; hu – humanised; PEM – polymorphic epithelial mucin; NHL – non-Hodgkins lymphoma; CLL – chronic lymphocytic leukaemia; FDA – approved for use by US food and drug administration

1.2.6.1 The antibody

The qualities required in an antibody for RAIT remain essentially the same as those for immunotherapy in general and discussed earlier. However antibodies which are internalised may have an advantage for RAIT as they would be expected also to do so for the delivery of cytotoxic drugs and immunoliposomes. An ideal antibody would be able

to target tumour rapidly, be internalised and retained within the tumour by target antigen re-expression so minimising exposure of normal tissue. The biggest problem remains that, as discussed above, only a tiny percentage of antibody reaches its target and so in clinical trials of solid tumours, the cumulative dose of radioactivity within a tumour is usually well below that required to achieve a therapeutic response. Increasing the tumour dose increases the tissue dose and this leads to toxic side effects (Goldenberg, 2002). The rate of clearance of the antibody is also of importance given the potential side effects. Clearance of whole antibody is slow and increasingly researchers looking at developing antibodies for RAIT have been looking to antibody fragments which are cleared more rapidly (see chapter 1.2.8).

1.2.6.2 The radionuclide

As table 1.ix shows, the choice of radionuclide in trials of RAIT has almost exclusively relied on two isotopes – ^{90}Y trium and ^{131}I odine. Both are β -emitting isotopes with reliable chemistry that lends itself to RAIT. Halogens, such as ^{131}I , are routinely introduced by direct halogenation of tyrosine residues of the protein. Metallic radionuclides, such as ^{111}In or ^{90}Y , require chelation of the metal through a suitable ligand. A considered advantage of β -emitters is that they are cytotoxic over a distance of numerous cell diameters (emission path length) and thus can exert a toxic effect into the substance of a poorly vascularised tumour. For example, ^{90}Y has an emission path length of 0.5-0.6mm which could penetrate a thickness of more than 200 cells. This long emission path length also helps deal with tumour heterogeneity of target antigen expression, as neighbouring cells which express the target antigen poorly or not at all, and perhaps would not bind antibody, are still killed by the radioactivity. However this same property also leads to limitations of use as it causes significant injury to non-tumour cells, especially haematological, accounting for much of the toxicity. Pretargeting strategies set out to reduce the collateral toxicity caused by RAIT. This will be discussed in more detail below (chapter 1.2.7.1)

^{131}I odine emits both β and γ radiation, has an 8 day half-life and can still be used on an outpatient basis although the γ emissions do present safety concerns. In addition there is rapid de-iodination of the antibody once internalised and a high enzymatic elimination

from tumours before the radioactive iodine can deposit all of its energy into the tumour (Foon et al., 1999). The γ -emission also allows the antibody to be imaged.

^{90}Y is a pure β emitter with an efficient labelling process and has a long maximum particle range, making it more suitable for the irradiation of larger tumours. The disadvantages include that, unlike ^{131}I , which has a γ -emitting component, ^{90}Y gives poor images, although this can be countered by using another radionuclide label to act as a tracer.

α -emitters have a much shorter emission path length but with a much higher energy transfer within that distance (high linear energy transfer) in the order of 500 times that of ^{90}Y . Hence they are highly cytotoxic to the immediate surroundings and they decay rapidly. This therefore would limit their use clinically to easily targeted, small, well vascularised tumours (eg micrometastatic disease) (Hartmann et al., 1994) where bystander cytotoxicity would be unhelpful.

1.2.6.3 Radioimmunotherapy and colorectal cancer

There have been many preclinical successes using RAIT to treat solid tumours (Axworthy et al., 2000; Koppe et al., 2003; Trail et al., 1993), including CRC but such success has not been replicated in the clinical arena. This failure serves to highlight the difficulty with xenograft models in antibody-based cancer therapy as well as the problems with RAIT in solid tumours. In a situation in which antibody targeting and clearance are vital, using a model with an increased number of NK cells and a tumour in a non-orthotopic site which is abnormally vascularised is clearly not a good representation of the solid tumour *in vivo*. There have however been some potential candidate radioconjugates for small volume metastatic disease in CRC. Behr et al (Behr et al., 2002) gave a ^{131}I -labelled humanised anti-CEA antibody, MN-14, to a heterogeneous group of patients who either had small volume metastatic disease (lesions <3cm) and who had failed first line therapy with fluorouracil, or had undergone a liver resection for metastases with curative intent. They noted an objective response of 16% in the group with non-operated metastasis and also found 7 of 9 patients of the operated patients were disease free at 3 years. This latter group who have had a potentially curative liver resection have been followed up and added to (23 in total) and the results published intermittently, most lately in 2007 (Liersch

et al., 2007). They recorded a median overall survival following treatment with ^{131}I -MN-14 of 58 months and a median disease-free survival of 18 months. They compared these figures to a comparable group who did not get given antibody who had survival data of 31 and 12 months respectively, although being unrandomised the statistical analysis should be read with caution. They estimated from Kaplan-Meier curves a 5-year survival rate of 42% which is certainly better than the national average of about 30% in the UK following such surgery and standard post-operative chemotherapy. Toxicity levels were acceptable. It remains to be seen what the benefits would have been with an unlabelled version of the same antibody.

There have however been many phase II and III trials which have shown no benefit in CRC with RAIT as is seen in table 1.x and this is typical for solid tumours in general.

Table 1.x: Radiolabelled mAb involved in clinical trials in the treatment of colorectal cancer

Antibody	Target	Radioisotope	Cancer	Response
A33 (Welt et al., 1994)	A33 antigen	^{131}I	Colorectal	PR 3/23
CC49 (Murray et al., 1994)	TAG72	^{131}I	Colorectal	NR 24/24
A5B7 (Lane et al., 1994)	CEA	^{131}I	Colorectal	1/9 CR (Fab ₂) 1/10 PR
Ch NR-LU-13 (Weiden et al., 1993)	EpCAM	^{186}Re	CRC, stomach, breast	9/9 NR
CC49 (Divgi et al., 1995)	TAG72	^{131}I	Colorectal	30/30 NR
Ch B72.3 (Meredith et al., 1992)	TAG72	^{131}I	Colorectal	4/12 SD

Abbreviations: PR – partial response; NR – no response; CR – complete response; SD – stable disease

1.2.7 Antibodies conjugated with other ligands

Early studies using non-radioactive ligands attempted to attach conventional chemotherapeutic agents such as doxorubicin to the tumour-specific antigen. However, given the tiny percentage of antibody (0.001 – 0.01%) reaching tumour antigen as discussed above, and the low molar toxicity of conventional chemotherapy, this technique was unsuccessful as it was impossible to expose enough tumour to the drug without causing significant systemic toxicity. For example the anti-sialyl Lewis-Y antigen mAb, BR96 was used as a doxorubicin-conjugated construct and found to be highly efficacious in xenograft studies (Trail et al., 1993) but demonstrated no effect in phase II trials for metastatic breast (Tolcher et al., 1999) and unresectable gastric (Ajani et al., 2000) cancers.

The next development has been the use of small molecule toxins such as calicheamicin which are 100-1000 times more potent than drugs such as doxorubicin and also have the advantage of being effectively inactive whilst bound to the antibody so, in effect, acting like a prodrug. Activation would ideally occur once the toxin was released from the antibody having bound to the tumour antigen and been internalised. This, therefore, limits the potential targets, requiring only targets that are internalised once bound by the antibody. These chemically-conjugated immunotoxins have further potential limitations in efficacy and safety in that the chemical linker used to join the antibody to the toxin may be subject to decay. Like RAIT, the only successes have been with haematogenous tumours, likely due to the more effective targeting by the antibody. This is aptly demonstrated by the anti-CD33-calicheamicin construct gemtuzumab ozogamicin which is licensed for use in patients with CD33⁺ acute myeloid leukaemia (AML) after the first relapse. It has a response rate of about 30% in clinical trials (Sievers et al., 2001).

Other toxins that have been used include the protein toxins: diphtheria toxin, *Pseudomonas* exotoxin A, *Staphylococcus* enterotoxin and ricin amongst others. These toxins often have to undergo modification of their binding sites to avoid their normal targets in non-tumour tissue. In addition, the use of these protein immunotoxins is further hampered by their relatively strong immunogenicity leading to a reduced serum half-life and, as a result, impaired effectiveness.

1.2.7.1 Pretargeting strategies

As has been discussed, the biggest obstacle to effective RAIT or immunoconjugate therapy has been the low percentage of antibody ever reaching the target, resulting in a low tumour to background ratio and, with this, the potential for systemic toxicity. Pretargeting strategies aim to increase tumour to background ratios by separating the targeting and active treatment steps of the process. The advantages over the direct arming approach include

- a. Using smaller carriers for the radionuclide or toxin that can therefore more easily target tumour but are also more rapidly cleared from the blood stream.
- b. Increasing tumour to non-tumour ratios by more than 10:1.
- c. For RAIT, the vehicle used for the radioisotope is often easier to label than an antibody, hence allowing a higher radioactive dose and a wider range of radioisotopes to be tried.
- d. For RAIT, reducing renal toxicity (for a given total radioactive dose) compared with using a radiometal-labelled antibody fragment. However as pretargeting strategies often employ higher doses of radioactivity, the risk of renal toxicity is often greater.

The two main techniques for non-radioactive conjugates have been to use bispecific or multispecific antibodies and secondly the use antibody-directed enzyme prodrug therapy (ADEPT).

In the first method, an engineered antibody which has for example dual valency like a normal antibody but with different specificities for the 2 Fab parts of the antibody (bispecific); one Fab with specificity for the tumour antigen and the other for some construct of the radioisotope or drug. A fraction of this unconjugated bivalent antibody binds to the tumour and the unbound majority is then cleared from the body, either naturally or with assistance from a clearing agent. The second step is then to administer the active radioisotope, drug or toxin which will either bind directly to the 2nd empty Fab, for which it has specificity, or the radioisotope / drug is administered bound to a protein for which the Fab is specific. Either way the active ligand does not require an antibody to get it to the target as the antibody is already in place, and any unbound ligand is more easily cleared than when bound to an antibody.

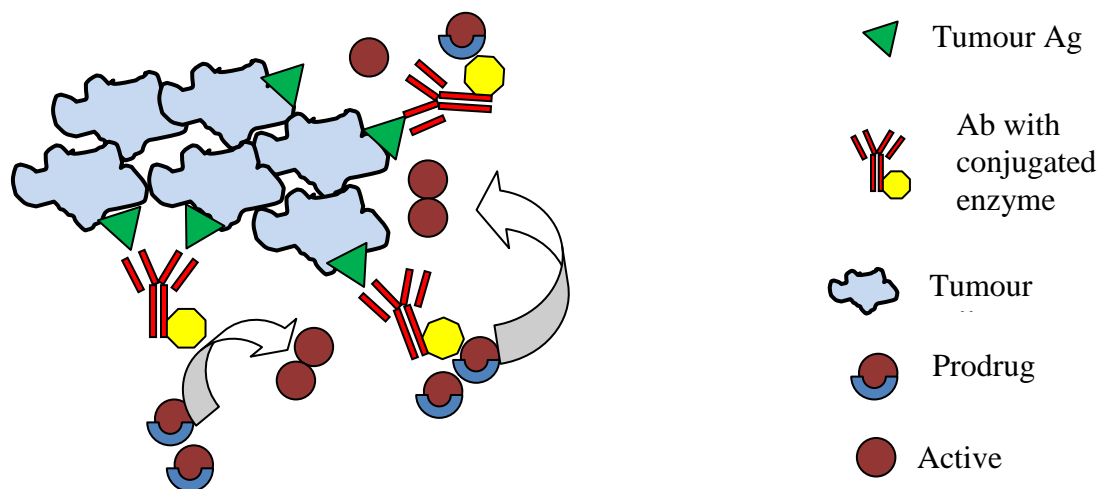
ADEPT is more straightforward and has the advantage of increasing drug concentration at the site of the tumour without relying on a high concentration of bound antibody or indeed a large number of target antigens to bind to (figure 1.xi). The targeting phase is much the same as with bispecific antibodies except that the antibody is unispecific for the target antigen but this time it is conjugated to an enzyme which will catalyse the conversion of a prodrug to its active form when it is injected in the 2nd phase of the process. As before, spare unbound antibody is cleared between the steps. The prodrug is thus converted to its active form at the site of the tumour, significantly increasing its local concentration over a single step process, especially as a single enzyme can convert many prodrug molecules to the active form, thus amplifying the effect. There is thus no requirement for all the cells within the tumour to express the target antigen as it will rely on a significant bystander effect to overcome this problem. As a result, an antibody – target complex that will not be internalised, or is internalised very slowly, is a prerequisite. The choice of enzymes range from non-mammalian enzymes with no mammalian homologue (class I) which are immunogenic but will not have any endogenous counterpart which will convert the prodrug to its active form in non-tumour tissues, to mammalian enzymes (class III) which, although only weakly immunogenic may have an endogenous form which will create active drug away from the tumour site. Class I enzymes are preferred on safety grounds but this is at the cost of efficacy as a result of immunogenicity. Like the techniques described previously, ADEPT has proven itself in xenografts (Syrigos and Epenetos, 1999) but no proven benefit exists in the clinic (Francis et al., 2002).

Pretargeting with radioimmunotherapy follows a similar principle. The commonest used method in mouse models and in clinical trials involves utilising the ultra-high affinity of streptavidin for biotin which has a dissociation constant of 10^{-15} , about 6 logs higher than most antibody-antigen interactions. Streptavidin has 4 subunits, each of which can bind one biotin molecule. In this method streptavidin is coupled to an anti-tumour IgG and this unlabelled construct is administered first. Once it has achieved its maximum uptake in the tumour (eg 1-2 days) it is then actively cleared from the blood to prevent non-specific binding to the radiolabelled biotin, as passive clearance of the antibody can take weeks.

The clearing agent uses a serum albumin construct bound to biotin to bind to the free unbound antibody. To prevent this clearing agent competing for the biotin sites on the tumour-bound antibody, a galactose molecule is coupled to the albumin-biotin so that it is avidly taken up by the liver and removed from the blood. Following a further 1-2 days, the active biotin-radionuclide construct is given which localises to the tumour.

Figure 1.xi: Antibody-directed enzyme prodrug therapy

The active drug is concentrated at the site of the tumour by a specific enzyme bound to the tumour-specific antibody which converts the prodrug to the active compound. Outside the tumour environment, the drug exists only as a prodrug.



A second method is to use the bispecific antibody techniques used for immunotoxins as described above, incorporating an anti-tumour arm and an anti-chelate metal for the radioisotope. Although the streptavidin-biotin system has a higher affinity between tumour antibody and radionuclide, studies have suggested that the dose-limiting response is in fact the tumour – antibody interaction and this should be the same in both systems. In addition streptavidin is immunogenic and thus precludes multiple doses.

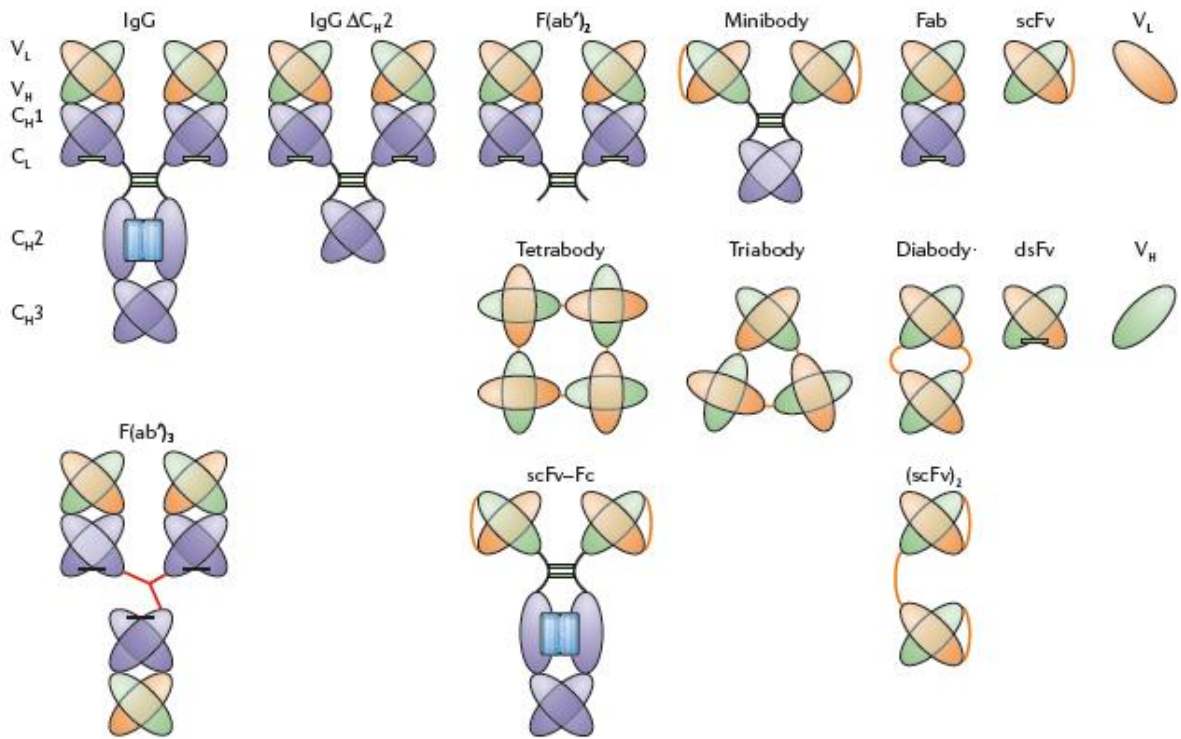
As mentioned in the chapter on RAIT above (chapter 1.2.6.1), pretargeting strategies have also looked to smaller antibody fragments to improve both diffusion into the tumour and clearance from the blood stream.

At the current time, again despite encouraging preclinical results (Sharkey et al., 2005), the use of pretargeting RAIT techniques has failed to show any benefit in solid tumours in clinical trials with the increase in radiation dose instead causing haematological and renal toxicity (Knox et al., 2000; Kraeber-Bodere et al., 2003). However there have been some reported improvements with pretargeting over direct arming in haematological malignancies (Press et al., 2001)

1.2.8 Immunotherapy with antibody fragments

Antibody engineering has led to a broad range of antibody fragment structures based essentially on the variable region of the antibody. Therefore they are universally without an Fc-region and as such forego the effector functions of this part of the molecule and with it the major mechanism for the majority of therapeutic antibodies, namely ADCC. The construct of the more common antibody fragments is given in figure 1.xii. These fragments have an enormous variability in size (12-200KDa) and valency (commonly between 1 and 4 binding sites). These binding sites can be for the same or different antigens or indeed different epitopes on the same antigen. The most common constructs are based around the scFv fragment which consists of the V_H and V_L domains of an antibody, connected together by a peptide linker. The majority of these fragments have a half-life which is too short to have any therapeutic application although they may be useful for imaging. The half-life however can be improved almost to that of a whole antibody, by binding it to an inert molecule such as polyethylene glycol (PEG). This so-called PEGylation has been used to good effect in an anti-TNF antibody used for the treatment of inflammatory bowel disease and connective-tissue diseases. Certolizumab pegol is a PEGylated humanised Fab of an anti-TNF antibody which has good efficacy *in vivo* (Sandborn et al., 2007) and is licensed by the FDA. It has a half-life of 14 days – similar to that of the parent IgG (Choy et al., 2002).

Figure 1.xii: Commonly used antibody fragments; after Carter (Carter, 2006)



The potential advantages of antibody fragments include

- Avoid Fc-related effector functions where they may be producing side effects. For example, the anti-CD3 antibody, muromonab, used in the prevention of rejection, acts by blocking CD3 and the Fc portion of the antibody is believed to be responsible for the viral-like symptoms caused by the T-cell proliferation and cytokine release following administration.
- Reasonable half-life after PEGylation.
- More efficient penetration of tumour which will improve tumour to non-tumour binding ratios.
- Faster clearance which again will improve binding ratios.
- May allow blockade of receptors without the concern of cross-linking and subsequent activation of the receptor, something which may happen with bivalent antibodies
- Potentially access areas of protein not accessible to whole antibody fragment

However in most instances the longer half-life of whole antibody means that overall tumour binding is better with whole antibodies than with their respective fragments.

Fragments that have been tested in clinical trials include scFv (Begent et al., 1996), Fab (Sandborn et al., 2007), F(ab')₂ (Rhee et al., 2000), minibody (Wong et al., 2004), (scFv)₂ (Schlereth et al., 2006) and scFv-Fc constructs. Despite this however, they have not performed sufficiently well in these trials to become licensed for use in cancer therapy. This is perhaps a reflection on their inability to initiate immune effector functions as mentioned above. Many continue in clinical trials however.

1.2.9 Models of colorectal cancer for antibody-based cancer therapy

1.2.9.1 Cell lines

Cell lines derived from human cancers have been vital in our understanding of the molecular pathophysiology of cancer and have been a frequently-used model to test potential treatments. The consistency of genetic and phenotypic data obtained from cell lines gives confidence to be able to examine the effect of antibodies against different cell lines and to equate that with the variability seen in the tumours of different individuals (Greshock et al., 2007). The cell lines used in this thesis have been well characterised and frequently checked for variability. Details of the cell lines can be found in the paper by Conaghan et al (Conaghan et al., 2008).

1.2.9.2 Animal models

These have often been seen as superior to *in vitro* work and there has been a heavy reliance on the xenograft in immunological therapies. However the discrepancy between results in xenografts and those in the clinic has led to suggestions that, for many anti-tumour therapies, *in vitro* work is more likely to correlate with effect in the clinical arena (Johnson et al., 2001). This is especially the case for antibody-based cancer therapy for which tumour targeting and clearance are of the ultimate importance in determining success of therapy.

1.2.9.2.1 Xenograft

The most commonly used animal model of CRC is the xenograft whereby a single cell suspension of the desired cancer is subcutaneously inoculated into an immunodeficient mouse. The nude mouse (*BALB/c nu/nu*) is deficient in T cells. These mice have a homozygous gene defect encoding for a transcription factor. This causes a failure of differentiation of certain types of epithelial cells that are required for the normal development of the thymus and hair follicles. Thus, these mice develop with neither a thymus nor body hair. The severe combined immunodeficiency (SCID) mouse is another immunocompromised mouse that can sustain a xenograft.

The inoculated xenograft tumour is able to grow without stimulating a host immune reaction. Although extensively used and validated, the absence of a competent immune system does not simulate the normal situation and for the investigation of immune-mediated responses and tumorigenesis, this type of animal model is inappropriate. Subcutaneous xenografts are located in an environment that is very different from the site from which they originate. Xenografts are often surrounded by a pseudocapsule, reducing the chances of the tumour spreading, and metastases from subcutaneously placed xenografts are rare. Xenografts also tend to have a significantly different vascular supply to spontaneously arising tumours, with an increased level of neovascularisation (Axworthy et al., 2000). This leads to increased levels of chemoradiotherapeutic agents and antibodies within the xenograft cancer with better than expected results (Behr et al., 1997; Tolcher et al., 1999).

1.2.9.2.2 Syngeneic model

The syngeneic mouse is a further mouse model that can be used. A murine tumour, either spontaneously arising or induced after chemotherapy or radiotherapy, is developed in an inbred mouse strain. This tumour is used to create a cell line that can be injected back into the original mouse strain to form a xenograft. Although this results in a mouse model that is immunocompetent, it still shares many of the problems of the xenograft model.

1.2.9.2.3 Transgenic model

These mice have been genetically modified by the insertion of human genes into the mouse genome. For the purposes of testing antibody-based cancer therapies, these genes usually encode for tumour associated antigens (TAA). Such mice allow, to some extent, the efficacy of mAbs to be assessed against tumour targets. Examples of such mice bearing human TAAs include the CEA.Tg (Eades-Perner et al., 1994) and MUC1.Tg (Peat et al., 1992) mice. Hence the mouse should show immunological tolerance to a subcutaneous injection of tumour expressing the TAA, often a murine tumour expressing the same transgene. These mice can then be used to test an antibody against the specific TAA without concern that the mouse will mount an independent immunological response against the TAA irrespective of mAb treatment. However this model still suffers from the inherent problem of a xenograft, namely, an unnatural tumour location, blood supply and pseudocapsule formation.

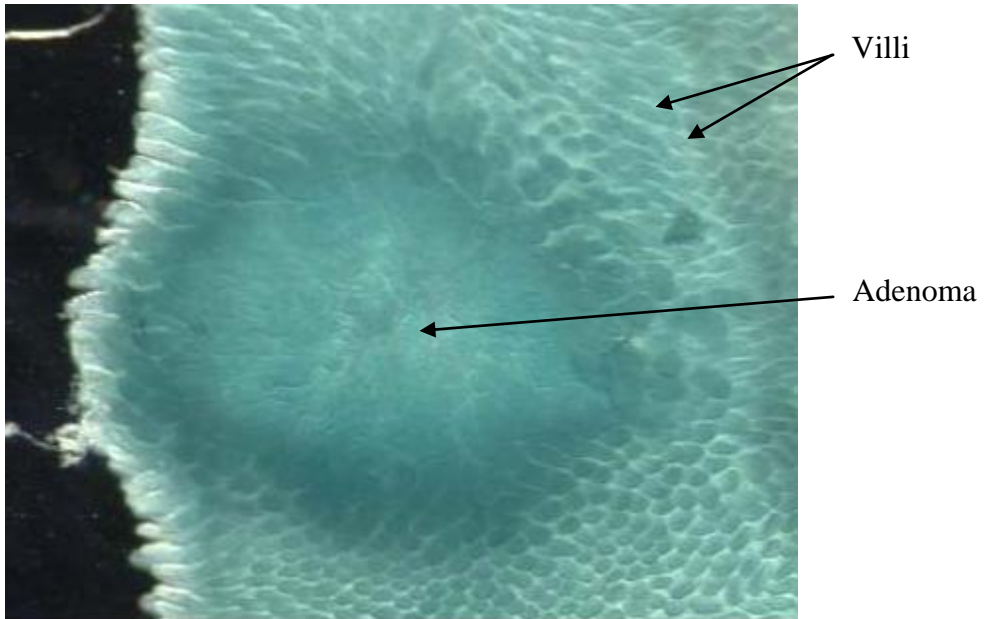
1.2.9.2.4 Ideal animal model

A transgenic mouse with spontaneously developing tumours with metastatic potential is likely to be the ideal murine model of testing immunological therapies in a preclinical situation, providing the germ line mutational spectrum is similar to that in humans. There has not, to date, been an adequate model of spontaneously arising CRC. However there are mice with APC (adenomatous polyposis coli) gene mutations which spontaneously develop adenomas (Su et al., 1992). One such strain, the MIN mouse (C57BL/6J APC^{Min/+}), develop between 50-300 adenomatous polyps, predominately in the small bowel with up to 10 adenomas in the large bowel (figure 1.xiii). Whilst these lesions are phenotypically and genotypically equivalent of the adenomas in humans, the adenomas in mice do not progress to carcinomas probably because the mice die before this can occur at approximately 120 days. Mice do not have a homologue of CEA. However a transgenic mouse expressing CEA has been crossed with the MIN mouse (Apc^{min/+} CEA.Tg^{+/-}), hereafter called the MINCEA mouse (Thompson et al., 1997). CEA.Tg mice have been shown to express CEA in the same spatiotemporal pattern as seen in humans (Eades-Perner et al., 1994). Interestingly the resulting adenomas in MINCEA mice express CEA with the same loss of polarisation seen in colorectal cancer (see chapter 1.4.2), hence

making the protein accessible to antibody-mediated therapy, as shown by studies using a¹²⁵I-labelled murine version of our antibody, PR1A3 (Wilkinson et al., 2001). Hence, this mouse model develops CEA-expressing tumours spontaneously in the bowel, with a tumour vasculature comparable to that seen in tumours in humans, and this in a mouse with an intact immune system. For CEA-based therapies, we believe this to be the best animal model for CRC, although achieving earlier carcinogenesis with metastatic potential is the next goal.

Figure 1.xiii:

A: Small bowel adenoma from the Min ($APC^{Min/+}$) mouse at 40x magnification



B: CEA distribution in an adenoma from the MINCEA mouse using immunohistochemistry (Wilkinson et al., 2001).

The CEA is stained black



1.3 Fc γ receptor family

The Fc receptors (FcRs) are type I transmembrane glycoproteins and belong to the Ig superfamily. They are encoded by genes clustered together on chromosome 1. The majority are low affinity receptors and consist of two extracellular Ig-like domains (D1 & D2) connected by a hinge whereas the high affinity Fc γ R1 contains an additional Ig-like domain (D3).

The FcRs as a group form part of the body's regulatory control for the immune system. It consists of a number of activating and inhibitory receptors found on a wide variety of haematopoietic and other cells. The FcRs for IgG (Fc γ R) provide the biggest subgroup of this receptor family and demonstrate how simultaneous triggering of activating and inhibitory signals can set limits for immune activation. Hence, when these signals become dysregulated, a state of autoimmunity or immunosuppression can result.

There are three different classes of Fc γ Rs in humans, each with its own subclasses, distribution patterns and binding affinities for various IgG subclasses (tables 1.viiB & 1.xi). In addition there is allelic variation within some receptor groups, such as IIA and IIIA (Clark et al., 1989; Wu et al., 1997) which further widens the potential repertoire. With the exception of Fc γ RIIb, which is an inhibitory receptor, and Fc γ RIIIb, which is GPI-linked and whose function is not clear, all other Fc γ Rs activate the immune response, either directly through an immunoreceptor tyrosine-based activating motif (ITAM) in their α -chain (Fc γ RIIa and Fc γ RIIc) or through association with an ITAM in separate γ - or δ -subunits (figure 1.xiv). Expression of these receptors is often reliant on expression of the γ -subunit (Clynes et al., 1998).

Table 1.xi: Human Fc-gamma Receptors

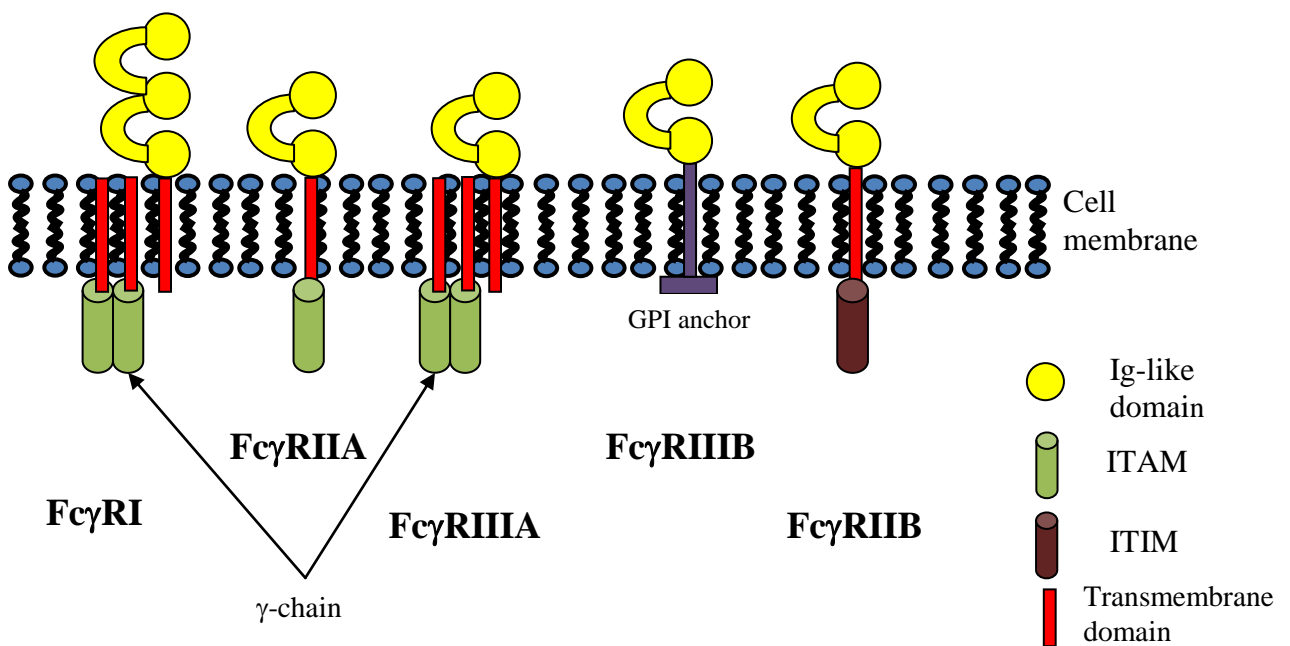
		I (CD64)	IIA (CD32)	IIB-1	IIB-2	IIIA (CD16)	IIIB
Affinity		High	Low-medium	Low-medium	Low-medium	Low-medium	Low-medium
Function		Myeloid cell activation	Myeloid cell activation	B-cell inhibition	Myeloid cell inhibition	Myeloid cell & NK activation	Unknown
Polymorphism		None	aa131 His-Arg	aa232 Ile-Thr	As for IIB-1	aa158 Val-Phe	NA1/NA2
Cellular expression	Mφ	+	+	-	+	+	-
	NK	-	-	-	-	+	-
	PMN	+	+	-	-	+	+
	DC	+	+	-	+	+	-
	B-cell	-	-	+	-	-	-
Endocytosis		Yes	Yes	No	Yes	Yes	No
Phagocytosis		Yes	Yes	No	No	Yes	No

Abbreviations: Mφ, macrophage; NK, natural killer cell; PMN, neutrophil; DC, dendritic cell; aa, amino acid; His, histidine; Arg, arginine; Ile, isoleucine; Thr, threonine, Val, valine; Phe, phenylalanine; NA, neutrophil antigen

The FcR family in mice has been well studied and there is significant homology with the human receptors, although not always with the similarly-numbered receptor in the different species and not with respect to cell-type specific expression patterns. For example, the closest homolog to the human FcγIIIA receptor is the murine FcγIV in terms of sequence similarity and genomic location (Mechetina et al., 2002). Like its human homolog, FcγRIV is the major Fc receptor used in murine ADCC but its cellular distribution is very different, being found on macrophages and neutrophils but not on NK

Figure 1.xiv: Selected members of the human Fc-gamma receptor family

Schematic diagram of some of the activating members of the Fc γ R family and the inhibitory Fc γ RIIB. The ITAM-containing gamma chain acts as the activating motif for all but the Fc γ RIIA, Fc γ RIIC (not shown) and Fc γ RIIB. The former 2 have an ITAM within the intracellular portion of their α -chain, whilst Fc γ RIIB has a GPI anchor attaching it to the cell membrane. The inhibitory Fc γ RIIB has an ITIM as part of its intracellular α -chain.



cells. It is interesting to note therefore that, unlike the human situation, NK cells are not believed to play a key role in ADCC in mice (Uchida et al., 2004). It would also explain the importance of the IgG2a isotype in ADCC in mice rather than the IgG1 required in humans. This is due to the differential binding of IgG2a to Fc γ RIV and the inhibitory Fc γ RIIb (table 1.vii) as discussed earlier in chapter 1.2.4.1. IgG2a binds to the low affinity Fc γ RIII in mice but also binds with 40 times higher affinity to Fc γ RIV. In addition IgG2a has a 70 times higher affinity for Fc γ RIV than for the inhibitory Fc

receptor Fc γ IIb. In contrast murine IgG1 binds to Fc γ RIII but not to Fc γ IV and in addition has a 10 times higher affinity for Fc γ IIb than for Fc γ RIII (Nimmerjahn and Ravetch, 2005). This would explain why IgG2a is best isotype for ADCC in mice.

Evidence increasingly demonstrates Fc γ Rs to be involved in a wide variety of immune functions which include

- Regulation of the innate immune system (Takai et al., 1994).
- Antigen uptake and presentation (Rafiq et al., 2002; Woelbing et al., 2006).
- Immune complex-mediated activation and maturation of dendritic cells (Regnault et al., 1999).
- Maintaining peripheral tolerance in the cellular immune system via Fc γ RIIB by preventing activation of dendritic cells by low levels of immune complexes in the serum (Dhodapkar et al., 2005).
- Regulation of B-cell activation (Tzeng et al., 2005) and plasma cell survival (Xiang et al., 2007) (thus regulating the production and specificity of their ligand – i.e. antibody).

One of the most important features of the Fc γ R system is the co-expression of activating and inhibitory receptors on the same cells with the exception of B-cells (Fc γ RIIB only) and NK cells (Fc γ RIIA only) (table 1.xi). The inhibitory receptors tend to dominate the response under normal circumstances thus preventing non-specific immune activation. The low affinity of all the receptors, except Fc γ RI, for antibody means that monomeric antibodies do not bind to activating receptors which bind only to immune complexes of antigen and antibody, thus again avoiding non-specific activation. Fc γ RI however is usually saturated with monomeric IgG and hence is not available under normal circumstances to bind immune complexes. It becomes available when local levels of Fc γ RI are increased by local inflammation (Bevaart et al., 2006). In such circumstances, other Fc γ Rs are also upregulated on effector cells by pro-inflammatory cytokines (e.g. IFN- γ & GM-CSF) (Schiff et al., 1997).

1.3.1 The FcγIIIa receptor in ADCC in humans

The FcγIIIa receptor is found predominantly on NK cells but also on macrophages, neutrophils and dendritic cells. Like most of the FcγR family it is a low affinity receptor for the Fc fragment of antibodies (10^{-6}) and consists of two extracellular Ig-like domains (figure 1.xiv). It is increasingly evident that mAbs exert their major anti-tumour effect through ADCC mediated by interaction of the antibody with the FcγIIIa receptor.

For example a number of studies have demonstrated a correlation between the clinical efficacy of Abs in humans and the affinity with which they bind to the FcγIIIa receptor. As highlighted in chapter 1.2.4.1 there is evidence to suggest that the interaction between the Fc fragment of the IgG antibody and the FcγIIIa receptor on effector cells, in particular NK cells, is influenced by polymorphisms in the gene encoding this receptor and this translates to differential activation in response to binding antibody (Koene et al., 1997; Wu et al., 1997). In particular a polymorphism in an area of the receptor responsible for antibody binding (at amino acid 158) leads to a higher binding FcγIIIa receptor where valine is used instead of phenylalanine. Three clinical studies have now shown that patients who are homozygous for the higher binding valine allele had better outcomes with the anti-CD20 antibody rituximab than heterozygotes and phenylalanine homozygotes (Cartron et al., 2002; Dall'Ozzo et al., 2004; Weng and Levy, 2003).

Secondly it has been shown by glycoform engineering (Schuster et al., 2005; Shields et al., 2002) and mutagenesis (Shields et al., 2001) that the affinity of interaction between Fc and the FcγIIIa receptor correlates with cytotoxicity in cell-based assays. Together these data suggest that an Ab with optimized affinity for the FcγIIIa receptor may be more effective against targeted cancer cells in patients, hence supporting the importance of this receptor and the process of ADCC in the mechanism of antibody action.

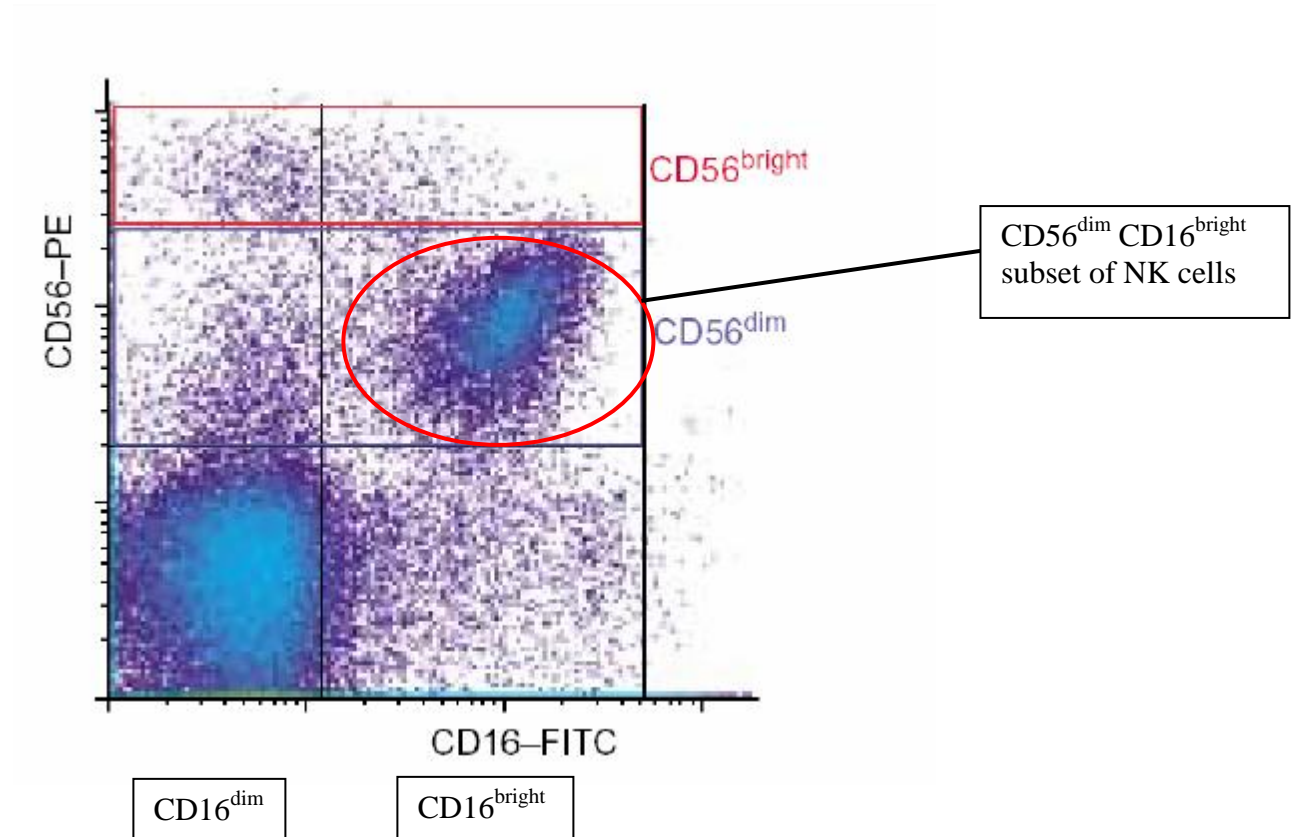
1.3.2 Role of NK cells in FcγIIIa-mediated cytotoxicity in humans

Human natural killer (NK) cells are lymphocytes, defined by the expression of CD56 (the neural cell adhesion molecule; NCAM) and CD16, and the absence of CD3. They constitute about 5-20% of all mononuclear cells. NK cells have a number of cell-surface receptors that help mediate the immune response, recognising ligands on cells, some of

which suppress NK-dependent cytotoxicity and some which cause NK activation. As part of the innate immune response, NK cells form part of the early recognition of abnormal cells, for example those which are virally infected or having undergone neoplastic transformation. Basic recognition of self is obviously vital in this respect and the major inhibitory signal to NK activation is through the interaction of the NK killer Ig-like receptors (KIRs) with self HLA class I on the potential target cells (Moretta and Moretta, 2004). This inhibitory signal overrides NK activating signals, hence suppressing NK-mediated cytotoxicity. The absence of this inhibitory signal enables NK activation using a variety of activating surface receptors that interact with different non-MHC ligands expressed on the target cells. This occurs, for example, when tumour cells downregulate MHC-I expression thus giving them an advantage against the adaptive immune response (Algarra et al., 2004). Tumour transformation can also lead to the upregulation or *de novo* expression of ligands for these activating receptors, especially through a group of receptors known as the natural cytotoxicity receptors (NCRs) (Bottino et al., 2005). These NCRs appear to activate the NK cells by associating with ITAMs in the cell membrane although the ligands activating them (NCR-Ls) are as yet unknown.

The other well recognised method of activation is via the Fc γ IIIa receptor (CD16A) which also uses the same ITAMs as the NCRs to initiate NK activation. In fact expression of CD16A is dependent upon the common gamma chain (Fc ϵ RI γ) expression (Lanier, 2001; Takai et al., 1994). NK cells are unique amongst the cells bearing activating Fc γ Rs in that they express only Fc γ IIIa (table 1.xi). The majority (90%) of circulating NK cells have low density expression of CD56 and high levels of Fc γ IIIa expression (CD56^{dim}, CD16^{bright}) whereas the remaining 10% are CD56^{bright}, CD16^{dim} (figure 1.xv). The former are more naturally cytotoxic whereas the CD56^{bright} group have a greater cytokine-producing capability following NK activation (Cooper et al., 2001b). Antibody-bound target cells cross-link CD16A on NK cells when the CD16A binds to the Fc-segment of the antibody, causing degranulation and cytokine production, possibly also accompanied by apoptosis of the NK cells (Ortaldo et al., 1995). The mechanism of cell lysis following NK activation by Fc γ IIIa is described above in chapter 1.2.4.1.

Figure 1.xv: FACS analysis of PBMC demonstrating that the majority of CD56^{dim} cells are CD16^{bright}; after Cooper (Cooper et al., 2001a). This is the subset of NK cells predominantly involved in ADCC.



The involvement of NK cells in human ADCC against tumours is crucial to the *in vivo* antibody response to tumours and this is mediated via the interaction of tumour-bound antibody with the FcγRIIIA receptor on the NK cell surface. Arnould et al (Arnould et al., 2006) demonstrated that neoadjuvant treatment of breast cancer patients with trastuzumab led to increased numbers of NK cells in the tumour compared to controls, as well as an increased expression of granzyme B and TiA1 (markers of cells with cytolytic activity), giving further weight to the importance of antibody-FcγRIIIA interactions on NK cells in antibody-based cancer therapy.

Kurai and colleagues showed that NK cells, isolated from patients with non-small cell lung cancer, were responsible for cetuximab-induced ADCC against lung cancer cell lines

and for the augmentation seen in response to stimulation of PBMCs with IL-2 (Kurai et al., 2007).

Interestingly, murine NK cells are not believed to play a direct cytotoxic role in ADCC. As in humans there is no Fc γ IIB receptor on NK cells, but in Fc γ IIB-deficient mice an enhanced antibody response is seen with trastuzumab and TA99. In addition, the B-cell clearance seen following treatment with anti-CD20 antibodies is not impaired in perforin-deficient or exocytosis-incapable mice (Uchida et al., 2004) which suggests that the NK perforin / granzyme system is not involved. However NK cells may play an indirect role through their production of activating cytokines (eg IFN- γ and TNF- α), given that NK-depleted mice, unlike wild-type mice, are not protected from melanoma metastases by the TA99 mAb (Hara et al., 1995). ADCC in mice is predominantly mediated through cells expressing the Fc γ IV receptor which is not found on NK cells but rather on neutrophils and macrophages (Nimmerjahn et al., 2005).

1.4 Carcinoembryonic Antigen (CEA)

1.4.1 CEA family of cell-surface glycoproteins

CEA is part of a larger family of related glycoproteins made up of two separate branches: the CEA cellular adhesion molecules, CEACAM, and the pregnancy-specific glycoprotein (PSG) subgroup. This CEA family belongs to the immunoglobulin (Ig) superfamily; hence all CEACAMs possess at least one immunoglobulin-like domain. Two types of immunoglobulin domains are found: an N-terminal domain of 108 amino acids homologous to the Ig variable domain and between 0-6 domains homologous to the Ig constant domains. The molecules of the CEA- and PSG-subgroups differ from each other at the C-terminal end. The CEA subgroup members are attached to the cell surface membrane while the PSGs are secreted molecules (Hammarstrom, 1999). Members of the CEACAM subgroup are expressed on the apical surface of enterocytes, although some members of the group have a much wider tissue distribution beyond the GI tract. The CEA protein itself is part of the CEACAM group and is a product of the *CEACAM5* gene (CD66e). Although the other proteins in the group tend to take the same name as their gene, the product of the *CEACAM5* gene is almost universally referred to as CEA because it was the first to be described.

A number of functions have been ascribed to the CEACAM subgroup. The potential functions of CEA itself will be discussed later in this chapter (chapter 1.4.3). CEACAM1, CEACAM6 and CEACAM8 as well as CEA, function as homophilic and heterophilic intercellular adhesion molecules *in vitro* (Beauchemin et al., 1999). CEACAM1, CEACAM3, CEA and CEACAM6 also act as receptors for *Neisseria* species, *Escherichia coli*, *Salmonella* and a number of other bacteria between them (Beauchemin et al., 1999; Hauck et al., 2006).

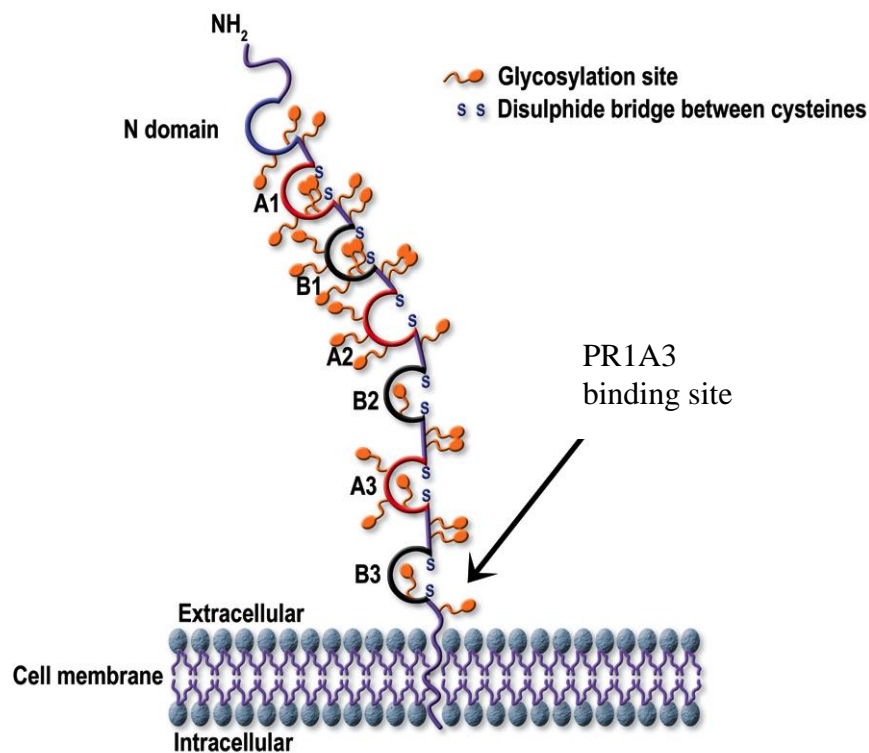
Apart from CEA, most of the research has been directed at CEACAM1. Homologues of CEACAM1 have been identified in rodents and a number of other mammals, making it more readily accessible to *in vivo* experimentation. The genetic deletion of CEACAM1 from the mouse genome and the generation of transgenic mice expressing defined mutants have now demonstrated a modulatory role for CEACAM1 in neovascularisation (Horst et al., 2006), insulin metabolism (Poy et al., 2002), T-cell regulation (Nakajima et

al., 2002) and possibly tumourigenesis (Leung et al., 2006). CEACAM1 seems to act as a tumour suppressor, although there is no definite increase in tumour formation in CEACAM1 knockout mice and they are both fertile and have normal life expectancy (Hemmila et al., 2004). However they do show an increase in tumours following treatment with carcinogens and the mouse colonocytes do show a reduced rate of apoptosis (Leung et al., 2006) both of which support a tumourogenic effect.

1.4.2 Expression of CEA in normal and neoplastic tissues

Carcinoembryonic antigen (CEA or CEACAM5) was first recognised as a potential human tumour-specific antigen in 1965 (Gold and Freedman, 1965). CEA is a 180KDa glycoprotein whose basic structure is shown in figure 1.xvi.

Figure 1.xvi: Structure of CEACAM5 (CEA) demonstrating the binding site for the monoclonal antibody PR1A3 (after Horig (Horig et al., 2000))



It consists of a single polypeptide chain, consisting of 108 amino acids at the N-terminal end followed by three highly homologous domains of 178 amino acids each (Oikawa et al., 1987). The protein core is attached to the cell membrane by a glycosyl phosphatidyl inositol (GPI) anchor (Hefta et al., 1988). There are carbohydrate side chains, forming over half of the molecular mass which are bound to the protein core via 28 asparagine-linked glycosylation sites (Benchimol et al., 1989).

In the colon, studies by immunoelectron microscopy show CEA to be specifically located within the apical glycocalyx, of the microvillous region of mature enterocytes (figure 1.xvii). When CEA is shed from the cell membrane, either the GPI anchor is disrupted, (Kuroki et al., 1992), or the CEA is released attached to a microvesicle having been pinched off with attached cell membrane. It is in the former situation, when the CEA is membrane-free, that the CEA molecule can be released into the blood stream. In this membrane-free state when disruption of the GPI anchor occurs, the molecule is presumed to undergo a conformational change that crucially prevents the binding of the antibody primarily examined in this thesis, PR1A3 (Durbin et al., 1994). A comparison of CEA shed into stool from normal or neoplastic colonic cells shows no significant difference in morphology between the two (Matsuoka et al., 1991).

CEA is produced in the foetal colon starting at week 9-13 (Nap et al., 1988). In the adult, normal expression of CEA is restricted to the luminal surface of epithelial cells, most commonly on the gastrointestinal tract, but also on other mucosal epithelia such as the nasopharynx, the lung and the urogenital tract as well as in sweat glands (Thompson, 1995). As yet, no homologues of CEA have been identified in non-primates.

CEA from normal and neoplastic cells is genetically indistinguishable (Fritsche and Mach, 1977) but its distribution is critically different. In contrast to the highly specific expression of CEA on the apical (luminal) surface of normal colonic columnar cells, adenoma and carcinoma cells lose their polarity and CEA is distributed on the basolateral as well as the apical surface of the cell (Hammarstrom, 1999) (figure 1.xviii). Intravenously administered antibody cannot access the luminal surface of enterocytes and hence anti-CEA antibodies do not target normal CEA-expressing tissue. It is also from the basolateral surface of the cell that CEA can be released firstly into the interstitial space and from there to the bloodstream. It is also this surface, being accessible to the

Figure 1.xvii: Electron micrograph of colonic luminal surface stained for CEA. The CEA is indicated by the arrowheads as it 'coats' the top of the microvilli.

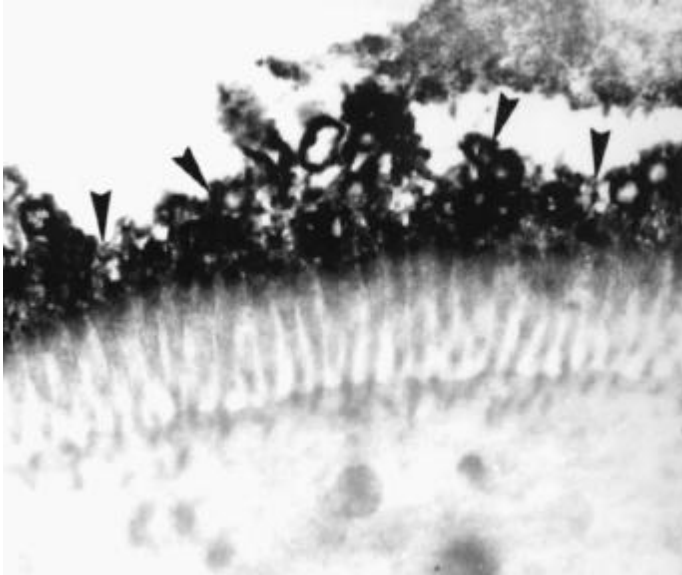
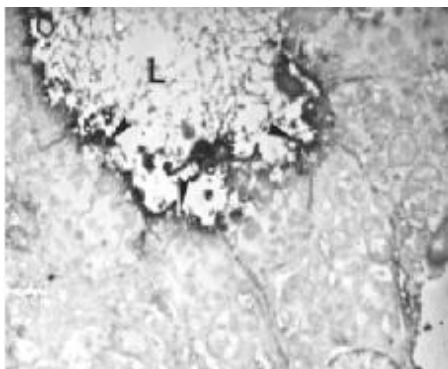
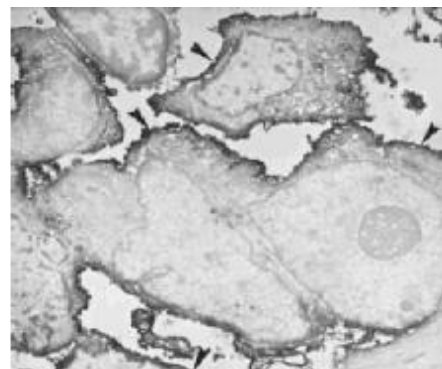


Figure 1.xviii: Immunoelectron microscopy of CEA in normal (A) and cancerous (B) colonic tissue with arrowheads pointing to the stained CEA in both sections. In A, CEA is visible only on the luminal surface whereas in B, CEA is clearly seen on all borders of the cell (from Hammatstrom (Hammarstrom, 1999))



A



B

bloodstream, that is available for antibody binding. Hence in individuals with a tumour poorly-expressing CEA, serum CEA levels are either undetectable or very low (<5ng/ml). Although a normal serum CEA level does not exclude the possibility of a CEA-expressing tumour, levels above 10ng/ml would strongly suggest the presence of such a tumour.

