Application of antibody engineering techniques to develop antibodies for detection of inflammatory and malignant disease

Rajinder Bhoday

Thesis submitted for the degree of
Doctor of Philosophy
for the
University of London

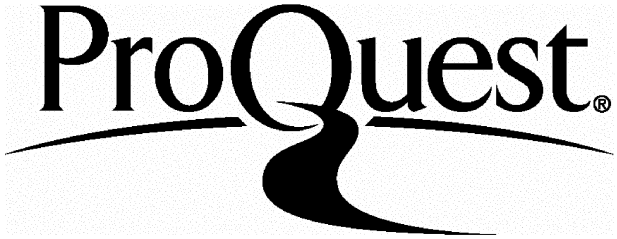
Royal Free and University College Medical School

ProQuest Number: 10014999

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.



ProQuest 10014999

Published by ProQuest LLC(2016). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code.
Microform Edition © ProQuest LLC.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

for my parents

Unless stated otherwise the research presented in this thesis is the result of original work conducted by myself under the supervision of Mr. C. Y. Yiu, Dr. P. J. Delves and Dr K. A. Chester

Rajinder Bhoday

Abstract

It is proposed that antibody engineering techniques can be used to generate clinically useful antibodies. These techniques provide a means of creating new antibodies of defined specificities and tailoring their properties as well as those of hybridoma-derived monoclonal antibodies.

In this thesis the potential of antibody engineering to produce reagents for the clinic has been explored. Three approaches have been taken.

First, construction of Fab and scFv antibody fragments from an established murine hybridoma. In this case the target antigen was NCA-95, a marker of inflammation. Functional expression of fragments could not be demonstrated so phage display technology with a murine scFv repertoire was successfully applied with this antigen.

A second approach, using synthetic carbohydrates, was to select antibodies against tumour associated glycoproteins. Naïve human scFv and Fab repertoires and a murine scFv repertoire were tested. A single human Fab fragment with binding to STn, a MUC1 tumour associated carbohydrate antigen, was isolated.

Finally, antibody engineering techniques were applied to rescue the binding regions of a human hybridoma which reacted with a (non-defined) tumour antigen on human colorectal cells. Nine different scFv fragments were generated. These were screened by immunohistochemistry against a range of normal and tumour human tissues. Specific reactivity was observed against a number of tumours including carcinoma of the colon, breast and ovary.

The work presented in this thesis has demonstrated the feasibility of isolating antibody fragments, against markers of inflammation and malignancy, by selection from antibody repertoires using phage display and rescue of V-genes from hybridomas.

Acknowledgements

I would like to thank my supervisors Chu Yiu, Peter Delves and Kerry Chester.

I am grateful to Chu for giving me the opportunity to continue my PhD in the Department of Surgery. Many thanks to Pete for his patience, support and continual encouragement over the years and to Kerry for her confidence in my abilities, her inspiration and guidance.

I would also like to thank Lynda Robson for her kind help and Geoff Boxer for guidance with the immunochemistry during my time in the Department of Clinical Oncology. Many thanks Hennie Hoogenboom for providing me with the opportunity to visit his laboratory.

Finally, I will forever be grateful to my Mum, Dad, Kirn, Cookie and Bobby for their precious love.

Table of contents

Abbreviations

ADCC	antibody-dependent cell mediated cytotoxicity
ADEPT	antibody-directed prodrug therapy
APC	adenomatous polyposis coli
bp	base pair
BIC	carbonate/bicarbonate buffer pH 6.9
BSA	bovine serum albumin
C5	complement component 5
$CaCl_2$	calcium chloride
CD4	cluster differentiation antigen 4
cDNA	complementary deoxynucleic acid
CDR	complementarity-determining region
CEA	carcinoembryonic antigen
CGM6	CEA gene family member 6
CH(1)	immunoglubulin heavy chain constant domain (1)
CIP	calf intestinal phosphatase
CL	light chain constant domain
CME	crude membrane extract
CPG_2	carboxypeptidase G_2
CsCl	caesium chloride
dATP	deoxyadenosine triphosphate
DAB	diaminobenzidine
dCTP	deoxycytosine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanidine triphosphate
DMEM	Dulbecco modified Eagle medium
DMSO	dimethylsulphoxide
DNA	deoxynucleic acid
DNase	deoxynuclease
D region	diversity region

dsDNA	double stranded DNA
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
E. coli	Escherichia coli
EDTA	ethylendiaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Fab	fragment antibody binding
Fc	fragment crystalisable
FcγR(II/III)	Fc gamma receptor (II/III)
FUO	fever of unknown origin
Fv	variable fragment
g(III/VIII)p	gene (III/VIII) protein
α-Gal	α-galactosyl antigen (Gal α1-3 Gal β1-4 GlcNAc)
Gal	Galactose
GalNAc	N-acetyl-D-galactosamine
Glc	β-D-glucose
GlcNAc	N-acetyl-D-glucosamine
GPI	glycophosphatidylinositol
H-chain	heavy chain
HCl	hydrochloric acid
HMy	LICR-LON-HMy2
HRP	horse radish peroxidase
HSA	human serum albumin
IgC	immunoglobulin constant region domain
IgV	immunoglubulin variable region domain
IL-8	interleukin-8
IMGT	International Immunogenetics database
IPTG	isopropyl-β-D-thio-galactopyranoside
J-region	joining region
K	kanamycin
Kd	kilodalton