CEA is over-expressed primarily by colorectal, gastric and pancreatic cancers with the vast majority of these tumours (>90%) expressing CEA (Wagener et al., 1984). In addition it is expressed by the majority of medullary thyroid cancers (Hamada and Hamada, 1977), more than 70% of non-small cell lung cancers (Wagener et al., 1984) and approximately 50% of breast cancers (Esteban et al., 1994). Reports of expression on many other cancers exist.

1.4.3 Proposed functions of CEA

Overexpression and loss of polarisation of CEA in colorectal carcinogenesis is an early event, being seen as early as in the aberrant crypt foci (Pretlow et al., 1994). Although a member of the Ig superfamily, CEA is linked to the cell membrane by a GPI anchor and thus has no direct intracellular signalling motif. The functional significance, if any, of its overexpression in colorectal and other cancers remains unclear. There is a wealth of potential functions of CEA gleaned from *in vitro* experiments but its distribution on the luminal surface of enterocytes and some other hollow organs would suggest that its primary function in normal enterocytes is the binding of pathogens, preventing access to the cell membrane, and then shedding into the gut lumen. This would explain the large daily turnover of more than 70mg of CEA found in the stool in adult humans (Matsuoka et al., 1991). The *in vitro* work, much of it from the Stanners group at McGill University, however requires discussion.

CEA can bind to itself (homotypic binding) or to one of several other CEACAM family members (heterotypic binding) thereby mediating cell to cell adhesion (Benchimol et al., 1989). These interactions are predominantly mediated by the N-terminal domain (i.e. furthest from the cell membrane; see figure 1.xvi). Early experiments using L6 rat

myoblasts which normally undergo myogenic differentiation in culture, demonstrated that when transfected with CEA, this differentiation was blocked (Eidelman et al., 1993). Overexpression of CEA to a level similar to that observed in human colorectal cancers or similar overexpression of CEACAM6, another GPI-linked CEACAM family member, have been shown to disturb the ordered tissue architecture that can be seen in 3D cultures with certain colon carcinoma cell lines (Ilantzis et al., 2002). These stably transfected cell lines, SW-1222 and Caco-2, failed to polarise and differentiate into glandular structures. In addition, transgenic mice that overexpress several human CEACAMs display increased colon tumour formation after treatment with azoxymethane, a carcinogen, compared to wild-type littermates (Chan et al., 2006). Over-expression of CEA has also been shown to be an inhibitor of anoikis *in vitro*. Anoikis is the process of apoptosis triggered when cells lose their attachment to the extracellular matrix. This obviously prevents cell migration, invasion and metastasis under normal circumstances. However, when transfected myoblasts expressing CEA or CEACAM6 on their surface were grown in the absence of matrix attachment, apoptosis was reduced, again arguing for a role of these CEACAM family members in promoting aberrant growth of adherent cells (Ordonez et al., 2000). As shown with CEA's potential involvement in tumourogenesis and disruption of tissue architecture (Taheri et al., 2003), the inhibition of apoptosis likewise required the N-terminal domain, suggesting that interactions mediated by this part of CEA are involved (Ordonez et al., 2000). However, despite these experiments, transgenic mice overexpressing CEA under its native control elements (CEA.tg) do not show an increased tumour incidence (Eades-Perner et al., 1994), even in the absence of a functional adenomatous polyposis coli (APC) protein (Thompson et al., 1997). Therefore, the physiological function of CEA and its potential involvement in tumour formation and progression are still uncertain. It is possible its over-expression in cancer is nothing more than a bystander event.

The proposed methods of signalling when CEA has no intracellular signalling motif have concentrated on the formation of "lipid rafts" where GPI-linked proteins are localised in membrane microdomains (Screaton et al., 2000). These rafts are freely mobile on the cell surface, enabling clustering with other lipid rafts, and contain subsets of signalling molecules which would enable clustering of GPI-linked proteins to signal through these

common pathways. CEA and CEACAM6 are co-localised with integrin $\alpha 5\beta 1$ in the same raft (Ordonez et al., 2007) and hence it has been proposed that clustering could activate the integrin signalling pathway (Camacho-Leal et al., 2007).

1.4.4 Advantages of CEA as a target for ADCC

Despite the large number of antibodies used in clinical trials, there are only a few targets which are consistently chosen. Almost 50% of such antibodies used only ten antigen targets, of which CEA was one (table 1.xii). An ideal target for ADCC has some of the following attributes:

- Accessible
- Abundant on the cell surface
- Homogeneous distribution
- Consistently present on tumour cell-surface
- Not expressed or not accessible on normal tissue
- Not shed from the cell surface or secreted by tumour
- High cell-surface density
- No internalisation
- Present on cancer stem cells

CEA has many of the properties one would look for in an ideal antigen target for antibody-based cancer therapy. Given that antibodies cross cell membranes poorly, the antigen requires abundant and accessible cell-surface expression, something which CEA has in tumour tissue. It is over-expressed in the vast majority of CRCs (Wagener et al., 1984) and whilst the CEA expressed on normal colonic epithelium is inaccessible to IgG antibody, being found only on the luminal surface of the cell, this expression pattern changes in the neoplastic cell so that CEA is also expressed on the basal and lateral membranes (Hammarstrom, 1999) making it accessible to blood-borne antibody. This means that not only does the antibody avoid getting sequestered by binding in abundance to non-tumour tissue, it also avoids the inappropriate cytotoxicity against normal tissue.

Table 1.xii: Ten most popular targets in clinical trials of antibody-based cancer therapy (Reichert and Valge-Archer, 2007)

Target	Number of antibodies used in trials
EpCAM	17
MUC-1	10
EGFR	12
CD20	10
CEA	9
HER2	9
CD22	6
CD33	6
Lewis Y	6
PSMA	6

Binding of antibody to many cell-surface targets results in internalisation of the complex, a process which inhibits ADCC which relies on continuous high-level expression. Thus a further advantage of CEA is that it is internalised very slowly if at all following binding (Conaghan et al., 2008; Schmidt et al., 2008) (see figure 1.xix).

Another advantage of CEA as a target for ADCC is the ability to influence its cell-surface expression. CEA has been shown to be upregulated by IFN- γ (Guadagni et al., 1990; Hauck and Stanners, 1991) and IL-6 (Ullmann et al., 1992), both cytokines released during immune activation which would be part of any anti-tumour response by the body, and hence potentiating any anti-CEA therapy. Furthermore CEA is upregulated by the commonest drug used in the neoadjuvant and adjuvant treatment of colorectal cancer, 5-fluorouracil (Aquino et al., 1998; Cappelletti et al., 2000; Correale et al., 2003; Prete et al., 1996) which would aid combination treatment of antibody therapy and standard chemotherapy.

The biggest disadvantage of CEA as a target is that it is readily cleaved from the cell surface and so shed into the blood stream from tumours, either directly or via the lymphatics, which is why the level of serum CEA has been used in the clinic as a marker for screening and recurrence, especially of colorectal cancer (Chau et al., 2004). Serum CEA binds to most of the currently used anti-CEA antibodies hindering them from reaching their target and so largely mitigating against any potential clinical effect. This problem however is overcome by the antibody PR1A3 which binds only to membrane-bound CEA as will be discussed later in chapter 1.5. In addition, it seems likely that CEA expression is not necessary for tumour progression or survival and hence negative variants may be easily selected for.

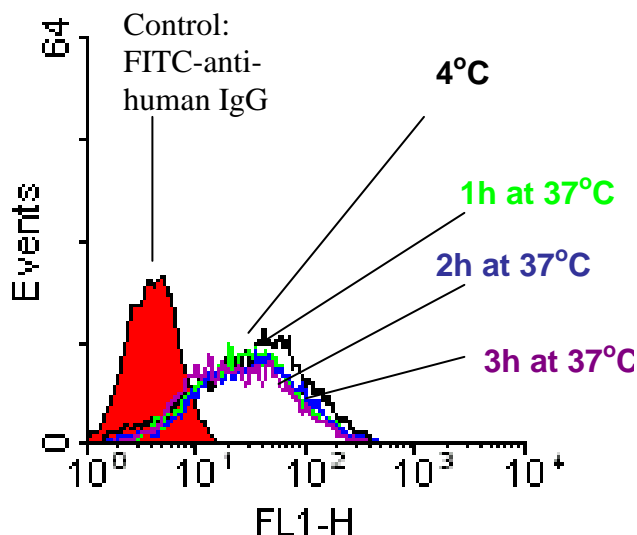
Figure 1.xix: Time-course FACS analysis plotting the binding of PR1A3 to CEA-expressing cells over time (Conaghan et al., 2008)

Time-course FACS analysis of hPR1A3 binding to CEA-expressing MKN45 cells. Cell line labelled with primary antibody (PR1A3 or AUA-1) at 4°C then incubated for varying times at 37°C before addition of secondary antibody (FITC-conjugated anti-human IgG or anti-mouse IgG respectively)

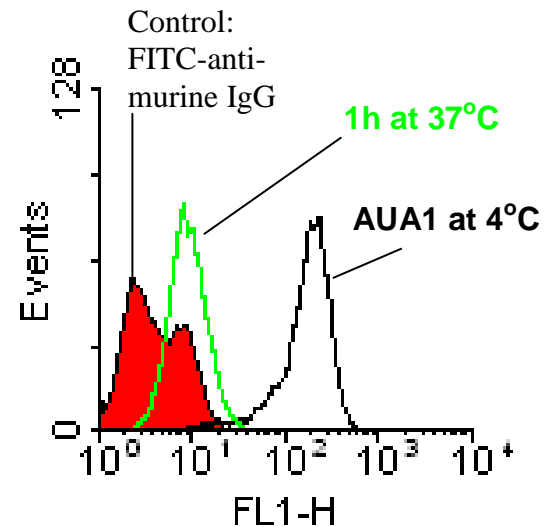
A: Analysis over time suggests that the CEA-PR1A3 complex is not internalised within 3 hours of binding CEA.

B: Binding of the monoclonal antibody AUA-1 to EpCAM on the surface of MKN45 cells leads to internalisation within one hour.

A: hPR1A3 treatment of MKN45 cells



B: AUA-1 treatment of MKN45 cells



1.5 The monoclonal antibody PR1A3

The IgG1 κ antibody PR1A3 (Richman and Bodmer, 1987) binds with high specificity to a distinctive determinant on membrane bound CEA that is not recognized by other anti-CEA antibodies (Durbin et al., 1994). In particular, PR1A3 binds little or no circulating CEA, in contrast with other anti-CEA antibodies (Durbin et al., 1994). PR1A3 has been used in humans as a radioimmunoscintigraphic guide for localizing metastatic deposits of CRC (Granowska et al., 1993) and shows strong immunohistochemical binding irrespective of the degree of differentiation of the tumour (Richman and Bodmer, 1987). These properties of PR1A3, together with the characteristics and distribution of CEA, make this antibody an ideal candidate for immunotherapy.

1.5.1 The biology of PR1A3

The mAb PR1A3 was selected from a panel of antibodies produced by the immunisation of mice with human colonic epithelia (Richman and Bodmer, 1987). Research with the original murine IgG1 mAb demonstrated that it bound specifically to CEA and did not show cross reactivity with other members of the CEACAM family (Durbin et al., 1994). Unlike other anti-CEA antibodies which readily complex with circulating CEA in CRC patients, PR1A3 does not bind to circulating CEA (Durbin et al., 1994) overcoming the biggest problem of CEA as a target in immunotherapy, as discussed above in chapter 1.4.4. This advantage arises from the epitope bound by PR1A3 (figure 1.xvi) which is lost or hidden when the glycoprotein is shed from the cell membrane. The epitope in question is formed from parts of the B3 domain and the GPI anchor (Durbin et al., 1994), an area near the site of cleavage of CEA from the cell surface. Removal of the B3/GPI anchor excluded the epitope, as did exchanging the GPI anchor for a CEACAM1 transmembrane domain (Durbin et al., 1994). PR1A3 binds, therefore, to both the B3 domain and elements of the GPI anchor. Loss of the hydrophobic anchor in circulating CEA may result in a conformational change that alters the spatial arrangement of the different epitope elements so that the PR1A3 epitope is sterically hidden (Durbin et al., 1994). In addition, membrane bound CEA is monomeric, whereas circulating CEA is likely to exist

as a dimer (Lisowska et al., 1983) which again may sterically hinder access to the epitope.

Hence the major advantage of PR1A3 over other anti-CEA antibodies is that it does not bind to free plasma CEA and so would not be sequestered by soluble CEA in patients with high plasma CEA levels (Durbin et al., 1994; Stewart et al., 1999). This has been confirmed in our recent studies (Conaghan et al., 2008).

PR1A3 has subsequently been humanised to overcome the immunogenicity of using murine antibodies *in vivo* (Stewart et al., 1999). Humanised PR1A3 was engineered in Chinese hamster ovary cells and retained its affinity for its epitope and also its specificity for membrane-bound CEA as distinct from soluble CEA.

Two binding affinities are quoted for PR1A3, the higher affinity at 1nM and the lower at 60nM (Stewart et al., 1999). These relate to different binding sites for the antibody on the gastric carcinoma cell line MKN45. Subsequent studies would suggest the lower of the two was more accurate given the relatively high concentrations of antibody required to effect ADCC *in vitro*, especially when compared to the anti-EGFR antibody, cetuximab (Conaghan et al., 2008). The chimeric cetuximab is quoted as having a binding affinity of approximately 0.2nM (Goldstein et al., 1995). Glycosylation and affinity maturation of PR1A3 is currently in progress and these techniques are expected to improve the binding affinity to maximise ADCC.

1.5.2 Binding of PR1A3 to colorectal tissue

The vast majority (>90%) of colorectal cancers express CEA (Wagener et al., 1984). Richman and Bodmer reported that 59 of 60 biopsies taken from colorectal adenocarcinomas bound PR1A3 regardless of their degree of differentiation (Richman and Bodmer, 1987). In 22 CRC cell lines investigated by Kim *et al*, all lines produced CEA with 9 producing more than 100ng/ml of CEA into the medium (Kim et al., 1999). As mentioned in chapter 1.4.2 when CEA is normally shed from the bowel wall, either the GPI anchor is disrupted (Kuroki et al., 1992), or the CEA is released attached to a

microvesicle having been pinched off with attached cell membrane. In this latter situation the CEA remains membrane-bound and staining of histological sections with PR1A3 thus show stained material in the bowel lumen and in the tumour gland lumen of neoplasms. The remnants of dead cell membranes also contribute to this staining.

As mentioned above in chapter 1.4.2, the expression of CEA in colorectal cancer differs from that on normal enterocytes. In contrast to the highly specific expression of CEA on the apical surface of normal colonic columnar cells, adenoma and carcinoma cells lose their polarity and CEA is distributed on the basolateral as well as the apical surface of the cell (Hammarstrom, 1999) (figure 1.xviii). Thus PR1A3, like other anti-CEA antibodies does not bind to normal enterocytes as antibodies are prevented from reaching the luminal surface by the intracellular tight junctions. However antibody can reach the basolateral surface of cells and hence can target neoplastic colorectal tissue. This loss of polarisation in neoplastic tissue is important in being able to target antibody to diseased rather than healthy tissue.

1.5.3 Animal studies using mPR1A3 in the *Apc^{min+/-}-CEA.tg^{+/-}* mouse model of colorectal cancer

Traditionally animal studies of tumour therapies are carried out on human xenografts implanted into immuno-incompetent mice (eg nude mice). In the setting of immune-based therapies such immunologically incompetent mice are unsuitable. In addition xenografts are abnormally vascularised and in a non-orthotopic site which makes interpretation of results difficult.

MIN (multiple intestinal neoplasia) mice have a truncating mutation in the *Apc* gene causing the development of multiple adenomatous polyps, predominately in the small bowel with up to 10 adenomas in the large bowel. These mice have been crossed with a mouse transgenic for the human CEA gene (Thompson et al., 1997) as described above (chapters 1.2.9.2.4 and 1.4.3). The resulting *APC^{min+/-}-CEA.tg^{+/-}* (MIN/CEA) mice express CEA in the same spatio-temporal pattern as in humans (Eades-Perner et al., 1994) and are

immunologically tolerant to the human protein (Clarke et al., 1998). In addition CEA expression in the murine polyps mimics the pattern seen in human CRC with CEA expressed abnormally on the basolateral surface of the cell. Radiolabelled PR1A3 has been used in the *MIN/CEA.Tg* mouse and this has shown a high sensitivity and specificity of the antibody for the adenomas (Wilkinson et al., 2001). This MIN/CEA mouse therefore represents a good model for the investigation of CEA-targeted immunotherapies for CRC.

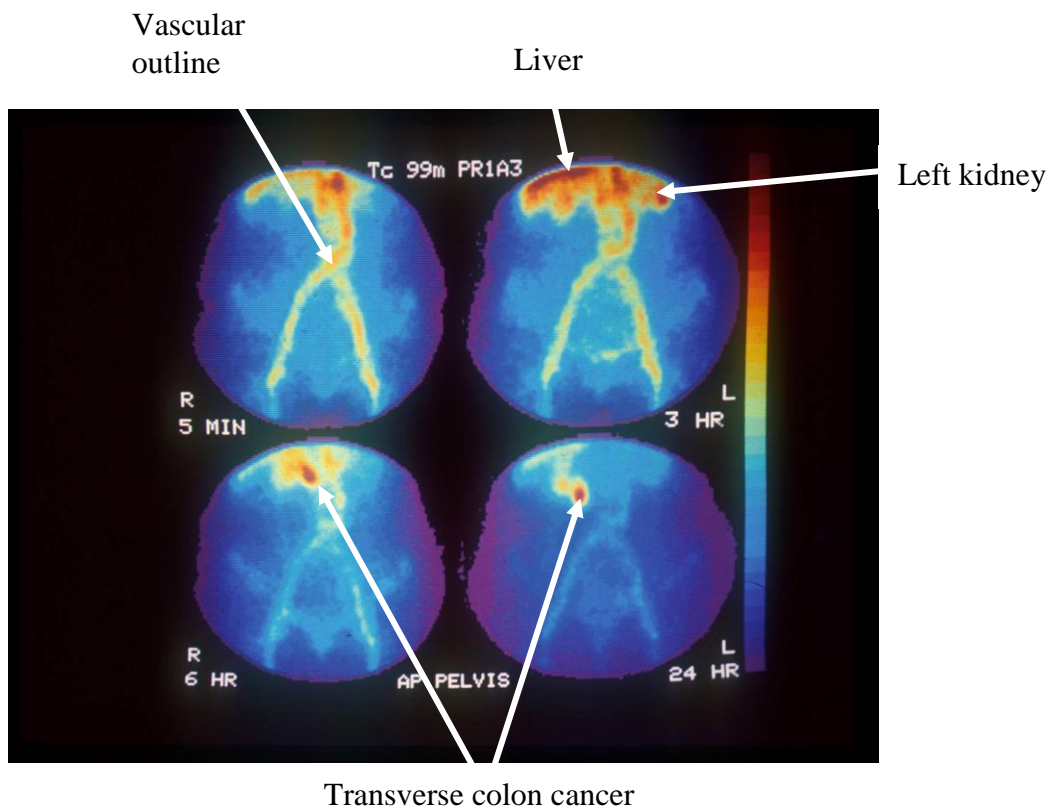
Our pilot study in 19 MIN/CEA mice used naked PR1A3 injected intraperitoneally twice weekly for 6 weeks from 6 weeks of age. All mice were killed at 12 weeks and the number and size of the adenomas were scored and compared to untreated MIN/CEA controls. Naked PR1A3 therapy resulted in a 15% ($p=0.08$) decrease in number and a 33% ($p=0.02$) decrease in burden (total surface area of adenomas). This study used the parent murine IgG1 antibody which is not the optimal isotype for ADCC in mice (Nimmerjahn and Ravetch, 2005). A larger study was planned to assess reproducibility, optimal dose and dosing schedule, pharmacokinetics and potential synergy with chemotherapy, and to compare these results to age-matched controls. However concerns that enforced changes to the animal laboratory diet might influence the phenotype, coupled with an observed change in polyp numbers without explanation, led to the abandonment of these plans and we have had to rederive the colonies with fresh stock.

1.5.4 Radioimmunoscintigraphy using PR1A3

An antibody that is used for radioimmunoscintigraphy (RIS) ideally needs to bind with high sensitivity and specificity to a cancer specific antigen. PR1A3 binding to membrane bound CEA of CRC cells, fulfils this requirement. Granowska *et al* reported the first use of Technetium-99m (^{99}Tc)-labelled PR1A3 for imaging CRC (Granowska et al., 1990) (figure 1.xx). They found the tumour to normal mucosa antibody uptake ratio was 63:1. In a further study, 85 patients with CRC were imaged with ^{99}Tc -labelled PR1A3 and all were found to be image positive (Granowska et al., 1993). In the assessment of recurrent CRC, the accuracy was 33/35 (94%). There was a positive predictive value for recurrence

of 92% and a negative predictive value of 100% at a prevalence of 66%. There was also a positive predictive value for liver metastases of 93% at a prevalence of 32%. This high level of accuracy, in particular the low false positive rate is likely due to the ability of PR1A3 to avoid binding to circulating CEA.

Figure 1.xx: Radioimmunoscintigraphy using ^{99}Tc -labelled PR1A3: a patient with a colorectal cancer of the transverse colon. Views at 5 minutes to 24 hours (Granowska et al., 1990)

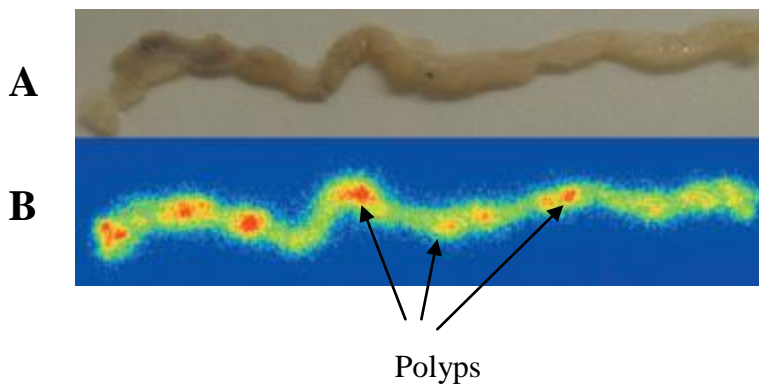


Radiolabelled PR1A3 has also been investigated in a murine model of CRC (Wilkinson et al., 2001). As discussed above in chapter 1.2.9.2.4 the MINCEA mouse develops CEA expressing bowel adenomas with the same spatial distribution of CEA as human colorectal adenomas. Eight to ten week old MIN and MINCEA mice were injected with iodine-125 (^{125}I)-radiolabelled PR1A3 (figure 1.xxi) or iso-matched non-specific antibody

as a control. In the MIN mice, which do not express CEA, the two antibodies had a similar distribution with the excretory organs showing significant uptake. In the MINCEA mice, however, there was significant difference between the antibodies, with PR1A3 uptake being twice that of the control antibody, with the uptake in the bowel higher than any other organ at all time points. The average ratio of uptake in the adenomas compared to the surrounding bowel mucosa was 1.6:1.

Figure 1.xxi: Small intestine from MIN.CEA.tg mouse injected 48hrs earlier with intravenous ^{125}I -PR1A3. Figure A shows the macroscopic specimen containing small bowel polyps and figure B shows the same polyps by immunoscintigraphy (Wilkinson et al., 2001).

This demonstrates the ability of PR1A3 to concentrate at the site of polyps and, given that the antibody was able to access the cellular CEA from the bloodstream, confirms that the distribution of CEA in MINCEA mice is the same as that in human colorectal tumours (i.e. basolateral expression as well as apical expression).



Other radiolabelled anti-CEA mAbs have been found to bind to shed CEA and this has given false positive uptake in normal lymph nodes, due to sequestered antigen (Kubo et al., 1992). This has not been seen with radiolabelled PR1A3, due to the antibody binding only to membrane-bound CEA.

1.5.5 Radioimmunoguided surgery

⁹⁹Tc-labelled PR1A3 was first used for radioimmunoguided surgery (RIGS) in 1995, where it was given to patients with CRC 24 hours before surgery. Intra-operatively a radioactivity-detecting probe was used to examine the tumour, normal bowel and any suspicious sites. An uptake criterion of 1.5 times the level of normal tissue was used and this resulted in a sensitivity for tumour of 66%. Once the suspicious specimen was removed and probed again, the sensitivity rose to 100%, hence providing an instant 'result' as to whether the tissue was involved with tumour without requiring pathological confirmation by frozen section (Howell et al., 1995).

Similar studies have been carried out in xenograft mice (Kim et al., 2000). ¹²⁵I-labelled antibody was given to nude mice with intra-abdominal CRC xenografts. The sensitivity and specificity of tumour localisation by RIGS was 71.4% and 91.4% respectively. The same group have also used a bispecific anti-CEA antibody for RIGS (Kim et al., 2002). This involved the cross linking of Fab' fragments from PR1A3 and another anti-CEA mAb, T84.66. The ¹²⁵I-labelled bispecific construct was used in nude mice with CRC xenografts implanted subcutaneously on their backs. The sensitivity and specificity of tumour localisation was 90.9% and 94.5% respectively. It was discussed that the bispecific mAb, using two anti-CEA mAb against different epitopes, achieved a greater affinity and avidity without increasing non-specific binding, hence leading to more accurate localisation of CRC.

1.5.6 PR1A3 and human trials

Although radiolabelled PR1A3 has been used in humans for RIS as described above, there are currently no unconjugated or 'naked' antibodies to CEA being used for the treatment of colorectal cancer. The anti-CEA antibodies that are currently used for therapy in pilot trials, are administered as radioconjugates (Liersch et al., 2007; Wong et al., 2004).

The experience of using naked PR1A3 is limited to using the murine parent antibody in 15 patients with advanced CRC (Zbar et al., 2005). The antibody was given in doses of 0.5-5mg intradermally at 4 weekly intervals for 3 months. The patients' serum was

assessed for anti-idiotypic (Ab2) antibodies, anti-anti-idiotypic antibodies (Ab3) and a HAMA response, and PBMC response to stimulation was assessed. No serious adverse effect occurred in any of the patients. After treatment 9 of the 15 patients showed Ab2 reactivity but there was no detectable Ab3 response. However there was a strong HAMA response in 7 out of 15 patients. Pretreatment PBMC proliferation was subnormal in all patients before treatment compared with normal controls, but became normal or increased in all patients after immunisation ($p < 0.001$). Other markers of PBMC activation were also raised following immunotherapy (eg IL-2 & IFN- γ secretion, IL-2 receptor expression). However all patients had CRC metastases and 14 of 15 patients had disease progression during the study period.

1.5.7 PR1A3 and antibody based cancer treatments

Humanised PR1A3 has many properties which would make it ideal for antibody-based cancer therapy.

Minimal immunogenicity

The humanisation process would minimise the immune response to the antibody. As this is yet to be used in human trials, the degree of anti-human antibody which would result is yet to be determined

No cross-reactivity with normal tissue

Intravenous antibody would not reach the luminal border of normal colorectal tissue where CEA is expressed (Hammarstrom, 1999), and hence should have minimal or no systemic toxicity.

Good targeting to tumour cells

More than 90% of colorectal tumours express CEA (Wagener et al., 1984).

Does not bind to circulating CEA

The biggest disadvantage of anti-CEA antibodies is sequestration by circulating CEA. PR1A3 binds only to membrane-bound CEA and as such is not affected by the serum CEA (Conaghan et al., 2008; Durbin et al., 1994).

Long antibody half-life

Like many antibodies, the clearance of ^{125}I -PR1A3 in wild type, CEA and MIN/CEA mice is relatively slow (Wilkinson et al., 2002; Wilkinson et al., 2001). In humans, ^{99}Tc -labelled murine PR1A3 had a biological half life of 24 hours with urinary activity of 10-12% per day (Granowska et al., 1990). The half life of the naked murine antibody in humans has not been ascertained but the use of humanised PR1A3 would certainly increase the expected half life by reducing the immunogenicity.

Internalisation of the antibody-antigen complex

This is ideal for ADCC as internalisation of the PR1A3-CEA complex is either very slow or absent (Conaghan et al., 2008), allowing maximal exposure of the antibody for interaction with immune cells, allowing ADCC and ADCP.

1.6 Factors influencing ADCC

The development of antibodies for antibody-based cancer therapy looks to improve the efficacy of the antibody (increasing the numbers of responders; extending the duration of response) whilst maintaining safety. Enhancing the potential of a therapeutic antibody to cause ADCC is the major goal for the majority of naked antibodies. This can be achieved by modifying the 3 factors involved in ADCC: the antibody, the effector cell, and the environment.

1.6.1 The antibody

1.6.1.1 Antibody isotype

Antibody Fc interactions with effector cell Fc receptors have been shown to be crucial in optimising ADCC (Nimmerjahn and Ravetch, 2005). In humans IgG1 and IgG3 are the most potent activators of effector functions (Carter, 2001) in particular ADCC and ADCP, although IgG3 is rarely used because of its longer hinge region making it more prone to proteolysis. This potency comes from the higher affinity of IgG1 and IgG3 for the FcγIIIa receptor.

1.6.1.2 Binding affinity with target and binding site barrier

Much of the *in vitro* evidence has shown that mAbs with higher affinities for their target are more effective at ADCC. However more recent *in vivo* studies have postulated that increasing antibody affinity for its target increases its biological potency only up to a given affinity, after which this affinity interferes with tumour penetration. This “binding-site barrier” impedes mAb penetration into a tumour mass because high-affinity interactions between the antigen and the mAb block the diffusion of the mAbs throughout the tumour mass. This was supported by Adams et al who compared high affinity scFvs for HER2 with those of lower affinity (Adams et al., 2001). The higher affinity scFvs were found around the periphery of the tumour and showed no improvement in tumour targeting, less specificity of targeting and less penetration into solid tumour masses.

Hence if rapid and complete tumour penetration is essential for Ab function, high affinity may be undesirable.

However, high affinity for the tumour antigen is generally beneficial for ADCC function and the binding site barrier is less relevant when micrometastases are considered, again highlighting the importance of antibody treatment at early stages of disease (i.e. adjuvant treatment rather than treatment of overt metastatic disease). The binding affinity of antibodies against solid tumours currently in clinical practice, seem to range from 0.1nM to 5nM.

Affinity maturation of antibodies by phage display (or more recently yeast- and ribosome-display) libraries or by clonal selection is now commonplace.

1.6.1.3 Glycosylation

The majority of antibodies produced by B-cells have carbohydrate side chains added post-translation. Some of these are particularly important in the affinity of the antibody for the relevant Fc receptors. For example, it is recognised that the cytotoxic activity of all human antibody isotypes can be increased by removal of the fucose from the branched-chain oligosaccharide attached to the conserved asparagine residue at position 297 in C_H2 of the antibody and this is directly related to the higher binding affinity to FcγRs (Nimmerjahn and Ravetch, 2005; Niwa et al., 2004b; Shields et al., 2002; Umana et al., 1999). This work was pioneered by Pablo Umana who produced recombinant mAbs in Chinese Hamster Ovary (CHO) cells. These CHO cells were engineered to over-express acetylglucosylaminyl transferase III (GnT III). The resulting mAbs had increased levels of bisected non-fucosylated oligosaccharides which had enhanced ADCC and reduced CDC (Schuster et al., 2005; Umana et al., 1999). This altered defucosylation of mAbs improves ADCC even in low-binding polymorphisms (Niwa et al., 2004a) but interestingly does not alter the binding to other activating FcγRs nor to the inhibitory FcγRIIB.

1.6.1.4 Fc sequence modification

Selective mutation of the Fc sequence has led to the screening of panels of mAb with higher binding affinity for the FcγIIIA receptor and lower binding to the FcγIIB receptor.

It has been found that altering 2-3 amino acids within the Fc region of the antibody produced antibody variants with binding affinities more than double that of the parent antibody. This enabled *in vitro* and *in vivo* cytotoxic activities with these variant antibodies even against cells expressing low levels of the target antigen and in conjunction with effector cells with a low-binding polymorphism for the Fc γ R11A (Lazar et al., 2006).

Mutational analysis has now also begun looking at maximising FcRn binding in addition to the high binding Fc γ 11A / low binding Fc γ 11B variants. This will be discussed further below in chapter 1.6.1.5.

However the techniques discussed in chapters 1.6.1.2 to 1.6.1.4 have to be weighed against the potentially deleterious effects of antibodies with excessively high affinity for both antigen and Fc receptors. The effect of the binding site barrier hindering tumour penetration by the antibody is one concern but of greater concern is the potential effect of widespread binding to activating Fc γ Rs in effector cells without the safety of Fc γ 11B binding, causing potentially dangerous over-stimulation.

1.6.1.5 Prolonging antibody half-life

Regulation of IgG concentration in the body is carried out to a large extent by the so-called neonatal FcRs (FcRn) (Junghans and Anderson, 1996). These FcRs are unlike others in the FcR family in that they are found across cells of diverse origin including endothelial cells, epithelial cells, dendritic cells and monocytes. The receptor is more akin to MHC class I than to other FcRs and again unlike other FcRs, binds to the Fc portion of the antibody in a pH-dependent manner. FcRns regulate IgG concentration by recycling antibody which it binds. IgG does not interact with FcRn on the cell surface where the pH is not sufficiently acidic (Raghavan et al., 1995). Instead, IgG is taken into the cells by pinocytosis and binds to FcRn in early endosomes at pH 6-6.5. This signals the endosomes away from becoming lysosomes where IgG would be subsequently degraded. Instead, endosomes in which IgG is bound to the FcRn migrate to the basolateral border of cells (transcytosis) and fuse with the membrane for exocytosis where the IgG is then released because the neutral pH then favours dissociation of the FcRn-IgG complex (Ober

et al., 2004). Hence the IgG is recycled when it is bound to FcRn. Increased IgG levels leads to saturation of these receptors and results in endosomes containing unbound IgG which subsequently follow the lysosomal pathway and are degraded, hence serving to reduce serum levels to normal. Decreased IgG levels leads to all the IgG being bound by FcRn and therefore recycled rather than degraded.

It is possible therefore to increase the half-life of therapeutic antibodies by designing antibodies with a higher affinity for FcRn. This can be achieved by site-directed mutagenesis of the therapeutic antibody, followed by selection of high binding clones. The antibody must retain its pH-dependent binding as failure to retain this may lead to failure to dissociate from the receptor on recycling. This technique for developing high-binding FcRn antibodies has already led to the demonstration of antibodies with longer half-lives (Hinton et al., 2006).

1.6.1.6 Internalisation of antibody-target complex

As described earlier, the choice of the tumour-associated target is important, not only if the intent is to block signalling from cell-surface receptors, but also for ADCC. Prolonged presence of the antibody on the cell surface without internalisation is important in allowing interaction with the effector cells. The big advantage of CEA as a target and the CEA-PR1A3 complex is that it is internalised slowly if at all (figure 1.xix).

1.6.2 Effector cell

1.6.2.1 Enhancing FcγIIIa receptor expression

Increasing the number of receptors on immune effector cells with which they can bind the Fc portion of the therapeutic antibody naturally increases the efficacy of the treatment. It is becoming increasingly apparent that antibody-based cancer therapy has an effect not only through stimulating the innate immune response but also by the induction of an adaptive immune response. The type of adaptive response produced can influence the expression of FcγIIIA receptors. For example the cytokines produced during a T_{H1} response such as GM-CSF and IFN-γ have been shown to upregulate activating FcRs whereas the T_{H2} cytokines IL4 and IL13 both upregulate the inhibitory FcγRIIB (Pricop et

al., 2001). The use of cytokines as adjuncts to antibody-based cancer treatments could be considered. This may also have the effect of activating or recruiting immune effector cells, further enhancing the effect of ADCC and the clinical response to the antibody. Thus, the magnitude of antibody-mediated effector responses is context dependent and can be regulated in tissues by locally produced cytokines.

1.6.2.2 High-binding FcγIIIa receptor polymorphism

This has been described above in chapters 1.2.4.1 and 1.3.1.

Three clinical studies have now shown that patients who are homozygous for the higher binding valine allele had better outcomes from treatment of their lymphoma with the anti-CD20 antibody rituximab than heterozygotes and phenylalanine homozygotes (Cartron et al., 2002; Dall'Ozzo et al., 2004; Weng and Levy, 2003). Genotyping of patients to select for valine homozygotes to receive antibody-based cancer therapy would certainly enhance its success rate.

1.6.2.3 Inhibiting FcγRIIB

The interaction between activating Fc-receptors and the inhibitory FcγRIIB receptor is known to be important in regulating ADCC (Nimmerjahn and Ravetch, 2005). The inhibitory FcγRIIB receptor is known to be a negative regulator of dendritic cell maturation and able to suppress the effect of cytotoxic antibodies. Hence, designing antibodies that avoid stimulation of FcγRIIB should lead to an increase in cytotoxic activity. Alternatively using antibodies to block FcγRIIB would also allow a transient increase in cytotoxic activity if given concomitantly with the cytotoxic antibody. Support for this concept comes from Clynes and colleagues who looked at mice deficient in the inhibitory FcγRIIB receptor. These mice had increased spontaneous tumour protection against a melanoma cell line and a potentiated antibody response, even at subclinical doses of anti-melanoma antibody (Clynes et al., 2000).

1.6.3 Local tumour environment

1.6.3.1 Other inhibitors of ADCC

The tumour itself creates an immunosuppressive microenvironment which can inhibit the effectiveness of cytotoxic antibodies. Theoretically this could be improved by blocking the negative regulators of the immune system. One such regulator is cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) which serves to prevent over-activation of the T-cell adaptive response. Blocking the activity of CTLA-4 allows greater expansion of all T-cell populations, presumably including those with antitumour reactivity. An anti-CTLA-4 antibody, ipilimumab has been used alone in a recent pilot study involving 14 patients with hormone-refractory metastatic prostate cancer (Small et al., 2007). Response was measured by a reduction in the prostate-specific antigen (PSA). Two patients had a reduction of more than 50% in the absence of any other treatment, and eight others had a reduction less than 50%.

1.6.3.2 Cytokines

A description of cytokine modulation of the immune response is beyond the scope of this thesis. However as has already been described above in chapter 1.6.2.1, the local cytokine environment can influence the immune response to any given antigen as well as influencing the cytotoxic activity of therapeutic antibodies. Certain cytokines released in this response (eg. IFN- γ & GM-CSF) can lead to upregulation of the expression of activating Fc γ Rs (Schiff et al., 1997) thus providing further opportunity for antibody-mediated toxicity to the tumour. In contrast the T_{H2} cytokines upregulate Fc γ RIIB which has the opposite effect. In mouse models of lymphoma, treatment with rituximab and the cytokine IL-2 improved survival compared with either agent alone (Eisenbeis et al., 2004). Early clinical trials of this combination in non-Hodgkins lymphoma have likewise shown some promise (Eisenbeis et al., 2004; Friedberg et al., 2002). However, similar early phase clinical trials using trastuzumab and IL-12 (Parihar et al., 2004) or IL-2 (Repka et al., 2003) have shown no improvement in clinical response despite demonstrating improved ADCC *ex vivo*.

1.6.3.3 Tumour vascularisation

Critical to the action of all chemotherapeutic agents, including mAbs, is the delivery of the drug to the tumour. This presents a problem in poorly vascularised tumours, a problem which is increasingly common with larger metastases. Being able to target tumour cells with antibody requires that the antibody is able to reach the target cells. In addition, tumour vasculature can also be important when antibodies are used to target the vasculature itself, either to improve delivery of other cytotoxic agents or to destroy the blood supply to the tumour. Major advantages of attacking the vasculature over the tumour itself include

- The vasculature more accessible than the tumour
- Vasculature damage has a multiplicative effect as many tumour cells are dependent on each capillary
- Since the vascular endothelial cells are in themselves not transformed, they are less likely to escape targeting and become resistant to therapy – a frequent problem with targeting the tumour

In addition, the tumour vasculature is different from normal vasculature thus providing novel targets. For example, ED-B domain of fibronectin is found in vessels of many solid tumours but not normal blood vessels. In a similar way strategies have been devised to disrupt pro-angiogenic signalling by targeting mAb against angiopoetin-1.

The ‘normalisation’ of tumour vasculature by the anti-VEGF antibody bevacizumab has already been discussed in chapter 1.2.4.6. It would seem sensible therefore to combine naked antibodies inducing ADCC with pre-treatment bevacizumab to enable better tumour penetration by the cytotoxic antibodies, especially as synergy has already been demonstrated with chemotherapy (Hurwitz et al., 2004).

It is worth noting at this point that, given that the environment of the tumour has such importance for treatment, the common preclinical use of xenografts may lead to spurious results. In a situation in which antibody targeting and clearance are vital, using a model with an increased number of NK cells and a tumour in a non-orthotopic site which is abnormally vascularised (Axworthy et al., 2000) is clearly not a good representation of the solid tumour *in vivo*. The xenograft cancers contain increased concentrations of

therapeutic antibody and chemotherapeutic agents, and this may result in falsely improved results (Behr et al., 1997; Tolcher et al., 1999).

1.7 Project Aims

The introduction has outlined the strategies available for colorectal cancer treatment and the possibilities available with immunotherapy. This thesis examines the potential efficacy of hPR1A3 as a future treatment for colorectal cancer by studying the expression of CEA on colorectal cancer cells and the ability of hPR1A3 to cause cancer cell death.

The aims of the thesis are as follows:

1. To examine the variability of expression of CEA on colorectal cancer cells and to determine factors that influence this expression
2. To examine the ability of hPR1A3 to act as a cytotoxic antibody.
3. To investigate the mechanism of hPR1A3 action *in vitro*.
4. To study the interaction of hPR1A3 with effector cells and, in particular, the FcγIIIa receptor
5. To investigate the potential for synergy between hPR1A3 and other chemotherapeutic agents.

CHAPTER 2

Materials and Methods

2.1 Cancer cell lines

2.1.1 Biography of cell lines used in the study

Eighteen immortalised colorectal cancer cell lines and one gastric cancer cell line were used in the following experiments and are listed with their origin and basic culture conditions in table 2.i. Culture properties of the cell lines are given in table 2.ii.

2.1.2 Cell culture conditions

All cell lines listed had been in the Cancer & Immunogenetics laboratory for more than 3 years and two of the lines, C70 and C99, had been generated within the laboratory.

The gastric carcinoma cell line MKN45 was obtained from Cell Services LIF, Cancer Research UK. HCT-116 and SKCO-1 were originally obtained from ATCC; C70 was established in the Cancer and Immunogenetics Laboratory; GP5d and HT55 were obtained from ECACC; LS174T was obtained from BH Tom, NW University Med Centre, Chicago, Ill, USA and PC/JW was obtained from C. Paraskeva, Directors Lab, CRUK, London.

All cell lines maintained in culture with E4 (Dulbecco's MEM) and 2% RPMI 1640 (RPMI) contained 1% L-glutamine and 10% foetal calf serum (FCS) and those cell lines maintained in ISCOVE'S (IMDM + L-glutamine + 25mM HEPES; Gibco) contained, 10% FCS and 1% by volume Streptomycin/Penicillin 10, 000U/ml (Gibco). Cells were incubated at 37°C in a humidified 10% CO₂ environment except those cultured in 2% RPMI which were incubated in a 5% CO₂ environment. Details of the media are given in appendix 1. E4 and RPMI were obtained through Cancer Research UK Cell Services.

Table 2.i: Cancer cell lines used in the study. For references see Conaghan et al (Conaghan et al., 2008)

Cell line	Origin	Additional information
MKN45	Gastric	
SKCO-1	Colorectal	65yo male. Ascitic cells from metastatic CRC
C99	Colorectal	Created 1993. 69yo male. Rectum. *Dukes' C1; ^φ Ly1; [‡] V1. Mod differentiated.
PC/JW	Colorectal	30yo female. FAP patient
LoVo	Colorectal	56yo male. Metastasis to subclavian lymph node; poorly differentiated
LS411	Colorectal	
HT55	Colorectal	Well differentiated rectal cancer
C70	Colorectal	Created 1991. 69yo female; Sigmoid colon. Dukes' B; V1, Ly1; moderately differentiated
SW403	Colorectal	51yo female. Dukes' C1. Mod differentiated
Car-1	Colorectal	
GP5d	Colorectal	71yo female. Local recurrence of resected sigmoid ca; Dukes' B. Poorly differentiated. Strong family history
HT29	Colorectal	44yo female. Metastatic. Moderately differentiated
Vaco-5	Colorectal	78yo female. Caecum. Dukes' C2. Poorly differentiated
NCI-H716	Colorectal	33yo male. Ascitic cells from metastatic caecal ca. Prior 5FU treatment
RKO	Colorectal	Colon. Poorly differentiated
HCT-116	Colorectal	Male. Colonic
LS174T	Colorectal	58yo female. Dukes' B colon. Trypsinised variant of LS180
SW1417	Colorectal	
SNU C2B	Colorectal	

*Dukes' staging: see table 1.iv and 1.v

[‡]V1 = vascular invasion

^φLy1 = lymphatic invasion

Table 2.ii: Culture characteristics of cancer cell lines used in the study

Cell line	Medium [‡]	Passage	Trypsinisation time*	Additional information
MKN45	E4	1:20 weekly	Moderate	
SKCO-1	E4	1:15 weekly	Moderate	
C99	Iscove's	1:15 weekly	Moderate	
PC/JW	E4	1:10 weekly	Long	
LoVo	E4	1:30 weekly	Short	
LS411	E4	1:10 weekly	Moderate	
HT55	E4	1:15 weekly	Long	
C70	E4	1:15 weekly	Moderate	Grows as clumps
SW403	E4	1:10 weekly	Short	
Car-1	Iscove's	1:15 weekly	Moderate	
GP5d	E4	1:10 weekly	Moderate	
HT29	E4	1:40 weekly	Short	
Vaco-5	Iscove's	1:10 weekly	Moderate	
NCI-H716	2% RPMI	1:15 weekly	None	Non-adherent line. Grows in suspension
RKO	2% RPMI	1:30 weekly	None	Loosely adherent. Removed with saline
HCT-116	E4	1:40 weekly	None	Loosely adherent. Removed with saline
LS174T	E4	1:20 weekly	Moderate	
SW1417	2% RPMI	1:10 weekly	Short	
SNU C2B	2% RPMI	1:10 weekly	Short	

*Short <30 seconds

Moderate 0.5-2 minutes

Long >2 minutes

[‡]Cells in E4 medium cultured in 10% CO₂

Cells in Iscove's medium cultured in 10% CO₂

Cells in 2% RPMI medium cultured in 5% CO₂

Media with added glutamine and FCS are referred to as “complete” (eg 2% RPMI 1640 with 1% glutamine and 10% FCS is referred to as RPMI complete). All cell lines were maintained in tissue culture flasks (BD Falcon; polystyrene base).

Preparation of foetal calf serum

Foetal calf serum was obtained from Bioclear, UK. It was heat-inactivated to destroy complement at 55°C for 30 minutes.

Passage details

All cell lines underwent passage approximately weekly. The cells were washed with phosphate-buffered saline (PBSA; appendix 1) to remove the old medium/FCS and then disaggregated with 0.04% trypsin in 0.2mM EDTA for varying lengths of time (see table 2.ii). The trypsin was then inactivated with complete medium and the cells were transferred to a Falcon tube and spun for 5 minutes at 500g. The medium was removed and the cell pellet resuspended in 5mls of complete medium. A fraction of the cells were then transferred to a fresh tissue-culture flask (see table 2.ii) and the medium made up to 10mls. The flasks were then returned to the incubator.

Cryopreservation

Those cell lines not in regular use were stored by cryopreservation. Cells from the flask were pelleted as described above. 2-5 million cells were then resuspended in cryopreservation medium of FCS containing 10% dimethylsulfoxide (DMSO) and stored in sterile cryopreservation vials. These were rapidly cooled by placing them over dry ice in a polystyrene box for 1 hour, then transferred to a -80°C freezer for 24 hours before being placed in liquid nitrogen (-200°C) for storage.

Cell lines were recovered by rapid defrosting to 37°C and immediate but cautious resuspension over 2 minutes with medium to a volume of 10mls. These cells were then spun at 500g for 5 minutes and the cell pellet resuspended and placed in a tissue-culture flask as for passage. The cell concentration in the flask was much higher than for routine passage to ensure cell survival and then passaged within the week. Cell lines were passaged at least twice before use in experiments.

Mycoplasma testing

Cell lines underwent routine mycoplasma testing before any was used for experimentation and at intervals during prolonged passage. Cells were tested at Cancer Research UK Cell Services by growth on PPLO medium, DNA staining and PCR detection.

Cell counting

In all experiments cell counting was carried out using a haemocytometer. For epithelial cells 50µl of cells were mixed with an equal volume of trypan blue in a microcentrifuge tube. 10µl of this was then placed under the coverslip of the haemocytometer and counted under a microscope. The number of living and dead cells was then recorded. For counting leucocytes, 20µl of cells were mixed with an equal volume of trypan blue and the volume then diluted to 160µl with PBSA to make visual counting possible. Cell viability above 80% was a prerequisite for use in the experiments.

2.2 Human effector cells

2.2.1 Peripheral blood mononuclear cells (PBMC)

PBMCs were obtained from either fresh whole blood from healthy laboratory volunteers or from single-donation leucodepletion filters (LDFs) obtained from Colindale National Blood Service, London.

2.2.1.1 Isolation of PBMCs from fresh whole blood

50mls of whole blood were collected and anticoagulated by mixing with an equal volume of ACD, an RPMI / citrate mixture (40ml 3.3% sodium citrate, 2ml 5mM 2-mercaptoethanol, 200ml RPMI 1640 + hepes). Blood was then layered onto Ficoll in a 50ml Falcon tube and centrifuged at 800g for 25 minutes, allowing the centrifuge to stop without the use of a brake. The interface formed contains the PBMC layer and this was aspirated with a Pasteur pipette and transferred to a fresh Falcon. This was washed to remove the Ficoll with an equal volume of RPMI and spun at 800g and the supernatant discarded. The pellet was resuspended and washed a second time with RPMI, spinning at 200g for 10mins to remove platelets. PBMCs were then resuspended in RPMI 1640 / 10% FCS / 1% glutamine (complete RPMI) and made up to a concentration of 1×10^7 /ml and used within 12 hours of preparation.

2.2.1.2 Isolation of PBMCs from LDFs

White cells were eluted from the filter in sterile conditions using 200mls 5mM EDTA. This was then layered onto Ficoll as described for fresh blood above and the same protocol followed.

2.2.2 Isolation of CD56+ CD3- cells (Natural killer subset)

White cells were eluted from an LDF filter as described above. 40mls of the 200ml solution were divided into 4 x 10ml aliquots. Each aliquot was mixed with 0.5mls of RosetteSep Human NK Cell Enrichment Cocktail (StemCell Technologies, London, UK) and incubated at room temperature for 20 minutes. This cocktail uses a series of

antibodies which crosslink the non-NK fraction of the PBMCs to red blood cells which are then pelleted along with red cells during density centrifugation, leaving NK cells in the buoyant interface normally containing PBMCs. LDFs still contain sufficient red blood cells for this cocktail to work (>30 red cells to every nucleated cell).

10mls of 2% FCS in PBSA was added to each of the 10ml aliquots and the blood mixture was then layered onto Ficoll and spun at 1200g for 25 minutes with the brake off. The interface formed should contain only NK cells and these are extracted with a Pasteur pipette as before. The rest of the preparation is carried out as for the general PBMC population described above in chapter 2.2.1.1.

2.3 Antibodies

2.3.1 ADCC assays

All antibodies for ADCC assays were diluted from stock concentrations to the desired concentration of the assay in 1% FCS in RPMI. The concentrations used are given in chapters 2.4.1.3 and 2.4.2.3 where the specific experiments are described.

PR1A3

As described in more detail in chapter 1.5, PR1A3 is an IgG1 κ monoclonal antibody to CEA originally derived as a murine antibody (IgG1) in the Cancer & Immunogenetics Laboratory (Richman and Bodmer, 1987) and later humanised (Stewart et al., 1999). Both murine (mPR1A3) and humanized (hPR1A3) antibody were obtained from the Biotherapeutics Development Unit, Clare Hall, Cancer Research UK, London, UK.

Anti-CD16

Two different clones of this antibody were used: MEM154 (Biovendor Laboratory Medicine Inc) and 3G8 (BD Biosciences, Pharmingen, USA). Both are monoclonal murine IgG1 κ antibodies against both A and B subtypes of the human Fc γ III receptor (CD16; Fc γ R3). F(ab')₂ derived from the 3G8 clone was obtained from Ansell Corp, Bayport, MN, USA.

Humanised A33 (hA33)

A33 is a monoclonal antibody developed in mice (IgG2a) and later humanised (IgG1). It binds to a 43KDa glycoprotein of unknown function whose expression is restricted to colorectal epithelium. It continues to be expressed homogeneously in more than 95% of colorectal cancers (Welt et al., 2003).

The humanised antibody (Welt et al., 2003) for use in the ADCC assay was kindly provided by Dr L. Old, Ludwig Institute for Cancer Research, New York, USA

Anti-Prostate Specific Membrane Antigen (PSMA)

Monoclonal antibody 107-1A4 to PSMA was kindly provided by Robert Vessella (Univ Washington, USA)

2.3.2 ELISA

All antibodies for ELISA assays were made to the desired concentration by serial dilution from stock concentrations in 1% FCS in RPMI.

Murine PR1A3 (mPR1A3)

As described above. It was used at concentrations of 1-60 μ g/ml.

Murine A33 (mA33)

As described for humanised A33. mA33 obtained from ATCC. It was used at a concentration of 6 μ g/ml.

AUA-1 (Epenetos et al., 1982) is a murine IgG1 antibody against the epithelial cell adhesion molecule, EpCAM. It was obtained from the Monoclonal Antibody Service, Clare Hall, Cancer Research UK, London, UK. It was used at a concentration of 1.5 μ g/ml.

DA2 is an IgG monoclonal antibody against a monomorphic determinant on HLA-class II (Brodsky et al., 1980). It was obtained from the Monoclonal Antibody Service, Clare Hall, Cancer Research UK, London, UK. It was used at a concentration of 1.5 μ g/ml.

Polyclonal rabbit anti-mouse antibody was obtained from DAKO A/S, Denmark, diluted 1:100 in RPMI 1640 / 1% FCS

Anti- β -galactosidase antibody, 4C7 clone (Durbin and Bodmer, 1987), was obtained from Clare Hall, Cancer Research UK, London, UK, and used at final concentration of 0.4 μ g/ml in RPMI 1640 / 1% FCS.

2.3.3 Fluorescence activated cell sorting (FACS) analysis

FITC-conjugated murine anti-human IgG and *FITC-conjugated rabbit anti-mouse IgG* were obtained from Sigma-Aldrich, Poole, UK, and used at a dilution of 1:50.

2.4 Antibody-dependent cellular cytotoxicity (ADCC) assays

Throughout the ADCC assays, the following terms are used and are defined as follows:

Background release

This refers to the amount of radioactivity or fluorescent compound released into the medium in the absence of effector cells or antibody. This might occur by passive cell death or active transport of the compounds out of the cell

Spontaneous killing/lysis

This refers to the active killing of target cells by effector cells but in the absence of antibody. This is a result of the donor and target cells coming from different individuals and, in these studies, the result of the target cells being neoplastic.

Maximum lysis

This refers to the reading obtained by lysing all the cells in a particular well with detergent so that all the radioactivity or fluorescent compound is released into the medium

% lysis

This is used as the outcome measure for the majority of the ADCC assays and appears on the y-axis of many of the graphs. This refers to the percentage of cells in a population killed by the effector cells. It is calculated as:

$$\frac{[\text{experimental release} - \text{background release}]}{[\text{maximum release} - \text{background release}]} \times 100\%$$

For most cell lines assayed, MKN45 was also used as a positive control and as a reference for inter-experimental analysis.

2.4.1 ⁵¹Chromium release assay

2.4.1.1 Target cell preparation

Cancer cell lines were extracted from their tissue-culture flasks and pelleted as previously described for the passage of cell lines (chapter 2.1.2). Cell lines were extracted at approximately 70-80% confluence for each experiment to avoid potential confluence-induced changes in the concentration of cell surface proteins. The cell pellet was resuspended in 5-10mls of RPMI complete and 50µl were taken for counting. 2×10^6 cells were taken from this suspension and spun down at 500g for 5 minutes in a 15ml Falcon. The supernatant was discarded and resuspended in 100µl ⁵¹Cr (200µCi / 7.4MBq). This was incubated for 60-100 minutes at 37°C depending on the optimal labeling time for the cell line (see below), following which the suspension was made up to 1ml with RPMI complete to wash and transferred to a microcentrifuge tube. The cells were spun down at 200g and then washed a second time in RPMI complete. Following repelleting the cells were resuspended in 1ml RPMI complete and counted. The cells were transferred back to a 15ml Falcon and made up to a concentration of 1×10^5 /ml.

2.4.1.2 Optimisation of labelling

Cells were pelleted and counted as above for target cell preparation. 5×10^6 cells were taken from the cell suspension and spun at 500g for 5 minutes in a 15ml Falcon. The supernatant was discarded and the pellet resuspended in 375µl of ⁵¹Cr (28MBq) and divided into 5 aliquots of 75µl. These were incubated at 37°C for differing times from 50-120 minutes. The cells were washed as per the ⁵¹Cr release protocol and made up in a solution of RPMI complete at 1×10^5 /ml

From each of the time periods, 100µl of cells were decanted into each of 3 wells and treated with the same volume of 5% Triton and this was compared with another 3 wells of cells that received RPMI medium instead of Triton as shown below

Plate set-up

Time labelled	Wells 1-3	Wells 4-6
A: 50mins	100µl	100µl
B: 70mins	cells +	cells +
C: 90mins	100µl	100µl
D: 100mins	medium	Triton
E: 120mins		

⁵¹Cr-release measured as per Cr-release protocols (chapter 2.4.1.4). The lysis:background release ratio was calculated and the labelling time with the highest ratio was chosen.

2.4.1.3 Effector cell and antibody preparation

These were prepared as described above in chapter 2.2 and 2.3. Only hPR1A3 was used in the Cr-release ADCC assays. A range of concentrations from 0.1µg/ml to 50µg/ml were used.

2.4.1.4 Assay conditions and measurement of cytotoxicity

Target cells were prepared at a concentration of 1×10^5 /ml and effector cells at a concentration of 1×10^7 /ml. The assay took place in microcentrifuge tubes which seemed to work better than 96-well tissue culture plates. Every condition was carried out in triplicate.

100µl of target cells (1×10^4 cells) were added to each tube. 20µl of hPR1A3 and 100µl of effector cells (1×10^6 cells) were added to the relevant tubes. 120µl of 5% Triton in RPMI was added to the lysis tubes and all other tubes were made up to 220µl with RPMI complete. Occasionally the antibody was preincubated with the target cells at 4°C on ice for 20 minutes prior to the addition of PBMCs (see chapter 4.2.2.4) but this made little difference to the results seen.

The microcentrifuge tubes were spun gently at 200g for 2 minutes to bring the cells into closer apposition for the assay incubation and then they were incubated at 37°C for 4 hours.

Following incubation the tubes were centrifuged at 500g for 5 minutes to pellet the cells and cellular debris.

100µl of Optiphase Supermix (Perkin Elmer, Boston, MA, USA) was added to each well of a clear 96-well PET round-bottomed microplate (Perkin Elmer). 35µl of the supernatant from the top of each microcentrifuge tube was added to the optiphase solution and mixed. The plate was then read by a Microbeta plate reader (Wallac1450 microbeta scintillation reader, Perkin Elmer) to determine the ⁵¹Cr concentration in each well.

2.4.2 Fluorescence-based assay using Europium-TDA

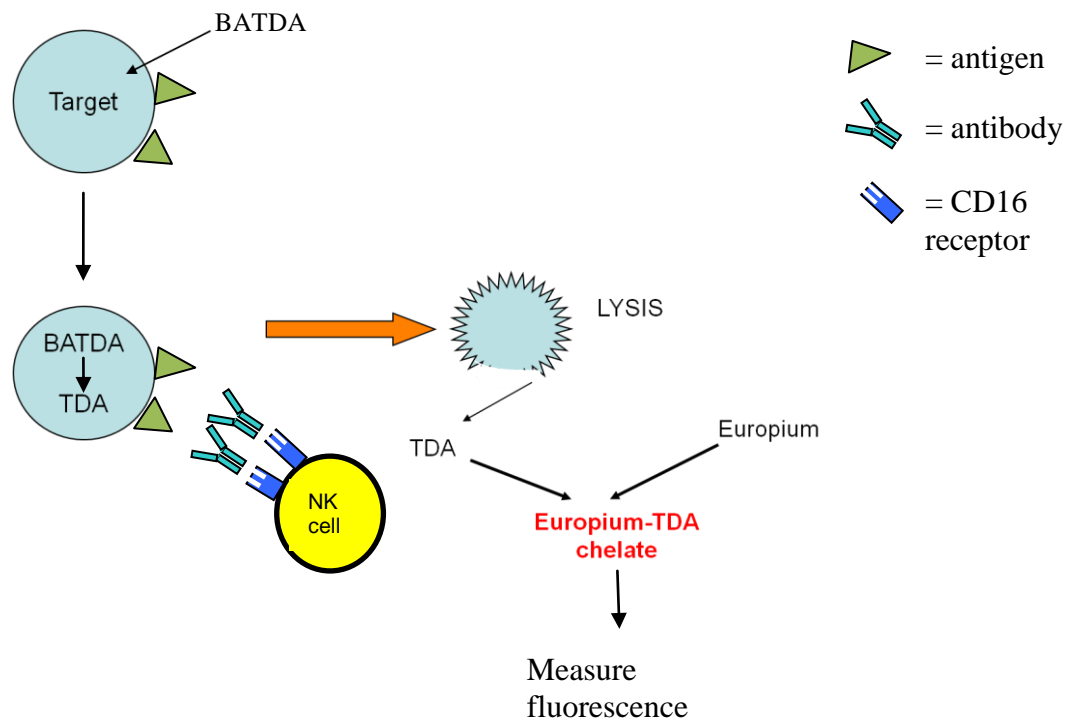
This assay was initially used in an attempt to find a reliable non-radioactive ADCC assay. The Europium-TDA assay is a fluorescence based assay which uses BATDA, a hydrophobic ester of the fluorescence-enhancing ligand terpyridine dicarboxylic acid (TDA). The hydrophobic BATDA crosses the cell membrane freely where, within the cell, the ester bonds are cleaved to produce the hydrophilic TDA which can no longer passively cross back out of the intact cell. Hence TDA is only detectable in the supernatant of the ADCC assay when the cell membrane has been perforated (figure 2.i)

2.4.2.1 Target cell preparation

Cancer cell lines were extracted from their tissue-culture flasks and pelleted as previously described for the passage of cell lines. The cell pellet was resuspended in 5-10mls of RPMI complete (or 2% RPMI with lower concentrations of FCS for certain experiments – chapter 5.2.2), counted and made up to a concentration of 1×10^6 cells/ml. 2×10^6 cells (2mls) were labelled with 6µl BATDA (Blomberg et al., 1996) (Perkin Elmer, Boston, MA, USA) and incubated for 10-25 minutes at 37°C depending on the optimal labelling time for the particular cell line (chapter 2.4.2.2). Following incubation the cells were spun at 500g for 5 minutes to pellet the cells and the supernatant discarded. The cells were resuspended in 5-7mls of RPMI and washed three times, discarding the supernatant on each occasion. Following the final wash the cells were resuspended in about 2mls RPMI complete and counted. They were then made up to a concentration of 1×10^5 cells/ml with further RPMI complete.

Figure 2.i: Process of cell-lysis detection by fluorescence-based Europium-TDA assay

The hydrophobic BATDA diffuses across cell membranes where it is converted to the hydrophilic TDA which is confined within the cell unless cell lysis occurs. Thus the detection of TDA in the medium is a measure of cell lysis. See text for more detailed explanation



2.4.2.2 Optimisation of labelling

Cells were pelleted and counted as above for target cell preparation. For each time and temperature studied, 2mls of a 1×10^6 cells/ml solution were labelled. The labelling times measured were 5 minute intervals from 5-30 minutes and the temperatures studied were 4°C, room temperature and 37°C.

Following incubation the cells were washed and recounted as for the target cell preparation above. For each of the time/temperature periods, 100µl of cells were decanted into each well and treated with either 100ul of 5% Triton to measure maximal lysis, or

with 100µl of RPMI to measure background release. Each condition was performed in triplicate. The DELFIA lysis buffer provided with the EuTDA assay was also tried instead of 5% Triton but gave no advantage to the assay. TDA release was measured in the same way as for the EuTDA assay (chapter 2.4.2.4).

The lysis : background release ratio was calculated and the labelling time with the highest ratio was chosen.

2.4.2.3 Effector cell and antibody preparation

These were prepared as described above in chapter 2.2 and 2.3

hPR1A3 concentrations from 0.1-50µg/ml

hA33 concentrations from 6-30µg/ml

anti-CD16 concentrations (MEM-154 clone) from 1-50µg/ml

anti-CD16 concentrations (3G8 clone) from 1-10µg/ml

anti-CD16 F(ab')₂ concentrations from 1-10µg/ml

2.4.2.4 Assay conditions and measurement of cytotoxicity

The concentrations of target cells and antibody in the microcentrifuge tubes were as for the ⁵¹Cr release assay but the effector concentration was occasionally varied to give varying effector : target ratios (chapter 4.4). After spinning at 200g for 2 mins the tubes were incubated at 37°C for 2 hours, in contrast to the 4 hours incubation for ⁵¹Cr-release assay. Following incubation, the tubes were spun at 500g for 5 minutes. 20µl of supernatant from the top of each microcentrifuge tube was added to 200µl of Europium in black 96-well plates and the resulting fluorescence read using an excitation wavelength of 340nm and an emission wavelength of 615nm in a Fluostar Optima time-resolved fluorometer (BMG Labtech, Aylesbury, UK).

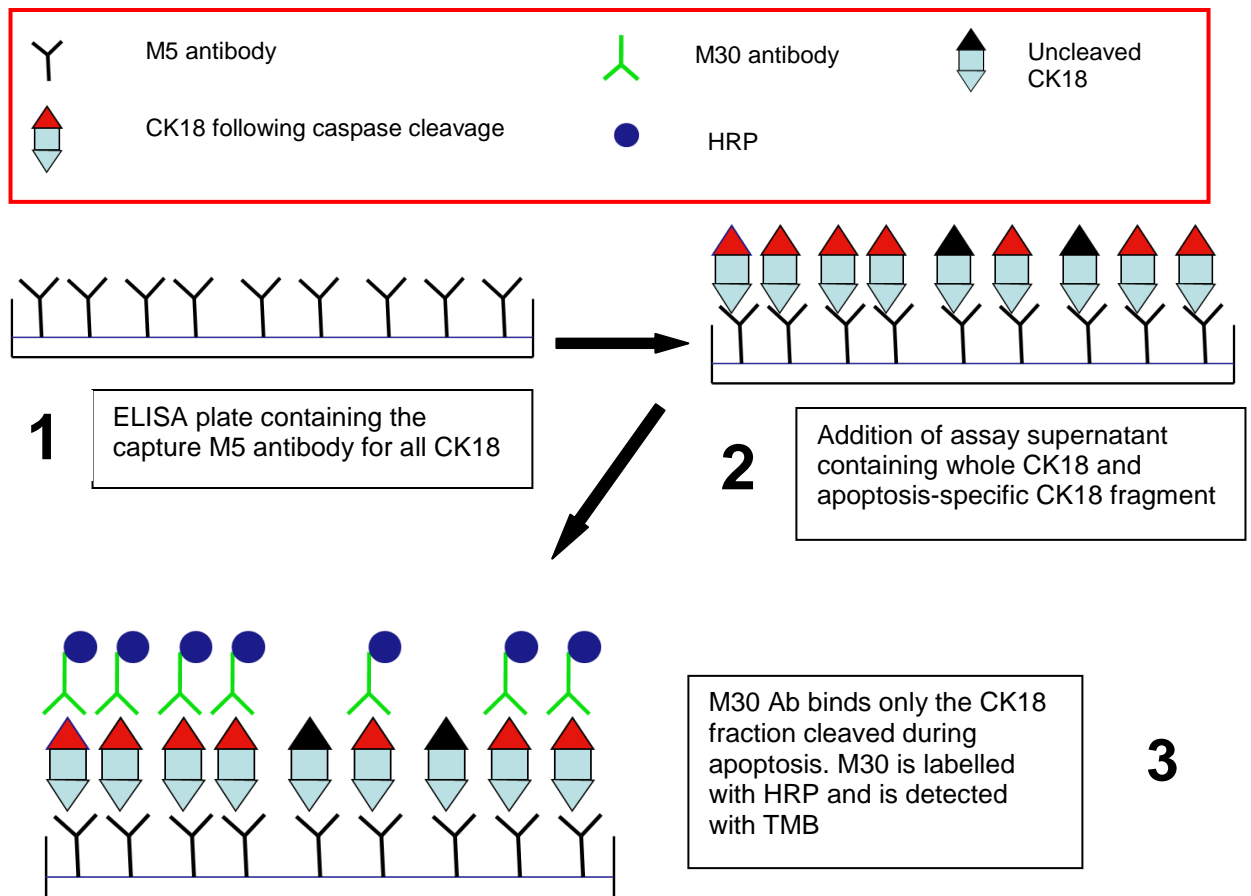
2.4.3 Epithelial cell apoptosis assay using M30 antibody

This assay was carried out using the M30-apotosense kit (Peviva, Bromma, Sweden). This is an ELISA for the quantitative detection of a caspase cleavage product of cytokeratin 18 (CK18), an intermediate filament protein specific to epithelial cells. The

M30 antibody recognizes a neo-epitope exposed after caspase cleavage of CK18 after the aspartic acid residue 396 (Leers et al., 1999). Cleavage at this position occurs during apoptosis by caspase-9 and then by caspase-3 and caspase-7 (Schutte et al., 2004). Hence this should enable quantification of epithelial cell apoptosis since it is specific both for epithelial cells (CK18) and apoptosis by detection of a specific caspase cleavage product (figure 2.ii).

Figure 2.ii: M30 cytotoxicity assay

See text for explanation



2.4.3.1 Assay conditions

Cancer cell lines were extracted from their tissue-culture flasks and pelleted as previously described for the passage of cell lines. The cell pellet was resuspended in 5-10mls of RPMI complete and counted and made up to a concentration of 1×10^5 cells/ml. No labelling was carried out. Effector cells and antibody were added as for EuTDA and ^{51}Cr -release assays. The microcentrifuge tubes were spun at 200g for 2 minutes and then incubated at 37°C for 4 hours. Following incubation the cells were spun at 18000g in a refrigerated centrifuge at 4°C. They were transferred to ice where the supernatant was removed and the cell pellet was lysed with 150µl of 0.5% NP40. The tubes were again spun at 18000g for 5 minutes and 100µl of the supernatant was removed and this was further spun at 18000g for 5 minutes. This is to avoid cellular debris within the assay. 70µl of the resultant supernatant was stored at -70°C for use in the M30 ELISA within one week.

2.4.3.2 M30 ELISA

Samples were defrosted on ice. 25µl of each microcentrifuge tube was added to one well of the M30 ELISA 96-well plate. M30 standards (solutions containing known concentrations of M30) were added to spare wells. This plate contained the capture antibody for all CK18 (M5 antibody), not just the caspase cleavage fragment. 75µl of HRP-conjugated M30 was then added to each well, without washing, to detect the caspase-cleavage fraction of CK18 (figure 2.ii).

The plate was placed on a plate-shaker at 600rpm for 4 hours at room temperature. Wells were then washed 5 times with 250µl of detergent-based wash solution provided with the M30 apoptosense kit. 200µl of tetramethylbenzidine (TMB) was added to each well to detect the HRP-M30 conjugate. The plate was incubated in the dark for 20 minutes at room temperature and then 50µl of stop solution was added to each well. The solution was placed on a plate shaker for 10 seconds to ensure the solutions were well mixed and to stop the reaction and then incubated for a further 5 minutes in the dark at room temperature.

The absorbance was read from the plate at 450nm in a microplate reader.

2.5 Evaluation of expression of CEA and other cell surface proteins on cancer cell lines

2.5.1 Enzyme linked immunosorbent assay (ELISA)

This technique was predominantly used to quantitate expression levels of CEA and other cell-surface proteins on cell lines after normal culture conditions. It was also used to assess the effects of changes in cell confluence and the administration to the culture medium of 5-fluorouracil and interferon- γ . The ELISA used is based on the technique of Durbin and Bodmer (Durbin and Bodmer, 1987) and is a solid-phase ELISA using a monoclonal murine anti- β -galactosidase antibody bound to its antigen, β -galactosidase (figure 2.iii). The cell line under investigation is bound to the plate and the cell-surface protein being assessed for expression is detected using a murine antibody as the first layer. The unbound primary antibody is then washed off. The amount of bound murine antibody is detected by a polyclonal rabbit anti-mouse antibody, which detects all classes of murine IgG, and again the unbound fraction is washed off. Specificity is increased by then adding a 3rd layer murine antibody which is specific for and bound to the β -galactosidase enzyme. Being a murine antibody, this also binds to the already-bound rabbit-anti-mouse antibody. Following washing, the amount of bound antibody is then detected by adding 4-methylumbelliferyl-B-D-galactoside (MUG) which is a substrate for β -galactosidase and the fluorescent product could be measured.

The advantage of this ELISA was that it was possible to detect any murine antibody, and hence the antigen to which they were bound, in a solid phase ELISA, with the cell lines being bound to the ELISA plate.

2.5.1.1 Cell preparation and attachment to plate

96-well clear bottomed plates (Nunc-Immuno PolySorp 96-well plates) were coated with 70 μ l 0.1mg/ml poly-l-lysine (PLL; Sigma-Aldrich, Poole, Dorset, UK) in PBSA. These were incubated overnight at 4°C. Cancer cell lines were extracted from their tissue-culture flasks and pelleted, as previously described for the passage of cell lines. The supernatant was discarded and the cell pellet was resuspended in 15-20mls of RPMI (without FCS)

for washing and centrifuged at 500g for 5 minutes. The supernatant was again discarded and the washing process repeated twice more. This process was to remove the FCS from the cells. The resulting cell pellet was resuspended in RPMI and made up to 5×10^5 /ml. The plates were flicked out to remove the spare PLL and were then washed twice with PBSA, blotting dry after each wash. 50µl of the cell solution was added to each well and then left still for 5 minutes at room temperature whilst the cells settled. The plates were spun at 500g for 7 minutes and then incubated at room temperature for 25 minutes. The wells were examined under the microscope for consistency of numbers and distribution throughout the wells.

Cells were fixed to the plate by adding 100µl of 0.025% glutaraldehyde in PBSA. This was slowly added down the sides of the wells to avoid dislodging the cells attached to the PLL. The plate was incubated at room temperature for 40 minutes. The supernatant was flicked out and the plate was then washed three times with PBSA, blotting dry after each wash. 200µl of 0.1% gelatin was then added to each well to bind to all spare PLL not bound by cells, thus avoiding the unattached PLL from binding to the antibodies. These plates were then stored at 4°C overnight.

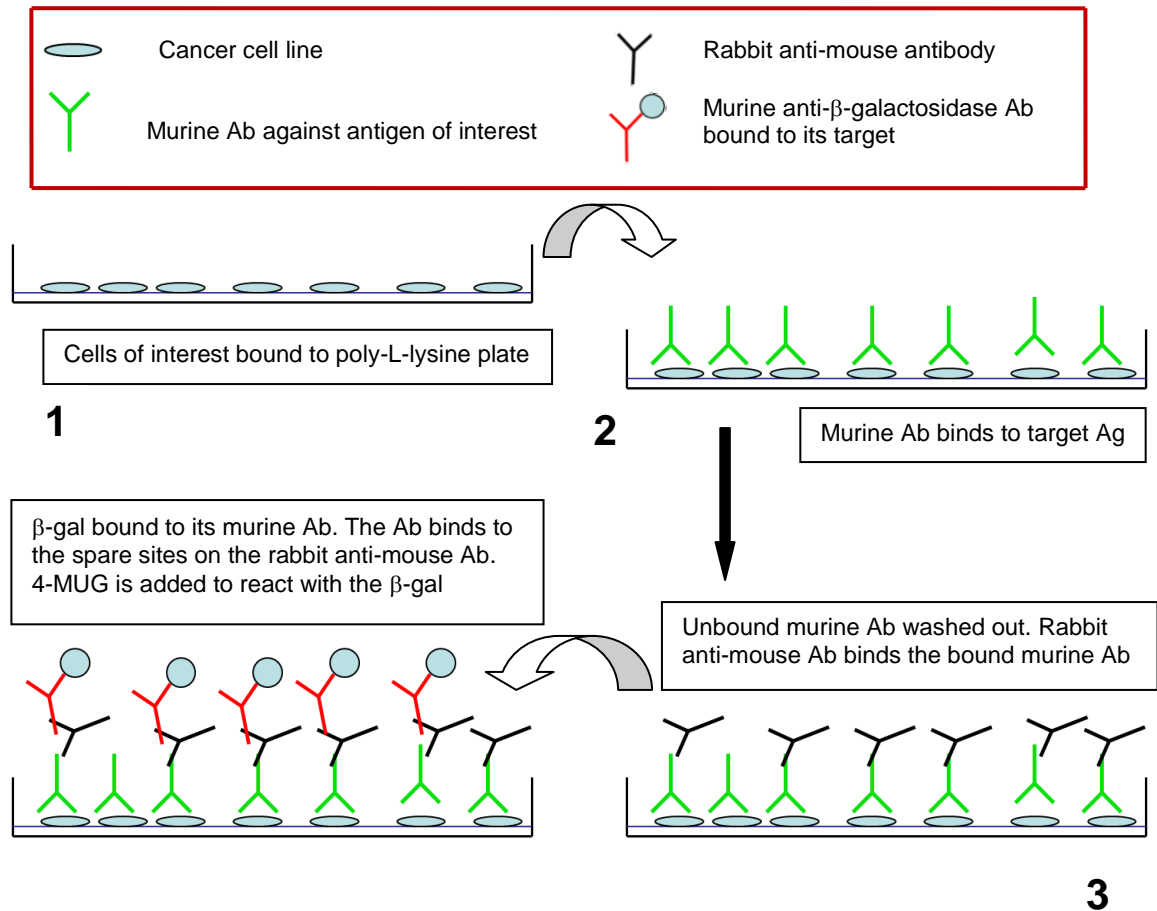
2.5.1.2 Cell confluence experiments

Cell lines were harvested from tissue-culture flasks at various levels of flask coverage and expressed as a percentage of how much of the flask surface was covered with cells. Confluence was defined as a time when the cells were no longer growing exponentially and had reached a stable number. For most cell lines this occurred when 100% coverage of the plate surface had been reached, although certain cell lines (e.g. C70) form clumps and so do not reach total surface coverage.

Three levels of flask coverage were examined: 40-60%; 70-90%; 10-14 days following the cells reaching 100% confluence (post-confluence). During incubation of cells to post-confluence the medium was replaced every 2 days.

Figure 2.iii: The β -galactosidase / anti- β -galactosidase ELISA

See text for explanation



2.5.1.3 5-fluorouracil treatment

5-fluorouracil (5-FU) (Sigma-Aldrich, Poole, UK; cat.no. 47576) solutions were made fresh from powder for each experiment by dissolving in distilled water. The desired concentrations were obtained by serial dilution.

Different cell lines were plated onto tissue-culture flasks at varying concentrations to reach 70-90% confluence by day 4. At day 4 the medium was removed and either replaced with fresh medium for control flasks or medium containing 150 μ g/ml 5-FU for 4 hours. The medium was removed and the cells were washed three times with PBSA

before adding further fresh medium. The cells were returned to incubation and harvested at day 6 for the ELISA.

The 5FU concentration was based on the upper end of the dose range in adults being treated on a once weekly basis. Since 5FU is evenly distributed throughout all compartments of the body, the steady-state plasma concentration was used which is approximately 150 μ g/ml (1.1mM) (CCO-Formulary, 2007). This does not take into account the levels of drug distributed into extravascular compartments and therefore in contact with tumour but it is a useful approximation. Similar experiments on cell lines using 5-FU from the literature uses doses ranging from 0.1 μ M to 8mM incubated over time periods from 1 hour to 3 days (Aquino et al., 1998; Correale et al., 2003; Ismail et al., 1998; Shimoyama et al., 2002).

2.5.1.4 Interferon-gamma treatment (IFN- γ)

Human recombinant IFN- γ (Sigma-Aldrich, Poole, UK) was reconstituted from a lyophilised powder in PBSA at a concentration of 1 μ g/ μ l. Desired concentrations were obtained by serial dilution in RPMI complete or E4 complete.

LS174T and HT55 were the cell lines used in these experiments. The cell lines were plated onto tissue-culture flasks at varying concentrations to reach 70-90% confluence by day 4. At day 4 the medium was removed and either replaced with fresh medium for control flasks or medium containing IFN- γ at concentrations of 50, 200 and 1000 units/ml (33, 133 and 667IU/ml respectively). Cells were harvested at 6 hours, 2 days and 4 days after the addition of IFN- γ and plated onto 96-well plates for the ELISA as described above (Aquino et al., 1998; Fichera et al., 1998).

2.5.1.5 Preparation of GAG complex

500 units of β -galactosidase was dissolved in 100 μ l of a solution of 100mM TRIS / 100mM MgCl / 100mM 2-mercaptoethanol in distilled water. This was added to 300 μ l of 1mg/ml purified anti- β -galactosidase antibody (4C7 clone) and 700 μ l of 200mM TRIS. The resulting β -galactosidase - anti- β -galactosidase complex, called the GAG complex, is

incubated overnight at 4°C before use. The GAG complex was used in the assay at a dilution of 1 in 750.

2.5.1.6 β -galactosidase/ anti- β -galactosidase enzyme-linked immunosorbent assay (GAG-ELISA)

Plates prepared for the ELISA were washed twice with a solution of 2g/L casein (Oxoid, Basingstoke, UK) in PBSA to remove all traces of gelatin, blotting the plates between washes. PR1A3 and other first layer antibodies were made up to varying concentrations in 2% RPMI containing 1% FCS. 50 μ l of the antibody dilution was added to each well. The plate was incubated at room temperature for 1 hour. The plates were then washed three times with a solution of PBSA containing 2g/L casein and 1% Tween-20 (Sigma-Aldrich, Poole, UK) to remove the unbound antibody.

Rabbit anti-mouse antibody (DAKO A/S, Denmark) was diluted 1:100 in RPMI / 1% FCS. 50 μ l of the rabbit anti-mouse dilution was added to each well and incubated at room temperature for 1 hour. The plates were again washed three times with the PBSA / casein / Tween solution to remove the unbound antibody.

The GAG complex (see chapter 2.5.1.5 above) was diluted 1:750 in RPMI / 1% FCS. 50 μ l of the GAG complex were then added to each of the wells and incubated at room temperature for 1 hour. The plates were again washed three times with the PBSA / casein / Tween solution to remove the unbound antibody.

100 μ l of a 4-methylumbelliferyl-B-D-galactoside (MUG), (Sigma-Aldrich, Poole, UK) solution was added to each well. MUG is the substrate for β -galactosidase and thus starts the reaction. The 4-MUG was made up in a buffer of 1mM MgCl₂ / 100mM 2-mercaptoethanol in PBSA. 0.3mg of MUG was added for each 1ml of buffer and the mixture stirred for 30 minutes at room temperature. This was then filtered to remove excess substrate. The MUG buffer was made up fresh for each experiment. Once 100 μ l of the MUG solution was added to each well the plates were incubated in the dark for 30 minutes at room temperature. The fluorescence from the plates were read on a Fluostar Optima microplate reader (BMG Labtech, Aylesbury, UK) using an excitation wavelength of 365nm and an emission wavelength of 445nm. The resulting fluorescence

reading is subtracted from the background reading. The process is summarised in figure 2.iii.

2.5.2 Fluorescence activated cell sorting (FACS)

Cancer cell lines were extracted from their tissue-culture flasks and pelleted, as previously described for the passage of cell lines. The supernatant was discarded and the cell pellet was resuspended in ice-cold PBSA containing 2% FCS (FACS wash), counted and the cell concentration was adjusted to 1×10^6 /ml. The cells were labelled on ice with 100 μ l of 20 μ g/ml PR1A3, or a similar volume of medium control, and incubated at 4°C for 30 minutes. The cells were then rewashed with FACS wash and labelled with 1:50 dilution of FITC-conjugated anti-human-IgG for PR1A3 in the dark at 4°C for 30mins before being washed again and resuspended in FACS wash. The cells were analysed on a FACS Calibur flow cytometer (BD Biosciences, Erembodegem, Belgium) and analysed using WinMDI software.

2.6 Genotyping of *FCGR3A* polymorphism

2.6.1 DNA extraction

DNA was extracted from the following sources:

- Fresh blood
- Stored peripheral blood mononuclear cells (PBMCs)
- Cell lines

Extraction was performed by using the DNeasy Blood and Tissue Kit (Qiagen, Crawley, UK)

Fresh blood

3-5mls blood mixed 1:1 with the ACD mixture for anticoagulation described in chapter 2.2.1.1. 10 μ l of blood were placed into a 1.5ml microcentrifuge tube and the following were added

- a) 20 μ l proteinase K
- b) 190 μ l of PBSA
- c) 200 μ l Qiagen buffer AL

The tube was mixed on a vortex and incubated at 70°C for 10 minutes. 200 μ l 100% ethanol was added and the sample mixed thoroughly. The solution was pipetted onto a DNeasy mini-spin filter column and spun at 8000g for one minute. The effluent was discarded and the collection tube replaced. 500 μ l Qiagen buffer AW1 was added and the sample spun at 8000g for one minute. The effluent was discarded and the collection tube replaced. 500 μ l Qiagen buffer AW2 was added and the sample spun at 20000g for 3 minutes to dry the filter membrane. The filter column was placed into a microcentrifuge tube and 100 μ l of Qiagen buffer AE was added directly onto the filter membrane and left for one minute at room temperature. The column was then spun at 8000g for one minute to elute the DNA.

Stored peripheral blood mononuclear cells (PBMCs)

PBMCs were defrosted at room temperature and transferred to 15ml Falcons and centrifuged at 900g for 10 minutes to pellet the cells. The supernatant was taken off and the cells resuspended in PBSA to an approximate concentration of at most 2.5×10^7 cells/ml. 20 μ l proteinase K and 200 μ l Qiagen buffer AL were added to 200 μ l of the cell suspension and the sample mixed by vortexing. The samples were incubated at 70°C for 10 minutes and then treated as per the protocol for fresh blood.

Cell lines

Cancer cell lines were extracted from their tissue-culture flasks and pelleted, as previously described for the passage of cell lines. The supernatant was discarded and the cell pellet was resuspended in PBSA, counted and the cell concentration was adjusted to an approximate concentration of at most 2.5×10^7 cells/ml. 20 μ l proteinase K and 200 μ l Qiagen buffer AL were added to 200 μ l of the cell suspension and the sample mixed by vortexing. The samples were incubated at 70°C for 10 minutes as for the protocol for fresh blood and then treated as for that protocol.

Estimates for the concentration of the DNA were made by measuring the optical density (OD) of the DNA in solution at an absorbance of 260nm, and checking the purity of the solution with the ratio of the OD at 260nm and 280nm (with contamination suspected for 260:280 ratios outside the range 1.8 - 2.0). This was carried out using a NanoDrop[®] ND-1000 Spectrophotometer.

2.6.2 Primer design

The primers required to amplify the Fc γ III A gene (Human Genome Nomenclature Committee designation, *FCGR3A*) needed to:

- Amplify only the *FCGR3A* gene and not the *FCGR3B* gene (forward primers)
- Distinguish between the valine and the phenylalanine polymorphisms at nucleotide 559 (reverse primers). Nucleotide 559 (transcript 003 from ensemble (Ensembl, 2009)) starting at the 5'UTR (untranslated region) forms the first base pair in a triad

that translates to phenylalanine or valine depending on whether it is a T or G (Wu et al., 1997). There is variability in the reporting of the position of this polymorphism and in the current ensemble transcript 1 sequence, the polymorphism is at amino acid 212 (nucleotide 634) of the coding sequence and nucleotide 818 of the transcript including the 5'UTR (Ensembl (Ensembl, 2009) transcript 001).

Given the close homology between the A and B genes, forward primers were designed to distinguish between these by including initially one, and later two, single nucleotide differences between the genes within the region to be amplified. In both forward and reverse primers one of the discriminating nucleotides was placed at the 3' end of the primer to increase the specificity of primer binding. For the forward primer this was to discriminate between the A and B versions of the gene. For the reverse primer, this was to discriminate between the two polymorphic nucleotides of the A gene. Forward primers used were:

Forward 1 (74 base-pair (bp) product): contained one single nucleotide difference from *FCGR3B* gene at the 3' end.

5' TCCAAAAGCCCACTCAAAGAC 3'

Forward 2 (134bp product): contained one single nucleotide differences from *FCGR3B* gene but amplifies a region containing two single nucleotide differences from *FCGR3B* gene. This provides added security that the correct gene has been amplified when sequencing.

5' CACATATTTACAGAATGGCAAAGG 3'

The reverse primers were used to create an allele-specific PCR to amplify only the T or the G allele and hence to distinguish between the two polymorphisms described above.

Reverse primer for 'T' allele:

CTGAAGACACATTTTTACTCCCAAA

Reverse primer for 'G' allele:

CTGAAGACACATTTTTACTCCCAAC

Figure 2.iv gives more details of the primer positions within the gene sequence.

Figure 2.iv: Comparison of cDNA sequence from the *FCGR3A* and *FCGR3B* genes taken from ensembl (Ensembl, 2009) transcript 001 for the *FCGR3A* gene and starting at nucleotide 428:

BOLD CAPITALS: sites of differences between the 2 genes

Red font: forward primer 1

Green font: forward primer 2

Blue font: reverse primer (nucleotide 634 underlined; T/G polymorphism given here as T allele)

***FCGR3A* gene**

EXON 4

gctggctgttgctccaggcccctcgggtgggtgttcaaggaggaagaccctattcacctgaggtgtcacag
ctggaagaacactgctctgcataaggt**caatattacagaatggcaaaGC**aggaagtatttcatcat
aattctgactt**TAC**at**tc****caaaagccacactcaaaGAC**agcggctcctacttctgcagggggct**TTG**
ggagtaaaaatgtgtctcagagactgtgaacatcaccatcactcaag

gtttggcagtgtaaccatctcatcattc**TTT**ccacctgggtaccaagtctcttctgcttggatggactcc
ttttgcagtggaacacaggactatatttctctgtgaagacaacattcgaagetcaacaagagactggaa
ggaccataaattaaatggagaaaggaccctcaagacaatgacccccatccatgggggtaataag
agcagtagcagcagcatctctgaacatttctctggatttgaacccccatcctcaggcctctc

***FCGR3B* gene**

EXON 4

gctggctgttgctccaggcccctcgggtgggtgttcaaggaggaagaccctattcacctgaggtgtcacag
ctggaagaacactgctctgcataaggtcacatattacagaatggcaaa**GAC**aggaagtatttcatcat
aattctgactt**CAC**attccaaaagccacactcaaa**GAT**agcggctcctacttctgcagggggct**GTT**
gggagtaaaaatgtgtctcagagactgtgaacatcaccatcactcaag

gtttggcagtgtaaccatctcatcattc**TCT**ccacctgggtaccaagtctcttctgcttggatggactc
cttttgcagtggaacacaggactatatttctctgtgaagacaacatttgaagetcaacaagagactggaa
ggaccataaacttaaatggagaaaggaccctcaagacaatgacccccatccatgggagtaataa
gagcagtggaacacaggactctctgaacatttctctggatttgaacccccatcctcaggcctctc

2.6.3 Polymerase chain reaction (PCR)

The *FCGR3A* gene was amplified in a 50µl reaction containing final concentrations of 1.5-2mM MgCl, 0.25mM dNTPs, 1 unit Qiagen Hotstar® Taq, 50-100ng DNA and 250uM of each primer. The reactions were performed on an MJR PTC-225 Tetrad thermal cycler (GMI Inc, Minnesota, USA) using the following PCR programme:

- a) Activate Taq for 15 minutes at 95°C
- b) Melt DNA – 94°C for 1 minute
- c) Anneal primers – 60°C for 1 minute (60-68°C used during the temperature gradient)
- d) Extend DNA -72°C for 1 min
- e) Repeat (b)- (d) 30 times
- f) Final extend step – 72°C for 10mins

PCR products were stored at -20°C if not used within 24 hours.

2.6.4 DNA gel electrophoresis

The PCR products were analysed by gel electrophoresis. 0.5µg/ml ethidium bromide was added to a 2% agarose gel in 1 x TBE (appendix 1). This was poured onto a flat-bed apparatus for electrophoresis. Once solid, the gel was covered in 1 x TBE. DNA samples were mixed with 6 x loading buffer (Orange G) in a ratio of 5 parts PCR reaction to 1 part loading buffer. 10µl of product were added to the wells in parallel with double-stranded DNA size markers – 1KBp, 500bp, 200bp sizes (ElectroFast Markers; ABGene, Surrey, UK) - and the gel run at 80V for 10-15 minutes. The DNA was visualised under ultraviolet light.

2.6.5 PCR product purification

This was carried out using the QIAquick PCR purification kit according to the manufacturer's instructions. 40µl of PCR product was added to 200µl of buffer PB1 and the sample placed onto a QIAquick spin column within a collection tube. The columns were spun at 16000g for 1 minute and the resulting effluent discarded. 0.75mls of buffer

PE were then added to the column and spun again at 16000g. The column was then placed in a microcentrifuge tube and the DNA eluted using 30µl of buffer EB.

2.7 Statistics

Statistical analysis was carried out with the aid of SPSS software (Version 12; SPSS Inc, Chicago, Illinois, USA) and Microsoft Excel 2007 (Microsoft Corporation, USA). Continuous data are presented as means (+/- standard error of the mean (SEM)). Continuous parametric data were analysed using a 2-tailed unpaired t-test for 2 group analysis and one-way ANOVA where comparison involved 3 groups. All significance tests were 2-tailed and considered statistically significant if the observed significance level was <0.05.

% lysis within cytotoxicity assays

This refers to the percentage of cells in a population killed by effector cells. It is calculated as:

$$\frac{[\text{experimental release} - \text{background release}]}{[\text{maximum release} - \text{background release}]} \times 100\%$$

All cytotoxicity assay experiments were carried out in triplicate and the mean (std error of mean) represented graphically.

CHAPTER 3

Assessment of CEA expression on colorectal cancer cell lines and factors influencing this expression

3.1 Expression of CEA on colorectal cancer cell lines

3.1.1 Introduction

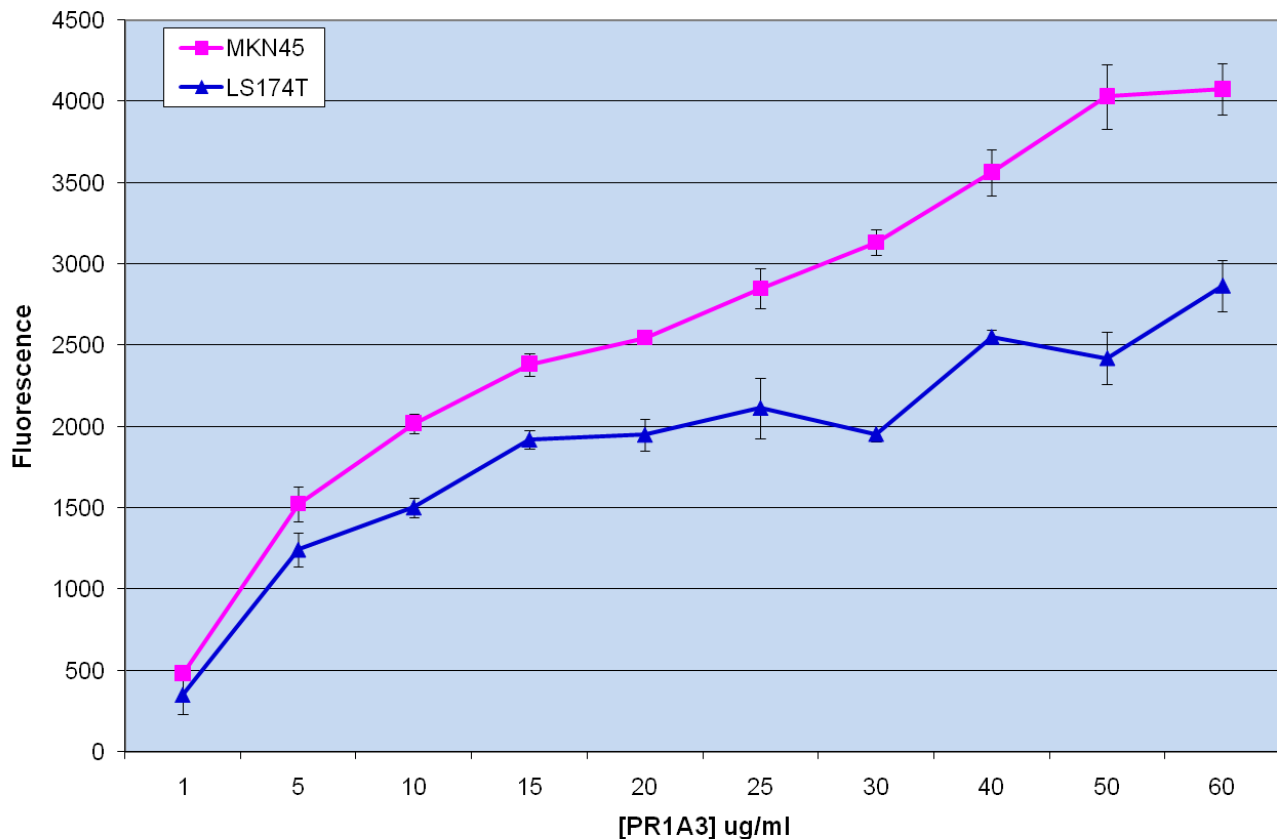
Comparisons of CEA expression on different cell lines and in different conditions were made predominantly using data from the GaG ELISA assay (chapter 2.5.1). Murine PR1A3 was used as the anti-CEA antibody in all the GaG ELISA studies described here. These results were supported by assessing CEA expression on certain cell lines by FACS analysis (chapter 2.5.2) and also by comparing results with data from the Cancer and Immunogenetics laboratory on CEA mRNA expression and microarray data. The GaG ELISA technique was also used to compare the expression of other cell-surface proteins on these same cell lines, generating data on other potential targets for therapeutic antibodies which might possibly have an additive or synergistic effect with PR1A3.

3.1.2 Antibody concentration optimisation

Optimisation of the antibody concentration for the ELISA was carried out using MKN45 as a known high-expressor of CEA from the initial ELISA experiments and compared with a moderate expressor LS174T. The two cell lines were exposed to different concentrations of mPR1A3 and the lowest concentration which could effectively differentiate between the cell lines was used in subsequent experiments to minimise non-specific binding in the CEA assays. At 20µg/ml mPR1A3 a clear distinction is seen in fluorescence between MKN45 and LS174T and so subsequent ELISAs for CEA expression were carried out using this concentration of antibody (figure 3.i).

Figure 3.i: Optimisation of PR1A3 concentration for use in ELISA studies. GaG ELISA using MKN45 and LS174T

Cell lines treated with various concentrations of mPR1A3. Each concentration tested in triplicate. Error bars represent standard error of the mean (SEM). All readings have had the background fluorescence reading (medium instead of first layer antibody) subtracted from them.



3.1.3 ELISA results

The differing levels of CEA expression are shown in figure 3.ii. The data were arbitrarily divided into high (24,000-34,000), moderate (14,000-16,000) and low (0-8,000) expressing cell lines for CEA (table 3.i) and these data were used to compare lines with differing levels of CEA expression in ADCC assays.

Figure 3.ii: CEA-expression of cell lines using GaG ELISA

Each value is the mean (+/- SEM) of 4 separate experiments with each run in triplicate. Cell lines were treated with 20µg/ml of mPR1A3 as the primary antibody. All readings have had the background fluorescence reading subtracted from them. Categorisation into high, moderate and low is an arbitrary one based on the data pattern observed.

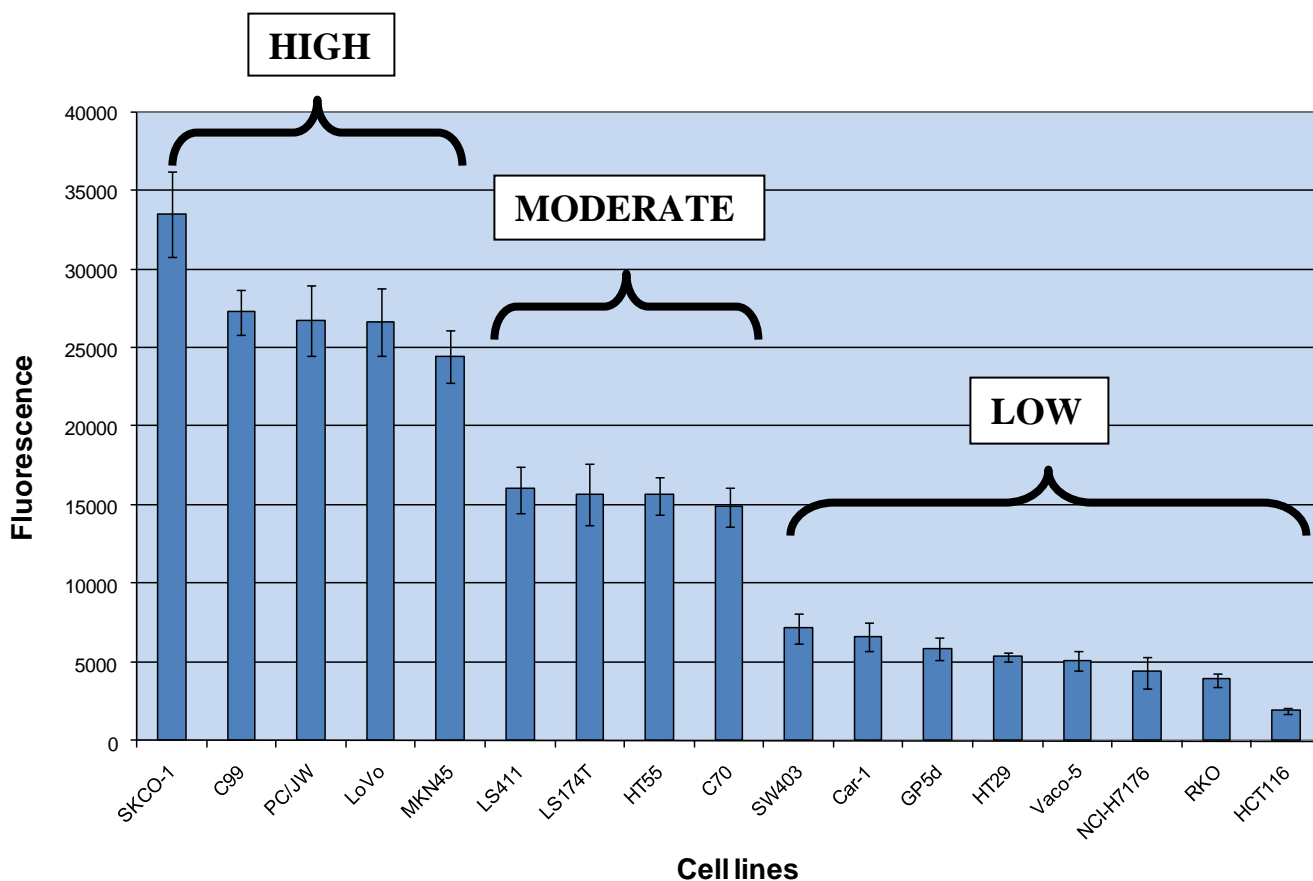


Table 3.i: Stratification of CEA expression on cell lines

CEA expression	Cell lines
High	SKCO-1, C99, PC-JW, LoVo, MKN45
Moderate	LS411, LS174T, HT55, C70
Low	SW403, Car-1, GP5d, HT29, Vaco, NCI-H716, RKO, HCT-116

Similar graphs are shown for expression of the highly gastrointestinal-specific peptide A33 (figure 3.iii), and the epithelial cellular adhesion molecule, EpCAM (figure 3.iv). The A33 graph can be arbitrarily divided into high and low expressors, as can EpCAM expression although the large majority are high expressors.

Although, as expected, no definite associations exist between the three cell-surface molecules, there are many cell lines which highly express two and some all three of the cell-surface molecules. Such data are useful for future planning of dual or poly antibody therapy to reduce the impact of antigen escape, a potential problem of antibody-based cancer therapy.

3.1.4 FACS results

Several cell lines were also examined for CEA expression using FACS analysis. Figure 3.v summarises these results with figure 3.vi providing a comparison of the various modalities for evaluating CEA expression. Although the FACS data largely correspond to the ELISA results demonstrating cell lines with high or low CEA expression, the low number of events with each of the cell lines does question how strong this is as supporting evidence. However MKN45, LoVo, LS174T and HT55 all show strong staining with PR1A3 whereas HCT116 and HT29 stained poorly.

3.1.5 Comparison with previous mRNA and microarray data

Previous work in the Cancer and Immunogenetics laboratory has looked at mRNA expression of the CEA transcript in a large panel of colorectal cell lines using RT-PCR. More recently the laboratory has also obtained affymetrix U133 + 2 expression microarray data on 30 of our panel of cell lines. Although these techniques do not look directly at cell-surface CEA expression they do give some indicator as to the likely level of expression. Again the correlation is good between these techniques and the ELISA (figure 3.vi).

Figure 3.iii: A33-expression of cell lines using GaG ELISA

Each cell line was tested in triplicate and is expressed as the mean (+/- SEM). Cell lines were treated with 6µg/ml of mA33 as the primary antibody. All readings have had the background fluorescence reading subtracted from them.

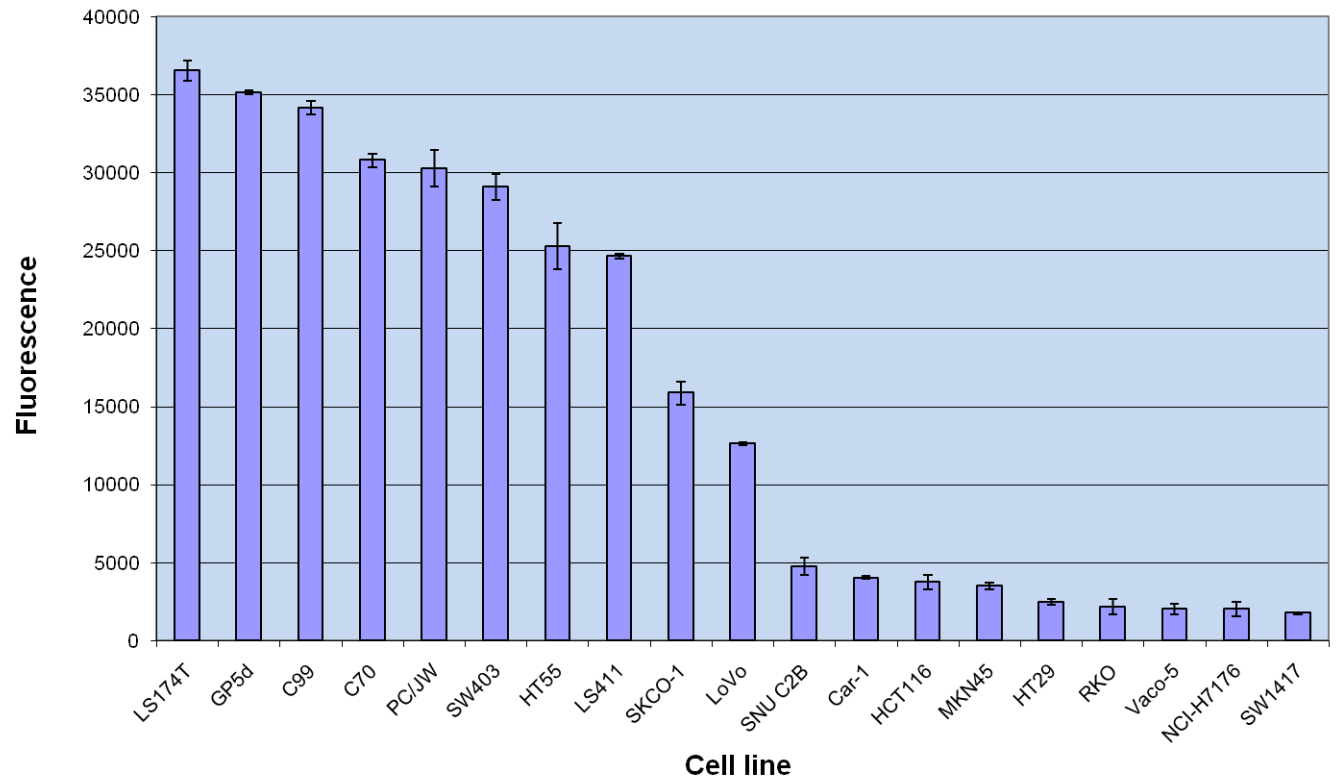


Figure 3.iv: EpCAM-expression of cell lines using GaG ELISA

Each value is the mean (\pm SEM) of 3 separate experiments with each run in triplicate. Cell lines were treated with $1\mu\text{g/ml}$ of AUA-1 (murine anti-EpCAM antibody) as the primary antibody. All readings have had the background fluorescence reading subtracted from them.