KH_2PO_4	potasium dihydrogen phosphate
KLH	keyhole lympet heamocyanin
LB	Luria broth
L-chain	light chain
Le^x	Lewis X antigen
MAR	Marvel skimmed milk powder
$MgCl_2$	magnesium chloride
MHC	major histocompatibility complex
M-LVRT	murine Leukemia reverse transcriptase
m.o.i	multiplicity of infection
mRNA	messenger RNA
MUC	mucin
NaCl	sodium chloride
Na_2HPO_4	di-sodium hydrogen phosphate
NaOH	sodium hydroxide
NCA(-95)	non-specific cross reactive antigen (95)
NeuAc	α-N-acetyl neuraminic acid (sialic acid)
PAA	poly [N-C_2-hydroxyethyl] acrylamide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PBS-Tween 0.05%	PBS with 0.05% (v/v) Tween 20
PCR	polymerase chain reaction
PEG	polyethylene glycol
PelB	pectate lyase protein
PMN	polymorphonuclear leucocyte
RAID	radioimmunodetection
RIGS	radioimmunoguided surgery
RIS	radioimmunoscintography
RNA	ribonucleic acid
scFv	single chain Fv
sdH_2O	sterile distilled water
ser	serine
SDS	sodium dodecyl sulphate

SLe^x	sialylated Lewis X antigen
ssDNA	single stranded DNA
STn	sialylated Tn (NeuAc α2-6 GalNAc)
T	Galβ1-3GalNacα1-O-Ser/Thr
TBS	Tris buffered saline
TE	10 mM Tris pH 7.4, 1 mM EDTA buffer
thr	threonine
Tn	α-N-acetyl-D-galactosamine
2TYAG	2xTY media with 100ug/ml ampicillin and 2% glucose
UV	ultraviolet
V-gene	variable region gene
VH	heavy chain variable region

List of Tables

Legends to Figures

Chapter 1

Figure 1.1 IgG and recombinant antibody fragments. VH and VL represent the variable heavy and light regions that together form the antigen binding site. CL represents the light chain constant region, with heavy chain constant region comprising of CH1, CH2 and CH3 domains. The CH2 and CH3 domains form the Fc fragment which is attached to CH1 via the hinge region. Both monomeric and dimeric forms of the engineered Fab and scFv antibody fragments are shown in which heavy and light chains are joined by peptide linkers.

Figure 1.2 Cloning V-genes and construction of scFv antibody fragment for display as an N-terminal gene III fusion protein on the surface of filamentous bacteriophage fd.

Figure 1.3 Selection of phage library by panning. The phage library is incubated with antigen and non-binding phage washed away. Specific phage are eluted and *E.coli* reinfected for amplification and rescue. Phage particles from this amplified library are used for the next round of selection. After 2-4 rounds of selection, individual (monoclonal) antibodies are analysed.

Figure 1.4 Diagrammatic representation of CEA, NCA and NCA-95 based on their cDNA sequence. The circles show segments that form IgV-like (N) domains and IgC-like (A and B) domains. The Gpi-linkage to the cell membrane is shown as ▼. Potential sites for N-linked glycosylation (Asn-X-Thr/Ser) are shown as ♦ (adapted from Hammarström *et al* 1998).

Figure 1.5 Schematic representation of expression of human mucin MUC1 on normal and transformed epithelium (adapted from Finn *et al* 1995).

Chapter 4

Chapter 5

Figure 5.5 LS174T tumour xenograft staining with fusion partner HMy, negative control for S2.5 showing absence of reactivity with malignant cells.

Figure 5.6 LS174T tumour xenograft staining with anti-CEA scFv MFE-23-myc, malignant glandular structures are uniformly positive for CEA.

Figure 5.7 LS174T tumour xenograft staining with scFv C9 S2.5 showing diffuse reactivity with malignant glandular structures.

Figure 5.8 LS174T tumour xenograft, negative omission control for scFv detection.

Figure 5.9 Human breast carcinoma staining with scFv C9 S2.5, cytoplasmic and membrane reactivity.

Figure 5.10 Human breast carcinoma, negative omission control for scFv detection.

Figure 5.11 Human carcinoma of the bladder staining with scFv C9 S2.5 showing heterogeneous reactivity with malignant epithelium.

Figure 5.12 Squamous cell carcinoma of the cervix, negative omission control.