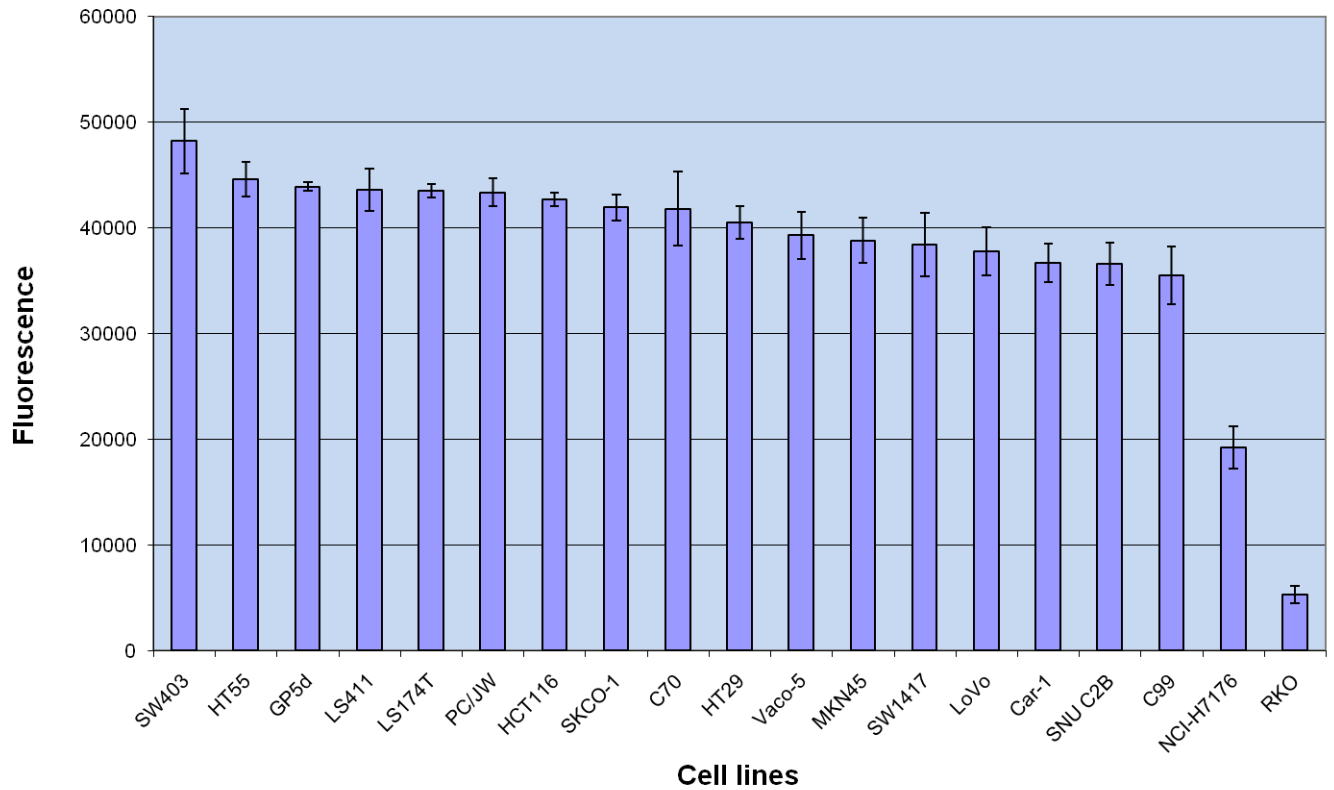
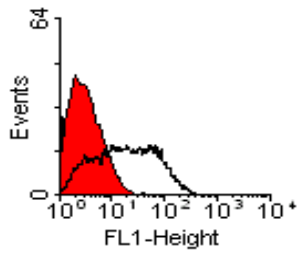


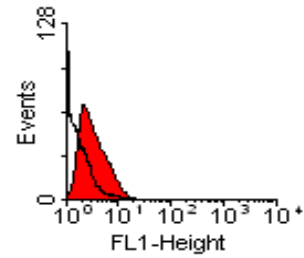
Figure 3.v: FACS analysis of cell lines used in ADCC assays showing levels of CEA expression.

All cell lines were treated with hPR1A3 (or medium control) as the primary antibody followed by FITC-conjugated anti-human IgG. The red-shaded curve is the control (no primary antibody).

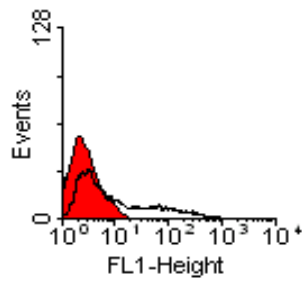
MKN45



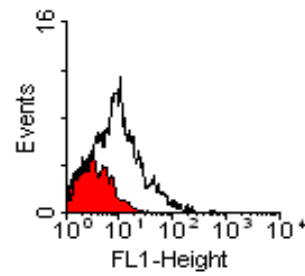
HT29



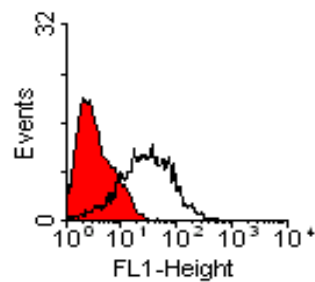
LoVo



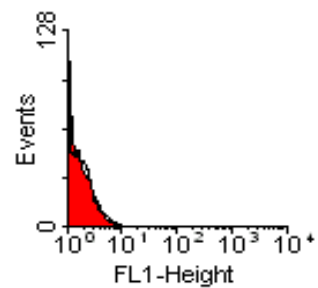
HT55



LS174T



HCT116



3.1.6 Discussion

The GaG ELISA assay was useful in determining the level of CEA expression in the cell lines examined. The data from this was supported by FACS analysis and by work done by others in the laboratory looking at mRNA expression and microarray analysis. From these results the cell lines could be clustered into high, moderate and low expressing cell lines. Hence, there was not a continuous spectrum of expression from the lowest to highest expressing cell lines.

The level of expression was not affected by the mode of extraction of the cells from the tissue culture flask. The standard extraction was by trypsin/EDTA and the measured level of expression was no different when compared with using EDTA alone (data not shown). Although the ELISA analysis was carried out using the parent murine antibody in contrast to the humanised antibody used in the FACS analysis, the two antibodies have been shown to have no difference in binding affinity or binding properties to CEA (Stewart et al., 1999).

The low level of expression in 8 of 17 cell lines examined should not be equated with the level of expression seen on normal tissue. Firstly the “low” level of expression mentioned is based on a comparison to other colorectal cancer cell lines and not to normal tissue. The facts that 90% of colorectal cancer samples bind anti-CEA antibodies (Wagener et al., 1984) and that targeting colorectal tumours with radioimmunoscinigraphy using mPR1A3 has already been successful (Granowska et al., 1993; Granowska et al., 1990), provide evidence that targeting CEA in the *in vivo* situation should be possible. Secondly the level of expression is not the key *in vivo* factor for ADCC, rather it is the aberrant expression that allows tumour cells to be targeted instead of normal tissue, where CEA is restricted to the luminal surface of the cell in the latter. However, the level of CEA expression is still a determinant of ADCC efficacy.

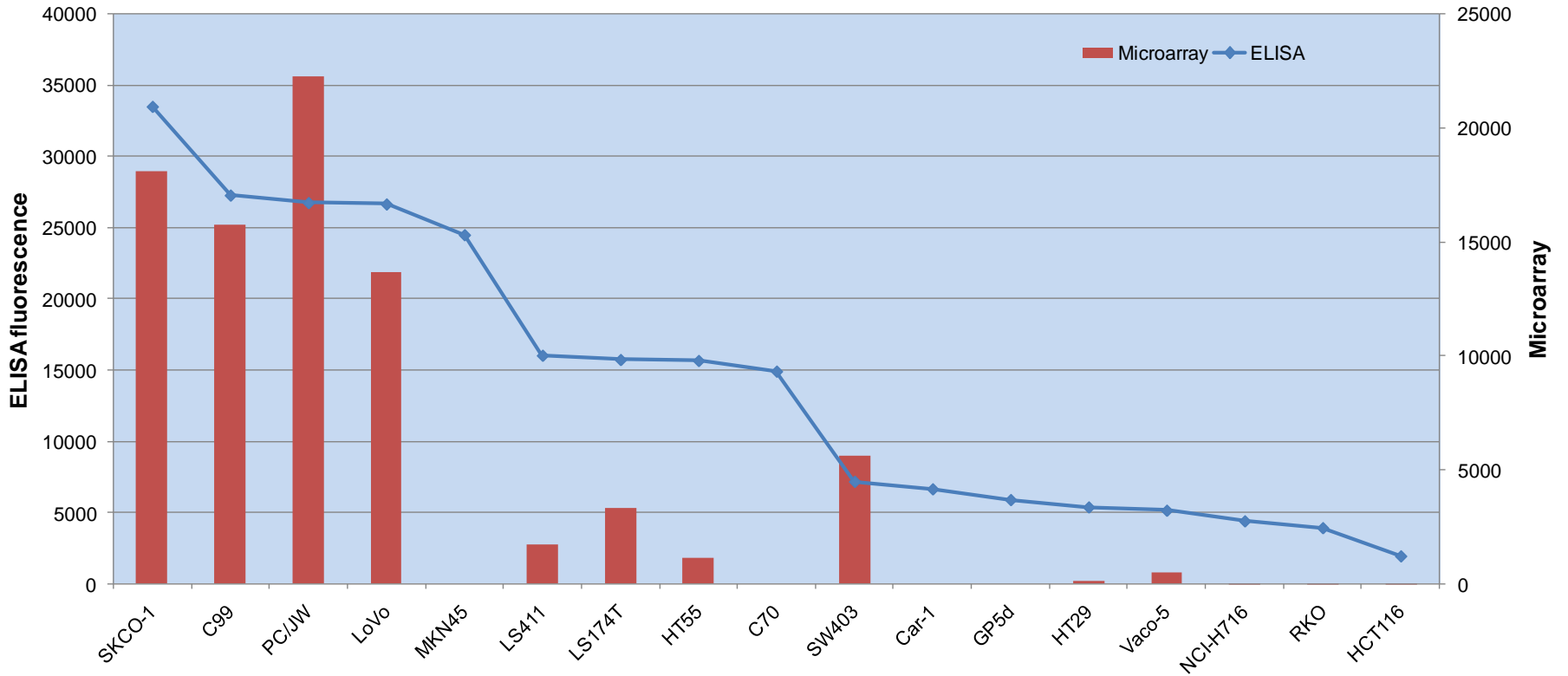
Throughout the ELISA experiments, cells at the edge of the plate showed consistently higher readings than identical wells inside the plate. This led to all experiments being designed with blank wells on the border of the plate which weren't considered for analysis. This ‘edge-effect’ is well described and is believed to be due to temperature or illumination differences between the central and peripheral wells, the former being more common. Despite incubation of the β -galactosidase / 4-methylumbelliferyl-B-D-

galactoside reaction taking place in the dark and all reactant liquids being applied at room temperature, the edge effect persisted.

Figure 3.vi: Summary of CEA expression on cell lines

Composite graph displaying CEA expression on 17 cell lines as determined by a combination of ELISA, microarray, RT-PCR and FACS analysis. MKN45 is a gastric cell line and was not included in the microarray analysis. Where cell lines were not tested by FACS or PCR the table box is left blank.

	SKCO	C99	PCJW	LoVo	MKN	LS411	LS174	HT55	C70	SW403	Car-1	GP5d	HT29	Vaco	NCI	RKO	HCT
PCR	+			+		+		++	++	+		+	+		-	-	-
FACS				++	++		++	+		-		+	-				-



3.2 Effect of cell confluence on CEA expression

3.2.1 Rationale

Factors increasing the expression of the target antigen are obviously of interest as they may naturally improve the efficacy of a therapeutic antibody or may suggest ways in which manipulation of the tumour environment might enable better targeting of the antibody and hence antibody efficacy. Confluence is one such factor which attempts to mimic the *in vivo* situation. Confluence was defined as the point where cell density was at its maximum on the plate and the cells were no longer growing exponentially and had reached a stable number.

3.2.2 Results

Five cell lines were examined representing a range from low- to high-expressing cell lines: C99, LoVo, MKN45, LS174T and HCT116. Cell lines were grown to varying levels of cell density on 75cm² tissue culture flasks before being harvested for the ELISA assay. The 3 levels of cell density examined were: 40-60% of total surface area occupied by the cells; 70-90%; 10-14 days post-confluence. During incubation of cells to post-confluence the medium was replaced every 2 days (chapter 2.5.1.2).

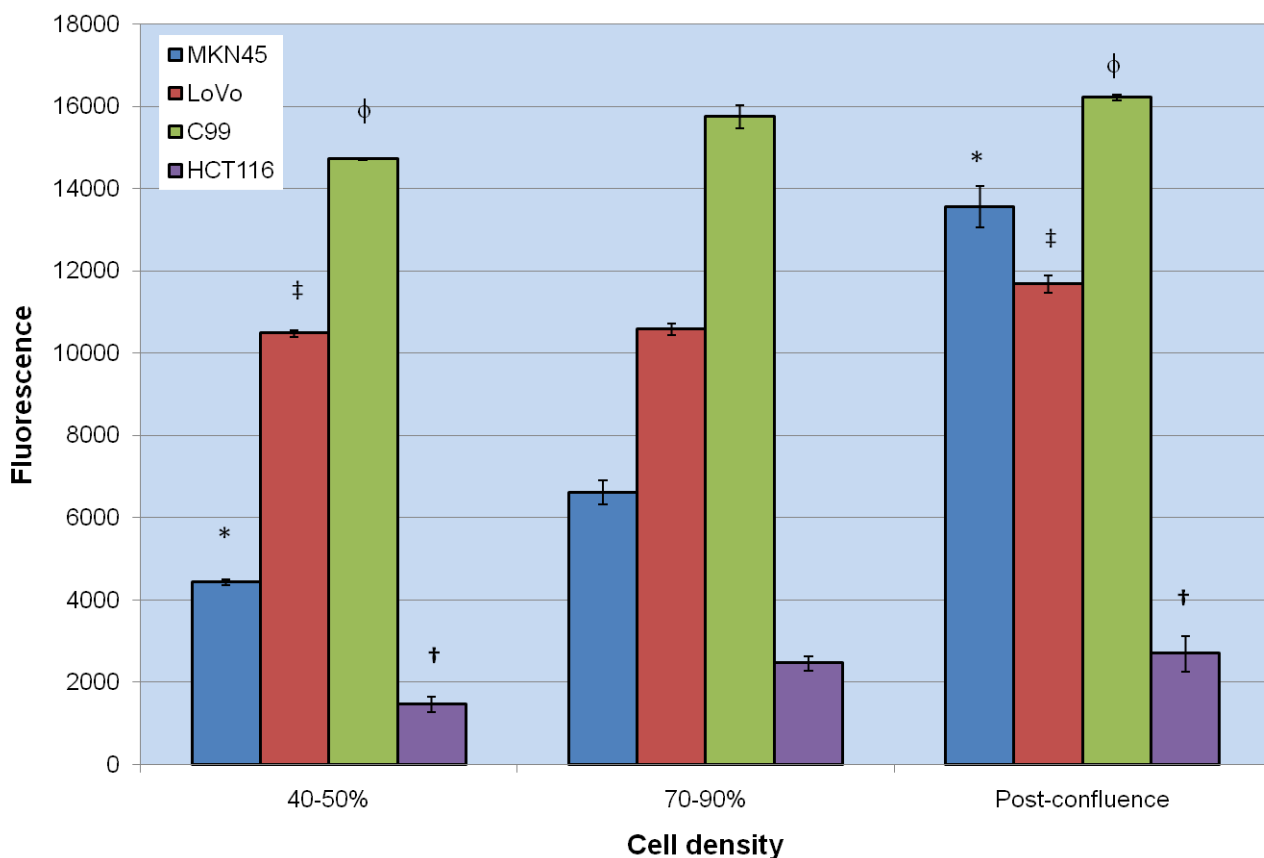
Only MKN45 and possibly HCT116 showed a statistically and clinically relevant rise in CEA expression when grown beyond confluence. These results are shown in figure 3.vii and table 3.ii. Two other cell lines (C99, LoVo) showed a statistically significant rise in CEA expression but the fold increase was so small to be clinically irrelevant. The 4 cell lines showing a statistically significant increase in CEA had varying levels of CEA expression in normal culture and are derived from tumours with varying levels of differentiation.

No similar increase in expression was seen during the same experiments for the A33 antigen (data not shown).

Figure 3.vii: Effect of increasing cell density and growing cells beyond confluence on CEA expression

CEA expression assessed by GaG ELISA. Each value is the mean (+/- SEM) of triplicate wells. All readings have had the background fluorescence reading subtracted from them. The 4 cell lines shown all reached statistical significance. Student t-test calculated significance between 40-50% cell density and post-confluence readings.

* p<0.001; ‡ p=0.03; φ p=0.02; †p=0.01



3.2.3 Discussion

The level of baseline CEA expression does not appear in itself to influence the confluence-induced increase in CEA expression seen above. However, MKN45 was the only cell line to show an obvious increase in expression. There was a moderate 1.8 fold increase in HCT-116 expression but the increase in LoVo and C99 was so small as to be of questionable significance clinically. Previous work (Hauck and Stanners, 1991) has

suggested that growing cancer cell lines to confluence encourages differentiation and that it is this process that drives the increase in CEA expression. However there is no obvious correlation between degree of differentiation of the original tumour and baseline CEA expression of the cell or the confluence-induced increase in CEA expression. From the information available in published work (Bicknell et al., 1994; Brattain et al., 1981; Drewinko et al., 1976; Motoyama, 1979; Tom et al., 1976) and from our own pathology archive in respect of C99, these cell lines encompass degrees of cellular differentiation from poor to well-differentiated tumours. In these experiments LS174T showed no significant change in CEA expression with changing level of confluence whereas MKN45, HCT-116, LoVo and C99 showed an increase in expression following 10-14 days of culture after they had reached confluence on the plate, perhaps mimicking more accurately the situation *in vivo* where tumour cells are tightly packed and compete for resources.

Repetition with a wider cell panel and an examination of reasons behind this variability would be useful.

Table 3.ii: Effect on CEA expression of growing cell lines beyond confluence

	Fold increase in CEA expression	p-value	Baseline CEA expression	Differentiation
MKN45	3.1	<0.001	High	Unknown
LoVo	1.2	0.03	High	Poor
HCT-116	1.8	0.01	Low	Unknown
C99	1.2	0.02	High	Moderate
LS174T	1.3	0.06	Moderate	Moderate

3.3 Effect of 5-fluorouracil (5FU) treatment on CEA expression

3.3.1 Rationale

5-fluorouracil (5FU) is the most commonly used drug in the adjuvant treatment of colorectal cancer (QUASAR-Collab-Gp, 2000). Use of monoclonal antibody therapy in combination with conventional chemotherapy in the adjuvant setting would be a logical introduction to the use of a new monoclonal agent providing there were no contraindications to combination therapy. Thus it would be important to establish that 5FU had no adverse effects on CEA expression. In fact several studies have shown that 5FU can increase CEA expression on colorectal cell lines (Aquino et al., 1998; Correale et al., 2003; Prete et al., 1996) and fresh tumour (Cappelletti et al., 2000). Hence there may be a potential synergistic effect in treatment with PR1A3 and 5FU.

3.3.2 Effect of p53 genotype on effect of 5FU

5FU resistance in colorectal cancer is a significant clinical problem. The tumour suppressor gene p53 is mutated in about 50% of colorectal cancers and studies have suggested that tumours with wild-type p53 are far less likely to show 5FU resistance (Ahnen et al., 1998; Bunz et al., 1999). Hence a comparison was made between the effect of 5FU on cell lines with mutant p53 and those with wild-type p53.

3.3.3 Results

From work done previously in the Cancer and Immunogenetics laboratory it was possible to select cell lines with wild-type p53 (MKN45, C99 and LS174T) and mutant p53 (HT55 and HT29). Cell lines were treated with 1.125mM 5FU dissolved in culture medium or a similar volume of medium alone. They were then harvested and CEA levels assessed by GaG-ELISA (chapter 2.5.1.3).

Four of the five cell lines tested showed an increase in CEA levels following treatment with 5FU compared to the untreated controls but the increase in most cases was small ranging from 1.2-fold to 2.9-fold. The results are summarised in figure 3.viii and table 3.iii. There was no obvious difference in the CEA response to 5FU between cell lines with wild-type p53 and those with mutant p53. The failure of C99 to show a statistically significant increase in CEA expression may be related to the variability between the triplicate wells than a peculiarity of the cell line, although the level of increase was again small.

Four other antibodies were used during these experiments to assess the effect on other cell surface markers:

A33 protein (A33 antibody)

EGF receptor (EGFR-1 antibody)

EpCAM (AUA-1 antibody)

E-cadherin (HECD-1 antibody)

No consistent change in the expression of any of the other cell-surface proteins was noted following treatment with 5FU (data not shown).

3.3.4 Discussion

5-fluorouracil is the primary drug used worldwide in the adjuvant treatment of colorectal cancer, either intravenously or as its oral prodrug, capecitabine. It acts as an analogue of uracil and is converted within cells to active metabolites which are incorporated into RNA and DNA, disrupting their synthesis, as well as inhibiting the nucleotide synthetic enzyme, thymidylate synthase (TS). The reaction catalysed by TS provides the only *de novo* source of thymidylate which is necessary for DNA replication and repair.

The effect of 5FU on CEA expression on colorectal tumours is advantageous for dual therapy with PR1A3, as it increases CEA expression on the cancer cells, allowing targeting by the antibody. The mechanism by which 5FU increases CEA expression is not clear, but from these experiments it does not seem to have a greater effect in cells with a wild-type p53. Possibilities might include the knock-on effects of 5FU action in DNA synthesis (Schuetz et al., 1986) and mRNA splicing (Lenz et al., 1994), causing structural

changes in transcription factors or the CEA promoter region, hence influencing the level of CEA expression.

The overall increase in CEA expression following 5-FU treatment was only modest. This may be a dosing effect and higher concentrations may prove to have better results. Although the upper dosing level of plasma concentrations was used to treat the cell lines it is known that 5-FU can be concentrated many fold in tumour tissue (Schuller et al., 2000).

Figure 3.viii: Effect of 5-fluorouracil on CEA expression using GaG-ELISA

CEA expression assessed by GaG ELISA. Each value is the mean (+/- SEM) of triplicate wells. All readings have had the background fluorescence reading subtracted from them. The increase seen in C99 did not reach statistical significance.

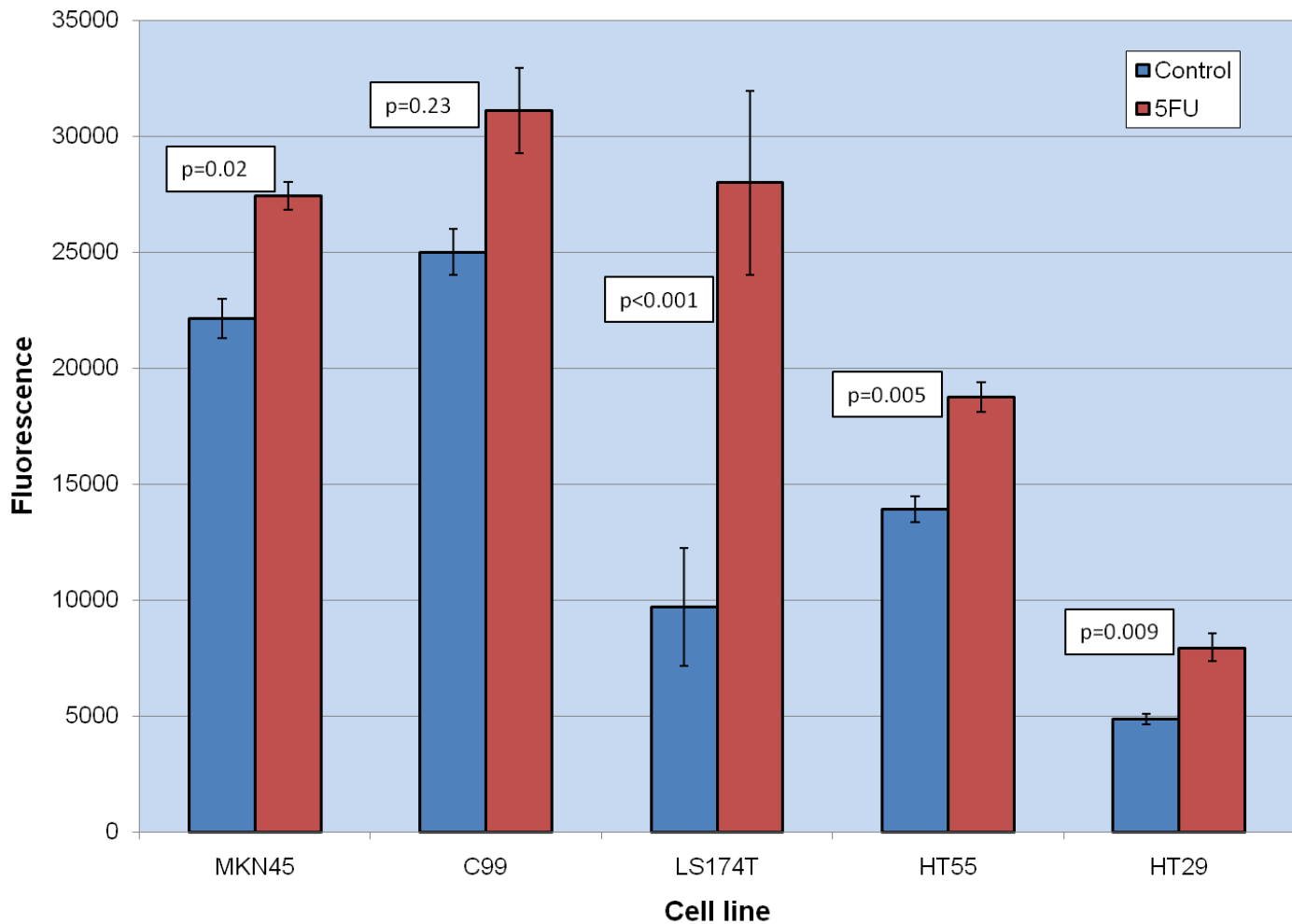


Table 3.iii: Effect of 5FU treatment on CEA expression

	Fold increase in CEA expression	p-value	p53 status	Baseline CEA expression
MKN45	1.20	0.02	Wild-type	High
C99	1.24	0.23	Wild-type	High
LS174T	2.88	<0.001	Wild-type	Moderate
HT55	1.35	0.005	Mutant	Moderate
HT29	1.64	0.009	Mutant	Low

There is conflicting information in the literature as to whether the p53-status of colorectal tumours influences firstly the response to adjuvant treatment and secondly the chances of both tumour recurrence and overall survival (Allegra et al., 2003; Liang et al., 2002; Paradiso et al., 2000; Russo et al., 2005; Westra et al., 2005). One of the major factors causing 5FU resistance is the over-expression of TS (Peters et al., 2002), the enzyme inhibited by 5FU. The wild-type p53 protein is believed to exert an inhibitory effect on the promoter of TS (Lee et al., 1997) and dysregulation of this control in p53 mutants leads to the over-expression of TS which contributes to 5FU resistance. However the control of TS levels within the cell involves a number of other regulatory genes which may be one of the reasons for the conflict in outcomes when comparing the clinical success of 5FU in tumours of differing p53 status. In addition, the various mutations in p53 make p53 mutants as a group functionally heterogeneous (Iacopetta et al., 2006) which may help explain the conflicting data with respect to 5-FU treatment.

Further experiments are required and LS174T would be a good candidate for such ADCC assays.

3.4 Effect of interferon-gamma (IFN- γ) on CEA-expression

3.4.1 Rationale

The cytokine IFN- γ is a well-researched activator of the immune response. It is secreted by and activates a variety of immune-effector cells, including those involved in ADCC. For this reason it has been used as an adjunct to immunological treatments in the treatment of colorectal cancer (Schwartzberg et al., 2002). Given that one advantage of antibody-based cancer therapy is the potential for a cascade of the immune response once cancer cells are lysed, enhancing that cascade by IFN- γ may also prove useful in therapy with PR1A3. In addition, previous reports have suggested that IFN- γ can increase expression of CEA on colorectal cell lines (Guadagni et al., 1990; Hauck and Stanners, 1991). Moreover there have been reports that it may act synergistically with 5FU in its ability to increase CEA expression on colorectal cell lines (Aquino et al., 1998) and, hence combined with the 5FU, the commonest drug used for adjuvant therapy in colorectal cancer, IFN- γ may further increase the available target population for anti-CEA therapy such as PR1A3.

3.4.2 Results

HT55 and LS174T, both moderate CEA expressors, were treated with different concentrations of IFN- γ or fresh medium as a control, with exposure times between 6 hours and 4 days (Aquino et al., 1998; Fichera et al., 1998) (chapter 2.5.1.4). The level of CEA assessed using PR1A3, and the level of MHC class II assessed using the DA2 antibody (as a positive control) were measured. MHC class II was used as a positive control as it is known to be upregulated by IFN- γ (Manyak et al., 1988). The plates were stored until all cells had been harvested and the various plates were analysed together.

Unusually high background readings were obtained for both cell lines at all time points and concentrations, and no difference was noted either in the level of CEA expression or in the level of class II expression (data not shown).

3.4.3 Discussion

Given the unusually high background fluorescence it is possible that this disguised any difference in fluorescence between the wells. The fact that the DA2 antibody did not detect any increase in class II expression suggests that these results are not reliable. Other workers have clearly shown an increase in class II expression on epithelial cancer cells after exposure to IFN- γ (Donnellan et al., 1995; Glimcher and Kara, 1992; Smith et al., 1989) within both the time frame and dose of IFN- γ used in these experiments. The increase in levels of IFN- γ at the site of an inflammatory response to a tumour would further enhance the effect of the PR1A3 antibody if IFN- γ , as well as activating other cells of the immune response, increased the expression of CEA on the tumour cells.

CHAPTER 4

Antibody-dependent cellular cytotoxicity (ADCC) of cancer cell lines using PR1A3

This chapter demonstrates the efficacy of PR1A3 as a potential therapeutic antibody in the treatment of colorectal cancer. Antibodies are reported to act via several distinct mechanisms *in vitro*, of which ADCC is of major importance. It will also show that PR1A3 does not work by blocking the function of CEA on the target cell, a mechanism which has been suggested for other anti-CEA antibodies (Taheri et al., 2003).

The humanised PR1A3 was used with human peripheral blood mononuclear cells (PBMC) against cell lines with varying levels of CEA expression as targets. Both chromium-release (chapter 2.4.1) and fluorescence-based assays (chapter 2.4.2) were used to assess the efficacy of the antibody in causing ADCC.

4.1 Optimisation of labelling with ^{51}Cr and BATDA

4.1.1 Rationale

In order to maximise the sensitivity of the labelled ADCC assays, it was necessary to maximise the amount of label taken up by the different cell lines. A panel of cell lines used within these ADCC experiments – SKCO-1, MKN45, LS174T, PC-JW, HT55, HT29, HCT-116 – were labelled for varying times with BATDA at different temperatures. Some of these cell lines were also used in the earlier Cr-release experiments and were similarly optimised for uptake with ^{51}Cr . The design of these experiments is given in chapters 2.4.1.2 and 2.4.2.2.

4.1.2 Results

Cell lines were compared following labelling with BATDA at various temperatures and for different time periods. Cells were analysed both straight after labelling and following a 2-hour incubation at 37°C to mimic the conditions of the ADCC assay. Good correlation between pre- and post-incubation results was noted in terms of which conditions provided the optimal uptake characteristics, and so later labelling optimisation looked only at the post-incubation differences. These experiments, however, were not cytotoxicity assays. They contained neither antibody nor effector cells. Instead they looked at the ratio between the maximum amount of label contained by the cells, measured by sampling the supernatant following lysis of the cells, and the spontaneous release of label from the cells during incubation, measured by sampling the supernatant above unlysed cells. Results are shown in tables 4.i & 4ii.

In the ⁵¹Cr-release assay, MKN45 had previously been optimised for 60mins incubation at 37°C and these results were not repeated. PC-JW was also optimised for the Cr-release assay using different time-points to determine the optimal labelling time (table 4.iii and figure 4.i).

4.1.3 Discussion

Without optimisation of labelling, the initial experiments comparing the effect of PR1A3 on different cell lines suffered from certain cell lines having very poor uptake or retention of label. This led to the difference between spontaneous release (amount of label in the supernatant of a suspension of a cell line) and maximal release (amount of label in the supernatant following lysis of all the cells in the suspension) being small, making it difficult to detect differences in the various treatments with any degree of confidence.

BATDA is a hydrophobic ester of the fluorescence-enhancing ligand terpyridine dicarboxylic acid (TDA). The hydrophobic BATDA crosses the cell membrane freely where, within the cell, the ester bonds are cleaved to produce the hydrophilic TDA which can no longer passively cross back out of the intact cell.

Tables 4.i-4.iii: Optimal BATDA labelling times of cell lines at different temperatures.

The numbers represent the fold increase in labelling from spontaneous release to maximal release (maximal release/spontaneous release). Results from optimal times are in bold.

i. Labelling of MKN45

Labelling time (mins)	Temperature		
	4°C	18°C	37°C
10	2.46	3.32	3.78
15			4.02
20	2.05	3.31	3.77
25			3.54
30	2.91	2.77	3.29

ii. Labelling of other cell lines (time chosen for experiments in bold)

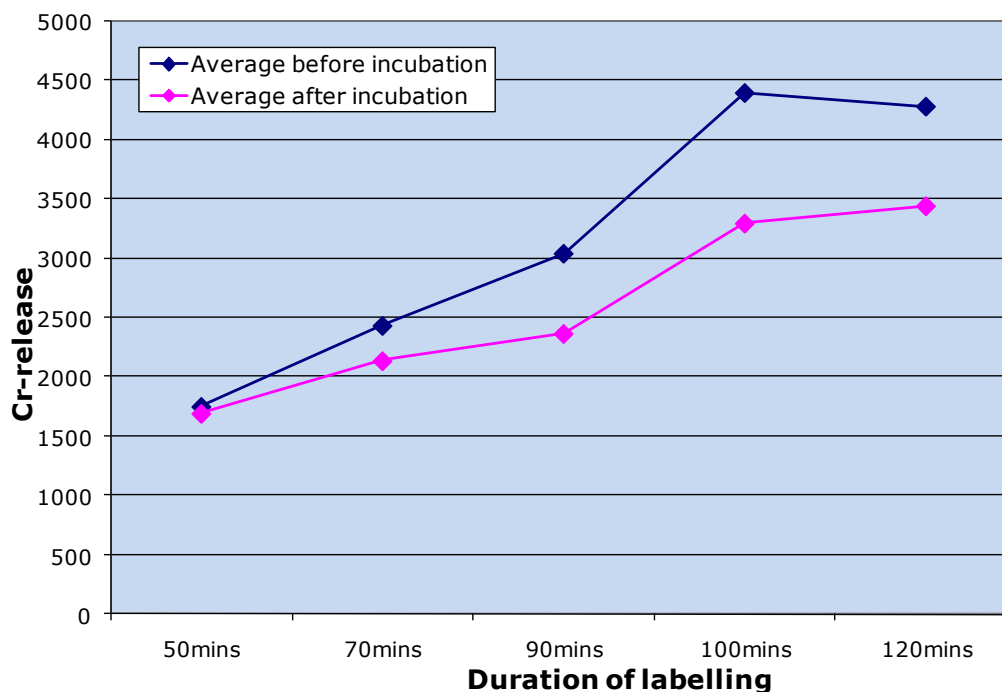
Labelling time (min)	HT29	HT55	HCT116	PC-JW	C99	SKCO-1
5	1.30		2.59	2.05		
10		2.50	3.59		2.28	2.86
15	2.00	2.27	3.64	2.96		
20	1.84	2.64	2.72		2.89	3.70
25	1.67			3.23		
30		3.20			2.59	3.82

(iii) Cr-release optimal labelling time for PC-JW

Incubation times (min)	Fold increase (max/spontaneous release)
50	2.50
70	3.43
90	3.84
100	6.16
120	2.74

Figure 4.i: Optimal labelling time of PC-JW cell line using ^{51}Cr

All points represent the mean of triplicate wells. The level of Cr-release shown is the result of maximum release (following cell lysis with detergent) minus the background release. The greatest difference between background release and maximum lysis (table 4.iii) was seen at 100 minutes both for measurements taken before incubation and after incubation.



There is a degree of variability amongst the cell lines in the retention of the BATDA label which leads to some cell lines having high levels of background release. The difference between background and maximal release ranges from between 2 and 4 fold in the cell lines we studied. There is no published information on why this occurs. One possibility is the over-expression of the MDR1 gene in some colorectal cell lines, which may provide a means of expelling the TDA label via the P-glycoprotein 170 pump.

4.2 Effect of humanised PR1A3 (hPR1A3) on the high CEA-expressing cell line MKN45

4.2.1 Rationale

The first step was to establish whether hPR1A3 could cause ADCC in human cancer cell lines. The antibody was used against the high CEA-expressing cell line, MKN45, in the presence of human PBMCs. Initially this was carried out using chromium-release assays and then later with a fluorescence-based technique using BADTA.

Due to the variability seen with labelling discussed in chapter 4.1.3, an attempt was made to develop an ADCC assay which would allow the specificity of measuring only epithelial cell lysis without the need to label the target cells. One possibility was to look at cytokeratin 18 (CK18), an epithelial-specific intermediate filament protein which is cleaved during the early stages of apoptosis by caspases 3,7 and 9 (Caulin et al., 1997; Ku et al., 1997; MacFarlane et al., 2000). During necrosis, the cytosolic pool of uncleaved CK18 is released from the cell, but following caspase-cleavage in apoptosis, CK18 is cleaved at Asp396 creating a neo-epitope which is detected by the commercially-available M30 antibody (with the CK18 cleavage product called the M30 antigen). The amount of M30 antigen can be measured in the supernatant by the M30 antibody by means of an ELISA. This technique was developed for screening chemotherapy agents for apoptotic activity in the sera of patients with cancer (Hagg et al., 2002) but seemed potentially suitable for assessing the level of apoptosis in an unlabelled ADCC assay (chapter 2.4.3).

4.2.2 Results

4.2.2.1 Chromium-release assay

In the absence of antibody, the PBMCs carried out a low level of spontaneous killing of the tumour cell line. The addition of hPR1A3 increased the level of killing. This effect was dose-dependent, with increasing concentrations of antibody causing increased killing.

Figure 4.ii shows combined results for 8 separate experiments involving the MKN45 cell line using the Cr-release assay.

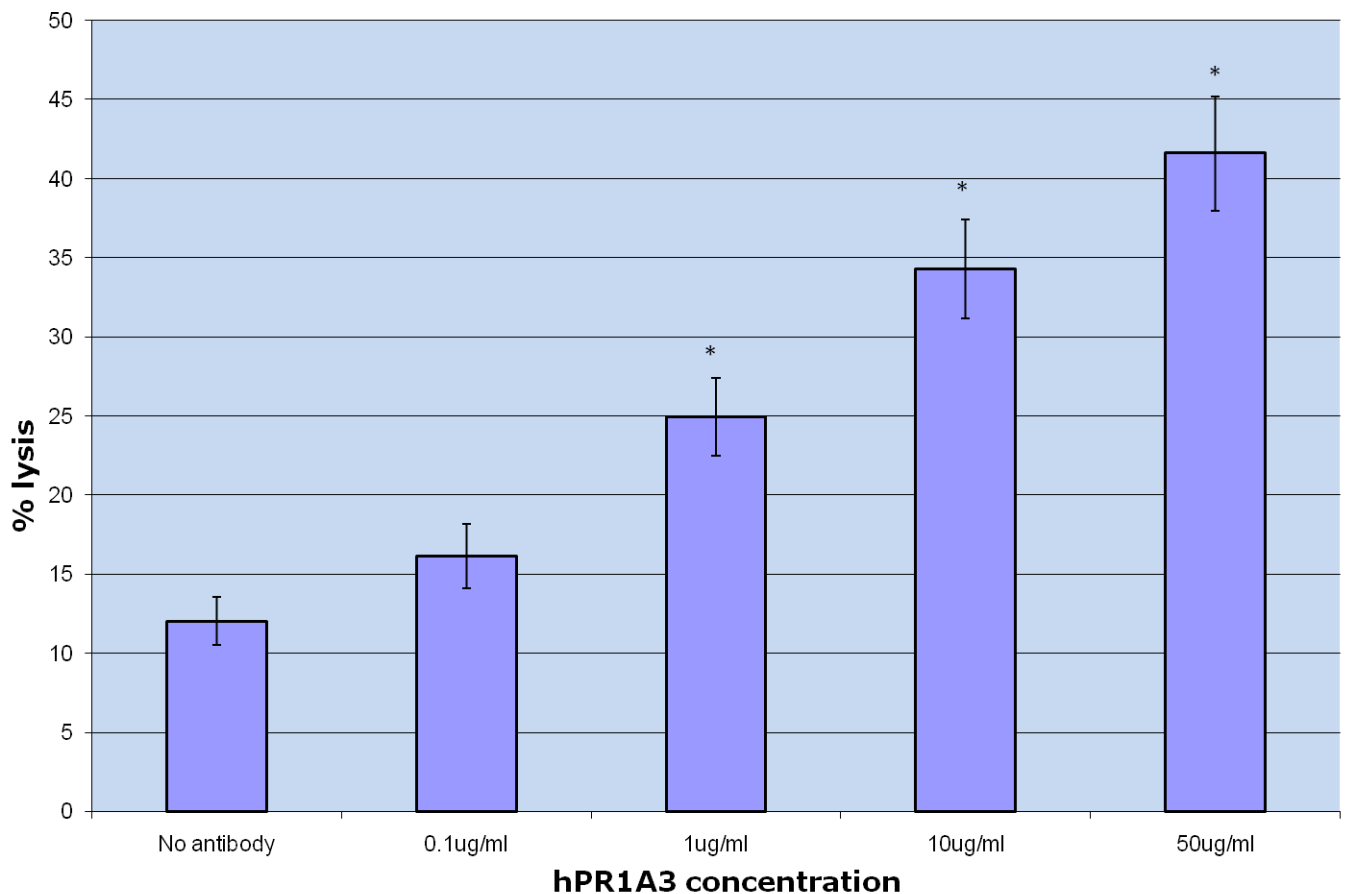
Figure 4.ii: Effect of increasing concentrations of hPR1A3 on killing of MKN45 cells in the presence of human PBMCs using ^{51}Cr assay.

Combined results of 8 separate experiments using the chromium-release assay

Cr-release ADCC assay using MKN45 as CEA-expressing target cell and human PBMC as effectors at ratio of 1:100. All columns represent wells containing target and effector cells with some containing varying concentrations of PR1A3 monoclonal antibody as shown.

Analysis from 8 separate experiments with each experiment using triplicate wells for each antibody concentration tested. Error bars indicate standard error of the mean (SEM). All significance testing was on comparison with the wells containing no antibody.

*p<0.001



4.2.2.2 BATDA assay

Similar results were obtained using the TDA-based assay as were seen for the Cr-release assay above in 4.2.2.1. Figure 4.iii shows combined results for 16 separate experiments involving the MKN45 cell line using the fluorescence assay.

4.2.2.3 M30 assay

The M30 assay gave similar results to the other 2 assays with an increase in cell lysis seen with increasing hPR1A3 concentration (figure 4.iv). However there was wide variability within the triplicates which would counsel caution in any interpretation of the results and this is reflected in only the higher two concentrations reaching significance when compared with the level of spontaneous killing.

4.2.2.4 Overall results for ADCC assays using MKN45 and hPR1A3

In all assays used, hPR1A3 caused a consistent increase in cell lysis using MKN45 in the presence of human PBMC. Using 50µg/ml in the standard assays, this increase was consistently more than three-fold above the level of spontaneous killing seen when effector cells are incubated with MKN45 without any antibody present.

There was no significant difference in results if hPR1A3 was pre-incubated with the cells before addition of the white cells (figure 4.v) and so this was not routinely carried out.

Figure 4.iii: Effect of increasing concentrations of hPR1A3 on killing of MKN45 cells in the presence of human PBMCs using EuTDA assay.

Combined results of 16 separate experiments using the fluorescence-based assay.

EuTDA ADCC assay using MKN45 as CEA-expressing target cell and human PBMC as effectors at ratio of 1:100. All columns represent wells containing target and effector cells with some containing varying concentrations of PR1A3 monoclonal antibody as shown.

Analysis from 16 separate experiments with each experiment using triplicate wells for each antibody concentration tested. Error bars indicate standard error of the mean (SEM). All significance testing was on comparison with the wells containing no antibody.

*p<0.001

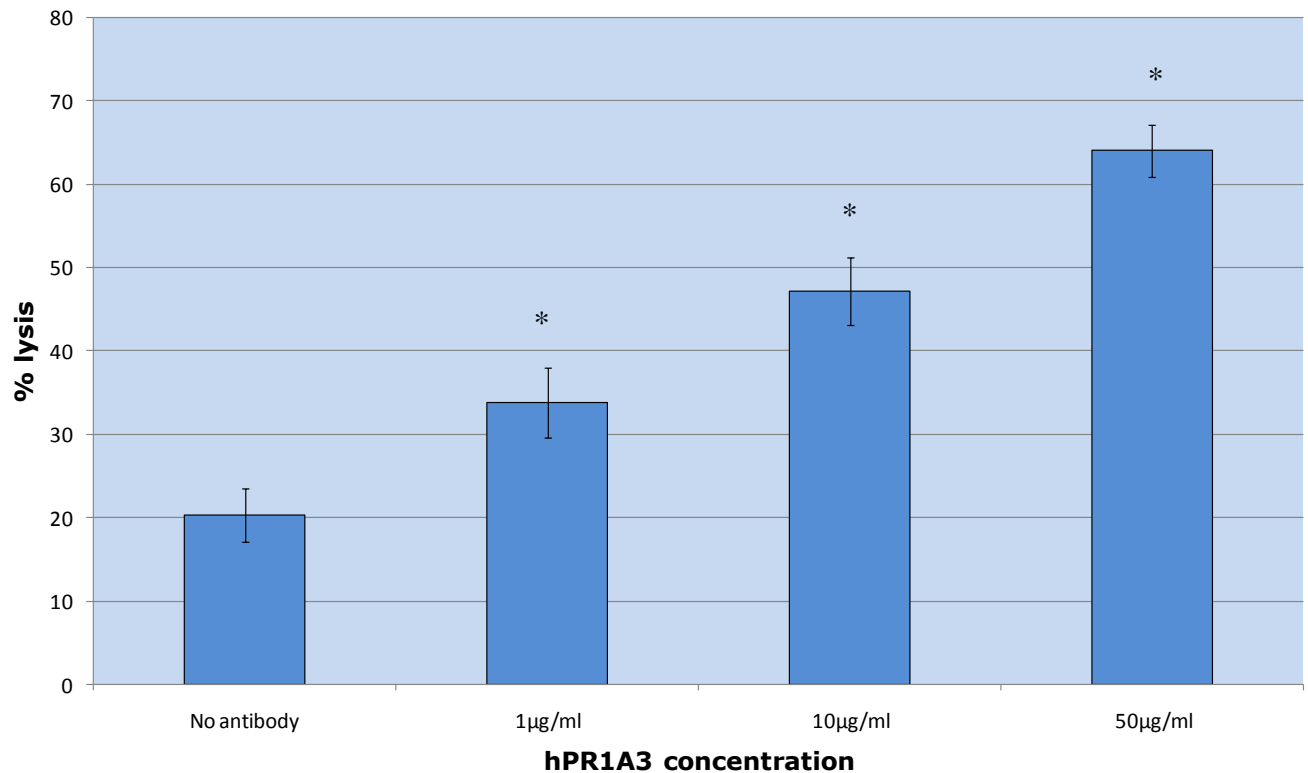


Figure 4.iv: Effect of increasing concentrations of hPR1A3 on killing of MKN45 cells in the presence of human PBMCs using a non-labelling technique; the M30 assay.

M30 assay using MKN45 as CEA-expressing target cell and human PBMC as effectors at ratio of 1:100. All columns represent wells containing target and effector cells with some containing varying concentrations of PR1A3 monoclonal antibody as shown.

Each antibody concentration tested was carried out in triplicate. Error bars indicate standard error of the mean (SEM). All significance testing was on comparison with the wells containing no antibody.

M5 antibody used for cytokeratin 18 capture from culture supernatants and then probed with HRP-labelled M30 antibody which was detected with TMB. Absorbance read at 450nm and converted to [M30] by way of measuring absorbance of standards of known M30 concentration.

*p=0.01

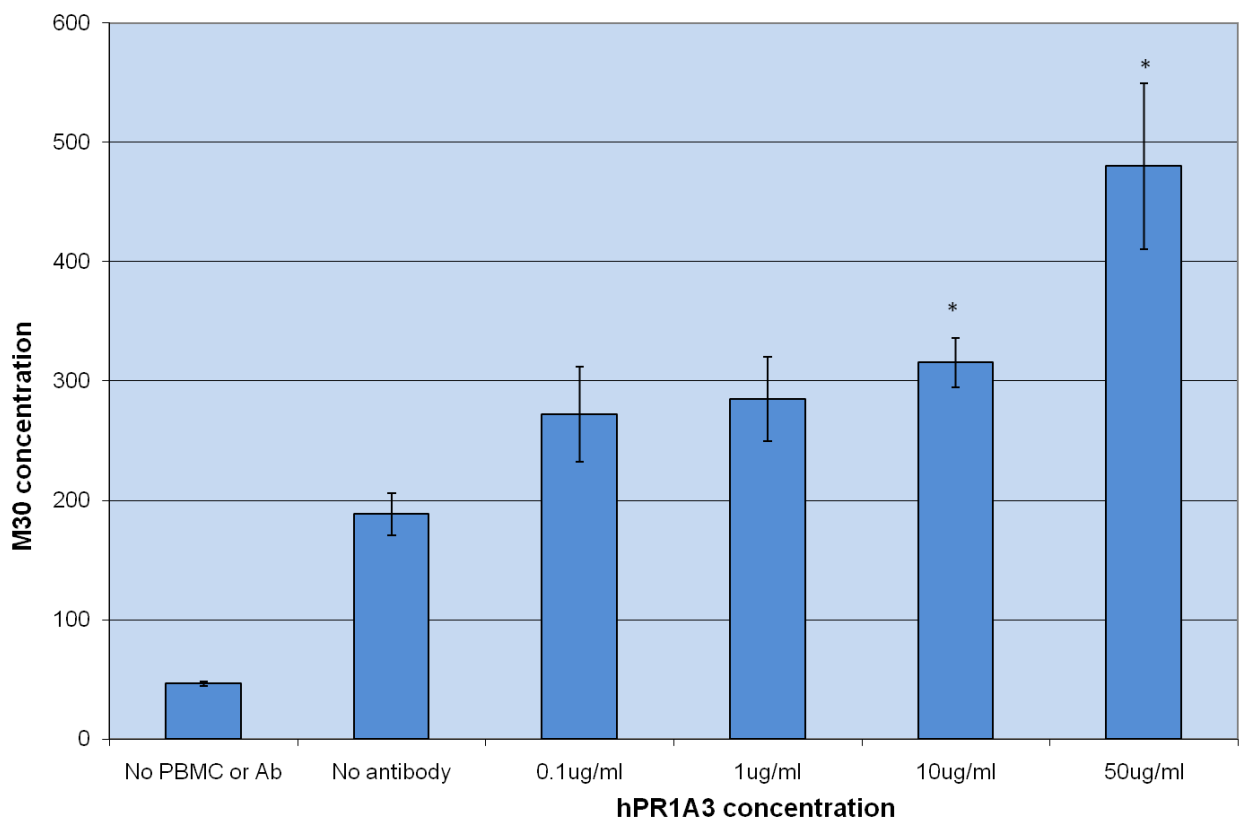
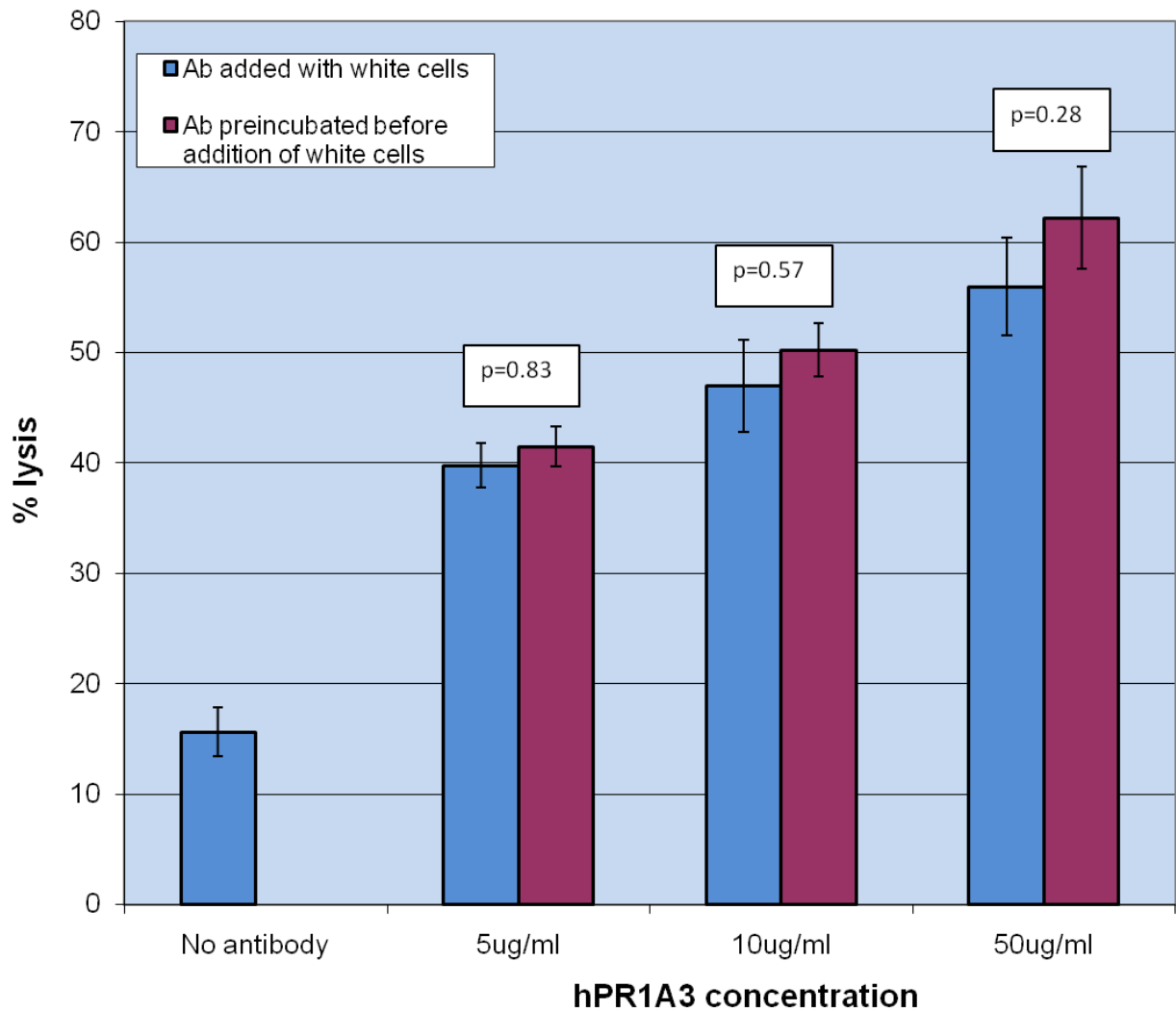


Figure 4.v: Comparison of preincubated antibody with standard technique in ADCC assay

EuTDA ADCC assay using MKN45 as CEA-expressing target cell and human PBMC as effectors at ratio of 1:100. All columns represent wells containing target and effector cells with some containing varying concentrations of PR1A3 monoclonal antibody as shown.

hPR1A3 was either incubated at 4°C for 20 minutes with MKN45 cells before the addition of the PBMCs (purple) or added to the target cells at the same time as the PBMC (blue). Each antibody concentration tested was carried out in triplicate. Error bars indicate standard error of the mean (SEM). Significance testing was between each pair using the same antibody concentration but with or without pre-incubation of the antibody. No difference between the pairs reached statistical significance.



4.2.3 Discussion

This result establishes that hPR1A3 enhances the killing of colorectal cancer cell lines in the presence of PBMC. The likeliest mechanism of action is via ADCC. There is no human serum in the assay and thus no complement to effect complement-dependent cytotoxicity. There is a level of spontaneous killing of MKN45 in the presence of the effector PBMCs alone as would be expected from mixing PBMCs, which include cytotoxic T-cells and NK cells, with epithelial cells from a different donor which thus would not be recognised as self. That the epithelial cells are neoplastic may serve to increase the level of spontaneous killing. The presentation of foreign antigens to the lymphocyte population would lead to an immune response against the tumour cells. In addition the cytotoxic activity of NK cells is regulated by the integration of stimulatory and inhibitory signals received from the surrounding environment. Many of the inhibitory receptors on NK cells recognise class I MHC which provides a further 'self' recognition mechanism to prevent indiscriminate NK killing. Downregulation of MHC class I as a means of avoiding recognition by cytotoxic T-cells would, however, make the tumour susceptible to NK detection. Some colorectal cancers and cancer cell lines (eg GP5d, LoVo) are known not to express class I MHC (Bicknell et al., 1994) due to a β 2-microglobulin mutation. This may leave them susceptible to NK-mediated lysis. None of the cell lines in this part of the study had such a known mutation.

The addition of hPR1A3 caused lysis above that experienced for spontaneous killing alone. This increase occurred in a dose-dependent manner up to the maximum dose given (50 μ g/ml). Hence hPR1A3 is shown to be an antibody with cytotoxic potential against human colorectal cancer cell lines.

The ^{51}Cr -release assay is the most widely used ADCC assay. It uses the gamma-emitter ^{51}Cr which has a physical half life of 28 days. Its kinetics and biodistribution within the cell are not well understood. It crosses the cell membrane through an ion channel, possibly an active process (Lilien et al., 1970), and once within the cell is not released. This is likely due to its incorporation into cytoplasmic proteins and likely also into nuclear proteins and DNA.

The EuTDA-based ADCC assay runs for 2 hours, a shorter time period than the corresponding 4 hour Cr-release assay. Running the assay for longer leads to higher level of spontaneous killing by the effector cells (ie effector cells lysing target cells in the absence of antibody). This may be partly the result of increased cell fragility having been incubated for longer periods with a BATDA reagent which is dissolved in DMSO. However the same problem with label extrusion discussed above is probably the major factor in limiting the incubation time. Despite this, an antibody-dependent and dose-dependent response was still observed.

The M30 assay allowed a return to the longer incubation time of 4 hours and it again showed a dose-dependent lysis of CEA-positive cells with hPR1A3. The assay was modified to minimise cellular debris within the supernatant used in the ELISA as this gave falsely high readings in all wells. Multiple washing and spinning of the sample gave more realistic readings but prolonged the extraction of a fragile peptide and may explain some of the variability in the triplicate wells and the low level of lysis. However the 4 hour incubation period may also allow degradation of this fragile peptide, as those cells killed early in the assay would release their CK18 hours before the peptide was extracted. It remains to be seen whether this will be a useful assay for ADCC.

It is interesting to note that the lysis caused by spontaneous killing seemed to be in a large part due to apoptosis, as given by the M30 reading. It is possible that a large part of the spontaneous component is due to NK cell detection and lysis of tumour cells as suggested above.

4.3 Effect of hPR1A3 on the low CEA-expressing cell line HCT116

4.3.1 Rationale

To ensure that the antibody was acting by specifically targeting CEA on the surface of the cancer cells in an antibody-antigen interaction and not by an epitope-independent mechanism, hPR1A3 was also used in ADCC assays with a cell line expressing only low levels of CEA. HCT-116, shown by ELISA, microarray and FACS to be either CEA negative or a very low CEA expressor, was used for this purpose.

4.3.2 Results

The fluorescence-based ADCC (EuTDA) assay was used, labelling HCT-116 cells with BATDA. MKN45 cells were used as a positive control and human PBMCs were used as effector cells with both cell lines. Whilst lysis of MKN45 cells followed the expected pattern seen in chapter 4.2, HCT-116 showed no evidence of antibody-mediated lysis. Spontaneous killing occurred as before in the absence of antibody (figure 4.vi).

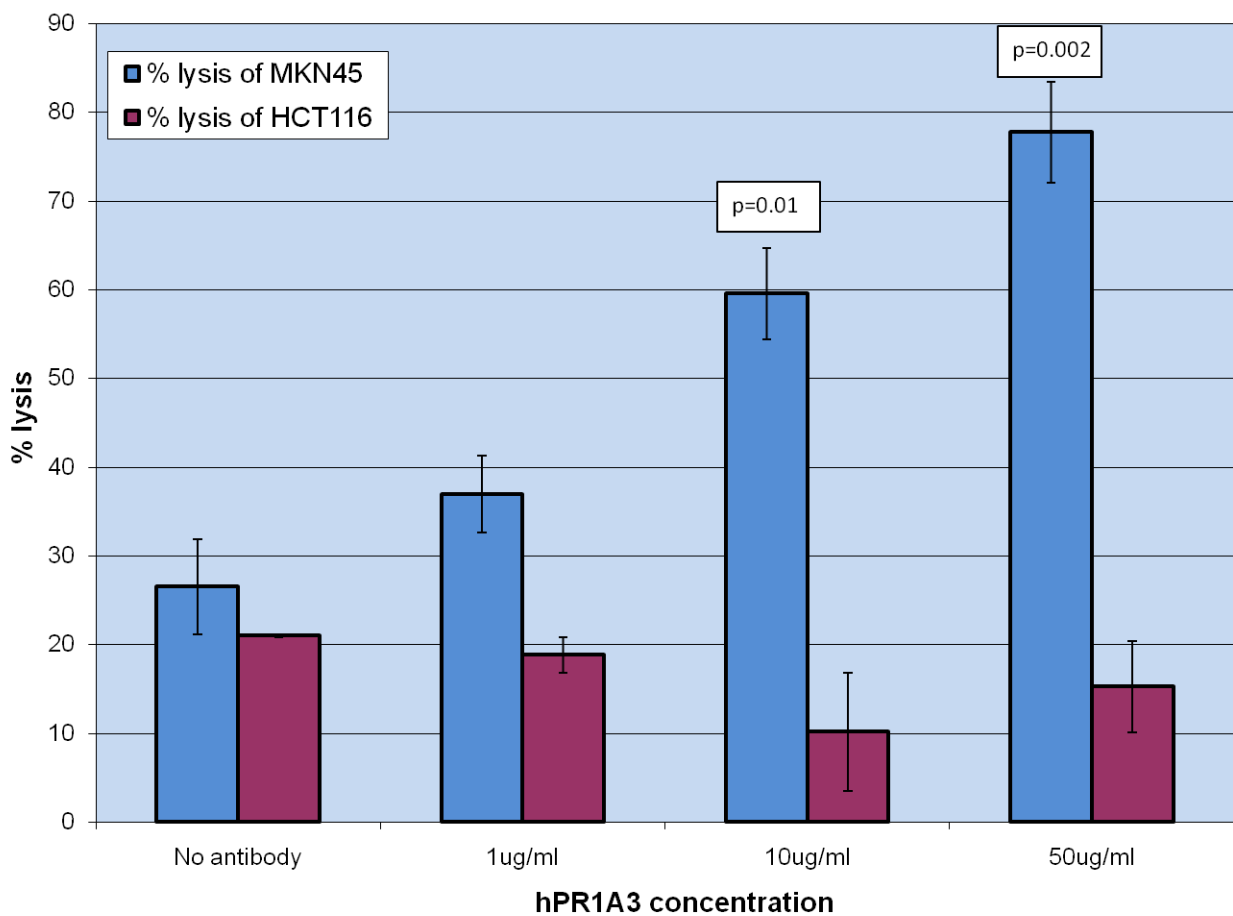
4.3.3 Discussion

The ability of hPR1A3 to cause killing of the CEA-positive MKN45 cell line but not of the CEA-negative HCT-116 cell line suggests that the antibody is acting by an epitope-specific mechanism by binding to CEA on the cell surface. CEA is expressed by virtually all CRC (Wagener et al., 1984) whereas about 5 of 30 cell lines tested by microarray were found to be CEA negative. The level of CEA needed to make a cancer susceptible to anti-CEA immunotherapy through ADCC is not known although PR1A3 has already been used in humans as a radioimmunoscentigraphic guide for localizing metastatic deposits of CRC (Granowska et al., 1993) and shows strong immunohistochemical binding irrespective of the degree of differentiation of the tumour (Richman and Bodmer, 1987). Hence PR1A3 would be expected to bind to the large majority of CRCs and with this would come the potential for ADCC

Figure 4.vi: Effect of increasing concentrations of hPR1A3 on high (MKN45) and low (HCT116) CEA-expressing cell lines

EuTDA ADCC assay using MKN45 as CEA-expressing target cell and human PBMC as effectors at ratio of 1:100. All columns represent wells containing target and effector cells with some containing varying concentrations of PR1A3 monoclonal antibody as shown.

Each antibody concentration tested was carried out in triplicate. Error bars indicate standard error of the mean (SEM). All significance testing was on comparison with the wells containing no antibody for the respective cell line with significant results ($p < 0.05$) shown.



4.4 Comparison of the effect of hPR1A3 on cell lines with different levels of CEA expression

4.4.1 Cell lines used

Further cell lines, encompassing a range of CEA cell-surface expression, were tested in the EuTDA ADCC assay with hPR1A3 and human PBMCs to ensure that these individual results were translatable to a variety of different cell lines. SKCO-1 was also tested with a range of effector : target ratios.

4.4.2 Results

SKCO-1, PC-JW, MKN45 (high expressors); LS174T, HT55 (moderate expressor); HT29 & HCT116 (low expressors) were compared for their vulnerability to lysis by PBMCs in the presence of hPR1A3. Results are summarised in figure 4.vii. The response of each cell line to treatment with hPR1A3 was approximately proportionate to the expression of CEA on the cell surface except for HT55 which seemed resistant to treatment with PR1A3 despite being a moderate expressor of CEA. This is discussed below.

In the second experiment using SKCO-1, lysis of this cell line was affected by the effector : target ratio as would be expected. The higher the effector : target ratio, the greater the level of cell lysis (figure 4.viii).

Figure 4.vii: Comparison of PR1A3-induced lysis in cell lines with different levels of ELISA CEA expression (shown in parentheses), expressed as % of the highest expressing cell line, SKCO-1.

Results from EuTDA-based ADCC assay using human PBMCs as effectors at ratio of 100:1 with the various target cell lines. Spontaneous killing levels have been subtracted to reflect antibody specific lysis only so that a more accurate comparison can be made. Each experiment carried out in triplicate.

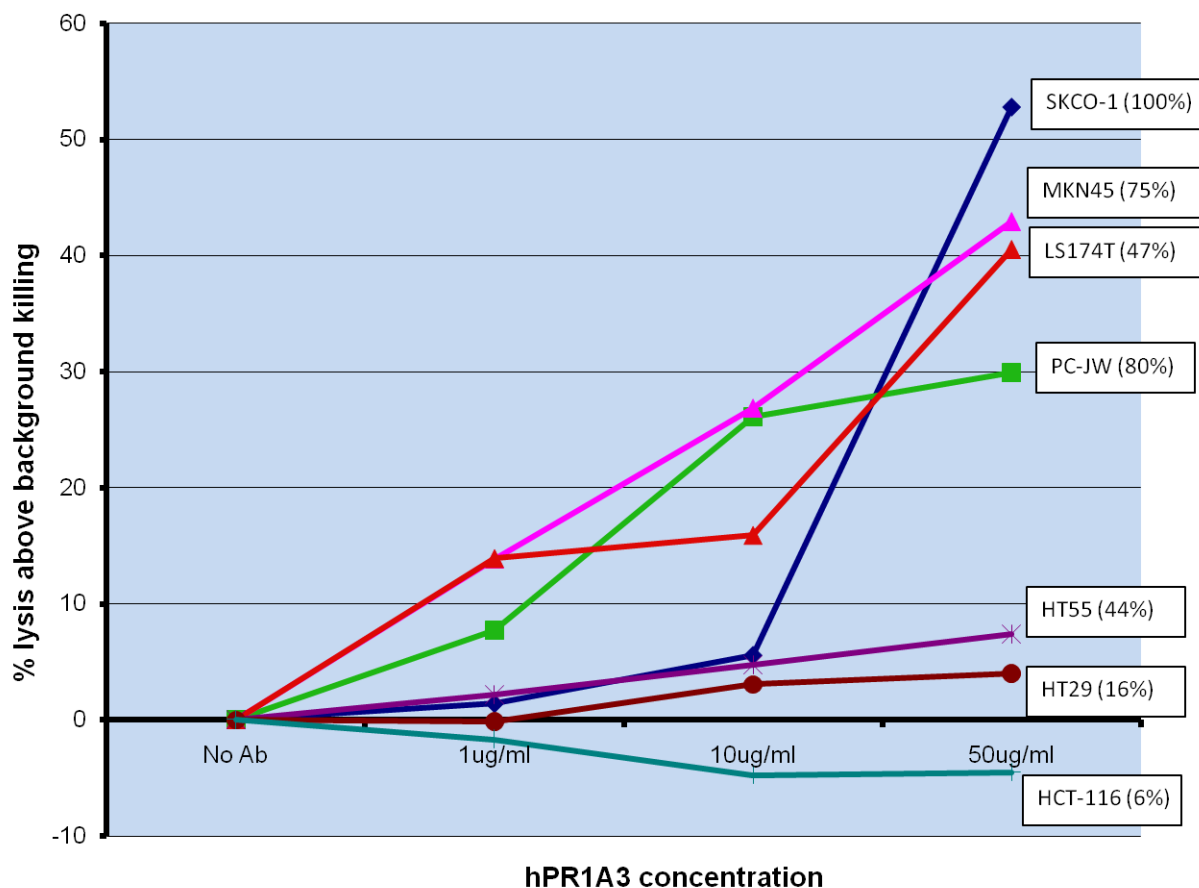
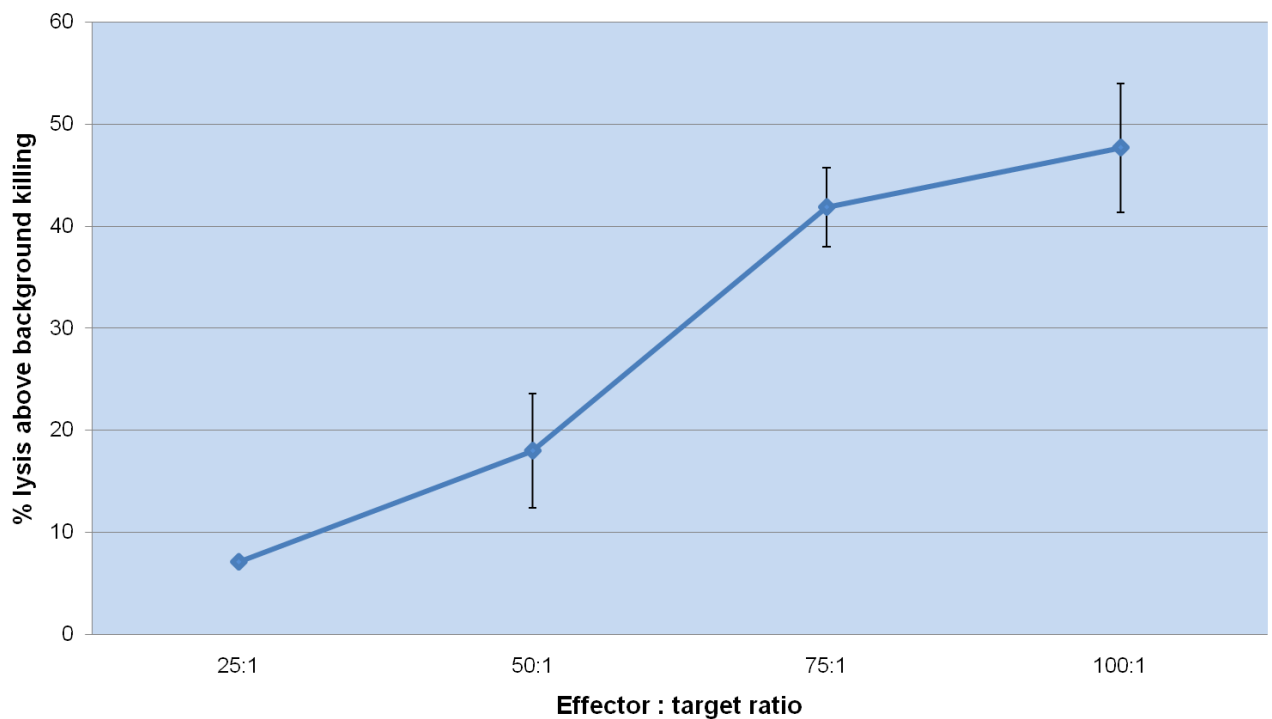


Figure 4.viii: The effect of increasing effector : target cell ratio on the level of target cell lysis in the presence of 50µg/ml hPR1A3.

EuTDA ADCC assay using SKCO-1 as CEA-expressing target cell and human PBMC as effectors at effector : target ratios varying from 25:1 to 100:1. All columns represent wells containing 50µg/ml hPR1A3 in addition to the target and effector cells.

Each effector : target ratio tested was carried out in triplicate. Error bars indicate standard error of the mean (SEM).

Spontaneous killing levels have been subtracted to reflect antibody specific lysis only.



4.4.3 Discussion

The conclusions of chapters 4.2 and 4.3 were tested in a wider panel of cell lines with a range of levels of CEA expression. These conclusions seem applicable to other colorectal cell lines, with hPR1A3 causing a dose-dependent level of cancer cell lysis in those lines with moderate-high CEA cell surface expression but not in those with low levels of

expression. Percentage lysis was not directly proportional to CEA expression however and this was expected given that the level of antigen expression required to induce ADCC is relatively low compared with other reported mechanisms of antibody action such as receptor blockade (Bleeker et al., 2004).

There were certain anomalies which would be prudent to look at again by repeating these experiments. The unresponsiveness of SKCO-1 to lysis by lower concentrations of antibody may simply be experimental error. It did show the highest level of lysis with 50µg/ml of antibody in keeping with its status as the highest CEA expressor, and it is likely that this reflects its true vulnerability to lysis by hPR1A3. The moderate CEA-expressing cell line HT55 does not seem as vulnerable to hPR1A3 as would be expected from its ELISA CEA expression. It is interesting to note that a discrepancy exists between the ELISA and microarray data for this cell line with the latter suggesting it to be a low CEA expressor. However, the ELISA data are more likely to be a true reflection of CEA cell-surface expression given that the microarray looks at gene expression rather than protein and, in addition, the ELISA also agrees with the FACS analysis which also looks at cell-surface expression. It would be interesting to look further at whether this resistance to PR1A3 is replicated with changes in culture conditions (eg PR1A3 incubation) and, if this resistance is consistent, whether it also shows resistance to other antibodies and to look at the mechanisms behind such resistance. It is interesting to note that HT55 also showed some resistance to the effect of an antibody against hepatocyte growth factor (a growth factor regulating cell growth, cell motility, and morphogenesis in epithelial and endothelial cells) despite the same antibody inhibiting cell motility in other colorectal cell lines (Jiang et al., 1993).

The overall level of cell lysis was slightly disappointing given that certain other therapeutic antibodies in the clinical arena such as trastuzumab, cetuximab and rituximab showed comparable levels of lysis at much lower concentrations of antibody (Carter et al., 1992; Kawaguchi et al., 2007; Kurai et al., 2007; Reff et al., 1994) . This leads to questions about the affinity of the antibody for its target, the condition or lack of preconditioning of the effector cells, and also the duration of the assay:

1) *Antibody affinity*. As discussed in the introduction (chapter 1.6.1.2) there is an optimum binding affinity for antibodies working by ADCC *in vivo*. However this does not translate to the *in vitro* ADCC assay where cells are in solution and the constraints of a binding site barrier, hypovascularisation or any other physiological barriers to antibody penetration do not apply. As a result, a discrepancy between optimum antibody concentration for efficacy *in vitro* and that *in vivo* may be understandable. In these experimental conditions, higher binding affinities generally lead to higher levels of cell lysis. As mentioned in chapter 1.5.1, two binding affinities are quoted for PR1A3 (Stewart et al., 1999), one of higher affinity (1nM) and the other at lower affinity (60nM). These relate to different binding sites for the antibody on the gastric carcinoma cell line MKN45. It may be, of course, that the lower affinity binding site predominates *in vivo* and that affinity maturation is required for optimal ADCC. Although some authorities quote a binding affinity of 1nM as optimal for ADCC, there is a considerable range (0.08-32nM) amongst the antibodies in clinical use (Carter, 2006).

2) *Preconditioning of PBMCs*. Many studies have used cytokine-stimulated PBMCs in ADCC assays (Carter et al., 1992; Reff et al., 1994). This causes both increased spontaneous killing but also increased antibody-specific lysis of cells. Interestingly however, although all NK cells express an intermediate-affinity IL-2 receptor (IL2R $\beta\gamma$), only the CD56^{bright} subset, which have a low concentration of the CD16 receptor mediating ADCC, constitutively express the high affinity IL-2 receptor (IL2 $\alpha\beta\gamma$) (Caligiuri et al., 1990). So although IL-2 may not directly activate the CD16^{bright} subset of NK cells, it may do so through the activation of CD56^{bright} NK cells, which then release cytokines such as gamma-interferon (IFN- γ) (Cooper et al., 2001b) resulting in an immune cascade and the proliferation and activation of CD16^{bright} cells. This thought is backed up by evidence demonstrating enhanced ADCC following IL-2 stimulation of NK cells (Kawaguchi et al., 2007). Hence this partially explains why the killing in these experiments is not as high as that seen in other studies.

3) *Duration of the assay*. The comparison between cell lines was carried out using the EuTDA assay. As mentioned above (chapter 4.2.3) this fluorescence-based assay ran for

half the time of a standard Cr-release ADCC assay (2hrs vs 4hrs) and this will, of course, have had a significant effect on the percentage of cells lysed during the assay.

4.5 Effect of hPR1A3 in the absence of effector cells

4.5.1 Introduction

The literature relating to the mechanism of naked antibody-based cancer therapy concentrates predominantly on antibodies binding to cell-surface receptors to cause a block of function, as proposed for cetuximab (Baselga, 2001) and trastuzumab (Baselga and Albanell, 2001; Lewis et al., 1993). To test this potential mechanism of action for PR1A3, the antibody was incubated with various cell lines in the absence of PBMCs to test for any direct cytotoxicity for the targets using PR1A3. The same conditions were used as for other ADCC assays using both Cr-release and BATDA assays.

4.5.2 Results

PR1A3 alone caused no direct cytotoxicity to the cell lines, irrespective of their level of CEA expression. The average of the MKN45 experiments is given in table 4.iv using 10µg/ml hPR1A3. Higher doses of antibody had no impact on the results in other experiments and similar results were seen using C70, PC-JW, LoVo and HCT-116 (table 4.v)

Table 4.iv: Effect of hPR1A3 on MKN45 in the absence of effector cells

ADCC assay involving MKN45 cells in the presence of hPR1A3 or PBMC or both. Average of 14 experiments.

	MKN45 + 10µg/ml hPR1A3	MKN45 + hPBMC	MKN45 + PBMC + 10µg/ml hPR1A3
% lysis of MKN45	0.15	16.47	41.01
SE mean	0.71	2.18	3.86

Table 4.v: Effect of hPR1A3 on colorectal cell lines in the absence of effector cells

		10µg/ml hPR1A3 but no PBMC	PBMC but no antibody	t-test
% lysis (+/- SEM)	C70	-1.9 (+/- 0.6)	7.9 (+/- 2.2)	p=0.008
	PC-JW	1.1 (+/- 1.0)	37.3 (+/- 3.5)	p=0.003
	LoVo	-1.8 (+/- 0.7)	19.8 (+/- 1.1)	p<0.001
	HCT-116	5 (+/- 3.1)	21.0 (+/- 0.2)	p=0.007

4.5.3 Discussion

The mechanism of action of hPR1A3 is likely to be by ADCC. Although a member of the Ig superfamily, CEA is linked to the cell membrane by a glycoposphatidylinositol (GPI) anchor (Thompson et al., 1991) and thus has no direct intracellular signaling motif. The function of CEA in both normal and colorectal cancer is not clear and the functional significance, if any, of its overexpression in colorectal cancer remains uncertain. Consequently it would seem unlikely that hPR1A3 binding to CEA could disrupt any vital cellular function. This is borne out by the results shown here which demonstrate no increase in tumour cell lysis in the presence of hPR1A3 except when PBMCs are also present.

However, these results are carried out with antibody incubated with the cell lines in 2 or 4 hour assays and an effect over a longer period of incubation cannot be excluded although this is unlikely. In addition these assays look only at cell lysis and do not take into account any effect that the antibody may have had on other tumourogenic factors such as tumour growth and blocking differentiation. In antibodies reporting these modes of action *in vitro*, the assays often involve incubation with the cell line over days (Huang et al., 1999; Petit et al., 1997).

4.6 Comparison of NK cells alone versus PBMC in ADCC assay with hPR1A3

4.6.1 Rationale

Natural killer cells are believed to play a primary role in ADCC *in vivo* (Clynes et al., 2000; Kawaguchi et al., 2007) and hence would be important in antibody-based cancer therapy. To assess whether NK cells can independently reproduce ADCC in cell lines which are consistently affected by the unselected PBMC population, it is necessary to isolate NK cells from the PBMC population. It would also provide further evidence that hPR1A3 acts by ADCC *in vitro* and demonstrate the contribution of NK cells to the spontaneous killing seen in the absence of antibody. Negative selection was used to avoid stimulation of the NK cells during the selection process (chapter 2.2.2). Since NK cells make up only about 4% of the white cell population, it is necessary to use a buffy coat or leucodepletion filter (LDFs) to achieve a reasonable harvest of NK cells after recovery.

4.6.2 Results

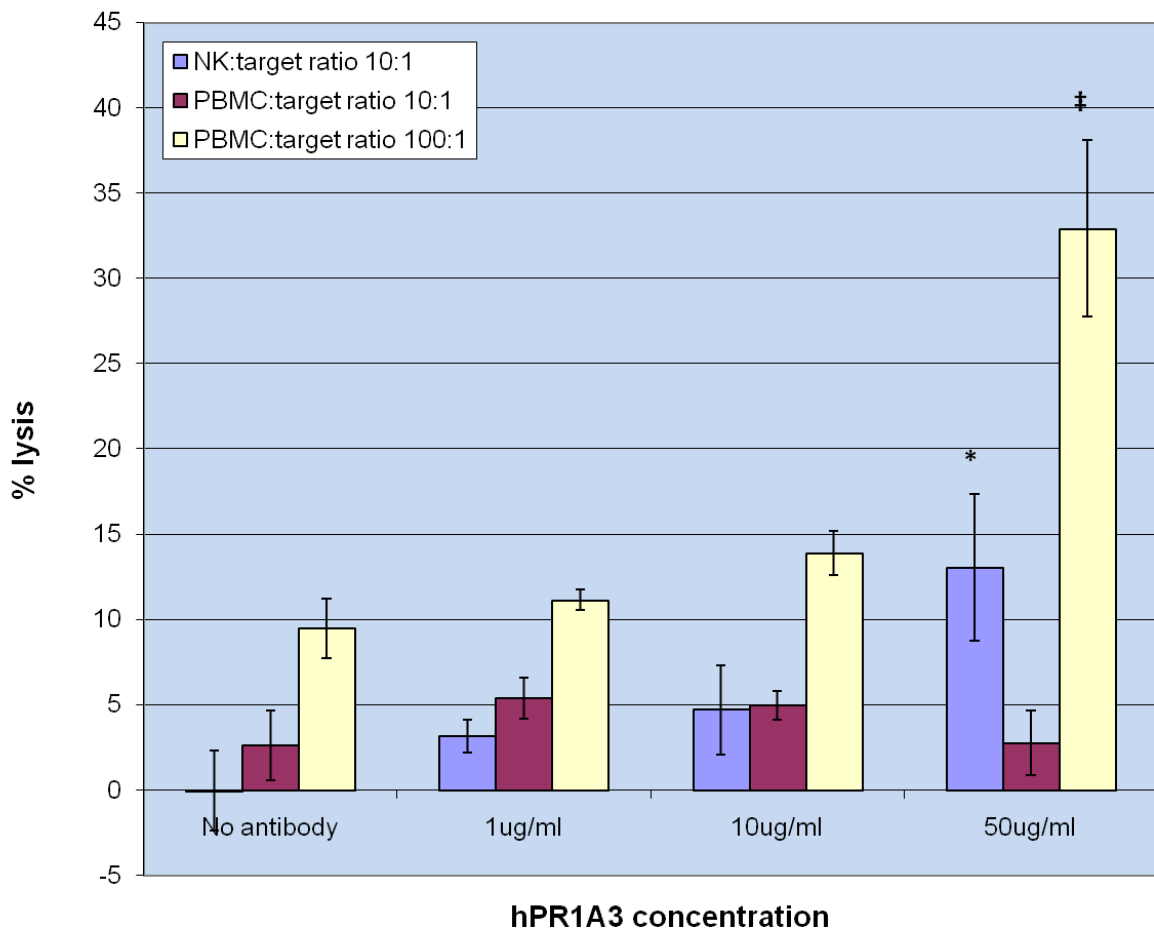
The harvest of cells following the negative selection was small (1.5×10^6 cells) and hence no characterisation of the recovered cells was possible since they were all used in the assay. The results shown in figure 4.ix must therefore be interpreted with caution, as the fold enrichment of NK cells was not determined. The results do show a difference between the selected cells and unselected cells at a ratio of 10 effectors : 1 target in that there was no obvious killing seen in any of the wells from the unselected population. However the level of killing from the wells using the NK-selected effector cells was poor.

Figure 4.ix: Effect of increasing concentrations of hPR1A3 on killing of MKN45 cells in the presence of NK cells alone or PBMCs at varying target : effector ratios.

EuTDA ADCC assay using MKN45 as CEA-expressing target cell and human NK-cell fraction or whole PBMC (from the same donor) as effectors at ratio of 1:10 and 1:100 as shown. All columns represent wells containing target and effector cells with some containing varying concentrations of PR1A3 monoclonal antibody as shown.

Each antibody concentration tested was carried out in triplicate. Error bars indicate standard error of the mean (SEM). All significance testing was on comparison with the respective wells containing no antibody.

* p=0.056; ‡ p=0.04



4.6.3 Discussion

The yield of NK cells from this technique was poor. From 40mls of blood eluted from an LDF filter, one could expect about 8×10^6 NK cells and recovery is expected to be at least 50% of this figure. However, recovery in this experiment yielded only 1.5×10^6 . The reasons for this are as yet unknown and may simply be experimental error. This prevented any analysis of the recovered population making the interpretation of the results difficult. However it is likely that the recovered population were at least NK-rich and may explain the small differences seen between the unselected and selected PBMC populations at the 10:1 effector : target ratio.

The quality of the cells from the LDF was less consistent than those from fresh blood. LDF blood was taken the day before at a nearby national blood service and then screened for pathogens before being separated into constituent fractions. This process meant that blood was only available the next morning, often about 24 hours after donation. Clearly this experiment needs to be repeated both to gain a greater yield of NK cells to enable characterisation of the recovered population and to be sure of the validity of the results.

The reason a negative selection method was used to isolate the NK population was in an attempt to avoid unrecognised activation or other stimulation of the recovered population.

CHAPTER 5

The importance of the Fc γ III A receptor and the antibody Fc-fragment in the action of PR1A3

An effective anti-tumour response or antibody-based cancer therapy requires not only recognition of the tumour by cellular or humoral components of the immune system or exogenous antibody, but also interaction of these with functioning effector cells. Failure at any one of these levels can lead to susceptibility to tumour growth or viable metastases and a failure to respond to antibody-based cancer therapy. However these interactions provide possibilities to enhance the antibody response. One of these important interactions is the coupling of the Fc-portion of the antibody to the Fc γ III A on the effector cell.

5.1 Effect of murine PR1A3 (mPR1A3) on the MKN45 cell line

5.1.1 Introduction

It has previously been shown that murine IgG2a is the major antibody for effecting ADCC responses in the mouse and that IgG2a binds with high affinity to human Fc γ receptors (Lubeck et al., 1985). However murine IgG1 binds to the human Fc γ receptors with an affinity 100-1000 times lower than the human IgG1 (Lubeck et al., 1985) and hence is poor at inducing ADCC responses from human effector cells. The binding affinity of murine and human PR1A3 to CEA, however, is the same (Stewart et al., 1999). Hence substituting the Fc segment of hPR1A3 for a murine IgG1 κ Fc segment would significantly diminish the interaction of the antibody with the Fc γ receptors on the human effector cells, and would thus determine whether ADCC was an important mechanism in the action of PR1A3.

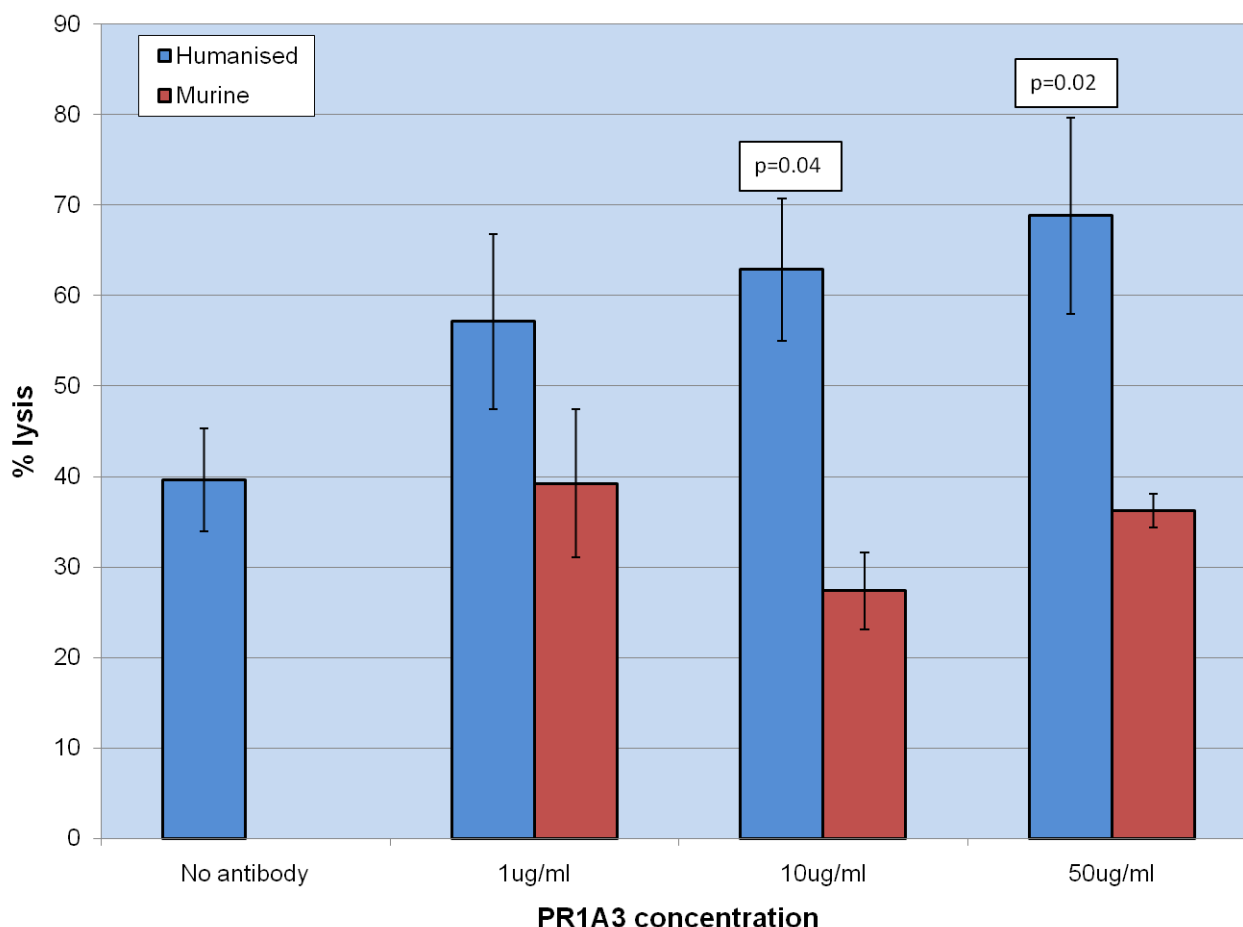
5.1.2 Comparison of mPR1A3 with hPR1A3

Whilst hPR1A3 caused dose-dependent lysis of the MKN45 cell line in the presence of human PBMCs, the murine Ab used at identical doses showed no ADCC. The results are summarised in figure 5.i.

Figure 5.i: Comparison of ADCC elicited by murine and humanised PR1A3.

EuTDA ADCC assay using MKN45 as CEA-expressing target cell and human PBMC as effectors at ratio of 1:100. All columns represent wells containing target and effector cells with some containing varying concentrations of either murine or humanised IgG1 PR1A3 monoclonal antibody as shown.

Each antibody concentration tested was carried out in triplicate. Error bars indicate standard error of the mean (SEM). All significance testing was on comparison between the wells containing murine and humanised antibody of the same concentration with significant results ($p < 0.05$) shown.



5.1.3 Discussion

Murine IgG1 is ineffective at eliciting ADCC from human PBMCs against CEA-positive cancer cells, despite equivalent affinity for its target. This is presumably because of the known poor interaction between the Fc portion of the murine IgG1 antibody and the human Fc receptors. This would add support to the hypothesis that PR1A3 has its effect *in vitro* by ADCC.

5.2 Effect of an antibody against the Fc γ IIIA (CD16) receptor on PR1A3-induced lysis

5.2.1 Rationale

Having demonstrated the importance of the antibody Fc portion in chapter 5.1, we then sought to disrupt the Fc-Fc γ receptor interaction by targeting the Fc γ IIIA receptor (CD16A) in order to further test the hypothesis that PR1A3 acts by ADCC. This was achieved by using anti-CD16 antibody as whole antibody and Fab fragments.

5.2.2 Effect of whole antibody of anti-CD16

In the standard fluorescence-based ADCC assay, various concentrations of a monoclonal antibody to CD16 (MEM-154 clone) were added to wells containing target and effector cells with or without 10 μ g/ml hPR1A3. Results are shown in figure 5.ii.

The hypothesis was that blocking the CD16 receptors on the effector cells would inhibit antibody-mediated killing but would not affect the spontaneous killing by the effector cells in the absence of antibody. Interestingly however, the data show that the anti-CD16 antibody blocked not only PR1A3-induced killing but also spontaneous killing.

To ensure that this result with the anti-CD16 antibody was not a clone-specific effect, an anti-CD16 from a second clone (3G8 clone) was used with identical results (figure 5.iii). To ensure also that this was a direct effect caused by blockade of the CD16 receptor and not a consequence of adding a second antibody to compete for Fc-receptor sites, a non-specific antibody against prostate specific membrane antigen (PSMA) was added. As expected, this did not affect the ability of PR1A3 to induce ADCC nor indeed of PBMCs to effect spontaneous killing of the target cells (figure 5.iii).

Figure: 5.ii: Effect of varying doses of anti-CD16 antibody (MEM154 clone) on Ab-dependent and Ab-independent lysis of CEA-positive target cells.

EuTDA ADCC assay using MKN45 as CEA-expressing target cell and human PBMC as effectors at ratio of 1:100. All columns represent wells containing target and effector cells with some containing varying concentrations of either anti-CD16 monoclonal antibody alone (blue) or the same anti-CD16 concentrations but also with 10 μ g/ml hPR1A3 (purple).

Each antibody concentration tested was carried out in triplicate. Error bars indicate standard error of the mean (SEM). All significance testing was on comparison between the wells containing hPR1A3 vs those without hPR1A3 for the same concentration of anti-CD16. The only significant result ($p < 0.05$) is shown.

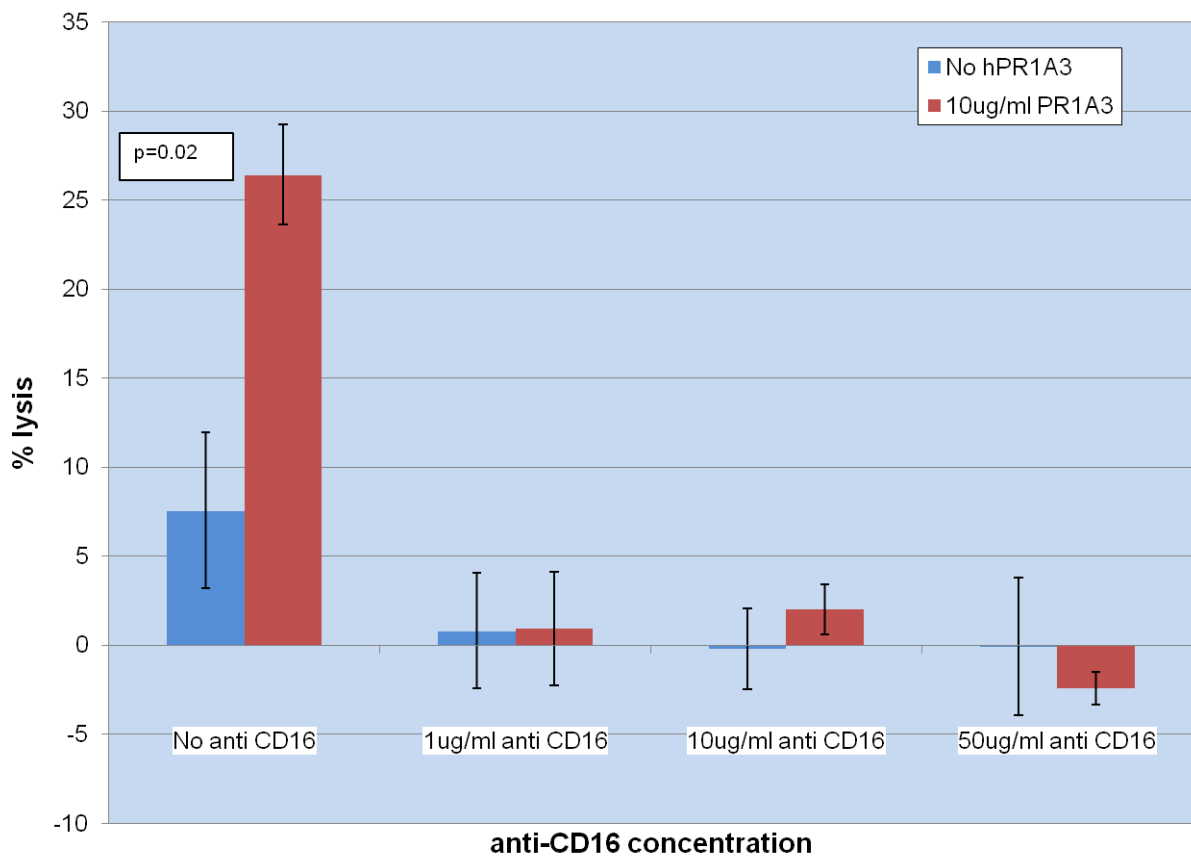
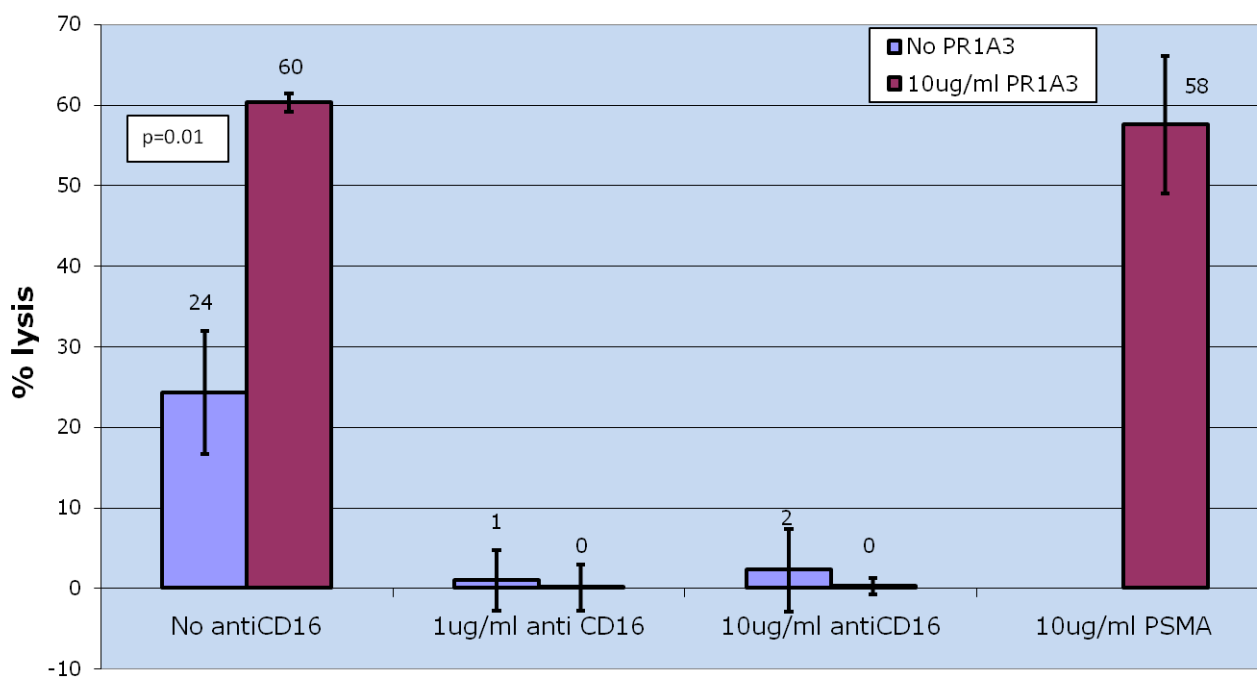


Figure 5.iii: Effect of varying doses of anti-CD16 antibody (3G8 clone) on Ab-dependent and Ab-independent lysis of CEA-positive target cells.

EuTDA ADCC assay using MKN45 as CEA-expressing target cell and human PBMC as effectors at ratio of 1:100. All columns represent wells containing target and effector cells with some containing varying concentrations of either anti-CD16 monoclonal antibody alone (blue) or the same anti-CD16 concentrations (or anti PSMA as an isotype control for anti-CD16) but also with 10µg/ml hPR1A3 (purple).

Each antibody concentration tested was carried out in triplicate. Error bars indicate standard error of the mean (SEM). All significance testing was on comparison between the wells containing hPR1A3 vs those without hPR1A3 for the same concentration of anti-CD16. The only significant result ($p < 0.05$) is shown.



To further investigate this effect whereby introducing anti-CD16 antibody abolished even spontaneous killing, the experiments were repeated with reducing amounts of foetal calf serum (FCS) in the assay media. A possibility was that FCS might be providing an additional source of antibody which might account for some part of the spontaneous killing seen in the absence of hPR1A3. If true, this would explain a possible mechanism

whereby spontaneous killing would be abrogated by blocking with anti-CD16. To test this hypothesis we compared different concentrations of FCS in the assay media (chapter 2.4.2.1) to investigate whether this would alter not only the level of spontaneous killing but also the effect that the anti-CD16 antibody has on this spontaneous component. However the level of spontaneous killing and the effect of the whole anti-CD16 antibody persist irrespective of the FCS concentration (figure 5.iv).

5.2.3 Effect of F(ab')₂ fragment of anti-CD16

Two other possible explanations were considered for the effect seen with whole anti-CD16 antibody. Firstly, since the anti-CD16 antibody binds to the NK cells and other CD16-bearing cells such as monocytes, these effector cells may then kill each other by ADCC via the anti-CD16 attached to their Fc γ receptors or form clusters of linked effector cells and antibody, so diminishing the number of effector cells to mediate the spontaneous killing. Secondly, we considered whether the anti-CD16 mAb was cross-linking the CD16 receptor on the surface of the effector cells causing activation in the absence of a bound target, degranulation and subsequent apoptosis of the effector (Azzoni et al., 1995; Ortaldo et al., 1995). To investigate both these possibilities a F(ab')₂ fragment of the anti-CD16 antibody was used instead of the whole antibody. This was made from the same 3G8 clone as one of the whole antibodies and used alongside it in an ADCC assay as comparison.

Unlike the whole antibody, the F(ab')₂ had no effect on the spontaneous killing. It did however abolish the antibody-dependent killing (figure 5.v). This demonstrates that the Fc γ III A (CD16) receptor is critical for the effect of hPR1A3 *in vitro* and provides further evidence that PR1A3 acts by ADCC and not by any block of function. This result may lend support to the idea that the loss of spontaneous killing seen with the whole anti-CD16 antibody is a result of the effector cells targeting each other for lysis. However, it refutes the other possibility that cross-linking the CD16 receptors was responsible for the loss of effector cells to carry out spontaneous killing, since cross-linking would still be possible with the F(ab')₂ as with the whole antibody.

Figure 5.iv: Effect of foetal calf serum concentration on spontaneous killing of MKN45 cells by PBMCs and on the inhibition of cell lysis caused by anti-CD16 antibody.

EuTDA ADCC assay using MKN45 as CEA-expressing target cell and human PBMC as effectors at ratio of 1:100. Different concentrations of foetal calf serum in the medium of the assay wells were used – 10%, 5% and 1%. All columns represent wells containing target and effector cells with some containing varying concentrations of either anti-CD16 monoclonal antibody alone (blue) or the same anti-CD16 concentrations but also with 10µg/ml hPR1A3 (purple).

Each antibody concentration tested was carried out in triplicate. Error bars indicate standard error of the mean (SEM). All significance testing was on comparison between the wells containing hPR1A3 vs those without hPR1A3 for the same concentration of anti-CD16. Only significant results ($p < 0.05$) are shown.

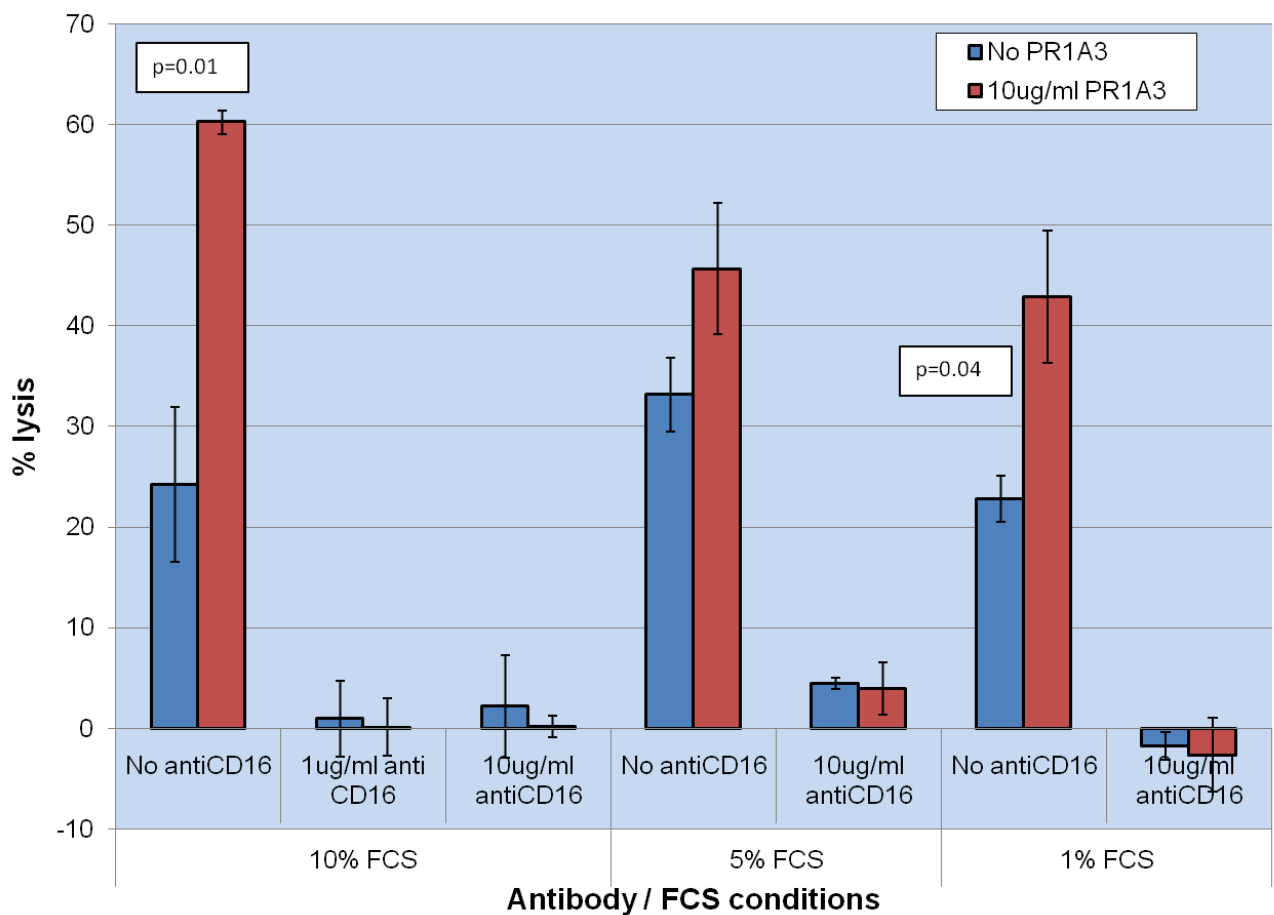
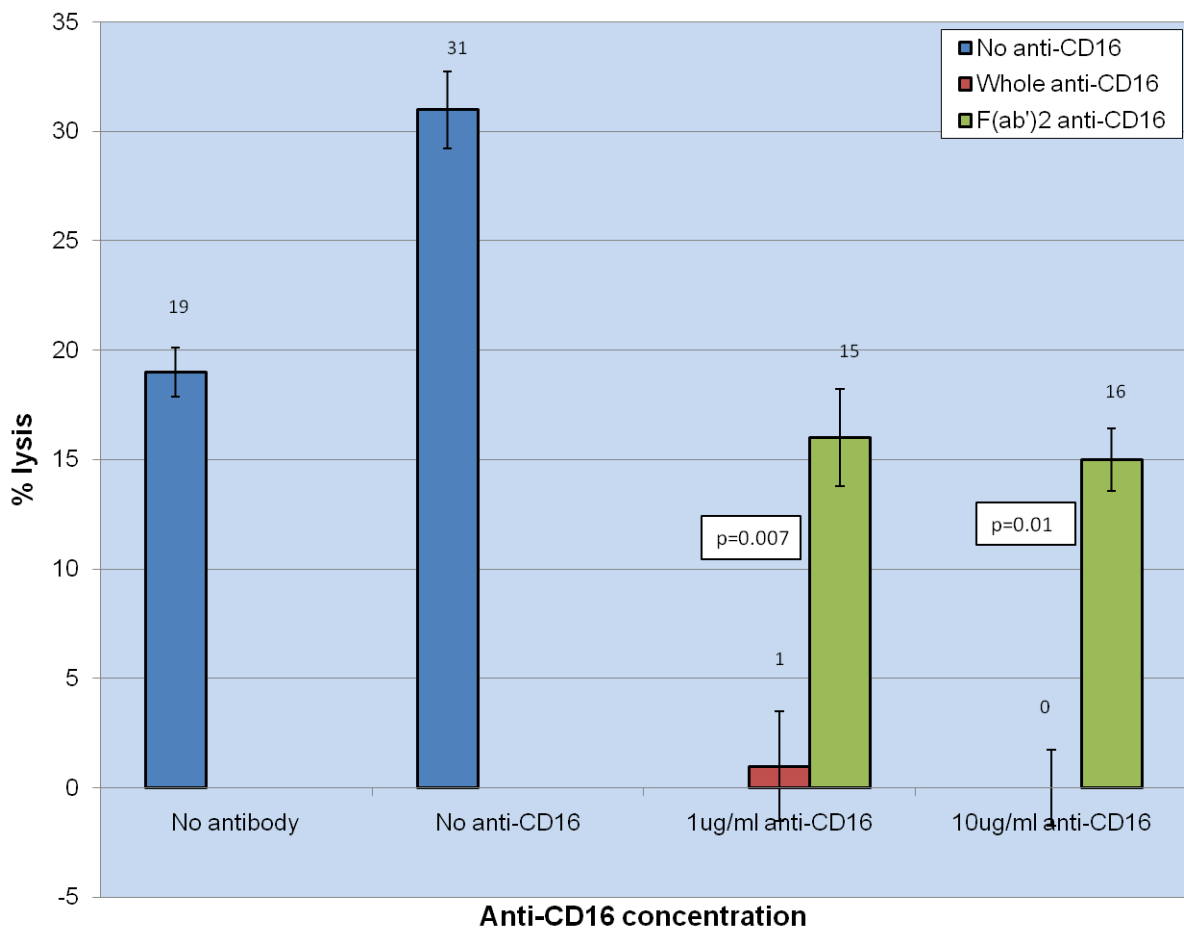


Figure 5.v: Effect of whole antibody and a F(ab')₂ fragment of anti-CD16 (3G8 clone) on PR1A3-induced lysis.

EuTDA ADCC assay using MKN45 as CEA-expressing target cell and human PBMC as effectors at ratio of 1:100. All columns represent wells containing target and effector cells with some also containing varying concentrations of either whole anti-CD16 monoclonal antibody (purple) or a F(ab')₂ of the same anti-CD16 clone (green). The anti-CD16 constructs were added at the same time as the effector cells without prior incubation. **Apart from the “No antibody” group, all wells also contain 10µg/ml hPR1A3.**

Each antibody concentration tested was carried out in triplicate. Error bars indicate standard error of the mean (SEM). All significance testing was on comparison between the wells containing whole anti-CD16 antibody vs those with the F(ab')₂ anti-CD16 of the same concentration. Only significant results (p<0.05) are shown.



5.2.4. Discussion

These experiments provide further evidence that interaction of hPR1A3 with the effector cell is critical to its action. Blockade of the interaction between antibody and effector cell by using an antibody to the Fc γ IIIa (CD16a) receptor (either whole antibody or F(ab')₂) led to an abolition of antibody-mediated response. Two different clones of anti-CD16 antibody were used. The 3G8 clone and MEM154 bind different epitopes on the receptor. There have been reports that the binding of certain clones is influenced by the polymorphism at aa158 which has been previously discussed (chapter 1.3.1). For example Koene et al (Koene et al., 1997) report that the binding of MEM154 required the presence of valine at position 158 and Wu et al (Wu et al., 1997) found the same trend with the 3G8 clone. A clear effect is seen with both antibodies in our study although they were not tried with effector cells from donors who were phenylalanine homozygotes at aa158.

The finding that whole anti-CD16 antibody caused the blockade of spontaneous killing, as well as the antibody-dependent killing, was unexpected and is not, as yet, fully explained. It does not appear to be discussed in the literature although it was a consistent finding in a number of repeated experiments. Given that spontaneous killing is restored when a F(ab')₂ fragment is used instead of the whole antibody led us to believe that interaction of the Fc-portion of the blocking antibody with effector cells was involved. Given that the blocking antibody would target all CD16⁺ cells, it may be that these cells themselves become targets for ADCC. It is also possible that CD16⁺ cells with bound anti-CD16 antibody form clusters of linked effector cells and antibody and as a result have reduced mobility in the wells. This would then impede their ability to carry out antibody-independent killing.

The persistence of the effect of whole anti-CD16 antibody irrespective of the FCS concentration reduces the possibility that FCS provides a source of additional antibody contributing to spontaneous killing.

The effect of the anti-CD16 antibodies was also seen at low concentrations. Full blockade of antibody-dependent and spontaneous killing was seen with concentrations of 1 μ g/ml. This is unusual in that one would have thought that saturation of the CD16 receptor was essential to fully block antibody-mediated killing. It could mean that blockade of some CD16 receptors allowed inhibitory signals to predominate and that it is a combination of

increased inhibitory signals and reduced ADCC which cause complete blockade at low concentrations. Alternatively if ADCC or immune clustering is the mechanism by which anti-CD16 inhibits spontaneous killing by targeting the effector cells themselves it may only require a low concentration of anti-CD16 to significantly diminish the activity of the effector cell population and effectively abolish spontaneous killing. This mechanism could be investigated by labelling the effector cells in an experiment using anti-CD16 to see if effector cell lysis occurs to any great extent.

Other groups have reported on the involvement of the Fc receptors in influencing active immunity and spontaneous killing. Clynes et al (Clynes et al., 2000) demonstrated that mice deficient in the FcγIIB receptor not only had a potentiated response to an anti-melanoma antibody in a metastatic melanoma model, they also had increased spontaneous tumour protection even in the absence of antibody treatment, leading to a reduced tumour burden. Bolland and Ravetch (Bolland and Ravetch, 1999) also implicated the inhibitory Fc-receptors in modulating cytotoxic responses against tumour cells for both antibody-independent as well as antibody-dependent pathways.

In earlier work, Clynes had used mice deficient in the FcγI and FcγIIIa receptors. They demonstrated that if nude mice were initially immunised with the gp75 protein (a melanoma target) to induce 'immunity' to future melanoma inoculation and then later inoculated with melanoma cells, the anti-tumour response was diminished in the FcγR deficient mice whereas there was a significant reduction in the number of lung metastases in the wild-type mice (Clynes et al., 1998). Hence the FcRs seem also to be required for active immunity and not just regulating the humoral antibody response.

5.3 Genetic typing of donors for a polymorphism in the FcγIIIa gene

5.3.1 Rationale

There is evidence to suggest that the interaction between the Fc fragment of the IgG antibody and the FcγIIIa receptor on effector cells, in particular NK cells, is influenced by polymorphisms in the gene encoding this receptor and this translates to differential activation in response to binding antibody (Cartron et al., 2002; Wu et al., 1997). The most commonly described is the T to G substitution at nucleotide 559 (exon 4) of the FcγRIIIa gene (*FCGR3A*) which changes the amino acid sequence of the receptor from phenylalanine to valine in an area of the receptor responsible for antibody binding (Tamm and Schmidt, 1996). If donors could be genotyped for this polymorphism, a comparison could be made on the magnitude of the ADCC response against a given cell line between donors with a high-binding polymorphism and those with the low-binding polymorphism. In the future this could be extended to the typing of *FCGR3A* in patients with CRC to assess their likelihood of response to anti-tumour antibody therapy in subsequent clinical trials.

5.3.2 Results

Blood from leucodepletion filters, fresh donors, cell lines and human random control DNA used in previous studies, were genotyped for the Fc-γIIIa receptor. This proved difficult due to the close homology of *FCGR3A* to the FcγR3b gene, *FCGR3B* (figures 2.iv and 5.vi). Forward primers were designed to discriminate between the 2 genes so as only to select the Fcgr3a gene and then reverse primers were used to discriminate between the T- and G-polymorphisms of the Fcgr3a gene in an allele-specific polymerase chain reaction (PCR).

Figure 5.vi: Comparison of amino acid sequence of the Fcgr3a and Fcgr3b proteins.

The polymorphism of interest in *FCGR3A* is at aa212 and is underlined and highlighted. It is listed as the more common phenylalanine (F) amino acid.

1	MGGG <u>A</u> GERLF <u>T</u> S <u>S</u> CLVGLVP LGLRISLVTC PLQCGIMWQL MGGG <u>T</u> GERLF <u>T</u> P <u>S</u> CLVGLVP LGLRISLVTC PLQCGIMWQL
41	LLPTALLLV SAGMRTEDLP KAVVFLEPQW Y <u>R</u> VLEKDSVT LLPTALLLV SAGMRTEDLP KAVVFLEPQW Y <u>S</u> VLEKDSVT
81	LKCQGAYSPE DNSTQWFHNE <u>S</u> LISSQASSY FIDAATV <u>D</u> DS LKCQGAYSPE DNSTQWFHNE <u>N</u> LISSQASSY FIDAATV <u>N</u> DS
121	GEYRCQTNLS TLDPVQLEV HIGWLLLQAP RWVFKEEDPI GEYRCQTNLS TLDPVQLEV HIGWLLLQAP RWVFKEEDPI
161	HLRCHSWKNT ALHKVTYLQN GK <u>G</u> RKYFHNN SDF <u>Y</u> IPKATL HLRCHSWKNT ALHKVTYLQN GK <u>D</u> RKYFHNN SDF <u>H</u> IPKATL
201	KDSGSYFCRG <u>L</u> <u>F</u> GSKNVSS TVNITITQGL AVSTISS <u>F</u> FP KDSGSYFCRG <u>L</u> <u>V</u> GSKNVSS TVNITITQGL AVSTISS <u>S</u> FP
241	PGYQVSFCLV MVLLFAVDTG LYFSVKTNIR SSTRDWKDHK PGYQVSFCLV MVLLFAVDTG LYFSVKTNI
281	FKWRKDPQDK

Black text: Fc-γIIIa receptor amino acid sequence
Red text: Fc-γIIIb receptor amino acid sequence
Blue text: Areas of variation between the 2 receptors (amino acid 212 underlined and highlighted; site of F/V polymorphism under investigation)

Optimising PCR

A temperature gradient was used to optimise the PCR using 8 temperatures from the range 60-68°C. Two different MgCl₂ concentrations were tested – 1.5mM and 2mM – and the samples were tried with and without the ‘Q-solution’ detergent provided by the Taq

manufacturers (Qiagen, Crawley, UK). The PCR deteriorated at the higher end of the temperature gradient especially for the T-allele and so the annealing temperature chosen was 60°C for the T-allele and 63°C for the G-allele, both with 2mM MgCl₂ and without Q-solution.

50-100ng DNA was added to each PCR reaction. Neat solutions were approximately 30ng/ml except where shown on the plate setup below. Fresh samples 2-5 however were weaker solutions (as shown) and contained only 30ng DNA. Samples were set up as below on the plate.

Filter 1 20ng/ml	Filter 2	Fresh sample 1	Fresh sample 2 5ng/ml	Fresh sample 3 5ng/ml	Fresh sample 4 5ng/ml	Fresh sample 5 5ng/ml	Vaco400
SW948	SW1417	LS411	SnuC2B	HCT116	MKN1	MKN28	MKN45
HRC1	HRC3	HRC4	HRC5	HRC6	HRC8	HRC9	HRC10
HRC13	HRC15	HRC19	HRC20	Blank			

HRC = human random control

Results for the T (phenylalanine) and the G (valine) polymorphism are shown in figure 5.vii and summarised in table 5.i. Variation in the DNA concentration from the fresh samples led to the variability seen in the intensity of the bands. The results show only 2 samples out of 28 which were homozygous for the valine polymorphism but with 17 heterozygotes, an allele frequency for the valine polymorphism of 0.38. The group however is heterogeneous, containing healthy volunteers, colorectal cell lines and random control DNA samples. Sadly neither of the valine homozygotes were from identifiable volunteers, being instead from the Vaco400 cell line and from one of the human random controls (HRC19).

Figure 5.vii: Genotyping of DNA samples obtained from stored human random controls, LDFs, fresh blood samples and cell lines. Allele-specific PCR typing for polymorphism in the *FCGR3A* gene coding for either phenylalanine or valine.

Forward primer: TCCAAAAGCCCACTCAAAGAC

Reverse primer: CTGAAGACACATTTTTACTCCCAA/C

Annealing temperature 60°C. All samples at approx 100ng DNA per PCR reaction apart from those shown

Samples only at 15-30ng DNA/50ul PCR reaction

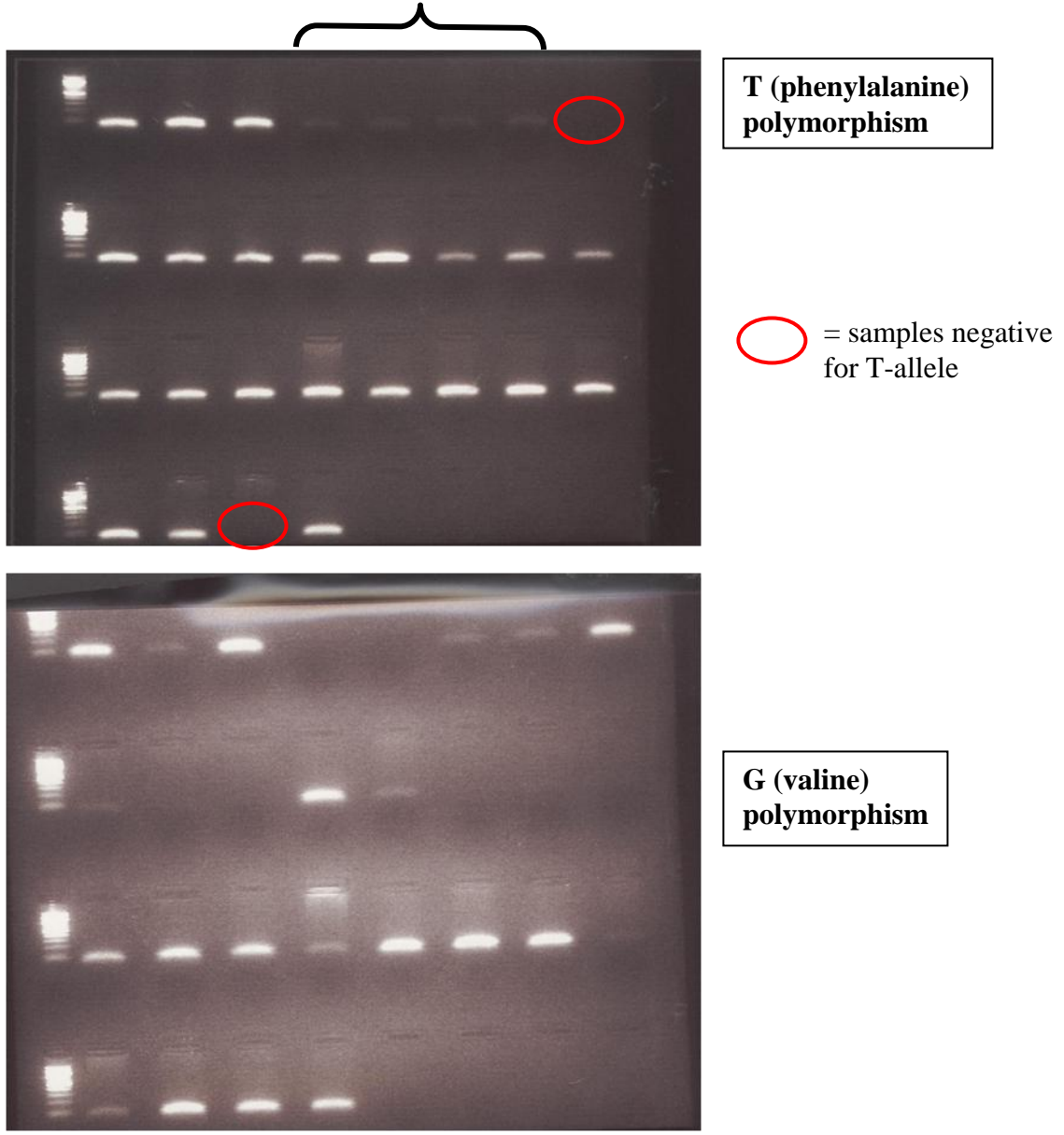


Table 5.i: Genotyping of DNA samples obtained from stored human random controls (HRC), leucodepletion filters, fresh blood samples and cell lines.

Allele-specific PCR typing for polymorphism in the *FCGR3A* gene coding for either phenylalanine (T allele) or valine (G allele).

Sample	Genotype	Sample	Genotype
Filter 1	TG	MKN28	TT
Filter 2	TG	MKN45	TT
Fresh sample 1	TG	HRC1	TG
Fresh sample 2	TT	HRC3	TG
Fresh sample 3	TT	HRV4	TG
Fresh sample 4	TG	HRC5	TG
Fresh sample 5	TG	HRC6	TG
Vaco400	GG	HRC8	TG
SW948	TT	HRC9	TG
SW1417	TT	HRC10	TT
LS411	TT	HRC13	TG
SNUC2B	TG	HRC15	TG
HCT116	TG	HRC19	GG
MKN1	TT	HRC20	TG

There is always the concern that with a second gene (*FCGR3B*) with such close homology to *FCGR3A*, it may be amplified within the PCR and so interfere with the data interpretation. However *FCGR3B* codes for G at position 634 and so any unintentional amplification of this gene would increase the apparent number of valine homozygotes and so this is unlikely to have happened within the study. Despite this the typing was repeated on a limited number of these same samples using a different forward primer which would amplify a region that contains a second base difference from *FCGR3B* and so was even less likely to amplify this gene. Vaco400 was included as it was one of only two samples from the first experiment which was potentially homozygous for the G-allele.

The samples were set up as below:

T-allele				G-allele			
60°C	61.3°C	62.3°C	63.5°C	60°C	61.3°C	62.3°C	63.5°C
Filter 2				Filter 2			
Vaco 400				Vaco 400			
Fresh sample 1				Fresh sample 1			
Fresh sample 4				Fresh sample 4			

The results were the same as with the original forward primer (figure v.iii).

5.3.3 Discussion

The failure to find any identifiable valine homozygote in the genotyping from whom we could obtain blood prevented any comparative ADCC study. It is possible that this paucity of valine homozygotes is influenced by technical error and chance and sequencing of the amplified sequence is necessary to be sure. From the published data the valine polymorphism allele frequency seems to vary from 0.15 to 0.55 (Cartron et al., 2002; Chen et al., 2006; Farag et al., 2004; Gruel et al., 2004; Koene et al., 1997; Niwa et al., 2004a; Treon et al., 2005; Weisenseel et al., 2007; Wu et al., 1997) although not all of these allele frequencies would fit within the Hardy-Weinberg equilibrium. Some however are taken from specific disease groups which may predispose patients towards these illnesses and so, in these patients, the frequency with which individual alleles are found may not follow a Mendelian pattern.

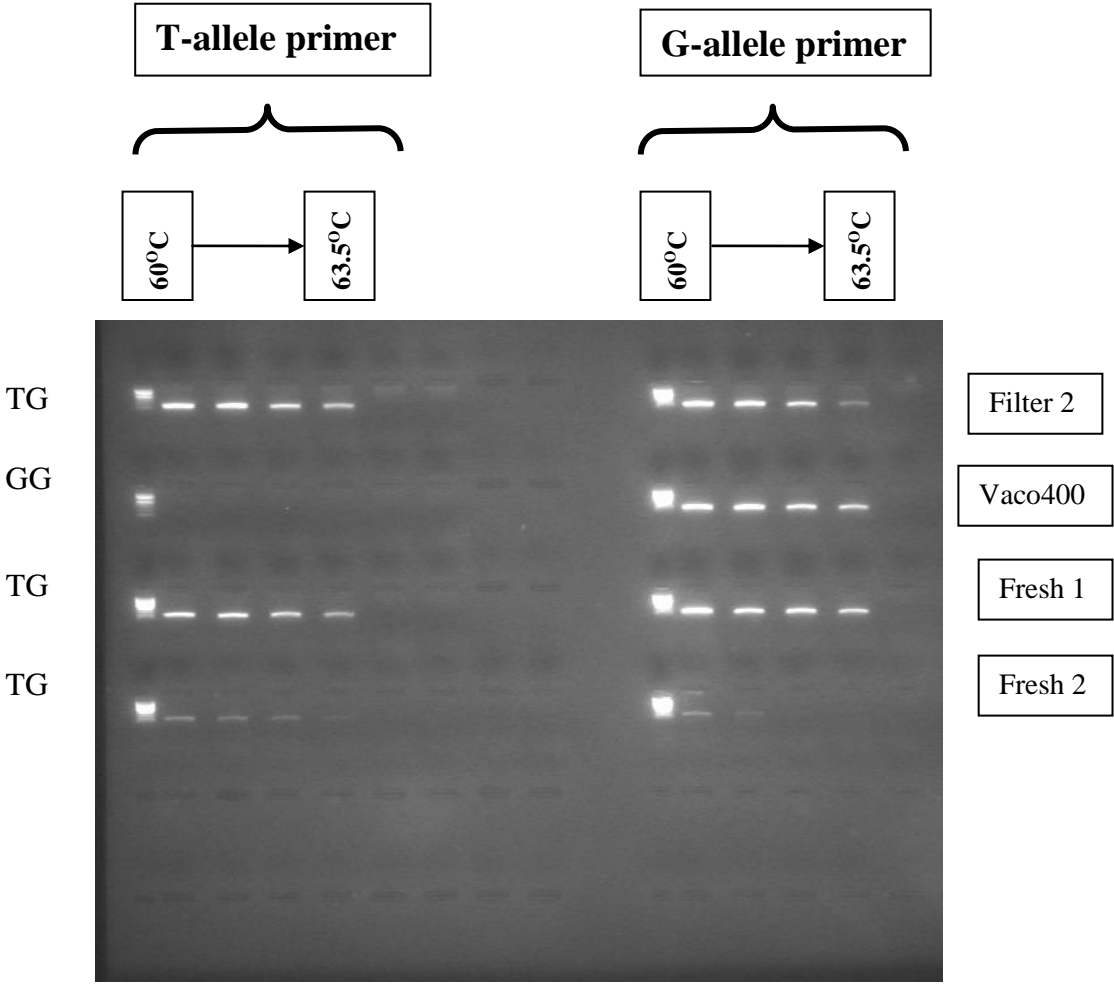
The T-G sequence polymorphism in the FcγIIIa receptor gene predicts a change from phenylalanine to valine in the membrane proximal extracellular domain EC2 of the

Figure 5.viii: Genotyping of DNA samples obtained from leucodepletion filters, fresh blood samples and cell lines. Allele-specific PCR typing for polymorphism in the *FCGR3A* gene coding for either phenylalanine or valine.

Temperature gradient from 60-63.5°C.

Forward primer: CACATATTTACAGAATGGCAAAGG

Reverse primers as for figure 5.vii: CTGAAGACACATTTTACTCCCAAA/C



receptor (nucleotide 559; exon 4). This is variably reported as amino acid 158 or 176 depending on the transcript sequence. The region in which this polymorphism is found is important in ligand binding (Tamm and Schmidt, 1996) and thus would further support the evidence that this polymorphism influences the interaction between the receptor and its ligand – the Fc portion of the antibody – and as a result may lead to differential results of antibody-based cancer therapy depending on the patient's genotype for the FcγIIIa receptor (Cartron et al., 2002; Treon et al., 2005). Hence further typing of volunteers and patients would be beneficial to identify more valine homozygotes with a view to examining the response of their PBMCs to hPR1A3 in an ADCC assay and comparing these with heterozygotes and phenylalanine homozygotes.

CHAPTER 6

Combination therapy: PR1A3 with other antibodies

6.1 PR1A3 and other antibodies

6.1.1 Rationale

6.1.1.1 Antigen escape

There are multiple mechanisms by which tumour cells could potentially evade either immune surveillance or attack. Antigen escape is one such mechanism in which a tumour being treated by antibody-based cancer therapy could, under selection pressure, increasingly consist of cells which have downregulated or lost the relevant tumour-associated antigen, hence making the tumour resistant to therapy or enabling it to evade immune detection. The best known example of antigen loss in colorectal cancer is the loss of HLA expression (Browning et al., 1993). In the case of treatment with PR1A3 this could lead to the emergence of a CEA-negative colorectal cancer over time.

One way of overcoming this problem is to combine PR1A3 treatment with either other antibodies or to combine it with other chemotherapy agents. This would minimise the chance of expansion of a cell population that had undergone antigen loss. In addition, a policy of using monoclonal antibodies to treat micrometastatic disease, in contrast to gross metastatic disease, reduces the risk of a surviving remnant of tumour cells with antigen-loss.

A dual antibody approach can also be useful in targeting non-tumour sites which are essential for tumour survival, such as angiogenesis.

6.1.1.2 Targeting complimentary non-tumour sites

There are certain non-tumour sites which are essential to tumour survival and hence serve as useful targets to treat together with a tumour-associated antigen. The most obvious site

is the blood supply to the tumour giving rise to the current interest in the anti-VEGF antibody, bevacizumab

6.1.1.3 Cost

Trials of dual antibody therapy are currently lacking as the cost of using 2 antibodies through the prolonged course of a clinical trial is often prohibitive and the place of single antibody therapy, particularly in the setting of adjuvant treatment rather than for the treatment of metastatic disease, is still being established. However if *in vitro* studies could show clear additive or synergistic effects then such trials may become viable.

6.1.2 The monoclonal antibody, A33

A33 is a monoclonal antibody developed in mice (IgG2a) and later humanised (IgG1). It binds to a 43KDa glycoprotein of unknown function whose expression is restricted to colorectal epithelium (Welt et al., 2003). It continues to be expressed homogeneously in more than 95% of colorectal cancers. A33 has been tested in a phase I clinical trial (Welt et al., 2003) although immunogenicity of the antibody prevented further evaluation. As shown in chapter 3.1.3, a panel of colorectal cell lines has been tested for A33 antigen expression using the murine parent of this antibody in an ELISA.

6.1.3 Dual treatment of a colorectal cell line with A33 and PR1A3

The cell line SKCO was used in this EuTDA ADCC assay. It is a high expressor of CEA (figure 3.ii) and a moderate expressor of A33 (figure 3.iii). These data are supported by our microarray results for A33 as also shown earlier for CEA. Antibodies were tested individually at 2 concentrations and then together at the lower concentration for each antibody. Results are shown in figure 6.i. As expected there was a dose-dependent increase for both antibodies when used individually. However there was also a possible synergistic effect when the antibodies were used in combination. At the lower dose of 10µg/ml hPR1A3 and 6µg/ml hA33, the antibodies alone lysed 9% and 32% of the cells respectively in an antibody-dependent fashion (i.e. lysis above the spontaneous killing

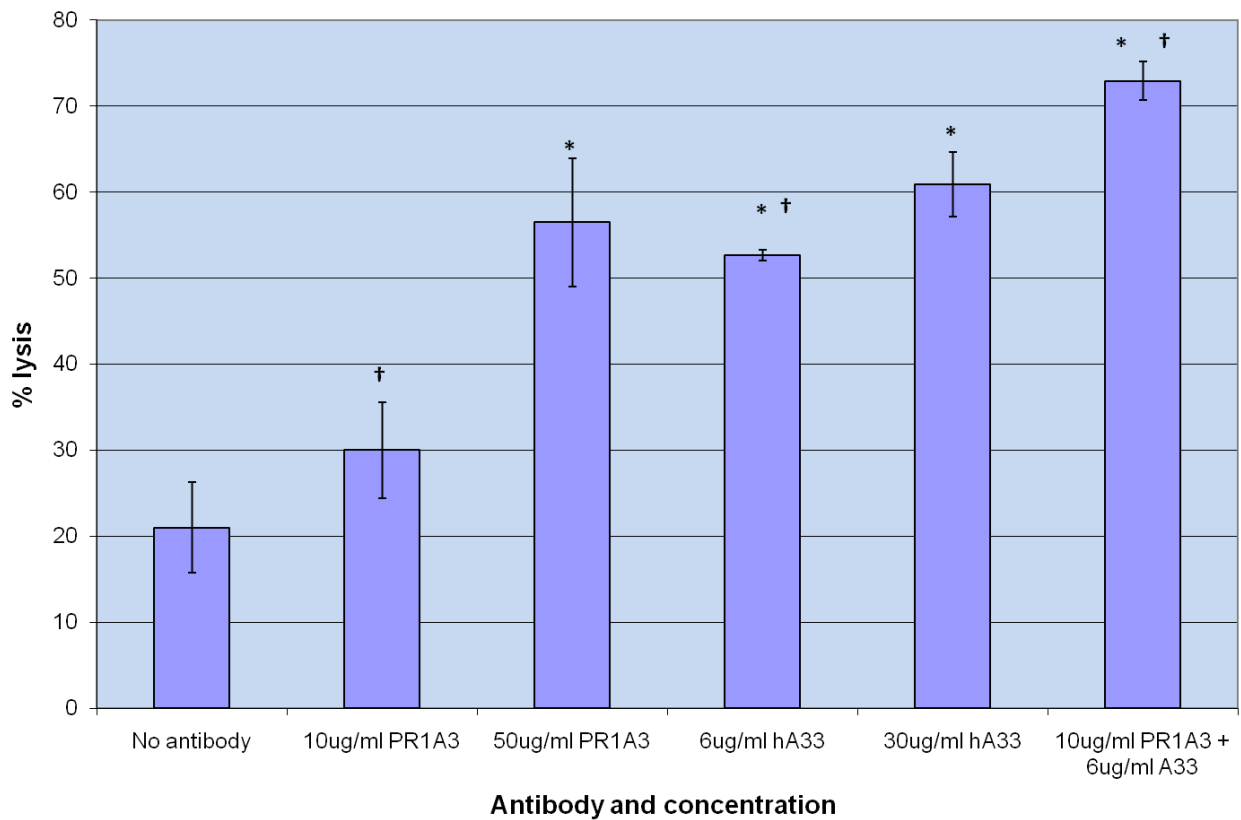
seen in the first column). However in combination at the same doses they achieved 52% lysis.

Figure 6.i: Effect of hPR1A3 and hA33 alone and in combination on the CEA positive and A33 antigen positive cell line, SKCO-1

EuTDA ADCC assay using SKCO-1 as A33- and CEA-expressing target cell and human PBMC as effectors at ratio of 1:100. All columns represent wells containing target and effector cells with some containing varying concentrations of either hPR1A3 or hA33 or both.

Each antibody concentration tested was carried out in triplicate. Error bars indicate standard error of the mean (SEM). Significance testing was on comparison between the wells containing antibody and those that contained neither (*) or between single antibody wells and the dual antibody wells († ; one way ANOVA) with significant results (p<0.05) shown.

*p<0.01 †p=0.001



6.1.4 Discussion

Combination antibody therapy is one of the potential solutions to the problem of antigen escape. hPR1A3 when combined with another cytotoxic antibody *in vitro* demonstrates an additive and possibly a small synergistic effect. To confirm which effect is occurring would require further testing at different doses of both antibodies. It is interesting to note that the cytotoxic effect of hA33 was greater than that of hPR1A3 but this may be related to the relative level of expression of the two proteins on the cell surface. Although SKCO-1 is only a moderate A33 expressor compared to other colorectal cell lines, the actual level of expression in terms of copy number on the cell surface, has not been evaluated. The proteins were assessed in the same ELISA assay on occasions but different concentrations of antibody were used and this makes a comparison between the two difficult with the data available, since this was not critical to the study. However it is more likely to be a consequence of better affinity for its target in the case of hA33 compared with hPR1A3.

It was important to establish that an additive and not inhibitory effect was seen when combining the antibodies and this is clear from the results. The results from the clinical trial of hA33 were disappointing in that in eight of the eleven patients, a significant immunogenic response (human anti-human antibodies; HAMA) was mounted against the antibody. Since the A33 antigen is also expressed homogeneously in normal colorectal epithelium, it remains to be seen whether this antibody will have significant gastrointestinal toxicity when a less immunogenic form of the antibody is developed.

Although SKCO-1 had differing expression levels for CEA and A33, a major benefit of dual antibody therapy may come in those tumours where the expression of the target antigens in question are at the opposite ends of the expression spectrum (e.g. high CEA expressors and low A33 protein expressors in the case of using PR1A3 and A33). This would ensure that any tumour cell population that evaded cytotoxic attack by one of the antibodies was more likely to be targeted by the second antibody.

CHAPTER 7

Future directions

7.1 Future directions of antibody-based cancer therapy

7.1.1 Optimising antibody binding to target and effector

Evidence from the success of various antibody constructs in clinical trials would suggest that the naked whole antibody is key to the clinical effect of monoclonal antibody therapy. The introduction above and the results of this thesis have set out the likely reasons behind this finding and I have postulated that the primary mechanism by which the majority of such naked antibodies have their effect is antibody-dependent cellular cytotoxicity (ADCC). Successful future direction is thus likely to concentrate on enhancing this mechanism. Optimising the affinity of antibodies for their target; enhancing the interaction between the Fc portion of the antibody and the activating Fc γ Rs, especially Fc γ R11A, whilst minimising their affinity for the inhibitory Fc γ Rs, especially Fc γ R11b; suppressing the inhibitors of ADCC and enhancing the potential of antibodies to stimulate the adaptive immune response are all potential pathways to improved antibody efficacy.

Alteration of the complementarity determining sequence by site-directed or random mutagenesis with display libraries (eg. phage, yeast, ribosome mRNA) is now commonplace although other novel approaches are now being suggested (Tanaka and Barbas, 2002).

The combination of mutagenesis of the Fc region with computer-based protein modelling can rapidly reduce the possible amino acid sequences to a size amenable to experimental screening to select for variants with optimal binding qualities, such as high affinity for activating Fc receptors and for FcRn, low affinity for inhibitory Fc receptors and ability to bind complement.

Selection can also be tailored to those antibodies which enhance antibody presentation by dendritic cells, based again on differential binding to FcRs so that such antibodies can promote cytotoxic T-cell responses. For example, an antibody that preferentially associates with the activating isoform FcγRIIIA is more likely to induce T_{H1}-dependent T-cell response, whereas those preferentially associating with FcγRIIB are more likely to induce T_{H2} responses.

7.1.2 Targeting small volume disease

It is likely that more antibodies will be tested in the adjuvant and neoadjuvant setting in the future. Although most new treatments are tested in the metastatic setting initially, as has been the case for mAbs, there are strong theoretical reasons why this is not their optimal setting and hence should only be used to test toxicity rather than efficacy. The naked antibody is a large 150KDa molecule which penetrates only slowly through the interstitium of a poorly vascularised tumour, as is the case in metastatic disease. The use of mAb in adjuvant therapy would allow their use in the setting of potential micrometastatic disease. In addition, the effect of the binding site barrier is diminished in small volume disease, allowing the use of higher affinity antibodies with their increased potential for ADCC without the concern of the therapeutic Ab being restricted to the periphery of the tumour.

The few trials looking at antibodies in these settings have shown improved outcomes compared to control groups. Trastuzumab was tested in such an environment in two randomised controlled trials (Piccart-Gebhart et al., 2005). Both compared trastuzumab in the adjuvant setting following locoregional control (surgery with or without radiotherapy). In the American trial, trastuzumab treatment following initial post-operative chemotherapy was associated with a 33% reduction in mortality risk compared to chemotherapy alone with an advantage in disease free survival (DFS) of 85.3% vs 67.1%. In the European study, analysis at one year showed no difference in overall survival as might be expected in such a time-frame but it did show an improved DFS in the group receiving trastuzumab following chemotherapy (or having had neoadjuvant chemotherapy) compared to the group having standard chemotherapy alone; 13% vs

7.5%. Hence it would seem that trastuzumab is beneficial in the adjuvant setting and not just in the context of metastatic disease. Trials of cetuximab (PETACC-8 trial, NCCTG-N0147) and bevacizumab (AVANT trial, QUASAR-2) in the adjuvant setting are already underway with some already finished recruiting.

7.1.3 Dual antibody therapy

There is also likely to be a future increase in combinatorial mAb treatment. Given the potential of tumours to select for antigen-loss variants, a dual antibody approach may seem sensible. In addition, treating micrometastatic disease, in contrast to gross metastatic disease, reduces the risk of a surviving remnant of tumour cells with antigen-loss. Targeting tumour vasculature as one arm of dual antibody therapy holds the advantages not only of reducing blood flow to the tumour, but also of targeting a non-neoplastic component of the tumour which is thus less prone to antigen-loss. The disadvantage of bevacizumab in dual Ab therapy is that it is not directly cytotoxic and so does not have the dual antigen capabilities in combination that two cytotoxic antibodies potentially have.

There have already been some early studies using antibody combinations. Following the results in the cetuximab trials, some researchers have tried bevacizumab and cetuximab together in the hope of further enhancing the effect of chemotherapy in metastatic CRC (Saltz et al., 2007).

7.1.4 Modulating immune responses to enhance the effect of cytotoxic antibodies

In addition to using therapy that would recruit the adaptive immune response, immune modulation could also be used to inhibit the natural suppressors of the immune system. Antibodies to block access to Fc γ RIIB and CTLA-4 have both been suggested and trials with the latter have shown early encouragement in potentiating the anti-tumour potential of the immune system alone (Small et al., 2007) or of an antibody-based vaccine (Phan et al., 2003). Among other immunomodulatory treatments in early development are anti-

CD25 antibodies for the depletion of regulatory T-cells (Murillo et al., 2003). In a similar way, boosting the activating arm of immune control has been revisited. Combining IL-2 (Friedberg et al., 2002) and IL-12 (Parihar et al., 2004) with antibody treatment has been tried with some early-trial success.

The obvious disadvantage of such treatment is the development of autoimmunity or, potentially, dangerous unregulated immune activation.

7.1.5 Development of antibody fragments

Despite the advancements in antibody technology, creating antibody fragments with multiple binding sites, occasionally for more than one antigen, and the development of radioactive and toxin conjugates, only the naked antibody shows any success in clinical trials. Although these newer constructs occasionally show benefits in mouse xenograft models (Trail et al., 1993), they fail to improve outcomes in clinical trials (Ajani et al., 2000; Tolcher et al., 1999).

There has been recent encouragement, however, with bispecific T-cell engager (BiTE) antibodies. These are single chain bispecific antibodies (2 scFv fragments) with specificity for the CD3 receptor on T-cells and a target cell antigen (eg EpCAM, CD19). By binding to the CD3 receptor on T-cells, these BiTE antibodies are able to transiently engage the T-cell with the target cell, despite the T-cell having no specificity for an antigen on the target cell and without reference to MHC class I. By this they are able to induce a polyclonal T-cell response that is not limited by the T-cell receptor (TCR) specificity, presence of MHC I, generation and presentation of antigen or the need for T-cell co-stimulation. Hence this construct overcomes the greatest disadvantage of antibody fragments – the inability to engage immune effector cells. Instead they have the potential to engage T_c and T_H cells which lack Fc receptors and hence are not normally engaged even by whole antibody. Early studies have been encouraging: a CD19 construct (blinatumomab) was used in late-stage non-Hodgkins lymphoma and shown to induce a partial or complete tumour response at very low concentrations (0.015-0.06mg/m²/day) in all patients examined (Bargou et al., 2008). Preclinical trials have also shown promise

with various other antigens including EpCAM (Brischwein et al., 2006) and CEA (Lutterbuese et al., 2009).

7.1.6 Development of conjugated antibodies

Recent attention has focussed on conjugating radioisotopes and drugs to established naked antibodies. For example *in vitro* studies are already underway using an α -emitter-trastuzumab conjugate in the hope that the shorter range of the α -emitters will reduce the bystander toxicity seen with β - and γ -emitters (Ballangrud et al., 2004). Trastuzumab has also been linked to a chemical conjugate, geldanamycin - a drug which acts as an inhibitor of several oncogenes by inducing their degradation. This construct has shown some good results in xenograft models using human breast carcinoma lines. The conjugated antibody achieved 69% tumour regression compared to 7% with trastuzumab alone (Mandler et al., 2004). One proposed method to reduce systemic toxicity caused by drug-antibody conjugates is to modify the linker connecting the two molecules so that clearance is accelerated. Liver uptake is a major source of hepatic toxicity and linkers have now been created which are cleavable by hepatocyte lysosome proteases which has the effect of speeding clearance from the liver. Preclinical evaluation of such radioimmunoconjugates has already been performed showing reduced hepatic radioactivity in both mice and humans using a cathepsin-degradable peptide linker (Richman et al., 2005). The ADEPT technique described in the introduction, which is also designed to reduce systemic toxicity, requires further evaluation in larger studies.

7.2 Future directions with PR1A3

7.2.1 Altered glycosylation of the Fc fragment and affinity maturation of the antibody

It is now recognised that the cytotoxic activity of all human antibody isotypes can be increased by modifying the glycosylation pattern of antibodies and this is directly related

to increasing their binding affinity to Fc γ Rs (Schuster et al., 2005; Shields et al., 2002; Umana et al., 1999). We have recently collaborated to produce a glycoengineered re-humanised IgG1 version of our antibody, hPR1A3. We will compare this against the unmodified antibody in ADCC assays as described in this thesis and would expect cytotoxic activity at lower concentrations of antibody *in vitro*.

This will be combined with affinity maturation of hPR1A3 to a level consistent with other naked antibodies in clinical use. This will maximise the potential for ADCC.

7.2.2 Isolation of subpopulations of peripheral blood mononuclear cells for testing in ADCC

In an attempt to further characterise the nature of PR1A3 activity in tumour cell lysis we plan to negatively select cell populations from the effector peripheral blood mononuclear cells (PBMC). We hope to assess whether NK cells, macrophages, and granulocytes can independently reproduce ADCC in cell lines which are consistently affected by the unselected PBMC population (eg MKN45).

Given the interaction believed to occur between innate and adaptive immune responses during antibody-based cancer therapy (Dhodapkar et al., 2005; Nimmerjahn and Ravetch, 2008), future work would involve analysing these interactions in antibody responses especially in cells such as macrophages and dendritic cells. We will be culturing monocyte-derived macrophages and dendritic cells under a variety of conditions to explore the circumstances under which induction of Fc γ RI and Fc γ RIIIa occurs. Cells would then be tested in standard cytotoxicity assays to ensure that this expression correlated with function.

Individual cell groups and PBMC as a whole will also be tested in the co-presence of hPR1A3 and complement given that complement may play at least some role in the mechanism of antibody action. Our colorectal panel will be screened for levels of expression of the membrane regulatory proteins which inhibit complement and then examined to see if the level of expression of these proteins influences the magnitude of the cytotoxic response to antibody for a given level of CEA.

These studies will enable a better understanding of the key components of ADCC and how this can be used to initiate a wider immune response.

7.2.3 FcγR studies with hPR1A3

As noted above, there is evidence to suggest that the interaction between the Fc fragment of the IgG antibody and the FcγIIIa receptor on effector cells, in particular NK cells, is influenced by a polymorphism in the gene encoding this receptor and this translates to differential magnitude of cytotoxicity in response to binding antibody (Koene et al., 1997; Wu et al., 1997) and in clinical response (Cartron et al., 2002; Dall'Ozzo et al., 2004; Weng and Levy, 2003). We plan to genotype the polymorphisms from the donor effector cells and compare this with donor variation in ADCC for a given cell-line. In the future this could be extended to the typing of FcγIIIa receptors in patients with CRC to assess their likelihood of response to anti-tumour antibody therapy in subsequent clinical trials. We will also further characterize the importance of stimulatory and inhibitory Fc-γ receptors by selectively blocking their function with anti-receptor antibodies. We would plan to investigate further the reason behind the loss of antibody-independent cytotoxicity during blockade of the FcγIIIa receptor with whole anti-receptor antibody and why this doesn't occur with a F(ab')₂ from the same clone.

7.2.4 Further combination studies

The possibility of enhancing ADCC will be tested by looking for synergy with other agents. We will examine the effect of adding immunomodulators such as gamma interferon, GM-CSF and IL-6 which have been shown either to upregulate the expression of CEA on CRC cells or to upregulate the expression of FcγIIIa on effector cells, as well as potentiating NK cell activation.

Antibodies such as cetuximab, trastuzumab and bevacizumab have all been used in combination with standard chemotherapy and it is envisaged that hPR1A3 will be used in a similar manner. The results above suggest that there may be some up-regulation of CEA

expression in response to 5-fluorouracil treatment. The next step would be to examine the effect of dual treatment in cytotoxicity assays.

7.2.5 *In vivo* studies using mPR1A3 in the $APC^{min+/-}CEA.tg^{+/-}$ mouse model of colorectal cancer

Traditionally animal studies of tumour therapies are carried out on human xenografts implanted into immuno-incompetent mice (eg nude mice). In the setting of immune-based therapies such immunologically incompetent mice are unsuitable. In addition xenografts are abnormally vascularised and in a non-orthotopic site which makes interpretation of results difficult.

As discussed in chapter 1.2.9.2.4, the $Apc^{min+/-}CEA.tg^{+/-}$ (MINCEA) mouse represents a good model for the investigation of CEA-targeted immunotherapies for CRC in that they

- express CEA in the same spatiotemporal pattern as seen in humans
- develop CEA-expressing tumours spontaneously in the bowel, with a tumour vasculature comparable to that seen in tumours in humans, and in a mouse with an intact immune system
- express CEA on such adenomas with the same loss of polarisation as seen in colorectal cancer, hence making the protein accessible to antibody-mediated therapy.

Following our pilot study (chapter 1.5.3), future work will now involve studies using an engineered murine IgG2a construct of the PR1A3 antibody. We will investigate the ability of the antibody to reduce the number and size of polyps in the MINCEA mice using both non-transgenic MIN mice treated with the same dose of antibody and untreated MINCEA mice as controls, the former to ensure there is no non-specific effect on polyp size and number from injecting antibody.

The assessment of potential synergy of antibody-mediated therapies with standard chemotherapeutic agents is an important step in progressing to clinical trials. Nearly all clinical trials of antibody-based therapies involved combination with another

chemotherapeutic agent. As such, we intend to compare the effectiveness of antibody or chemotherapy monotherapy with the combination of the two treatments in this mouse model. In addition dual antibody treatment as suggested for the *in vitro* work would be explored.

7.2.6 Phase I clinical trial

There is already strong interest in the development of targeted antibody therapy for the palliative treatment of cancer and now also for use as adjuvant treatment of micrometastases. I have outlined the ideal nature of PR1A3 as a potential antibody-based therapy and the initial success in both *in vitro* and *in vivo* studies.

Studies involving the early use of hPR1A3 in the adjuvant treatment of CRC would be the ideal setting for such a clinical study. Optimising the affinity of the antibody for its target and the Fc γ IIIa receptor as highlighted in chapter 7.2.1 is a prerequisite before such a trial is undertaken.

CHAPTER 8

Conclusions

The results of this thesis clearly demonstrate that humanised PR1A3 (hPR1A3) can mediate the killing of colorectal cancer cell lines expressing CEA and that the level of cytotoxicity is proportional to the level of CEA expression on the cell line. Although this work demonstrates a wide variability in CEA expression amongst different colorectal cell lines, it is known from previous work on colorectal cancer specimens that the majority of colorectal cancers express CEA to a level which makes them detectable to antibody (Wagener et al., 1984) and that PR1A3 is able to bind specifically to the large majority of colorectal tumours *in vivo* (Granowska et al., 1993). In addition, CEA also fulfils many of the properties of an ideal tumour-associated antigen. The major disadvantage has been that CEA is shed from the cell surface and circulates free in the lymphatic system and blood stream where it can be detected in high concentrations (Kubo et al., 1992; Wiratkapun et al., 2001). The advantage of PR1A3 over previous anti-CEA antibodies is that PR1A3 only binds to membrane-bound CEA as shown *in vitro* (Conaghan et al., 2008; Durbin et al., 1994) and demonstrated *in vivo* by its low false positive rate for lymph node metastases when used in immunoscintigraphy (Granowska et al., 1993).

This thesis has demonstrated further advantages of CEA as a target for immunotherapy in colorectal cancer. It has shown that CEA levels on colorectal cancer cells are upregulated by 5-fluorouracil, the most commonly used drug in adjuvant and neoadjuvant treatment of this disease. The increase in expression was modest although it is likely that the *in vivo* levels within the tumour will be higher than those used in these experiments which were based on average plasma concentrations. This thesis has also shown that CEA levels increase with increasing cell confluence, although I would maintain the stance that immunotherapy is best served in micrometastatic disease. Although not confirmed by our results, other workers have shown that CEA is upregulated by cytokines commonly found in the microenvironment of an anti-tumour response. These include IFN- γ (Guadagni et

al., 1990; Hauck and Stanners, 1991) and IL-6 (Ullmann et al., 1992) and such a response would serve to increase the cytotoxic potential of PR1A3.

In terms of antibody mechanism this thesis has strongly suggested that PR1A3 acts *in vitro* by ADCC and has no ability to block any putative function of CEA. ADCC is likely to be the mode of action *in vivo*. This assertion is based on the following findings:

1. hPR1A3 has no cytotoxic activity in the absence of effector cells.
2. Changing the Fc portion of PR1A3 to a murine IgG1 results in the antibody losing cytotoxicity because murine IgG1 binds poorly with the Fc γ IIIa receptor on the effector cell.
3. The addition of anti-CD16 antibody completely abolishes the cytotoxic activity of hPR1A3
4. hPR1A3 works at a lower effector : target cell concentration if NK cells alone are used as the effector instead of unfractionated PBMC. NK cells have a high concentration of Fc γ IIIa on their surface and are the major effector involved in ADCC in humans.

I have made the case that ADCC is likely to be the main mode of action of the majority of naked antibodies in clinical use.

This thesis has demonstrated that the interaction of the Fc portion of the antibody with the human Fc γ IIIa receptor is critical for antibody function. Genotyping of a polymorphism in the binding site of the Fc γ RIIIa may be of benefit in tailoring antibody treatment to individuals as it influences the strength of effector-antibody binding and influences the response to antibody therapy (Cartron et al., 2002). Identifying a valine homozygote would allow some comparative ADCC studies with lower binding polymorphisms.

This thesis has demonstrated an additive effect between hPR1A3 and a non-CEA antibody, A33. Combination antibody therapy is one of the potential solutions to the problem of antigen escape. The ability of the A33 antibody and hPR1A3 to combine their cytotoxic potential whilst targeting different antigens is encouraging and there was no inhibition of hPR1A3 activity by the addition of a second antibody. Although we have not

yet demonstrated a synergistic action of 5-FU with PR1A3, the upregulation of CEA by 5-FU discussed above, gives us hope that such synergy is possible.

Experimentally we have also explored novel ways of measuring cytotoxic activity *in vitro*. There are well-recognised problems in targeting cells which have been in some way been exposed to a labelling process. The concern is that the labelling itself may influence its response to the antibody treatment. Chromium-labelling has the added disadvantage of the radioactive risk to the investigator. We designed a label-free cytotoxic assay based on the apoptosis marker, M30. We were able to demonstrate a typical cytotoxic graph of increasing lysis (M30 concentration) with increasing levels of hPR1A3 in a label free cytotoxicity assay. However this assay was by no means optimised and further work is required before we can assess whether it will be useful in routine use.

The cytotoxic assays used within this thesis are all done with unstimulated white cells. Most studies in the literature use preconditioned effector cells which have been stimulated with IFN- γ or IL-2. This may indeed reflect more accurately the *in vivo* conditions where immune activation leads to local pro-inflammatory cytokine release. However it may also lead to hyper-responsiveness both to spontaneous killing and to antibody therapy. This could either give a false impression about an antibody's potential or indeed obscure its potential by significantly increasing the level of spontaneous killing. The lack of preconditioning however may account for some of the differences noted such as the low level of killing in occasional assays, especially when using cells from leucodepletion filters, rather than whole blood, the former having been through a cell sorting process and then compacted within the filter for about 18 hours before use, hence potentially losing some of their responsiveness.

Further improvements in the cytotoxicity of PR1A3 will undoubtedly be seen following affinity maturation and glycosylation of the antibody as described above.

In summary, the work that is presented here underlines the potential application of PR1A3, a monoclonal antibody targeting CEA, in the adjuvant treatment of colorectal cancer.

References

- Aaltonen, L., Johns, L., Jarvinen, H., Mecklin, J. P., and Houlston, R. (2007). Explaining the familial colorectal cancer risk associated with mismatch repair (MMR)-deficient and MMR-stable tumors. *Clin Cancer Res* 13, 356-361.
- ACPGBI (2007). Guidelines for the management of colorectal cancer (The Association of Coloproctology of GB & Ireland).
- Adams, G. P., Schier, R., McCall, A. M., Simmons, H. H., Horak, E. M., Alpaugh, R. K., Marks, J. D., and Weiner, L. M. (2001). High affinity restricts the localization and tumor penetration of single-chain fv antibody molecules. *Cancer Res* 61, 4750-4755.
- Adams, G. P., and Weiner, L. M. (2005). Monoclonal antibody therapy of cancer. *Nat Biotechnol* 23, 1147-1157.
- Ahnen, D. J., Feigl, P., Quan, G., Fenoglio-Preiser, C., Lovato, L. C., Bunn, P. A., Jr., Stemmerman, G., Wells, J. D., Macdonald, J. S., and Meyskens, F. L., Jr. (1998). Ki-ras mutation and p53 overexpression predict the clinical behavior of colorectal cancer: a Southwest Oncology Group study. *Cancer Res* 58, 1149-1158.
- Ajani, J. A., Kelsen, D. P., Haller, D., Hargraves, K., and Healey, D. (2000). A multi-institutional phase II study of BMS-182248-01 (BR96-doxorubicin conjugate) administered every 21 days in patients with advanced gastric adenocarcinoma. *Cancer J* 6, 78-81.
- Akiyama, K., Ebihara, S., Yada, A., Matsumura, K., Aiba, S., Nukiwa, T., and Takai, T. (2003). Targeting apoptotic tumor cells to Fc gamma R provides efficient and versatile vaccination against tumors by dendritic cells. *J Immunol* 170, 1641-1648.
- Albert, D., Dunham, J., Khan, S., Stansberry, J., Kolasinski, S., Tsai, D., Pullman-Moore, S., Barnack, F., Striebich, C., Looney, R. J., *et al.* (2008). Variability in the biological response to anti-CD20 B-cell depletion in SLE. *Ann Rheum Dis*.
- Algarra, I., Garcia-Lora, A., Cabrera, T., Ruiz-Cabello, F., and Garrido, F. (2004). The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. *Cancer Immunol Immunother* 53, 904-910.
- Allegra, C. J., Paik, S., Colangelo, L. H., Parr, A. L., Kirsch, I., Kim, G., Klein, P., Johnston, P. G., Wolmark, N., and Wieand, H. S. (2003). Prognostic value of thymidylate synthase, Ki-67, and p53 in patients with Dukes' B and C colon cancer: a National Cancer

Institute-National Surgical Adjuvant Breast and Bowel Project collaborative study. *J Clin Oncol* 21, 241-250.

Andre, T., Boni, C., Mounedji-Boudiaf, L., Navarro, M., Taberero, J., Hickish, T., Topham, C., Zaninelli, M., Clingan, P., Bridgewater, J., *et al.* (2004). Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. *N Engl J Med* 350, 2343-2351.

Andreyev, H. J., Norman, A. R., Cunningham, D., Oates, J., Dix, B. R., Iacopetta, B. J., Young, J., Walsh, T., Ward, R., Hawkins, N., *et al.* (2001). Kirsten ras mutations in patients with colorectal cancer: the 'RASCAL II' study. *Br J Cancer* 85, 692-696.

Anonymous (1985). A randomized clinical trial of OKT3 monoclonal antibody for acute rejection of cadaveric renal transplants. Ortho Multicenter Transplant Study Group. *N Engl J Med* 313, 337-342.

Anonymous (2004). A comparison of laparoscopically assisted and open colectomy for colon cancer. *N Engl J Med* 350, 2050-2059.

Aquino, A., Prete, S. P., Greiner, J. W., Giuliani, A., Graziani, G., Turriziani, M., De Filippi, R., Masci, G., Bonmassar, E., and De Vecchis, L. (1998). Effect of the combined treatment with 5-fluorouracil, gamma-interferon or folinic acid on carcinoembryonic antigen expression in colon cancer cells. *Clin Cancer Res* 4, 2473-2481.

Arber, N., Eagle, C. J., Spicak, J., Racz, I., Dite, P., Hajer, J., Zavoral, M., Lechuga, M. J., Gerletti, P., Tang, J., *et al.* (2006). Celecoxib for the prevention of colorectal adenomatous polyps. *N Engl J Med* 355, 885-895.

Armstrong, B., and Doll, R. (1975). Environmental factors and cancer incidence and mortality in different countries, with special reference to dietary practices. *Int J Cancer* 15, 617-631.

Arnould, L., Gelly, M., Penault-Llorca, F., Benoit, L., Bonnetain, F., Migeon, C., Cabaret, V., Fermeaux, V., Bertheau, P., Garnier, J., *et al.* (2006). Trastuzumab-based treatment of HER2-positive breast cancer: an antibody-dependent cellular cytotoxicity mechanism? *Br J Cancer* 94, 259-267.

Axworthy, D. B., Reno, J. M., Hylarides, M. D., Mallett, R. W., Theodore, L. J., Gustavson, L. M., Su, F., Hobson, L. J., Beaumier, P. L., and Fritzberg, A. R. (2000). Cure of human carcinoma xenografts by a single dose of pretargeted yttrium-90 with negligible toxicity. *Proc Natl Acad Sci U S A* 97, 1802-1807.

Azzoni, L., Anegon, I., Calabretta, B., and Perussia, B. (1995). Ligand binding to Fc gamma R induces c-myc-dependent apoptosis in IL-2-stimulated NK cells. *J Immunol* *154*, 491-499.

Baker, S. J., Preisinger, A. C., Jessup, J. M., Paraskeva, C., Markowitz, S., Willson, J. K., Hamilton, S., and Vogelstein, B. (1990). p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. *Cancer Res* *50*, 7717-7722.

Ballangrud, A. M., Yang, W. H., Palm, S., Enmon, R., Borchardt, P. E., Pellegrini, V. A., McDevitt, M. R., Scheinberg, D. A., and Sgouros, G. (2004). Alpha-particle emitting atomic generator (Actinium-225)-labeled trastuzumab (herceptin) targeting of breast cancer spheroids: efficacy versus HER2/neu expression. *Clin Cancer Res* *10*, 4489-4497.

Bargou, R., Leo, E., Zugmaier, G., Klinger, M., Goebeler, M., Knop, S., Noppeney, R., Viardot, A., Hess, G., Schuler, M., *et al.* (2008). Tumor regression in cancer patients by very low doses of a T cell-engaging antibody. *Science* *321*, 974-977.

Baron, J. A., Cole, B. F., Sandler, R. S., Haile, R. W., Ahnen, D., Bresalier, R., McKeown-Eyssen, G., Summers, R. W., Rothstein, R., Burke, C. A., *et al.* (2003). A randomized trial of aspirin to prevent colorectal adenomas. *N Engl J Med* *348*, 891-899.

Baron, J. A., Sandler, R. S., Bresalier, R. S., Quan, H., Riddell, R., Lanas, A., Bolognese, J. A., Oxenius, B., Horgan, K., Loftus, S., and Morton, D. G. (2006). A randomized trial of rofecoxib for the chemoprevention of colorectal adenomas. *Gastroenterology* *131*, 1674-1682.

Baselga, J. (2001). The EGFR as a target for anticancer therapy--focus on cetuximab. *Eur J Cancer* *37 Suppl 4*, S16-22.

Baselga, J., and Albanell, J. (2001). Mechanism of action of anti-HER2 monoclonal antibodies. *Ann Oncol* *12 Suppl 1*, S35-41.

Beauchemin, N., Draber, P., Dveksler, G., Gold, P., Gray-Owen, S., Grunert, F., Hammarstrom, S., Holmes, K. V., Karlsson, A., Kuroki, M., *et al.* (1999). Redefined nomenclature for members of the carcinoembryonic antigen family. *Exp Cell Res* *252*, 243-249.

Begent, R. H., Verhaar, M. J., Chester, K. A., Casey, J. L., Green, A. J., Napier, M. P., Hope-Stone, L. D., Cushen, N., Keep, P. A., Johnson, C. J., *et al.* (1996). Clinical evidence of efficient tumor targeting based on single-chain Fv antibody selected from a combinatorial library. *Nat Med* *2*, 979-984.

Behr, T. M., Liersch, T., Greiner-Bechert, L., Griesinger, F., Behe, M., Markus, P. M., Gratz, S., Angerstein, C., Brittinger, G., Becker, H., *et al.* (2002). Radioimmunotherapy of small-volume disease of metastatic colorectal cancer. *Cancer* 94, 1373-1381.

Behr, T. M., Sharkey, R. M., Juweid, M. E., Dunn, R. M., Ying, Z., Zhang, C. H., Siegel, J. A., and Goldenberg, D. M. (1997). Variables influencing tumor dosimetry in radioimmunotherapy of CEA-expressing cancers with anti-CEA and antimucin monoclonal antibodies. *J Nucl Med* 38, 409-418.

Benchimol, S., Fuks, A., Jothy, S., Beauchemin, N., Shirota, K., and Stanners, C. P. (1989). Carcinoembryonic antigen, a human tumor marker, functions as an intercellular adhesion molecule. *Cell* 57, 327-334.

Bertagnolli, M. M., Eagle, C. J., Zauber, A. G., Redston, M., Solomon, S. D., Kim, K., Tang, J., Rosenstein, R. B., Wittes, J., Corle, D., *et al.* (2006). Celecoxib for the prevention of sporadic colorectal adenomas. *N Engl J Med* 355, 873-884.

Bevaart, L., Jansen, M. J., van Vugt, M. J., Verbeek, J. S., van de Winkel, J. G., and Leusen, J. H. (2006). The high-affinity IgG receptor, FcγRI, plays a central role in antibody therapy of experimental melanoma. *Cancer Res* 66, 1261-1264.

Bicknell, D. C., Rowan, A., and Bodmer, W. F. (1994). Beta 2-microglobulin gene mutations: a study of established colorectal cell lines and fresh tumors. *Proc Natl Acad Sci U S A* 91, 4751-4755.

Bingham, S. A., Day, N. E., Luben, R., Ferrari, P., Slimani, N., Norat, T., Clavel-Chapelon, F., Kesse, E., Nieters, A., Boeing, H., *et al.* (2003). Dietary fibre in food and protection against colorectal cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC): an observational study. *Lancet* 361, 1496-1501.

Birgisson, H., Pahlman, L., Gunnarsson, U., and Glimelius, B. (2008). Late gastrointestinal disorders after rectal cancer surgery with and without preoperative radiation therapy. *Br J Surg* 95, 206-213.

Bleeker, W. K., Lammerts van Bueren, J. J., van Ojik, H. H., Gerritsen, A. F., Pluyter, M., Houtkamp, M., Halk, E., Goldstein, J., Schuurman, J., van Dijk, M. A., *et al.* (2004). Dual mode of action of a human anti-epidermal growth factor receptor monoclonal antibody for cancer therapy. *J Immunol* 173, 4699-4707.

Blomberg, K., Hautala, R., Lovgren, J., Mukkala, V. M., Lindqvist, C., and Akerman, K. (1996). Time-resolved fluorometric assay for natural killer activity using target cells labelled with a fluorescence enhancing ligand. *J Immunol Methods* 193, 199-206.

Bodmer, W., Bielas, J. H., and Beckman, R. A. (2008). Genetic instability is not a requirement for tumor development. *Cancer Res* 68, 3558-3560; discussion 3560-3551.

Bolland, S., and Ravetch, J. V. (1999). Inhibitory pathways triggered by ITIM-containing receptors. *Adv Immunol* 72, 149-177.

Borner, M., Koeberle, D., Von Moos, R., Saletti, P., Rauch, D., Hess, V., Trojan, A., Helbling, D., Pestalozzi, B., Caspar, C., *et al.* (2008). Adding cetuximab to capecitabine plus oxaliplatin (XELOX) in first-line treatment of metastatic colorectal cancer: a randomized phase II trial of the Swiss Group for Clinical Cancer Research SAKK. *Ann Oncol* 19, 1288-1292.

Bottino, C., Castriconi, R., Moretta, L., and Moretta, A. (2005). Cellular ligands of activating NK receptors. *Trends Immunol* 26, 221-226.

Brattain, M. G., Fine, W. D., Khaled, F. M., Thompson, J., and Brattain, D. E. (1981). Heterogeneity of malignant cells from a human colonic carcinoma. *Cancer Res* 41, 1751-1756.

Breukink, S., Pierie, J., and Wiggers, T. (2006). Laparoscopic versus open total mesorectal excision for rectal cancer. *Cochrane Database Syst Rev*, CD005200.

Brischwein, K., Schlereth, B., Guller, B., Steiger, C., Wolf, A., Lutterbuese, R., Offner, S., Locher, M., Urbig, T., Raum, T., *et al.* (2006). MT110: a novel bispecific single-chain antibody construct with high efficacy in eradicating established tumors. *Mol Immunol* 43, 1129-1143.

Brodsky, F. M., Parham, P., and Bodmer, W. F. (1980). Monoclonal antibodies to HLA--DRw determinants. *Tissue Antigens* 16, 30-48.

Browning, M. J., Krausa, P., Rowan, A., Hill, A. B., Bicknell, D. C., Bodmer, J. G., and Bodmer, W. F. (1993). Loss of human leukocyte antigen expression on colorectal tumor cell lines: implications for anti-tumor immunity and immunotherapy. *J Immunother* 14, 163-168.

Bunz, F., Hwang, P. M., Torrance, C., Waldman, T., Zhang, Y., Dillehay, L., Williams, J., Lengauer, C., Kinzler, K. W., and Vogelstein, B. (1999). Disruption of p53 in human cancer cells alters the responses to therapeutic agents. *J Clin Invest* 104, 263-269.

Cairns, S., and Scholefield, J. H. (2002). Guidelines for colorectal cancer screening in high risk groups. *Gut* 51, v1-2.

Caligiuri, M. A., Zmuidzinas, A., Manley, T. J., Levine, H., Smith, K. A., and Ritz, J. (1990). Functional consequences of interleukin 2 receptor expression on resting human lymphocytes. Identification of a novel natural killer cell subset with high affinity receptors. *J Exp Med* *171*, 1509-1526.

Camacho-Leal, P., Zhai, A. B., and Stanners, C. P. (2007). A co-clustering model involving alpha5beta1 integrin for the biological effects of GPI-anchored human carcinoembryonic antigen (CEA). *J Cell Physiol* *211*, 791-802.

Cancer-Research-UK (2007). Cancer Research UK. Statistics on colorectal cancer (<http://www.info.cancerresearchuk.org/cancerstats/types/bowel/incidence/>).

Cappelletti, D., Cardillo, A., Bonanno, E., Prete, S. P., Cucchiara, G., Turriziani, M., Greiner, J. W., Cottarelli, A., Breda, E., Aquino, A., *et al.* (2000). Drug-induced modulation of carcinoembryonic antigen (CEA) expression in neoplastic cells from a patient with rectal cancer. *J Exp Clin Cancer Res* *19*, 467-469.

Carter, P. (2001). Improving the efficacy of antibody-based cancer therapies. *Nat Rev Cancer* *1*, 118-129.

Carter, P., Presta, L., Gorman, C. M., Ridgway, J. B., Henner, D., Wong, W. L., Rowland, A. M., Kotts, C., Carver, M. E., and Shepard, H. M. (1992). Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc Natl Acad Sci U S A* *89*, 4285-4289.

Carter, P. J. (2006). Potent antibody therapeutics by design. *Nat Rev Immunol* *6*, 343-357.

Cartron, G., Dacheux, L., Salles, G., Solal-Celigny, P., Bardos, P., Colombat, P., and Watier, H. (2002). Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. *Blood* *99*, 754-758.

Caulin, C., Salvesen, G. S., and Oshima, R. G. (1997). Caspase cleavage of keratin 18 and reorganization of intermediate filaments during epithelial cell apoptosis. *J Cell Biol* *138*, 1379-1394.

CCO-Formulary (2007). CCO Formulary, In Fluorouracil.

Chan, A. T., Giovannucci, E. L., Meyerhardt, J. A., Schernhammer, E. S., Curhan, G. C., and Fuchs, C. S. (2005). Long-term use of aspirin and nonsteroidal anti-inflammatory drugs and risk of colorectal cancer. *Jama* *294*, 914-923.

Chan, C. H., Cook, D., and Stanners, C. P. (2006). Increased colon tumor susceptibility in azoxymethane treated CEABAC transgenic mice. *Carcinogenesis* 27, 1909-1916.

Chau, I., Allen, M. J., Cunningham, D., Norman, A. R., Brown, G., Ford, H. E., Tebbutt, N., Tait, D., Hill, M., Ross, P. J., and Oates, J. (2004). The value of routine serum carcino-embryonic antigen measurement and computed tomography in the surveillance of patients after adjuvant chemotherapy for colorectal cancer. *J Clin Oncol* 22, 1420-1429.

Chen, J. Y., Wang, C. M., Wu, J. M., Ho, H. H., and Luo, S. F. (2006). Association of rheumatoid factor production with Fcγ3 polymorphism in Taiwanese rheumatoid arthritis. *Clin Exp Immunol* 144, 10-16.

Chong, G., Lee, F. T., Hopkins, W., Tebbutt, N., Cebon, J. S., Mountain, A. J., Chappell, B., Papenfuss, A., Schleyer, P., U, P., *et al.* (2005). Phase I trial of 131I-huA33 in patients with advanced colorectal carcinoma. *Clin Cancer Res* 11, 4818-4826.

Choy, E. H., Hazleman, B., Smith, M., Moss, K., Lisi, L., Scott, D. G., Patel, J., Sopwith, M., and Isenberg, D. A. (2002). Efficacy of a novel PEGylated humanized anti-TNF fragment (CDP870) in patients with rheumatoid arthritis: a phase II double-blinded, randomized, dose-escalating trial. *Rheumatology (Oxford)* 41, 1133-1137.

Chu, D. Z., Giacco, G., Martin, R. G., and Guinee, V. F. (1986). The significance of synchronous carcinoma and polyps in the colon and rectum. *Cancer* 57, 445-450.

Chulada, P. C., Thompson, M. B., Mahler, J. F., Doyle, C. M., Gaul, B. W., Lee, C., Tiano, H. F., Morham, S. G., Smithies, O., and Langenbach, R. (2000). Genetic disruption of Ptg-1, as well as Ptg-2, reduces intestinal tumorigenesis in Min mice. *Cancer Res* 60, 4705-4708.

Clark, M. R., Clarkson, S. B., Ory, P. A., Stollman, N., and Goldstein, I. M. (1989). Molecular basis for a polymorphism involving Fc receptor II on human monocytes. *J Immunol* 143, 1731-1734.

Clarke, P., Mann, J., Simpson, J. F., Rickard-Dickson, K., and Primus, F. J. (1998). Mice transgenic for human carcinoembryonic antigen as a model for immunotherapy. *Cancer Res* 58, 1469-1477.

Clynes, R. (2006). Antitumor antibodies in the treatment of cancer: Fc receptors link opsonic antibody with cellular immunity. *Hematol Oncol Clin North Am* 20, 585-612.

Clynes, R., Takechi, Y., Moroi, Y., Houghton, A., and Ravetch, J. V. (1998). Fc receptors are required in passive and active immunity to melanoma. *Proc Natl Acad Sci U S A* 95, 652-656.

Clynes, R. A., Towers, T. L., Presta, L. G., and Ravetch, J. V. (2000). Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nat Med* 6, 443-446.

Coiffier, B., Lefebvre, S., Pedersen, L. M., Gadeberg, O., Fredriksen, H., van Oers, M. H., Wooldridge, J., Kloczko, J., Holowiecki, J., Hellmann, A., *et al.* (2008). Safety and efficacy of ofatumumab, a fully human monoclonal anti-CD20 antibody, in patients with relapsed or refractory B-cell chronic lymphocytic leukemia: a phase 1-2 study. *Blood* 111, 1094-1100.

Conaghan, P., Ashraf, S., Tytherleigh, M., Wilding, J., Tchilian, E., Bicknell, D., Mortensen, N. J., and Bodmer, W. (2008). Targeted killing of colorectal cancer cell lines by a humanised IgG1 monoclonal antibody that binds to membrane-bound carcinoembryonic antigen. *Br J Cancer* 98, 1217-1225.

Conlin, A., Smith, G., Carey, F. A., Wolf, C. R., and Steele, R. J. (2005). The prognostic significance of K-ras, p53, and APC mutations in colorectal carcinoma. *Gut* 54, 1283-1286.

Cooper, M. A., Fehniger, T. A., and Caligiuri, M. A. (2001a). The biology of human natural killer-cell subsets. *Trends Immunol* 22, 633-640.

Cooper, M. A., Fehniger, T. A., Turner, S. C., Chen, K. S., Ghaheri, B. A., Ghayur, T., Carson, W. E., and Caligiuri, M. A. (2001b). Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood* 97, 3146-3151.

Correale, P., Aquino, A., Giuliani, A., Pellegrini, M., Micheli, L., Cusi, M. G., Nencini, C., Petrioli, R., Prete, S. P., De Vecchis, L., *et al.* (2003). Treatment of colon and breast carcinoma cells with 5-fluorouracil enhances expression of carcinoembryonic antigen and susceptibility to HLA-A(*)02.01 restricted, CEA-peptide-specific cytotoxic T cells in vitro. *Int J Cancer* 104, 437-445.

Cunningham, D., Humblet, Y., Siena, S., Khayat, D., Bleiberg, H., Santoro, A., Bets, D., Mueser, M., Harstrick, A., Verslype, C., *et al.* (2004). Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med* 351, 337-345.

Czito, B. G., Bendell, J. C., Willett, C. G., Morse, M. A., Blobe, G. C., Tyler, D. S., Thomas, J., Ludwig, K. A., Mantyh, C. R., Ashton, J., *et al.* (2007). Bevacizumab, oxaliplatin, and capecitabine with radiation therapy in rectal cancer: Phase I trial results. *Int J Radiat Oncol Biol Phys* 68, 472-478.

Czuczman, M. S., Weaver, R., Alkuzweny, B., Berlfein, J., and Grillo-Lopez, A. J. (2004). Prolonged clinical and molecular remission in patients with low-grade or

follicular non-Hodgkin's lymphoma treated with rituximab plus CHOP chemotherapy: 9-year follow-up. *J Clin Oncol* 22, 4711-4716.

Dall'Ozzo, S., Tartas, S., Piantaud, G., Cartron, G., Colombat, P., Bardos, P., Watier, H., and Thibault, G. (2004). Rituximab-dependent cytotoxicity by natural killer cells: influence of FCGR3A polymorphism on the concentration-effect relationship. *Cancer Res* 64, 4664-4669.

Davis, T. A., Grillo-Lopez, A. J., White, C. A., McLaughlin, P., Czuczman, M. S., Link, B. K., Maloney, D. G., Weaver, R. L., Rosenberg, J., and Levy, R. (2000). Rituximab anti-CD20 monoclonal antibody therapy in non-Hodgkin's lymphoma: safety and efficacy of re-treatment. *J Clin Oncol* 18, 3135-3143.

Dhodapkar, K. M., and Dhodapkar, M. V. (2005). Recruiting dendritic cells to improve antibody therapy of cancer. *Proc Natl Acad Sci U S A* 102, 6243-6244.

Dhodapkar, K. M., Kaufman, J. L., Ehlers, M., Banerjee, D. K., Bonvini, E., Koenig, S., Steinman, R. M., Ravetch, J. V., and Dhodapkar, M. V. (2005). Selective blockade of inhibitory Fcγ receptor enables human dendritic cell maturation with IL-12p70 production and immunity to antibody-coated tumor cells. *Proc Natl Acad Sci U S A* 102, 2910-2915.

Di Gaetano, N., Cittera, E., Nota, R., Vecchi, A., Grieco, V., Scanziani, E., Botto, M., Introna, M., and Golay, J. (2003). Complement activation determines the therapeutic activity of rituximab in vivo. *J Immunol* 171, 1581-1587.

Diep, C. B., Thorstensen, L., Meling, G. I., Skovlund, E., Rognum, T. O., and Lothe, R. A. (2003). Genetic tumor markers with prognostic impact in Dukes' stages B and C colorectal cancer patients. *J Clin Oncol* 21, 820-829.

Divgi, C. R., Scott, A. M., Dantis, L., Capitelli, P., Siler, K., Hilton, S., Finn, R. D., Kemeny, N., Kelsen, D., Kostakoglu, L., and et al. (1995). Phase I radioimmunotherapy trial with iodine-131-CC49 in metastatic colon carcinoma. *J Nucl Med* 36, 586-592.

Donnellan, I., Cantrill, J., Fraser, I., and Morris, A. (1995). Activation by interferon-gamma of expression of ICAM-1 and MHC class II antigens in tumour cells from colorectal carcinomas. *Clin Mol Pathol* 48, M40-M45.

Douillard, J. Y., Cunningham, D., Roth, A. D., Navarro, M., James, R. D., Karasek, P., Jandik, P., Iveson, T., Carmichael, J., Alakl, M., et al. (2000). Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. *Lancet* 355, 1041-1047.

Drewinko, B., Romsdahl, M. M., Yang, L. Y., Ahearn, M. J., and Trujillo, J. M. (1976). Establishment of a human carcinoembryonic antigen-producing colon adenocarcinoma cell line. *Cancer Res* 36, 467-475.

Dukes, C. E. (1949). The Surgical Pathology of Rectal Cancer. *J Clin Pathol* 2, 95-98.

Durbin, H., and Bodmer, W. F. (1987). A sensitive micro-immunoassay using beta-galactosidase/anti-beta-galactosidase complexes. *J Immunol Methods* 97, 19-27.

Durbin, H., Young, S., Stewart, L. M., Wrba, F., Rowan, A. J., Snary, D., and Bodmer, W. F. (1994). An epitope on carcinoembryonic antigen defined by the clinically relevant antibody PR1A3. *Proc Natl Acad Sci U S A* 91, 4313-4317.

Eades-Perner, A. M., van der Putten, H., Hirth, A., Thompson, J., Neumaier, M., von Kleist, S., and Zimmermann, W. (1994). Mice transgenic for the human carcinoembryonic antigen gene maintain its spatiotemporal expression pattern. *Cancer Res* 54, 4169-4176.

Eberhart, C. E., Coffey, R. J., Radhika, A., Giardiello, F. M., Ferrenbach, S., and DuBois, R. N. (1994). Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 107, 1183-1188.

Eidelman, F. J., Fuks, A., DeMarte, L., Taheri, M., and Stanners, C. P. (1993). Human carcinoembryonic antigen, an intercellular adhesion molecule, blocks fusion and differentiation of rat myoblasts. *J Cell Biol* 123, 467-475.

Eisenbeis, C. F., Grainger, A., Fischer, B., Baiocchi, R. A., Carrodegua, L., Roychowdhury, S., Chen, L., Banks, A. L., Davis, T., Young, D., *et al.* (2004). Combination immunotherapy of B-cell non-Hodgkin's lymphoma with rituximab and interleukin-2: a preclinical and phase I study. *Clin Cancer Res* 10, 6101-6110.

Ensembl (2009). <http://www.ensembl.org/index.html>.

Epenetos, A. A., Canti, G., Taylor-Papadimitriou, J., Curling, M., and Bodmer, W. F. (1982). Use of two epithelium-specific monoclonal antibodies for diagnosis of malignancy in serous effusions. *Lancet* 2, 1004-1006.

Epenetos, A. A., Munro, A. J., Stewart, S., Rampling, R., Lambert, H. E., McKenzie, C. G., Soutter, P., Rahemtulla, A., Hooker, G., Sivolapenko, G. B., and *et al.* (1987). Antibody-guided irradiation of advanced ovarian cancer with intraperitoneally administered radiolabeled monoclonal antibodies. *J Clin Oncol* 5, 1890-1899.

- Esteban, J. M., Felder, B., Ahn, C., Simpson, J. F., Battifora, H., and Shively, J. E. (1994). Prognostic relevance of carcinoembryonic antigen and estrogen receptor status in breast cancer patients. *Cancer* *74*, 1575-1583.
- Fan, Z., Masui, H., Altas, I., and Mendelsohn, J. (1993). Blockade of epidermal growth factor receptor function by bivalent and monovalent fragments of 225 anti-epidermal growth factor receptor monoclonal antibodies. *Cancer Res* *53*, 4322-4328.
- Farag, S. S., Flinn, I. W., Modali, R., Lehman, T. A., Young, D., and Byrd, J. C. (2004). Fc gamma RIIIa and Fc gamma RIIa polymorphisms do not predict response to rituximab in B-cell chronic lymphocytic leukemia. *Blood* *103*, 1472-1474.
- Fearnhead, N. S., Britton, M. P., and Bodmer, W. F. (2001). The ABC of APC. *Hum Mol Genet* *10*, 721-733.
- Fearnhead, N. S., Wilding, J. L., Winney, B., Tonks, S., Bartlett, S., Bicknell, D. C., Tomlinson, I. P., Mortensen, N. J., and Bodmer, W. F. (2004). Multiple rare variants in different genes account for multifactorial inherited susceptibility to colorectal adenomas. *Proc Natl Acad Sci U S A* *101*, 15992-15997.
- Fichera, A., Michelassi, F., and Arenas, R. B. (1998). Selective expression of carcinoembryonic antigen promoter in cancer cell lines: targeting strategy for gene therapy in colorectal cancer. *Dis Colon Rectum* *41*, 747-754.
- Firestein, R., Bass, A. J., Kim, S. Y., Dunn, I. F., Silver, S. J., Guney, I., Freed, E., Ligon, A. H., Vena, N., Ogino, S., *et al.* (2008). CDK8 is a colorectal cancer oncogene that regulates beta-catenin activity. *Nature* *455*, 547-551.
- Foon, K. A., Yannelli, J., and Bhattacharya-Chatterjee, M. (1999). Colorectal cancer as a model for immunotherapy. *Clin Cancer Res* *5*, 225-236.
- Forslund, A., Lonroth, C., Andersson, M., Brevinge, H., and Lundholm, K. (2001). Mutations and allelic loss of p53 in primary tumor DNA from potentially cured patients with colorectal carcinoma. *J Clin Oncol* *19*, 2829-2836.
- Francis, R. J., Sharma, S. K., Springer, C., Green, A. J., Hope-Stone, L. D., Sena, L., Martin, J., Adamson, K. L., Robbins, A., Gumbrell, L., *et al.* (2002). A phase I trial of antibody directed enzyme prodrug therapy (ADEPT) in patients with advanced colorectal carcinoma or other CEA producing tumours. *Br J Cancer* *87*, 600-607.
- Franklin, M. C., Carey, K. D., Vajdos, F. F., Leahy, D. J., de Vos, A. M., and Sliwkowski, M. X. (2004). Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. *Cancer Cell* *5*, 317-328.

Friedberg, J. W., Neuberg, D., Gribben, J. G., Fisher, D. C., Canning, C., Koval, M., Poor, C. M., Green, L. M., Daley, J., Soiffer, R., *et al.* (2002). Combination immunotherapy with rituximab and interleukin 2 in patients with relapsed or refractory follicular non-Hodgkin's lymphoma. *Br J Haematol* *117*, 828-834.

Fritsche, R., and Mach, J. P. (1977). Isolation and characterization of carcinoembryonic antigen (CEA) extracted from normal human colon mucosa. *Immunochemistry* *14*, 119-127.

Gennari, R., Menard, S., Fagnoni, F., Ponchio, L., Scelsi, M., Tagliabue, E., Castiglioni, F., Villani, L., Magalotti, C., Gibelli, N., *et al.* (2004). Pilot study of the mechanism of action of preoperative trastuzumab in patients with primary operable breast tumors overexpressing HER2. *Clin Cancer Res* *10*, 5650-5655.

Giusti, R. M., Shastri, K., Pilaro, A. M., Fuchs, C., Cordoba-Rodriguez, R., Koti, K., Rothmann, M., Men, A. Y., Zhao, H., Hughes, M., *et al.* (2008). U.S. Food and Drug Administration approval: panitumumab for epidermal growth factor receptor-expressing metastatic colorectal carcinoma with progression following fluoropyrimidine-, oxaliplatin-, and irinotecan-containing chemotherapy regimens. *Clin Cancer Res* *14*, 1296-1302.

Glimcher, L. H., and Kara, C. J. (1992). Sequences and factors: a guide to MHC class-II transcription. *Annu Rev Immunol* *10*, 13-49.

Gold, P., and Freedman, S. O. (1965). Specific carcinoembryonic antigens of the human digestive system. *J Exp Med* *122*, 467-481.

Goldenberg, D. M. (2002). Targeted therapy of cancer with radiolabeled antibodies. *J Nucl Med* *43*, 693-713.

Goldenberg, M. M. (1999). Trastuzumab, a recombinant DNA-derived humanized monoclonal antibody, a novel agent for the treatment of metastatic breast cancer. *Clin Ther* *21*, 309-318.

Goldstein, N. I., Prewett, M., Zuklys, K., Rockwell, P., and Mendelsohn, J. (1995). Biological efficacy of a chimeric antibody to the epidermal growth factor receptor in a human tumor xenograft model. *Clin Cancer Res* *1*, 1311-1318.

Golfinopoulos, V., Salanti, G., Pavlidis, N., and Ioannidis, J. P. (2007). Survival and disease-progression benefits with treatment regimens for advanced colorectal cancer: a meta-analysis. *Lancet Oncol* *8*, 898-911.

Gordon, L. I., Witzig, T., Molina, A., Czuczman, M., Emmanouilides, C., Joyce, R., Vo, K., Theuer, C., Pohlman, B., Bartlett, N., *et al.* (2004). Yttrium 90-labeled ibritumomab

tiuxetan radioimmunotherapy produces high response rates and durable remissions in patients with previously treated B-cell lymphoma. *Clin Lymphoma* 5, 98-101.

Gorter, A., and Meri, S. (1999). Immune evasion of tumor cells using membrane-bound complement regulatory proteins. *Immunol Today* 20, 576-582.

Granowska, M., Britton, K. E., Mather, S. J., Morris, G., Ellison, D., Soobramoney, S., Talbot, I. C., and Northover, J. M. (1993). Radioimmunoscinigraphy with technetium-99m labelled monoclonal antibody, 1A3, in colorectal cancer. *Eur J Nucl Med* 20, 690-698.

Granowska, M., Mather, S. J., Britton, K. E., Bentley, S., Richman, P., Phillips, R. K., and Northover, J. M. (1990). 99mTc radioimmunoscinigraphy of colorectal cancer. *Br J Cancer Suppl* 10, 30-33.

Granqvist, S. (1981). Distribution of polyps in the large bowel in relation to age. A colonoscopic study. *Scand J Gastroenterol* 16, 1025-1031.

Grau, M. V., Baron, J. A., Sandler, R. S., Wallace, K., Haile, R. W., Church, T. R., Beck, G. J., Summers, R. W., Barry, E. L., Cole, B. F., *et al.* (2007). Prolonged effect of calcium supplementation on risk of colorectal adenomas in a randomized trial. *J Natl Cancer Inst* 99, 129-136.

Greshock, J., Nathanson, K., Martin, A. M., Zhang, L., Coukos, G., Weber, B. L., and Zaks, T. Z. (2007). Cancer cell lines as genetic models of their parent histology: analyses based on array comparative genomic hybridization. *Cancer Res* 67, 3594-3600.

Gruel, Y., Pouplard, C., Lasne, D., Magdelaine-Beuzelin, C., Charroing, C., and Watier, H. (2004). The homozygous FcγRIIIa-158V genotype is a risk factor for heparin-induced thrombocytopenia in patients with antibodies to heparin-platelet factor 4 complexes. *Blood* 104, 2791-2793.

Guadagni, F., Witt, P. L., Robbins, P. F., Schlom, J., and Greiner, J. W. (1990). Regulation of carcinoembryonic antigen expression in different human colorectal tumor cells by interferon-gamma. *Cancer Res* 50, 6248-6255.

Guillou, P. J., Quirke, P., Thorpe, H., Walker, J., Jayne, D. G., Smith, A. M., Heath, R. M., and Brown, J. M. (2005). Short-term endpoints of conventional versus laparoscopic-assisted surgery in patients with colorectal cancer (MRC CLASICC trial): multicentre, randomised controlled trial. *Lancet* 365, 1718-1726.

Habr-Gama, A., Perez, R. O., Nadalin, W., Nahas, S. C., Ribeiro, U., Jr., Silva, E. S. A. H., Jr., Campos, F. G., Kiss, D. R., and Gama-Rodrigues, J. (2005). Long-term results of

preoperative chemoradiation for distal rectal cancer correlation between final stage and survival. *J Gastrointest Surg* 9, 90-99; discussion 99-101.

Habr-Gama, A., Perez, R. O., Nadalin, W., Sabbaga, J., Ribeiro, U., Jr., Silva e Sousa, A. H., Jr., Campos, F. G., Kiss, D. R., and Gama-Rodrigues, J. (2004). Operative versus nonoperative treatment for stage 0 distal rectal cancer following chemoradiation therapy: long-term results. *Ann Surg* 240, 711-717; discussion 717-718.

Hagg, M., Biven, K., Ueno, T., Rydlander, L., Bjorklund, P., Wiman, K. G., Shoshan, M., and Linder, S. (2002). A novel high-through-put assay for screening of pro-apoptotic drugs. *Invest New Drugs* 20, 253-259.

Hamada, S., and Hamada, S. (1977). Localization of carcinoembryonic antigen in medullary thyroid carcinoma by immunofluorescent techniques. *Br J Cancer* 36, 572-576.

Hammarstrom, S. (1999). The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues. *Semin Cancer Biol* 9, 67-81.

Hara, I., Takechi, Y., and Houghton, A. N. (1995). Implicating a role for immune recognition of self in tumor rejection: passive immunization against the brown locus protein. *J Exp Med* 182, 1609-1614.

Hardcastle, J. D., Chamberlain, J. O., Robinson, M. H., Moss, S. M., Amar, S. S., Balfour, T. W., James, P. D., and Mangham, C. M. (1996). Randomised controlled trial of faecal-occult-blood screening for colorectal cancer. *Lancet* 348, 1472-1477.

Hartmann, F., Horak, E. M., Garmestani, K., Wu, C., Brechbiel, M. W., Kozak, R. W., Tso, J., Kostein, S. A., Gansow, O. A., Nelson, D. L., and et al. (1994). Radioimmunotherapy of nude mice bearing a human interleukin 2 receptor alpha-expressing lymphoma utilizing the alpha-emitting radionuclide-conjugated monoclonal antibody 212Bi-anti-Tac. *Cancer Res* 54, 4362-4370.

Hauck, C. R., Agerer, F., Muenzner, P., and Schmitter, T. (2006). Cellular adhesion molecules as targets for bacterial infection. *Eur J Cell Biol* 85, 235-242.

Hauck, W., and Stanners, C. P. (1991). Control of carcinoembryonic antigen gene family expression in a differentiating colon carcinoma cell line, Caco-2. *Cancer Res* 51, 3526-3533.

Hawkins, N. J., Tomlinson, I., Meagher, A., and Ward, R. L. (2001). Microsatellite-stable diploid carcinoma: a biologically distinct and aggressive subset of sporadic colorectal cancer. *Br J Cancer* 84, 232-236.

Hefta, S. A., Hefta, L. J., Lee, T. D., Paxton, R. J., and Shively, J. E. (1988). Carcinoembryonic antigen is anchored to membranes by covalent attachment to a glycosylphosphatidylinositol moiety: identification of the ethanolamine linkage site. *Proc Natl Acad Sci U S A* 85, 4648-4652.

Hemmila, E., Turbide, C., Olson, M., Jothy, S., Holmes, K. V., and Beauchemin, N. (2004). Ceacam1a^{-/-} mice are completely resistant to infection by murine coronavirus mouse hepatitis virus A59. *J Virol* 78, 10156-10165.

Hiddemann, W., Kneba, M., Dreyling, M., Schmitz, N., Lengfelder, E., Schmits, R., Reiser, M., Metzner, B., Harder, H., Hegewisch-Becker, S., *et al.* (2005). Frontline therapy with rituximab added to the combination of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) significantly improves the outcome for patients with advanced-stage follicular lymphoma compared with therapy with CHOP alone: results of a prospective randomized study of the German Low-Grade Lymphoma Study Group. *Blood* 106, 3725-3732.

Hinton, P. R., Xiong, J. M., Johlfs, M. G., Tang, M. T., Keller, S., and Tsurushita, N. (2006). An engineered human IgG1 antibody with longer serum half-life. *J Immunol* 176, 346-356.

Horig, H., Medina, F. A., Conkright, W. A., and Kaufman, H. L. (2000). Strategies for cancer therapy using carcinoembryonic antigen vaccines. *Expert Rev Mol Med* 2000, 1-24.

Horst, A. K., Ito, W. D., Dabelstein, J., Schumacher, U., Sander, H., Turbide, C., Brummer, J., Meinertz, T., Beauchemin, N., and Wagener, C. (2006). Carcinoembryonic antigen-related cell adhesion molecule 1 modulates vascular remodeling in vitro and in vivo. *J Clin Invest* 116, 1596-1605.

Howell, R., Hawley, P. R., Granowska, M., Morris, G., and Britton, K. E. (1995). Peroperative radioimmunodetection, PROD, of colorectal cancer using ^{99m}Tc PR1A3 monoclonal antibody. *Tumori* 81, 107-108.

Huang, S. M., Bock, J. M., and Harari, P. M. (1999). Epidermal growth factor receptor blockade with C225 modulates proliferation, apoptosis, and radiosensitivity in squamous cell carcinomas of the head and neck. *Cancer Res* 59, 1935-1940.

Huang, S. M., and Harari, P. M. (2000). Modulation of radiation response after epidermal growth factor receptor blockade in squamous cell carcinomas: inhibition of damage repair, cell cycle kinetics, and tumor angiogenesis. *Clin Cancer Res* 6, 2166-2174.

Huber, V. C., Lynch, J. M., Bucher, D. J., Le, J., and Metzger, D. W. (2001). Fc receptor-mediated phagocytosis makes a significant contribution to clearance of influenza virus infections. *J Immunol* *166*, 7381-7388.

Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., Berlin, J., Baron, A., Griffing, S., Holmgren, E., *et al.* (2004). Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* *350*, 2335-2342.

Iacopetta, B., Russo, A., Bazan, V., Dardanoni, G., Gebbia, N., Soussi, T., Kerr, D., Elsaleh, H., Soong, R., Kandioler, D., *et al.* (2006). Functional categories of TP53 mutation in colorectal cancer: results of an International Collaborative Study. *Ann Oncol* *17*, 842-847.

Ilantzis, C., DeMarte, L., Screatton, R. A., and Stanners, C. P. (2002). Deregulated expression of the human tumor marker CEA and CEA family member CEACAM6 disrupts tissue architecture and blocks colonocyte differentiation. *Neoplasia* *4*, 151-163.

IMPACT-B2-Investigators (1999). Efficacy of adjuvant fluorouracil and folinic acid in B2 colon cancer. International Multicentre Pooled Analysis of B2 Colon Cancer Trials (IMPACT B2) Investigators. *J Clin Oncol* *17*, 1356-1363.

Imray, C. H., Radley, S., Davis, A., Barker, G., Hendrickse, C. W., Donovan, I. A., Lawson, A. M., Baker, P. R., and Neoptolemos, J. P. (1992). Faecal unconjugated bile acids in patients with colorectal cancer or polyps. *Gut* *33*, 1239-1245.

Inoges, S., Rodriguez-Calvillo, M., Zabalegui, N., Lopez-Diaz de Cerio, A., Villanueva, H., Soria, E., Suarez, L., Rodriguez-Caballero, A., Pastor, F., Garcia-Munoz, R., *et al.* (2006). Clinical benefit associated with idiotypic vaccination in patients with follicular lymphoma. *J Natl Cancer Inst* *98*, 1292-1301.

Inoue, K., Slaton, J. W., Perrotte, P., Davis, D. W., Bruns, C. J., Hicklin, D. J., McConkey, D. J., Sweeney, P., Radinsky, R., and Dinney, C. P. (2000). Paclitaxel enhances the effects of the anti-epidermal growth factor receptor monoclonal antibody ImClone C225 in mice with metastatic human bladder transitional cell carcinoma. *Clin Cancer Res* *6*, 4874-4884.

Ismail, A., Van Groeningen, C. J., Hardcastle, A., Ren, Q., Aherne, G. W., Geoffroy, F., Allegra, C. J., and Grem, J. L. (1998). Modulation of fluorouracil cytotoxicity by interferon-alpha and -gamma. *Mol Pharmacol* *53*, 252-261.

Jayne, D. G., Guillou, P. J., Thorpe, H., Quirke, P., Copeland, J., Smith, A. M., Heath, R. M., and Brown, J. M. (2007). Randomized trial of laparoscopic-assisted resection of

colorectal carcinoma: 3-year results of the UK MRC CLASICC Trial Group. *J Clin Oncol* 25, 3061-3068.

Jiang, W. G., Lloyds, D., Puntis, M. C., Nakamura, T., and Hallett, M. B. (1993). Regulation of spreading and growth of colon cancer cells by hepatocyte growth factor. *Clin Exp Metastasis* 11, 235-242.

Johnson, J. I., Decker, S., Zaharevitz, D., Rubinstein, L. V., Venditti, J. M., Schepartz, S., Kalyandrug, S., Christian, M., Arbuck, S., Hollingshead, M., and Sausville, E. A. (2001). Relationships between drug activity in NCI preclinical in vitro and in vivo models and early clinical trials. *Br J Cancer* 84, 1424-1431.

Jones, P. T., Dear, P. H., Foote, J., Neuberger, M. S., and Winter, G. (1986). Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature* 321, 522-525.

Jonker, D. J., O'Callaghan, C. J., Karapetis, C. S., Zalberg, J. R., Tu, D., Au, H. J., Berry, S. R., Krahn, M., Price, T., Simes, R. J., *et al.* (2007). Cetuximab for the treatment of colorectal cancer. *N Engl J Med* 357, 2040-2048.

Jubb, A. M., Hurwitz, H. I., Bai, W., Holmgren, E. B., Tobin, P., Guerrero, A. S., Kabbinavar, F., Holden, S. N., Novotny, W. F., Frantz, G. D., *et al.* (2006). Impact of vascular endothelial growth factor-A expression, thrombospondin-2 expression, and microvessel density on the treatment effect of bevacizumab in metastatic colorectal cancer. *J Clin Oncol* 24, 217-227.

Junghans, R. P., and Anderson, C. L. (1996). The protection receptor for IgG catabolism is the beta2-microglobulin-containing neonatal intestinal transport receptor. *Proc Natl Acad Sci U S A* 93, 5512-5516.

Kabbinavar, F. F., Hambleton, J., Mass, R. D., Hurwitz, H. I., Bergsland, E., and Sarkar, S. (2005a). Combined analysis of efficacy: the addition of bevacizumab to fluorouracil/leucovorin improves survival for patients with metastatic colorectal cancer. *J Clin Oncol* 23, 3706-3712.

Kabbinavar, F. F., Schulz, J., McCleod, M., Patel, T., Hamm, J. T., Hecht, J. R., Mass, R., Perrou, B., Nelson, B., and Novotny, W. F. (2005b). Addition of bevacizumab to bolus fluorouracil and leucovorin in first-line metastatic colorectal cancer: results of a randomized phase II trial. *J Clin Oncol* 23, 3697-3705.

Kapiteijn, E., Marijnen, C. A., Nagtegaal, I. D., Putter, H., Steup, W. H., Wiggers, T., Rutten, H. J., Pahlman, L., Glimelius, B., van Krieken, J. H., *et al.* (2001). Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. *N Engl J Med* 345, 638-646.

Karanjia, N. D., Schache, D. J., North, W. R., and Heald, R. J. (1990). 'Close shave' in anterior resection. *Br J Surg* 77, 510-512.

Karapetis, C. S., Khambata-Ford, S., Jonker, D. J., O'Callaghan, C. J., Tu, D., Tebbutt, N. C., Simes, R. J., Chalchal, H., Shapiro, J. D., Robitaille, S., *et al.* (2008). K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med* 359, 1757-1765.

Kawaguchi, Y., Kono, K., Mimura, K., Sugai, H., Akaike, H., and Fujii, H. (2007). Cetuximab induce antibody-dependent cellular cytotoxicity against EGFR-expressing esophageal squamous cell carcinoma. *Int J Cancer* 120, 781-787.

Kikuchi, R., Takano, M., Takagi, K., Fujimoto, N., Nozaki, R., Fujiyoshi, T., and Uchida, Y. (1995). Management of early invasive colorectal cancer. Risk of recurrence and clinical guidelines. *Dis Colon Rectum* 38, 1286-1295.

Kim, J. C., Kim, W. S., Ryu, J. S., Oh, S. J., Lee, D. H., Koo, K. H., Roh, S. A., Kim, H. C., Yu, C. S., Kang, G. H., and Bodmer, W. F. (2000). Applicability of carcinoembryonic antigen-specific monoclonal antibodies to radioimmunoguided surgery for human colorectal carcinoma. *Cancer Res* 60, 4825-4829.

Kim, J. C., Koo, K. H., Kim, B. S., Park, K. C., Bicknell, D. C., and Bodmer, W. F. (1999). Carcino-embryonic antigen may function as a chemo-attractant in colorectal-carcinoma cell lines. *Int J Cancer* 82, 880-885.

Kim, J. C., Roh, S. A., Koo, K. H., Ryu, J. H., Hong, H. K., Oh, S. J., Ryu, J. S., Kim, H. J., and Bodmer, W. F. (2002). Enhancement of colorectal tumor targeting using a novel biparatopic monoclonal antibody against carcinoembryonic antigen in experimental radioimmunoguided surgery. *Int J Cancer* 97, 542-547.

Kipps, T. J., Parham, P., Punt, J., and Herzenberg, L. A. (1985). Importance of immunoglobulin isotype in human antibody-dependent, cell-mediated cytotoxicity directed by murine monoclonal antibodies. *J Exp Med* 161, 1-17.

Klapper, L. N., Waterman, H., Sela, M., and Yarden, Y. (2000). Tumor-inhibitory antibodies to HER-2/ErbB-2 may act by recruiting c-Cbl and enhancing ubiquitination of HER-2. *Cancer Res* 60, 3384-3388.

Knox, S. J., Goris, M. L., Tempero, M., Weiden, P. L., Gentner, L., Breitz, H., Adams, G. P., Axworthy, D., Gaffigan, S., Bryan, K., *et al.* (2000). Phase II trial of yttrium-90-DOTA-biotin pretargeted by NR-LU-10 antibody/streptavidin in patients with metastatic colon cancer. *Clin Cancer Res* 6, 406-414.

Koene, H. R., Kleijer, M., Algra, J., Roos, D., von dem Borne, A. E., and de Haas, M. (1997). Fc gammaRIIIa-158V/F polymorphism influences the binding of IgG by natural

killer cell Fc gammaRIIIa, independently of the Fc gammaRIIIa-48L/R/H phenotype. *Blood* 90, 1109-1114.

Kohler, G., and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256, 495-497.

Koppe, M. J., Soede, A. C., Pels, W., Oyen, W. J., Goldenberg, D. M., Bleichrodt, R. P., and Boerman, O. C. (2003). Experimental radioimmunotherapy of small peritoneal metastases of colorectal origin. *Int J Cancer* 106, 965-972.

Kraeber-Bodere, F., Faivre-Chauvet, A., Ferrer, L., Vuillez, J. P., Brard, P. Y., Rousseau, C., Resche, I., Devillers, A., Laffont, S., Bardies, M., *et al.* (2003). Pharmacokinetics and dosimetry studies for optimization of anti-carcinoembryonic antigen x anti-hapten bispecific antibody-mediated pretargeting of Iodine-131-labeled hapten in a phase I radioimmunotherapy trial. *Clin Cancer Res* 9, 3973S-3981S.

Kronborg, O., Fenger, C., Olsen, J., Jorgensen, O. D., and Sondergaard, O. (1996). Randomised study of screening for colorectal cancer with faecal-occult-blood test. *Lancet* 348, 1467-1471.

Kronborg, O., Hage, E., Fenger, C., and Deichgraeber, E. (1986). Do synchronous adenomas influence prognosis after radical surgery for colorectal carcinoma? A prospective study. *Int J Colorectal Dis* 1, 99-103.

Ku, N. O., Liao, J., and Omary, M. B. (1997). Apoptosis generates stable fragments of human type I keratins. *J Biol Chem* 272, 33197-33203.

Kubo, A., Nakamura, K., Katayama, M., Hashimoto, S., Teramoto, T., and Kodaira, S. (1992). Pharmacokinetic analysis of antibody localization in human colon cancer: comparison with immunoscintigraphy. *Ann Nucl Med* 6, 21-27.

Kuo, T., Cho, C. D., Halsey, J., Wakelee, H. A., Advani, R. H., Ford, J. M., Fisher, G. A., and Sikic, B. I. (2005). Phase II study of gefitinib, fluorouracil, leucovorin, and oxaliplatin therapy in previously treated patients with metastatic colorectal cancer. *J Clin Oncol* 23, 5613-5619.

Kurai, J., Chikumi, H., Hashimoto, K., Yamaguchi, K., Yamasaki, A., Sako, T., Touge, H., Makino, H., Takata, M., Miyata, M., *et al.* (2007). Antibody-dependent cellular cytotoxicity mediated by cetuximab against lung cancer cell lines. *Clin Cancer Res* 13, 1552-1561.

Kuroki, M., Murakami, M., Wakisaka, M., Krop-Watorek, A., Oikawa, S., Nakazato, H., Kosaki, G., and Matsuoka, Y. (1992). Epitopes predominantly retained on the

carcinoembryonic antigen molecules in plasma of patients with malignant tumors but not on those in plasma of normal individuals. *Jpn J Cancer Res* 83, 505-514.

Lane, D. M., Eagle, K. F., Begent, R. H., Hope-Stone, L. D., Green, A. J., Casey, J. L., Keep, P. A., Kelly, A. M., Ledermann, J. A., Glaser, M. G., and et al. (1994). Radioimmunotherapy of metastatic colorectal tumours with iodine-131-labelled antibody to carcinoembryonic antigen: phase I/II study with comparative biodistribution of intact and F(ab')₂ antibodies. *Br J Cancer* 70, 521-525.

Lanier, L. L. (2001). On guard--activating NK cell receptors. *Nat Immunol* 2, 23-27.

Lazar, G. A., Dang, W., Karki, S., Vafa, O., Peng, J. S., Hyun, L., Chan, C., Chung, H. S., Eivazi, A., Yoder, S. C., et al. (2006). Engineered antibody Fc variants with enhanced effector function. *Proc Natl Acad Sci U S A* 103, 4005-4010.

Lee, Y., Chen, Y., Chang, L. S., and Johnson, L. F. (1997). Inhibition of mouse thymidylate synthase promoter activity by the wild-type p53 tumor suppressor protein. *Exp Cell Res* 234, 270-276.

Leers, M. P., Kolgen, W., Bjorklund, V., Bergman, T., Tribbick, G., Persson, B., Bjorklund, P., Ramaekers, F. C., Bjorklund, B., Nap, M., et al. (1999). Immunocytochemical detection and mapping of a cytokeratin 18 neo-epitope exposed during early apoptosis. *J Pathol* 187, 567-572.

Lenz, H. J., Manno, D. J., Danenberg, K. D., and Danenberg, P. V. (1994). Incorporation of 5-fluorouracil into U2 and U6 snRNA inhibits mRNA precursor splicing. *J Biol Chem* 269, 31962-31968.

Lepourcelet, M., Chen, Y. N., France, D. S., Wang, H., Crews, P., Petersen, F., Bruseo, C., Wood, A. W., and Shivdasani, R. A. (2004). Small-molecule antagonists of the oncogenic Tcf/beta-catenin protein complex. *Cancer Cell* 5, 91-102.

Leung, N., Turbide, C., Olson, M., Marcus, V., Jothy, S., and Beauchemin, N. (2006). Deletion of the carcinoembryonic antigen-related cell adhesion molecule 1 (Ceacam1) gene contributes to colon tumor progression in a murine model of carcinogenesis. *Oncogene* 25, 5527-5536.

Lewis, G. D., Figari, I., Fendly, B., Wong, W. L., Carter, P., Gorman, C., and Shepard, H. M. (1993). Differential responses of human tumor cell lines to anti-p185HER2 monoclonal antibodies. *Cancer Immunol Immunother* 37, 255-263.

Li, S., Schmitz, K. R., Jeffrey, P. D., Wiltzius, J. J., Kussie, P., and Ferguson, K. M. (2005). Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. *Cancer Cell* 7, 301-311.

Liang, J. T., Huang, K. C., Cheng, Y. M., Hsu, H. C., Cheng, A. L., Hsu, C. H., Yeh, K. H., Wang, S. M., and Chang, K. J. (2002). P53 overexpression predicts poor chemosensitivity to high-dose 5-fluorouracil plus leucovorin chemotherapy for stage IV colorectal cancers after palliative bowel resection. *Int J Cancer* 97, 451-457.

Lichtenstein, P., Holm, N. V., Verkasalo, P. K., Iliadou, A., Kaprio, J., Koskenvuo, M., Pukkala, E., Skytthe, A., and Hemminki, K. (2000). Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland. *N Engl J Med* 343, 78-85.

Liersch, T., Meller, J., Bittrich, M., Kulle, B., Becker, H., and Goldenberg, D. M. (2007). Update of carcinoembryonic antigen radioimmunotherapy with (131)I-labetuzumab after salvage resection of colorectal liver metastases: comparison of outcome to a contemporaneous control group. *Ann Surg Oncol* 14, 2577-2590.

Lilien, D. L., Spivak, J. L., and Goldman, I. D. (1970). Chromate transport in human leukocytes. *J Clin Invest* 49, 1551-1557.

Linardou, H., Dahabreh, I. J., Kanaloupiti, D., Siannis, F., Bafaloukos, D., Kosmidis, P., Papadimitriou, C. A., and Murray, S. (2008). Assessment of somatic k-RAS mutations as a mechanism associated with resistance to EGFR-targeted agents: a systematic review and meta-analysis of studies in advanced non-small-cell lung cancer and metastatic colorectal cancer. *Lancet Oncol* 9, 962-972.

Lisowska, E., Krop-Watorek, A., and Sedlaczek, P. (1983). The dimeric structure of carcinoembryonic antigen (CEA). *Biochem Biophys Res Commun* 115, 206-211.

Liu, B., Fang, M., Schmidt, M., Lu, Y., Mendelsohn, J., and Fan, Z. (2000). Induction of apoptosis and activation of the caspase cascade by anti-EGF receptor monoclonal antibodies in DiFi human colon cancer cells do not involve the c-jun N-terminal kinase activity. *Br J Cancer* 82, 1991-1999.

Lonberg, N., Taylor, L. D., Harding, F. A., Trounstein, M., Higgins, K. M., Schramm, S. R., Kuo, C. C., Mashayekh, R., Wymore, K., McCabe, J. G., and et al. (1994). Antigen-specific human antibodies from mice comprising four distinct genetic modifications. *Nature* 368, 856-859.

Lubeck, M. D., Steplewski, Z., Baglia, F., Klein, M. H., Dorrington, K. J., and Koprowski, H. (1985). The interaction of murine IgG subclass proteins with human monocyte Fc receptors. *J Immunol* 135, 1299-1304.

Lutterbuese, R., Raum, T., Kischel, R., Lutterbuese, P., Schlereth, B., Schaller, E., Mangold, S., Rau, D., Meier, P., Kiener, P. A., et al. (2009). Potent control of tumor

growth by CEA/CD3-bispecific single-chain antibody constructs that are not competitively inhibited by soluble CEA. *J Immunother* 32, 341-352.

MacFarlane, M., Merrison, W., Dinsdale, D., and Cohen, G. M. (2000). Active caspases and cleaved cytokeratins are sequestered into cytoplasmic inclusions in TRAIL-induced apoptosis. *J Cell Biol* 148, 1239-1254.

Mach, J. P., Carrel, S., Merenda, C., Sordat, B., and Cerottini, J. C. (1974a). In vivo localisation of radiolabelled antibodies to carcinoembryonic antigen in human colon carcinoma grafted into nude mice. *Nature* 248, 704-706.

Mach, J. P., Jaeger, P., Bertholet, M. M., Rueggsegger, C. H., Loosli, R. M., and Pettavel, J. (1974b). Detection of recurrence of large-bowel carcinoma by radioimmunoassay of circulating carcinoembryonic antigen (C.E.A.). *Lancet* 2, 535-540.

Mahnke, K., Schonfeld, K., Fondel, S., Ring, S., Karakhanova, S., Wiedemeyer, K., Bedke, T., Johnson, T. S., Storn, V., Schallenberg, S., and Enk, A. H. (2007). Depletion of CD4+CD25+ human regulatory T cells in vivo: kinetics of Treg depletion and alterations in immune functions in vivo and in vitro. *Int J Cancer* 120, 2723-2733.

Maloney, D. G. (2005). Concepts in radiotherapy and immunotherapy: anti-CD20 mechanisms of action and targets. *Semin Oncol* 32, S19-26.

Maloney, D. G., Grillo-Lopez, A. J., White, C. A., Bodkin, D., Schilder, R. J., Neidhart, J. A., Janakiraman, N., Foon, K. A., Liles, T. M., Dallaire, B. K., *et al.* (1997). IDEC-C2B8 (Rituximab) anti-CD20 monoclonal antibody therapy in patients with relapsed low-grade non-Hodgkin's lymphoma. *Blood* 90, 2188-2195.

Mandel, J. S., Church, T. R., Bond, J. H., Ederer, F., Geisser, M. S., Mongin, S. J., Snover, D. C., and Schuman, L. M. (2000). The effect of fecal occult-blood screening on the incidence of colorectal cancer. *N Engl J Med* 343, 1603-1607.

Mandel, J. S., Church, T. R., Ederer, F., and Bond, J. H. (1999). Colorectal cancer mortality: effectiveness of biennial screening for fecal occult blood. *J Natl Cancer Inst* 91, 434-437.

Mandler, R., Kobayashi, H., Hinson, E. R., Brechbiel, M. W., and Waldmann, T. A. (2004). Herceptin-geldanamycin immunoconjugates: pharmacokinetics, biodistribution, and enhanced antitumor activity. *Cancer Res* 64, 1460-1467.

Manyak, C. L., Tse, H., Fischer, P., Coker, L., Sigal, N. H., and Koo, G. C. (1988). Regulation of class II MHC molecules on human endothelial cells. Effects of IFN and dexamethasone. *J Immunol* 140, 3817-3821.

- Matsuoka, Y., Matsuo, Y., Okamoto, N., Kuroki, M., Kuroki, M., and Ikehara, Y. (1991). Highly effective extraction of carcinoembryonic antigen with phosphatidylinositol-specific phospholipase C. *Tumour Biol* 12, 91-98.
- Mazelin, L., Bernet, A., Bonod-Bidaud, C., Pays, L., Arnaud, S., Gespach, C., Bredesen, D. E., Scoazec, J. Y., and Mehlen, P. (2004). Netrin-1 controls colorectal tumorigenesis by regulating apoptosis. *Nature* 431, 80-84.
- McCafferty, J., Griffiths, A. D., Winter, G., and Chiswell, D. J. (1990). Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 348, 552-554.
- McLaughlin, P., Grillo-Lopez, A. J., Link, B. K., Levy, R., Czuczman, M. S., Williams, M. E., Heyman, M. R., Bence-Bruckler, I., White, C. A., Cabanillas, F., *et al.* (1998). Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. *J Clin Oncol* 16, 2825-2833.
- McMichael, A. J., McCall, M. G., Hartshorne, J. M., and Woodings, T. L. (1980). Patterns of gastro-intestinal cancer in European migrants to Australia: the role of dietary change. *Int J Cancer* 25, 431-437.
- Mechetina, L. V., Najakshin, A. M., Alabyev, B. Y., Chikaev, N. A., and Taranin, A. V. (2002). Identification of CD16-2, a novel mouse receptor homologous to CD16/Fc gamma RIII. *Immunogenetics* 54, 463-468.
- Mella, J., Biffin, A., Radcliffe, A. G., Stamatakis, J. D., and Steele, R. J. (1997). Population-based audit of colorectal cancer management in two UK health regions. Colorectal Cancer Working Group, Royal College of Surgeons of England Clinical Epidemiology and Audit Unit. *Br J Surg* 84, 1731-1736.
- Meredith, R. F., Khazaeli, M. B., Plott, W. E., Saleh, M. N., Liu, T., Allen, L. F., Russell, C. D., Orr, R. A., Colcher, D., Schlom, J., and *et al.* (1992). Phase I trial of iodine-131-chimeric B72.3 (human IgG4) in metastatic colorectal cancer. *J Nucl Med* 33, 23-29.
- Milenic, D. E., Brady, E. D., and Brechbiel, M. W. (2004). Antibody-targeted radiation cancer therapy. *Nat Rev Drug Discov* 3, 488-499.
- Mohsin, S. K., Weiss, H. L., Gutierrez, M. C., Chamness, G. C., Schiff, R., Digiovanna, M. P., Wang, C. X., Hilsenbeck, S. G., Osborne, C. K., Allred, D. C., *et al.* (2005). Neoadjuvant trastuzumab induces apoptosis in primary breast cancers. *J Clin Oncol* 23, 2460-2468.
- Moretta, L., and Moretta, A. (2004). Killer immunoglobulin-like receptors. *Curr Opin Immunol* 16, 626-633.

Morris, E. J., Maughan, N. J., Forman, D., and Quirke, P. (2007). Who to treat with adjuvant therapy in Dukes B/stage II colorectal cancer? The need for high quality pathology. *Gut* 56, 1419-1425.

Morrison, S. L., Johnson, M. J., Herzenberg, L. A., and Oi, V. T. (1984). Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. *Proc Natl Acad Sci U S A* 81, 6851-6855.

Morson, B. C. (1966). Factors influencing the prognosis of early cancer of the rectum. *Proc R Soc Med* 59, 607-608.

Motoyama, T., Hojo, H., Suzuki, T., and Oboshi, S (1979). Evaluation of the regrowth assay method as an in vitro drug sensitivity test and its application to cultured human gastric cancer cell lines. . *Acta Medica et Biologica* 27, 49-63.

Murillo, O., Arina, A., Tirapu, I., Alfaro, C., Mazzolini, G., Palencia, B., Lopez-Diaz De Cerio, A., Prieto, J., Bendandi, M., and Melero, I. (2003). Potentiation of therapeutic immune responses against malignancies with monoclonal antibodies. *Clin Cancer Res* 9, 5454-5464.

Murray, J. L., Macey, D. J., Kasi, L. P., Rieger, P., Cunningham, J., Bhadkamkar, V., Zhang, H. Z., Schlom, J., Rosenblum, M. G., and Podoloff, D. A. (1994). Phase II radioimmunotherapy trial with 131I-CC49 in colorectal cancer. *Cancer* 73, 1057-1066.

Musolino, A., Naldi, N., Bortesi, B., Pezzuolo, D., Capelletti, M., Missale, G., Laccabue, D., Zerbini, A., Camisa, R., Bisagni, G., *et al.* (2008). Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/neu-positive metastatic breast cancer. *J Clin Oncol* 26, 1789-1796.

Muto, T., Bussey, H. J., and Morson, B. C. (1975). The evolution of cancer of the colon and rectum. *Cancer* 36, 2251-2270.

Nakajima, A., Iijima, H., Neurath, M. F., Nagaishi, T., Nieuwenhuis, E. E., Raychowdhury, R., Glickman, J., Blau, D. M., Russell, S., Holmes, K. V., and Blumberg, R. S. (2002). Activation-induced expression of carcinoembryonic antigen-cell adhesion molecule 1 regulates mouse T lymphocyte function. *J Immunol* 168, 1028-1035.

Nap, M., Mollgard, K., Burtin, P., and Fleuren, G. J. (1988). Immunohistochemistry of carcino-embryonic antigen in the embryo, fetus and adult. *Tumour Biol* 9, 145-153.

Nascimbeni, R., Burgart, L. J., Nivatvongs, S., and Larson, D. R. (2002). Risk of lymph node metastasis in T1 carcinoma of the colon and rectum. *Dis Colon Rectum* 45, 200-206.

NHS-Screening-Pilot (2004). Results of the first round of a demonstration pilot of screening for colorectal cancer in the United Kingdom. *Bmj* 329, 133.

Nimmerjahn, F., Bruhns, P., Horiuchi, K., and Ravetch, J. V. (2005). FcgammaRIV: a novel FcR with distinct IgG subclass specificity. *Immunity* 23, 41-51.

Nimmerjahn, F., and Ravetch, J. V. (2005). Divergent immunoglobulin g subclass activity through selective Fc receptor binding. *Science* 310, 1510-1512.

Nimmerjahn, F., and Ravetch, J. V. (2008). Fcgamma receptors as regulators of immune responses. *Nat Rev Immunol* 8, 34-47.

Niwa, R., Hatanaka, S., Shoji-Hosaka, E., Sakurada, M., Kobayashi, Y., Uehara, A., Yokoi, H., Nakamura, K., and Shitara, K. (2004a). Enhancement of the antibody-dependent cellular cytotoxicity of low-fucose IgG1 is independent of FcgammaRIIIa functional polymorphism. *Clin Cancer Res* 10, 6248-6255.

Niwa, R., Shoji-Hosaka, E., Sakurada, M., Shinkawa, T., Uchida, K., Nakamura, K., Matsushima, K., Ueda, R., Hanai, N., and Shitara, K. (2004b). Defucosylated chimeric anti-CC chemokine receptor 4 IgG1 with enhanced antibody-dependent cellular cytotoxicity shows potent therapeutic activity to T-cell leukemia and lymphoma. *Cancer Res* 64, 2127-2133.

O'Connell, M. J., Laurie, J. A., Kahn, M., Fitzgibbons, R. J., Jr., Erlichman, C., Shepherd, L., Moertel, C. G., Kocha, W. I., Pazdur, R., Wieand, H. S., *et al.* (1998). Prospectively randomized trial of postoperative adjuvant chemotherapy in patients with high-risk colon cancer. *J Clin Oncol* 16, 295-300.

Ober, R. J., Martinez, C., Lai, X., Zhou, J., and Ward, E. S. (2004). Exocytosis of IgG as mediated by the receptor, FcRn: an analysis at the single-molecule level. *Proc Natl Acad Sci U S A* 101, 11076-11081.

Oikawa, S., Nakazato, H., and Kosaki, G. (1987). Primary structure of human carcinoembryonic antigen (CEA) deduced from cDNA sequence. *Biochem Biophys Res Commun* 142, 511-518.

Ordonez, C., Sreaton, R. A., Ilantzis, C., and Stanners, C. P. (2000). Human carcinoembryonic antigen functions as a general inhibitor of anoikis. *Cancer Res* 60, 3419-3424.

Ordonez, C., Zhai, A. B., Camacho-Leal, P., Demarte, L., Fan, M. M., and Stanners, C. P. (2007). GPI-anchored CEA family glycoproteins CEA and CEACAM6 mediate their biological effects through enhanced integrin alpha5beta1-fibronectin interaction. *J Cell Physiol* 210, 757-765.

Ortaldo, J. R., Mason, A. T., and O'Shea, J. J. (1995). Receptor-induced death in human natural killer cells: involvement of CD16. *J Exp Med* 181, 339-344.

Paradiso, A., Simone, G., Petroni, S., Leone, B., Vallejo, C., Lacava, J., Romero, A., Machiavelli, M., De Lena, M., Allegra, C. J., and Johnston, P. G. (2000). Thymidilate synthase and p53 primary tumour expression as predictive factors for advanced colorectal cancer patients. *Br J Cancer* 82, 560-567.

Parihar, R., Nadella, P., Lewis, A., Jensen, R., De Hoff, C., Dierksheide, J. E., VanBuskirk, A. M., Magro, C. M., Young, D. C., Shapiro, C. L., and Carson, W. E., 3rd (2004). A phase I study of interleukin 12 with trastuzumab in patients with human epidermal growth factor receptor-2-overexpressing malignancies: analysis of sustained interferon gamma production in a subset of patients. *Clin Cancer Res* 10, 5027-5037.

Parkin, D. M., Bray, F., Ferlay, J., and Pisani, P. (2005). Global cancer statistics, 2002. *CA Cancer J Clin* 55, 74-108.

Peat, N., Gendler, S. J., Lalani, N., Duhig, T., and Taylor-Papadimitriou, J. (1992). Tissue-specific expression of a human polymorphic epithelial mucin (MUC1) in transgenic mice. *Cancer Res* 52, 1954-1960.

Peeters, K. C., van de Velde, C. J., Leer, J. W., Martijn, H., Junggebur, J. M., Kranenbarg, E. K., Steup, W. H., Wiggers, T., Rutten, H. J., and Marijnen, C. A. (2005). Late side effects of short-course preoperative radiotherapy combined with total mesorectal excision for rectal cancer: increased bowel dysfunction in irradiated patients--a Dutch colorectal cancer group study. *J Clin Oncol* 23, 6199-6206.

Perrotte, P., Matsumoto, T., Inoue, K., Kuniyasu, H., Eve, B. Y., Hicklin, D. J., Radinsky, R., and Dinney, C. P. (1999). Anti-epidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. *Clin Cancer Res* 5, 257-265.

Peters, G. J., Backus, H. H., Freemantle, S., van Triest, B., Codacci-Pisanelli, G., van der Wilt, C. L., Smid, K., Lunec, J., Calvert, A. H., Marsh, S., *et al.* (2002). Induction of thymidylate synthase as a 5-fluorouracil resistance mechanism. *Biochim Biophys Acta* 1587, 194-205.

Peters, U., Sinha, R., Chatterjee, N., Subar, A. F., Ziegler, R. G., Kulldorff, M., Bresalier, R., Weissfeld, J. L., Flood, A., Schatzkin, A., and Hayes, R. B. (2003). Dietary fibre and colorectal adenoma in a colorectal cancer early detection programme. *Lancet* 361, 1491-1495.

Petersen, V. C., Baxter, K. J., Love, S. B., and Shepherd, N. A. (2002). Identification of objective pathological prognostic determinants and models of prognosis in Dukes' B colon cancer. *Gut* 51, 65-69.

Petit, A. M., Rak, J., Hung, M. C., Rockwell, P., Goldstein, N., Fendly, B., and Kerbel, R. S. (1997). Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. *Am J Pathol* 151, 1523-1530.

Phan, G. Q., Yang, J. C., Sherry, R. M., Hwu, P., Topalian, S. L., Schwartzentruber, D. J., Restifo, N. P., Haworth, L. R., Seipp, C. A., Freezer, L. J., *et al.* (2003). Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc Natl Acad Sci U S A* 100, 8372-8377.

Piccart-Gebhart, M. J., Procter, M., Leyland-Jones, B., Goldhirsch, A., Untch, M., Smith, I., Gianni, L., Baselga, J., Bell, R., Jackisch, C., *et al.* (2005). Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *N Engl J Med* 353, 1659-1672.

Posner, M. C., Niedzwiecki, D., Venook, A. P., Hollis, D. R., Kindler, H. L., Martin, E. W., Schilsky, R. L., and Goldberg, R. M. (2008). A phase II prospective multi-institutional trial of adjuvant active specific immunotherapy following curative resection of colorectal cancer hepatic metastases: cancer and leukemia group B study 89903. *Ann Surg Oncol* 15, 158-164.

Poy, M. N., Yang, Y., Rezaei, K., Fernstrom, M. A., Lee, A. D., Kido, Y., Erickson, S. K., and Najjar, S. M. (2002). CEACAM1 regulates insulin clearance in liver. *Nat Genet* 30, 270-276.

Press, O. W., Corcoran, M., Subbiah, K., Hamlin, D. K., Wilbur, D. S., Johnson, T., Theodore, L., Yau, E., Mallett, R., Meyer, D. L., and Axworthy, D. (2001). A comparative evaluation of conventional and pretargeted radioimmunotherapy of CD20-expressing lymphoma xenografts. *Blood* 98, 2535-2543.

Prete, S. P., Aquino, A., Masci, G., Orlando, L., Giuliani, A., De Santis, S., De Vecchis, L., De Filippi, R., Greiner, J. W., Bonmassar, E., and Graziani, G. (1996). Drug-induced changes of carcinoembryonic antigen expression in human cancer cells: effect of 5-fluorouracil. *J Pharmacol Exp Ther* 279, 1574-1581.

Pretlow, T. P., Roukhadze, E. V., O'Riordan, M. A., Chan, J. C., Amini, S. B., and Stellato, T. A. (1994). Carcinoembryonic antigen in human colonic aberrant crypt foci. *Gastroenterology* 107, 1719-1725.

Pricop, L., Redecha, P., Teillaud, J. L., Frey, J., Fridman, W. H., Sautes-Fridman, C., and Salmon, J. E. (2001). Differential modulation of stimulatory and inhibitory Fc gamma receptors on human monocytes by Th1 and Th2 cytokines. *J Immunol* 166, 531-537.

QUASAR-Collab-Gp (2000). Comparison of fluorouracil with additional levamisole, higher-dose folinic acid, or both, as adjuvant chemotherapy for colorectal cancer: a randomised trial. QUASAR Collaborative Group. *Lancet* 355, 1588-1596.

Rafiq, K., Bergtold, A., and Clynes, R. (2002). Immune complex-mediated antigen presentation induces tumor immunity. *J Clin Invest* 110, 71-79.

Raghavan, M., Bonagura, V. R., Morrison, S. L., and Bjorkman, P. J. (1995). Analysis of the pH dependence of the neonatal Fc receptor/immunoglobulin G interaction using antibody and receptor variants. *Biochemistry* 34, 14649-14657.

Rao, S., Cunningham, D., de Gramont, A., Scheithauer, W., Smakal, M., Humblet, Y., Kourteva, G., Iveson, T., Andre, T., Dostalova, J., *et al.* (2004). Phase III double-blind placebo-controlled study of farnesyl transferase inhibitor R115777 in patients with refractory advanced colorectal cancer. *J Clin Oncol* 22, 3950-3957.

Redfern, C. H., Guthrie, T. H., Bessudo, A., Densmore, J. J., Holman, P. R., Janakiraman, N., Leonard, J. P., Levy, R. L., Just, R. G., Smith, M. R., *et al.* (2006). Phase II trial of idiotype vaccination in previously treated patients with indolent non-Hodgkin's lymphoma resulting in durable clinical responses. *J Clin Oncol* 24, 3107-3112.

Reff, M. E., Carner, K., Chambers, K. S., Chinn, P. C., Leonard, J. E., Raab, R., Newman, R. A., Hanna, N., and Anderson, D. R. (1994). Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. *Blood* 83, 435-445.

Regnault, A., Lankar, D., Lacabanne, V., Rodriguez, A., They, C., Rescigno, M., Saito, T., Verbeek, S., Bonnerot, C., Ricciardi-Castagnoli, P., and Amigorena, S. (1999). Fc gamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. *J Exp Med* 189, 371-380.

Reichert, J. M., and Valge-Archer, V. E. (2007). Development trends for monoclonal antibody cancer therapeutics. *Nat Rev Drug Discov* 6, 349-356.

Rembacken, B. J., Fujii, T., Cairns, A., Dixon, M. F., Yoshida, S., Chalmers, D. M., and Axon, A. T. (2000). Flat and depressed colonic neoplasms: a prospective study of 1000 colonoscopies in the UK. *Lancet* 355, 1211-1214.

Repka, T., Chiorean, E. G., Gay, J., Herwig, K. E., Kohl, V. K., Yee, D., and Miller, J. S. (2003). Trastuzumab and interleukin-2 in HER2-positive metastatic breast cancer: a pilot study. *Clin Cancer Res* 9, 2440-2446.

Rhee, P., Morris, J., Durham, R., Hauser, C., Cipolle, M., Wilson, R., Luchette, F., McSwain, N., and Miller, R. (2000). Recombinant humanized monoclonal antibody against CD18 (rhuMAb CD18) in traumatic hemorrhagic shock: results of a phase II clinical trial. Traumatic Shock Group. *J Trauma* 49, 611-619; discussion 619-620.

Richman, C. M., Denardo, S. J., O'Donnell, R. T., Yuan, A., Shen, S., Goldstein, D. S., Tuscano, J. M., Wun, T., Chew, H. K., Lara, P. N., *et al.* (2005). High-dose radioimmunotherapy combined with fixed, low-dose paclitaxel in metastatic prostate and breast cancer by using a MUC-1 monoclonal antibody, m170, linked to indium-111/yttrium-90 via a cathepsin cleavable linker with cyclosporine to prevent human anti-mouse antibody. *Clin Cancer Res* 11, 5920-5927.

Richman, P. I., and Bodmer, W. F. (1987). Monoclonal antibodies to human colorectal epithelium: markers for differentiation and tumour characterization. *Int J Cancer* 39, 317-328.

Riethmuller, G., Holz, E., Schlimok, G., Schmiegel, W., Raab, R., Hoffken, K., Gruber, R., Funke, I., Pichlmaier, H., Hirche, H., *et al.* (1998). Monoclonal antibody therapy for resected Dukes' C colorectal cancer: seven-year outcome of a multicenter randomized trial. *J Clin Oncol* 16, 1788-1794.

Roitt (2001). *Essential Immunology*, 10th edn (Oxford: Blackwell Science Ltd).

Romond, E. H., Perez, E. A., Bryant, J., Suman, V. J., Geyer, C. E., Jr., Davidson, N. E., Tan-Chiu, E., Martino, S., Paik, S., Kaufman, P. A., *et al.* (2005). Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N Engl J Med* 353, 1673-1684.

Rosen, L. S. (2002). Clinical experience with angiogenesis signaling inhibitors: focus on vascular endothelial growth factor (VEGF) blockers. *Cancer Control* 9, 36-44.

Russo, A., Bazan, V., Iacopetta, B., Kerr, D., Soussi, T., and Gebbia, N. (2005). The TP53 colorectal cancer international collaborative study on the prognostic and predictive significance of p53 mutation: influence of tumor site, type of mutation, and adjuvant treatment. *J Clin Oncol* 23, 7518-7528.

Saltz, L. B., Clarke, S., Diaz-Rubio, E., Scheithauer, W., Figer, A., Wong, R., Koski, S., Lichinitser, M., Yang, T. S., Rivera, F., *et al.* (2008). Bevacizumab in combination with oxaliplatin-based chemotherapy as first-line therapy in metastatic colorectal cancer: a randomized phase III study. *J Clin Oncol* 26, 2013-2019.

Saltz, L. B., Cox, J. V., Blanke, C., Rosen, L. S., Fehrenbacher, L., Moore, M. J., Maroun, J. A., Ackland, S. P., Locker, P. K., Pirodda, N., *et al.* (2000). Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. Irinotecan Study Group. *N Engl J Med* 343, 905-914.

Saltz, L. B., Lenz, H. J., Kindler, H. L., Hochster, H. S., Wadler, S., Hoff, P. M., Kemeny, N. E., Hollywood, E. M., Gonen, M., Quinones, M., *et al.* (2007). Randomized phase II trial of cetuximab, bevacizumab, and irinotecan compared with cetuximab and bevacizumab alone in irinotecan-refractory colorectal cancer: the BOND-2 study. *J Clin Oncol* 25, 4557-4561.

Saltz, L. B., Meropol, N. J., Loehrer, P. J., Sr., Needle, M. N., Kopit, J., and Mayer, R. J. (2004). Phase II trial of cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. *J Clin Oncol* 22, 1201-1208.

Sandborn, W. J., Feagan, B. G., Stoinov, S., Honiball, P. J., Rutgeerts, P., Mason, D., Bloomfield, R., and Schreiber, S. (2007). Certolizumab pegol for the treatment of Crohn's disease. *N Engl J Med* 357, 228-238.

Sandler, R. S., Halabi, S., Baron, J. A., Budinger, S., Paskett, E., Keresztes, R., Petrelli, N., Pipas, J. M., Karp, D. D., Loprinzi, C. L., *et al.* (2003). A randomized trial of aspirin to prevent colorectal adenomas in patients with previous colorectal cancer. *N Engl J Med* 348, 883-890.

Sato, J. D., Kawamoto, T., Le, A. D., Mendelsohn, J., Polikoff, J., and Sato, G. H. (1983). Biological effects in vitro of monoclonal antibodies to human epidermal growth factor receptors. *Mol Biol Med* 1, 511-529.

Scaltriti, M., Verma, C., Guzman, M., Jimenez, J., Parra, J. L., Pedersen, K., Smith, D. J., Landolfi, S., Ramon y Cajal, S., Arribas, J., and Baselga, J. (2009). Lapatinib, a HER2 tyrosine kinase inhibitor, induces stabilization and accumulation of HER2 and potentiates trastuzumab-dependent cell cytotoxicity. *Oncogene* 28, 803-814.

Scheele, J., Stang, R., Altendorf-Hofmann, A., and Paul, M. (1995). Resection of colorectal liver metastases. *World J Surg* 19, 59-71.

Schiff, D. E., Rae, J., Martin, T. R., Davis, B. H., and Curnutte, J. T. (1997). Increased phagocyte Fc gammaRI expression and improved Fc gamma-receptor-mediated phagocytosis after in vivo recombinant human interferon-gamma treatment of normal human subjects. *Blood* 90, 3187-3194.

Schlereth, B., Quadt, C., Dreier, T., Kufer, P., Lorenczewski, G., Prang, N., Brandl, C., Lippold, S., Cobb, K., Brasky, K., *et al.* (2006). T-cell activation and B-cell depletion in

chimpanzees treated with a bispecific anti-CD19/anti-CD3 single-chain antibody construct. *Cancer Immunol Immunother* 55, 503-514.

Schmidt, M. M., Thurber, G. M., and Wittrup, K. D. (2008). Kinetics of anti-carcinoembryonic antigen antibody internalization: effects of affinity, bivalency, and stability. *Cancer Immunol Immunother*.

Schmidt, R. E., and Gessner, J. E. (2005). Fc receptors and their interaction with complement in autoimmunity. *Immunol Lett* 100, 56-67.

Schottenfeld, D., and Winawer, S. J. (1983). Cholecystectomy and colorectal cancer. *Gastroenterology* 85, 966-967.

Schuetz, J. D., Collins, J. M., Wallace, H. J., and Diasio, R. B. (1986). Alteration of the secondary structure of newly synthesized DNA from murine bone marrow cells by 5-fluorouracil. *Cancer Res* 46, 119-123.

Schuller, J., Cassidy, J., Dumont, E., Roos, B., Durston, S., Banken, L., Utoh, M., Mori, K., Weidekamm, E., and Reigner, B. (2000). Preferential activation of capecitabine in tumor following oral administration to colorectal cancer patients. *Cancer Chemother Pharmacol* 45, 291-297.

Schulz, H., Bohlius, J., Skoetz, N., Trelle, S., Kober, T., Reiser, M., Dreyling, M., Herold, M., Schwarzer, G., Hallek, M., and Engert, A. (2007). Chemotherapy plus Rituximab versus chemotherapy alone for B-cell non-Hodgkin's lymphoma. *Cochrane Database Syst Rev*, CD003805.

Schuster, M., Umana, P., Ferrara, C., Brunker, P., Gerdes, C., Waxenecker, G., Wiederkum, S., Schwager, C., Loibner, H., Himmler, G., and Mudde, G. C. (2005). Improved effector functions of a therapeutic monoclonal Lewis Y-specific antibody by glycoform engineering. *Cancer Res* 65, 7934-7941.

Schutte, B., Henfling, M., Kolgen, W., Bouman, M., Meex, S., Leers, M. P., Nap, M., Bjorklund, V., Bjorklund, P., Bjorklund, B., *et al.* (2004). Keratin 8/18 breakdown and reorganization during apoptosis. *Exp Cell Res* 297, 11-26.

Schwartzberg, L. S., Petak, I., Stewart, C., Turner, P. K., Ashley, J., Tillman, D. M., Douglas, L., Tan, M., Billups, C., Mihalik, R., *et al.* (2002). Modulation of the Fas signaling pathway by IFN-gamma in therapy of colon cancer: phase I trial and correlative studies of IFN-gamma, 5-fluorouracil, and leucovorin. *Clin Cancer Res* 8, 2488-2498.

Scott, A. M., Lee, F. T., Jones, R., Hopkins, W., MacGregor, D., Cebon, J. S., Hannah, A., Chong, G., U, P., Papenfuss, A., *et al.* (2005). A phase I trial of humanized

monoclonal antibody A33 in patients with colorectal carcinoma: biodistribution, pharmacokinetics, and quantitative tumor uptake. *Clin Cancer Res* 11, 4810-4817.

Screaton, R. A., DeMarte, L., Draber, P., and Stanners, C. P. (2000). The specificity for the differentiation blocking activity of carcinoembryonic antigen resides in its glycoposphatidyl-inositol anchor. *J Cell Biol* 150, 613-626.

Sebag-Montefiore, D., Stephens, R. J., Steele, R., Monson, J., Grieve, R., Khanna, S., Quirke, P., Couture, J., de Metz, C., Myint, A. S., *et al.* (2009). Preoperative radiotherapy versus selective postoperative chemoradiotherapy in patients with rectal cancer (MRC CR07 and NCIC-CTG C016): a multicentre, randomised trial. *Lancet* 373, 811-820.

Sebastian, S., Johnston, S., Geoghegan, T., Torreggiani, W., and Buckley, M. (2004). Pooled analysis of the efficacy and safety of self-expanding metal stenting in malignant colorectal obstruction. *Am J Gastroenterol* 99, 2051-2057.

Shan, D., Ledbetter, J. A., and Press, O. W. (1998). Apoptosis of malignant human B cells by ligation of CD20 with monoclonal antibodies. *Blood* 91, 1644-1652.

Sharkey, R. M., and Goldenberg, D. M. (2005). Perspectives on cancer therapy with radiolabeled monoclonal antibodies. *J Nucl Med* 46 *Suppl 1*, 115S-127S.

Sharkey, R. M., Karacay, H., Cardillo, T. M., Chang, C. H., McBride, W. J., Rossi, E. A., Horak, I. D., and Goldenberg, D. M. (2005). Improving the delivery of radionuclides for imaging and therapy of cancer using pretargeting methods. *Clin Cancer Res* 11, 7109s-7121s.

Shields, R. L., Lai, J., Keck, R., O'Connell, L. Y., Hong, K., Meng, Y. G., Weikert, S. H., and Presta, L. G. (2002). Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcγ₃ and antibody-dependent cellular toxicity. *J Biol Chem* 277, 26733-26740.

Shields, R. L., Namenuk, A. K., Hong, K., Meng, Y. G., Rae, J., Briggs, J., Xie, D., Lai, J., Stadlen, A., Li, B., *et al.* (2001). High resolution mapping of the binding site on human IgG1 for Fc γ₁, Fc γ₂, Fc γ₃, and FcRn and design of IgG1 variants with improved binding to the Fc γ₃. *J Biol Chem* 276, 6591-6604.

Shimoyama, S., Mochizuki, Y., Kusada, O., and Kaminishi, M. (2002). Supra-additive antitumor activity of 5FU with tumor necrosis factor-related apoptosis-inducing ligand on gastric and colon cancers in vitro. *Int J Oncol* 21, 643-648.

Sieber, O. M., Heinemann, K., and Tomlinson, I. P. (2003). Genomic instability--the engine of tumorigenesis? *Nat Rev Cancer* 3, 701-708.

Sievers, E. L., Larson, R. A., Stadtmauer, E. A., Estey, E., Lowenberg, B., Dombret, H., Karanes, C., Theobald, M., Bennett, J. M., Sherman, M. L., *et al.* (2001). Efficacy and safety of gemtuzumab ozogamicin in patients with CD33-positive acute myeloid leukemia in first relapse. *J Clin Oncol* *19*, 3244-3254.

Simmonds, P. C., Primrose, J. N., Colquitt, J. L., Garden, O. J., Poston, G. J., and Rees, M. (2006). Surgical resection of hepatic metastases from colorectal cancer: a systematic review of published studies. *Br J Cancer* *94*, 982-999.

Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* *235*, 177-182.

Slamon, D. J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., *et al.* (2001). Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* *344*, 783-792.

Small, E. J., Tchekmedyian, N. S., Rini, B. I., Fong, L., Lowy, I., and Allison, J. P. (2007). A pilot trial of CTLA-4 blockade with human anti-CTLA-4 in patients with hormone-refractory prostate cancer. *Clin Cancer Res* *13*, 1810-1815.

Smith, G., Carey, F. A., Beattie, J., Wilkie, M. J., Lightfoot, T. J., Coxhead, J., Garner, R. C., Steele, R. J., and Wolf, C. R. (2002). Mutations in APC, Kirsten-ras, and p53--alternative genetic pathways to colorectal cancer. *Proc Natl Acad Sci U S A* *99*, 9433-9438.

Smith, M. E., Bodmer, J. G., Kelly, A. P., Trowsdale, J., Kirkland, S. C., and Bodmer, W. F. (1989). Variation in HLA expression on tumors: an escape from immune response. *Cold Spring Harb Symp Quant Biol* *54 Pt 1*, 581-586.

Sobin, L. H., and Wittekind, C. (2002). TNM classification of malignant tumours, 6th ed. / edited by L.H. Sobin and Ch. Wittekind. edn (New York ; [Chichester]: Wiley-Liss).

Stangl, R., Altendorf-Hofmann, A., Charnley, R. M., and Scheele, J. (1994). Factors influencing the natural history of colorectal liver metastases. *Lancet* *343*, 1405-1410.

Steplewski, Z., Lubeck, M. D., and Koprowski, H. (1983). Human macrophages armed with murine immunoglobulin G2a antibodies to tumors destroy human cancer cells. *Science* *221*, 865-867.

Stewart, L. M., Young, S., Watson, G., Mather, S. J., Bates, P. A., Band, H. A., Wilkinson, R. W., Ross, E. L., and Snary, D. (1999). Humanisation and characterisation

of PR1A3, a monoclonal antibody specific for cell-bound carcinoembryonic antigen. *Cancer Immunol Immunother* 47, 299-306.

Su, L. K., Kinzler, K. W., Vogelstein, B., Preisinger, A. C., Moser, A. R., Luongo, C., Gould, K. A., and Dove, W. F. (1992). Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. *Science* 256, 668-670.

Sunada, H., Magun, B. E., Mendelsohn, J., and MacLeod, C. L. (1986). Monoclonal antibody against epidermal growth factor receptor is internalized without stimulating receptor phosphorylation. *Proc Natl Acad Sci U S A* 83, 3825-3829.

Suntharalingam, G., Perry, M. R., Ward, S., Brett, S. J., Castello-Cortes, A., Brunner, M. D., and Panoskaltsis, N. (2006). Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. *N Engl J Med* 355, 1018-1028.

Syrigos, K. N., and Epenetos, A. A. (1999). Antibody directed enzyme prodrug therapy (ADEPT): a review of the experimental and clinical considerations. *Anticancer Res* 19, 605-613.

Taheri, M., Saragovi, H. U., and Stanners, C. P. (2003). The adhesion and differentiation-inhibitory activities of the immunoglobulin superfamily member, carcinoembryonic antigen, can be independently blocked. *J Biol Chem* 278, 14632-14639.

Takai, T., Li, M., Sylvestre, D., Clynes, R., and Ravetch, J. V. (1994). FcR gamma chain deletion results in pleiotropic effector cell defects. *Cell* 76, 519-529.

Tamm, A., and Schmidt, R. E. (1996). The binding epitopes of human CD16 (Fc gamma RIII) monoclonal antibodies. Implications for ligand binding. *J Immunol* 157, 1576-1581.

Tanaka, F., and Barbas, C. F., 3rd (2002). Reactive immunization: a unique approach to catalytic antibodies. *J Immunol Methods* 269, 67-79.

Taylor, C., Hershman, D., Shah, N., Suci-Foca, N., Petrylak, D. P., Taub, R., Vahdat, L., Cheng, B., Pegram, M., Knutson, K. L., and Clynes, R. (2007). Augmented HER-2 specific immunity during treatment with trastuzumab and chemotherapy. *Clin Cancer Res* 13, 5133-5143.

Tempero, M., Leichner, P., Baranowska-Kortylewicz, J., Harrison, K., Augustine, S., Schlom, J., Anderson, J., Wisecarver, J., and Colcher, D. (2000). High-dose therapy with ⁹⁰Yttrium-labeled monoclonal antibody CC49: a phase I trial. *Clin Cancer Res* 6, 3095-3102.

Thiagalingam, S., Lengauer, C., Leach, F. S., Schutte, M., Hahn, S. A., Overhauser, J., Willson, J. K., Markowitz, S., Hamilton, S. R., Kern, S. E., *et al.* (1996). Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers. *Nat Genet* 13, 343-346.

Thompson, J. A. (1995). Molecular cloning and expression of carcinoembryonic antigen gene family members. *Tumour Biol* 16, 10-16.

Thompson, J. A., Eades-Perner, A. M., Ditter, M., Muller, W. J., and Zimmermann, W. (1997). Expression of transgenic carcinoembryonic antigen (CEA) in tumor-prone mice: an animal model for CEA-directed tumor immunotherapy. *Int J Cancer* 72, 197-202.

Thompson, J. A., Grunert, F., and Zimmermann, W. (1991). Carcinoembryonic antigen gene family: molecular biology and clinical perspectives. *J Clin Lab Anal* 5, 344-366.

Tolcher, A. W., Sugarman, S., Gelmon, K. A., Cohen, R., Saleh, M., Isaacs, C., Young, L., Healey, D., Onetto, N., and Slichenmyer, W. (1999). Randomized phase II study of BR96-doxorubicin conjugate in patients with metastatic breast cancer. *J Clin Oncol* 17, 478-484.

Tom, B. H., Rutzky, L. P., Jakstys, M. M., Oyasu, R., Kaye, C. I., and Kahan, B. D. (1976). Human colonic adenocarcinoma cells. I. Establishment and description of a new line. *In Vitro* 12, 180-191.

Tomizuka, K., Shinohara, T., Yoshida, H., Uejima, H., Ohguma, A., Tanaka, S., Sato, K., Oshimura, M., and Ishida, I. (2000). Double trans-chromosomic mice: maintenance of two individual human chromosome fragments containing Ig heavy and kappa loci and expression of fully human antibodies. *Proc Natl Acad Sci U S A* 97, 722-727.

Tomlinson, I., Webb, E., Carvajal-Carmona, L., Broderick, P., Kemp, Z., Spain, S., Penegar, S., Chandler, I., Gorman, M., Wood, W., *et al.* (2007). A genome-wide association scan of tag SNPs identifies a susceptibility variant for colorectal cancer at 8q24.21. *Nat Genet*.

Trail, P. A., Willner, D., Lasch, S. J., Henderson, A. J., Hofstead, S., Casazza, A. M., Firestone, R. A., Hellstrom, I., and Hellstrom, K. E. (1993). Cure of xenografted human carcinomas by BR96-doxorubicin immunoconjugates. *Science* 261, 212-215.

Treon, S. P., Hansen, M., Branagan, A. R., Verselis, S., Emmanouilides, C., Kimby, E., Frankel, S. R., Touroutoglou, N., Turnbull, B., Anderson, K. C., *et al.* (2005). Polymorphisms in FcγRIIIA (CD16) receptor expression are associated with clinical response to rituximab in Waldenstrom's macroglobulinemia. *J Clin Oncol* 23, 474-481.

Tzeng, S. J., Bolland, S., Inabe, K., Kurosaki, T., and Pierce, S. K. (2005). The B cell inhibitory Fc receptor triggers apoptosis by a novel c-Abl family kinase-dependent pathway. *J Biol Chem* 280, 35247-35254.

Uchida, J., Hamaguchi, Y., Oliver, J. A., Ravetch, J. V., Poe, J. C., Haas, K. M., and Tedder, T. F. (2004). The innate mononuclear phagocyte network depletes B lymphocytes through Fc receptor-dependent mechanisms during anti-CD20 antibody immunotherapy. *J Exp Med* 199, 1659-1669.

Ullmann, C. D., Schlom, J., and Greiner, J. W. (1992). Interleukin-6 increases carcinoembryonic antigen and histocompatibility leukocyte antigen expression on the surface of human colorectal carcinoma cells. *J Immunother* (1991) 12, 231-241.

Ulrich, C. M., Whitton, J., Yu, J. H., Sibert, J., Sparks, R., Potter, J. D., and Bigler, J. (2005). PTGS2 (COX-2) -765G > C promoter variant reduces risk of colorectal adenoma among nonusers of nonsteroidal anti-inflammatory drugs. *Cancer Epidemiol Biomarkers Prev* 14, 616-619.

Umana, P., Jean-Mairet, J., Moudry, R., Amstutz, H., and Bailey, J. E. (1999). Engineered glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. *Nat Biotechnol* 17, 176-180.

Van Cutsem, E., Peeters, M., Siena, S., Humblet, Y., Hendlisz, A., Neyns, B., Canon, J. L., Van Laethem, J. L., Maurel, J., Richardson, G., *et al.* (2007). Open-label phase III trial of panitumumab plus best supportive care compared with best supportive care alone in patients with chemotherapy-refractory metastatic colorectal cancer. *J Clin Oncol* 25, 1658-1664.

van Hooft, J. E., Fockens, P., Marinelli, A. W., Timmer, R., van Berkel, A. M., Bossuyt, P. M., and Bemelman, W. A. (2008). Early closure of a multicenter randomized clinical trial of endoscopic stenting versus surgery for stage IV left-sided colorectal cancer. *Endoscopy* 40, 184-191.

van Meerten, T., van Rijn, R. S., Hol, S., Hagenbeek, A., and Ebeling, S. B. (2006). Complement-induced cell death by rituximab depends on CD20 expression level and acts complementary to antibody-dependent cellular cytotoxicity. *Clin Cancer Res* 12, 4027-4035.

van Oers, M. H., Klasa, R., Marcus, R. E., Wolf, M., Kimby, E., Gascoyne, R. D., Jack, A., Van't Veer, M., Vranovsky, A., Holte, H., *et al.* (2006). Rituximab maintenance improves clinical outcome of relapsed/resistant follicular non-Hodgkin lymphoma in patients both with and without rituximab during induction: results of a prospective randomized phase 3 intergroup trial. *Blood* 108, 3295-3301.

van Spruiel, A. B., Leusen, J. H., van Egmond, M., Dijkman, H. B., Assmann, K. J., Mayadas, T. N., and van de Winkel, J. G. (2001). Mac-1 (CD11b/CD18) is essential for Fc receptor-mediated neutrophil cytotoxicity and immunologic synapse formation. *Blood* 97, 2478-2486.

Varey, A. H., Rennel, E. S., Qiu, Y., Bevan, H. S., Perrin, R. M., Raffy, S., Dixon, A. R., Paraskeva, C., Zaccheo, O., Hassan, A. B., *et al.* (2008). VEGF 165 b, an antiangiogenic VEGF-A isoform, binds and inhibits bevacizumab treatment in experimental colorectal carcinoma: balance of pro- and antiangiogenic VEGF-A isoforms has implications for therapy. *Br J Cancer* 98, 1366-1379.

Vasen, H. F., Watson, P., Mecklin, J. P., and Lynch, H. T. (1999). New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. *Gastroenterology* 116, 1453-1456.

Vasovic, L. V., Dyall, R., Clynes, R. A., Ravetch, J. V., and Nikolic-Zugic, J. (1997). Synergy between an antibody and CD8+ cells in eliminating an established tumor. *Eur J Immunol* 27, 374-382.

Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smits, A. M., and Bos, J. L. (1988). Genetic alterations during colorectal-tumor development. *N Engl J Med* 319, 525-532.

Wagener, C., Petzold, P., Kohler, W., and Totovic, V. (1984). Binding of five monoclonal anti-CEA antibodies with different epitope specificities to various carcinoma tissues. *Int J Cancer* 33, 469-475.

Walport, M. J. (2001). Complement. First of two parts. *N Engl J Med* 344, 1058-1066.

Weiden, P. L., Breitz, H. B., Seiler, C. A., Bjorn, M. J., Ratliff, B. A., Mallett, R., Beaumier, P. L., Appelbaum, J. W., Fritzberg, A. R., and Salk, D. (1993). Rhenium-186-labeled chimeric antibody NR-LU-13: pharmacokinetics, biodistribution and immunogenicity relative to murine analog NR-LU-10. *J Nucl Med* 34, 2111-2119.

Weisenseel, P., Martin, S., Partsch, K., Messer, G., and Prinz, J. C. (2007). Relevance of the low-affinity type of the Fcγ-receptor IIIa-polymorphism in bullous pemphigoid. *Arch Dermatol Res* 299, 163-164.

Welt, S., Divgi, C. R., Kemeny, N., Finn, R. D., Scott, A. M., Graham, M., Germain, J. S., Richards, E. C., Larson, S. M., Oettgen, H. F., and *et al.* (1994). Phase I/II study of iodine 131-labeled monoclonal antibody A33 in patients with advanced colon cancer. *J Clin Oncol* 12, 1561-1571.

Welt, S., Divgi, C. R., Real, F. X., Yeh, S. D., Garin-Chesa, P., Finstad, C. L., Sakamoto, J., Cohen, A., Sigurdson, E. R., Kemeny, N., and et al. (1990). Quantitative analysis of antibody localization in human metastatic colon cancer: a phase I study of monoclonal antibody A33. *J Clin Oncol* 8, 1894-1906.

Welt, S., Ritter, G., Williams, C., Jr., Cohen, L. S., John, M., Jungbluth, A., Richards, E. A., Old, L. J., and Kemeny, N. E. (2003). Phase I study of anticolon cancer humanized antibody A33. *Clin Cancer Res* 9, 1338-1346.

Weng, W. K., and Levy, R. (2001). Expression of complement inhibitors CD46, CD55, and CD59 on tumor cells does not predict clinical outcome after rituximab treatment in follicular non-Hodgkin lymphoma. *Blood* 98, 1352-1357.

Weng, W. K., and Levy, R. (2003). Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. *J Clin Oncol* 21, 3940-3947.

Westra, J. L., Schaapveld, M., Hollema, H., de Boer, J. P., Kraak, M. M., de Jong, D., ter Elst, A., Mulder, N. H., Buys, C. H., Hofstra, R. M., and Plukker, J. T. (2005). Determination of TP53 mutation is more relevant than microsatellite instability status for the prediction of disease-free survival in adjuvant-treated stage III colon cancer patients. *J Clin Oncol* 23, 5635-5643.

Wilkinson, R. W., Ross, E. L., Ellison, D., Zimmermann, W., Snary, D., and Mather, S. J. (2002). Evaluation of a transgenic mouse model for anti-human CEA radioimmunotherapeutics. *J Nucl Med* 43, 1368-1376.

Wilkinson, R. W., Ross, E. L., Poulson, R., Ilyas, M., Straub, J., Snary, D., Bodmer, W. F., and Mather, S. J. (2001). Antibody targeting studies in a transgenic murine model of spontaneous colorectal tumors. *Proc Natl Acad Sci U S A* 98, 10256-10260.

Willett, C. G., Boucher, Y., di Tomaso, E., Duda, D. G., Munn, L. L., Tong, R. T., Chung, D. C., Sahani, D. V., Kalva, S. P., Kozin, S. V., *et al.* (2004). Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. *Nat Med* 10, 145-147.

Willett, W. C., Stampfer, M. J., Colditz, G. A., Rosner, B. A., and Speizer, F. E. (1990). Relation of meat, fat, and fiber intake to the risk of colon cancer in a prospective study among women. *N Engl J Med* 323, 1664-1672.

Winkler, F., Kozin, S. V., Tong, R. T., Chae, S. S., Booth, M. F., Garkavtsev, I., Xu, L., Hicklin, D. J., Fukumura, D., di Tomaso, E., *et al.* (2004). Kinetics of vascular normalization by VEGFR2 blockade governs brain tumor response to radiation: role of oxygenation, angiopoietin-1, and matrix metalloproteinases. *Cancer Cell* 6, 553-563.

Wiratkapun, S., Kraemer, M., Seow-Choen, F., Ho, Y. H., and Eu, K. W. (2001). High preoperative serum carcinoembryonic antigen predicts metastatic recurrence in potentially curative colonic cancer: results of a five-year study. *Dis Colon Rectum* 44, 231-235.

Witzig, T. E., Vukov, A. M., Habermann, T. M., Geyer, S., Kurtin, P. J., Friedenberg, W. R., White, W. L., Chalchal, H. I., Flynn, P. J., Fitch, T. R., and Welker, D. A. (2005). Rituximab therapy for patients with newly diagnosed, advanced-stage, follicular grade I non-Hodgkin's lymphoma: a phase II trial in the North Central Cancer Treatment Group. *J Clin Oncol* 23, 1103-1108.

Woelbing, F., Kostka, S. L., Moelle, K., Belkaid, Y., Sunderkoetter, C., Verbeek, S., Waisman, A., Nigg, A. P., Knop, J., Udey, M. C., and von Stebut, E. (2006). Uptake of *Leishmania major* by dendritic cells is mediated by Fcγ receptors and facilitates acquisition of protective immunity. *J Exp Med* 203, 177-188.

Wong, J. Y., Chu, D. Z., Williams, L. E., Yamauchi, D. M., Ikle, D. N., Kwok, C. S., Liu, A., Wilczynski, S., Colcher, D., Yazaki, P. J., *et al.* (2004). Pilot trial evaluating an 123I-labeled 80-kilodalton engineered anticarcinoembryonic antigen antibody fragment (cT84.66 minibody) in patients with colorectal cancer. *Clin Cancer Res* 10, 5014-5021.

Woodford-Richens, K. L., Rowan, A. J., Gorman, P., Halford, S., Bicknell, D. C., Wasan, H. S., Roylance, R. R., Bodmer, W. F., and Tomlinson, I. P. (2001). SMAD4 mutations in colorectal cancer probably occur before chromosomal instability, but after divergence of the microsatellite instability pathway. *Proc Natl Acad Sci U S A* 98, 9719-9723.

Wu, J., Edberg, J. C., Redecha, P. B., Bansal, V., Guyre, P. M., Coleman, K., Salmon, J. E., and Kimberly, R. P. (1997). A novel polymorphism of FcγRIIIa (CD16) alters receptor function and predisposes to autoimmune disease. *J Clin Invest* 100, 1059-1070.

Xiang, Z., Cutler, A. J., Brownlie, R. J., Fairfax, K., Lawlor, K. E., Severinson, E., Walker, E. U., Manz, R. A., Tarlinton, D. M., and Smith, K. G. (2007). FcγRIIb controls bone marrow plasma cell persistence and apoptosis. *Nat Immunol* 8, 419-429.

Yang, X. D., Jia, X. C., Corvalan, J. R., Wang, P., Davis, C. G., and Jakobovits, A. (1999). Eradication of established tumors by a fully human monoclonal antibody to the epidermal growth factor receptor without concomitant chemotherapy. *Cancer Res* 59, 1236-1243.

Zbar, A. P., Thomas, H., Wilkinson, R. W., Wadhwa, M., Syrigos, K. N., Ross, E. L., Dilger, P., Allen-Mersh, T. G., Kmiot, W. A., Epenetos, A. A., *et al.* (2005). Immune responses in advanced colorectal cancer following repeated intradermal vaccination with the anti-CEA murine monoclonal antibody, PR1A3: results of a phase I study. *Int J Colorectal Dis* 20, 403-414.

APPENDIX 1

E4 MEDIUM (DULBECCO'S MODIFICATION OF EAGLE'S MEDIUM)

	mg/litre
Inorganic Salts	
CaCl ₂ .2H ₂ O	265.00
Fe(NO ₃)	3 0.1
KCl	400.00
MgSO ₄ . 7H ₂ O	200.00
NaCl	6400.00
NaH ₂ PO ₄ .2H ₂ O	140.00
Amino Acids	
L- Arginine mono.HCl	84.00
L-Cystine	48.00
L-Histidine mono.HCl	42.00
L-Glutamine	584.00
Glycine	30.00
L-Isoleucine	104.80
L-Leucine	104.80
L-Lysine mono.HCl	146.20
L-Methionine	30.00
L-Phenylalanine	66.00
L-Serine	42.00
L-Threonine	95.20
L-Tryptophan	16.00
L-Tyrosine	72.40
L-Valine	93.60
Inositol	7.00
Vitamins	
Choline Chloride	4.00
Folic acid	4.00

Nicotinamide	4.00
DL-pantothenic acid, Ca salt	4.00
Pyridoxal HCl	4.00
Riboflavin	0.40
Thiamine HCl (aneurine)	4.00
Other Compounds	
D-Glucose	4500.00
Phenol red	15.00
NaHCO ₃	3700.00
Penicillin	100,000 units
Streptomycin Sulphate	100.00
Sodium pyruvate	110.00
Antimycotic (Butyl-p-hydroxybenzoate)	0.2

2% RPMI (ROSWELL PARK MEMORIAL INSTITUTE)

	mg/litre
Inorganic Salts mg/litre	
Ca (NO ₃) ₂ . 4H ₂ O	100.00
KCl	400.00
MgSO ₄	48.84
NaCl	6000.00
Na ₂ HPO ₄ (anhydrous)	800.00
Amino Acids	
L-Arginine (free base)	200.00
L-Asparagine	50.00
L-Aspartic acid	20.00
L-Cystine (2HCl)	65.15
L-Glutamic acid	20.00
L-Glutamine	300.00
Glycine	10.00
L-Histidine (free base)	15.00
L-Hydroxyproline	20.00
L-Isoleucine (Allo free)	50.00
L-Leucine (Methionine free)	50.00
L-Lysine HCl	40.00
L-Methionine	15.00
L-Phenylalanine	15.00
L-Proline (Hydroxy L-Proline free)	20.00
L-Serine	30.00
L-Threonine (Allo free)	20.00
L-Tryptophan	5.00
L-Tyrosine (Na salt)	28.83
L-Valine	20.00
Vitamins	
Biotin	0.20
D-Ca Panthothenate	0.25
Choline Chloride	3.00

Folic acid	1.00
i-Inositol	35.00
Nicotinamide	1.00
Para-aminobenzoic acid	1.00
Pyridoxine HCl	1.00
Riboflavin	0.20
Thiamine HCl	1.00
Vitamin B12	0.005
Other Compounds	
D-Glucose	2000.00
Glutathione (reduced)	1.00
Phenol red	5.00
NaHCO ₃	2000.00
Penicillin	100,000 units
Streptomycin	100.00

ISCOVE'S MODIFIED DULBECCO'S MEDIUM (IMDM)

E4 medium

25mM HEPES

3.024mg/L NaHCO₃

PHOSPHATE-BUFFERED SALINE (PBSA)

The following salts dissolved in distilled water:

NaCl 8g/L

KCl 0.25g/L

Na₂HPO₄ 1.43g/L

KH₂PO₄ 0.25g/L

TRIS / BORATE / EDTA (TBE) BUFFER

Tris base 10.8g/L

Boric acid 5.5g/L

Dissolved in 2mM EDTA (pH 8.0)