Figure 5.13 Squamous cell carcinoma of the cervix staining with scFv C9S2.5, diffuse cytoplasmic reactivity and with more intense reactivity with keratinising epithelium.

Figure 5.14 Human squamous cell carcinoma of the cervix staining with scFv C8

Figure 5.15 Human colorectal adenocarcinoma staining with scFv C9 S2.5 showing binding to malignant glandular structures, some luminal surfaces are highlighted.

Figure 5.16 Normal human colonic mucosa staining with scFv C9 S2.5 showing reactivity at basal aspect of crypt epithelium.

Figure 5.17 Human colorectal adenocarcinoma staining with scFv MFE-23, malignant glandular structures are uniformly positive for CEA.

Figure 5.18 Human lung squamous cell carcinoma staining with scFv C9 S2.5 showing reactivity with malignant cells.

Figure 5.19 Human papillary serous adenocarcinoma of the ovary staining with scFv C9 S2.5 showing reactivity with malignant glands, strong luminal staining with some cytoplasmic reactivity.

Figure 5.20 Human papillary serous adenocarcinoma of the ovary, negative omission control

Figure 5.21 Sequence alignment of cloned VK gene of scFv clone C9 derived from the human monoclonal antibody S2.5.

Figure 5.22 Sequence alignment of cloned VH gene of scFv clone C9 derived from the human monoclonal antibody S2.5.

Chapter 1

General Introduction

Introduction

Hybridoma technology (Kohler and Milstein, 1975), allows the isolation of cell lines secreting antibody of single specificity. This has enabled the inherent specificity of antibodies to be exploited to provide invaluable diagnostic tools in research, medicine and industry. For therapy, the ultimate goal is to use antibodies as 'magic bullets' as originally proposed by Erlich (Erlich, 1957), that is reagents that have the ability to selectively destroy their target without any detrimental effect on the host. Monoclonal antibodies have been used as carriers for radionuclides, drugs, enzymes and toxins in an attempt to produce such reagents. For human therapy however, efficacy has been limited by a number of factors including the induction of a human anti-mouse antibody (HAMA) response. The affect of HAMA on treatment is particularly apparent where repeated administration of antibody is required, as is the case with the treatment of cancer. The production of human monoclonal antibodies should eliminate many of the problems of immunogenicity associated with rodent antibodies. However the production of human monoclonal antibodies by the hybridoma technique has been difficult. This coupled with the constraints imposed by the inability to immunise has meant that human monoclonal antibodies are not yet viable alternatives for human therapy.

The antibody (IgG) molecule is a heterodimer of two identical heavy and identical two light chains. The heavy and light chains are composed of constant and variable region domains that are linked by short peptides that allow elbow bending. The Fab fragment contains the complete light chain (VLCL) and the first 2 domains (VHCH1) of the heavy chain and is linked by a hinge region to the constant fragment (Fc) composed of the CH3 and CH4 domains of the heavy chains. The Fab fragment is responsible for antigen binding with the antibody binding site formed by the heavy (VH) and light (VL) chain variable region domains (Fv) [Figure 1.1]. Each antibody domain is composed of 2 layers of beta sheets that are linked

by a disulphide bridge. At the top of each variable domain the beta strands are linked by 3 loops which are hypervariable in length and sequence and are known as the complementarity determining regions (CDRs). The residues within these loops make the majority of contacts with antigen and form the antigen binding site of the Fv. Binding of antibody to antigen triggers effector mechanisms such as activation of complement, phagocytosis or antibody-dependant cell-mediated cytotoxicity (ADCC), which are mediated by the Fc domain. The domain structure of antibodies has facilitated engineering of these molecules allowing cloning of functional domains carrying binding specificity or effector functions.

1.1 Engineered antibodies

Recent advances in molecular biology have allowed the cloning of antibody genes to engineer a new generation of antibodies. Chimaeric antibodies where murine constant regions are replaced with human constant regions have been made as a means of reducing the immunogenicity of rodent antibodies as well as improving/changing effector function by fusion with appropriate isotype domains (Morrison, 1992; Boulianne *et al.*, 1984). To further decrease the immunogenicity of the therapeutic antibody, V-region antigenicity may be minimised by humanisation. Murine CDR sequences that form the antigen binding site are incorporated into a human framework by grafting CDRs onto a structurally complementary human β-sheet scaffold (Jones *et al.*, 1986; Riechmann, 1988). The success of these techniques in generating chimeric and humanised antibodies with desired specificities and reduced immunogenicity has been demonstrated by their approval for therapy as well as many others showing clinical potential (Glennie and Johnson, 2000). Disadvantages associated with this method are that it is time consuming and does not guarantee loss of immunogenicity and, where framework residues are involved, can result in loss of binding (Riechmann, 1998; Queen *et al.*, 1989; Gorman *et al.*, 1991).

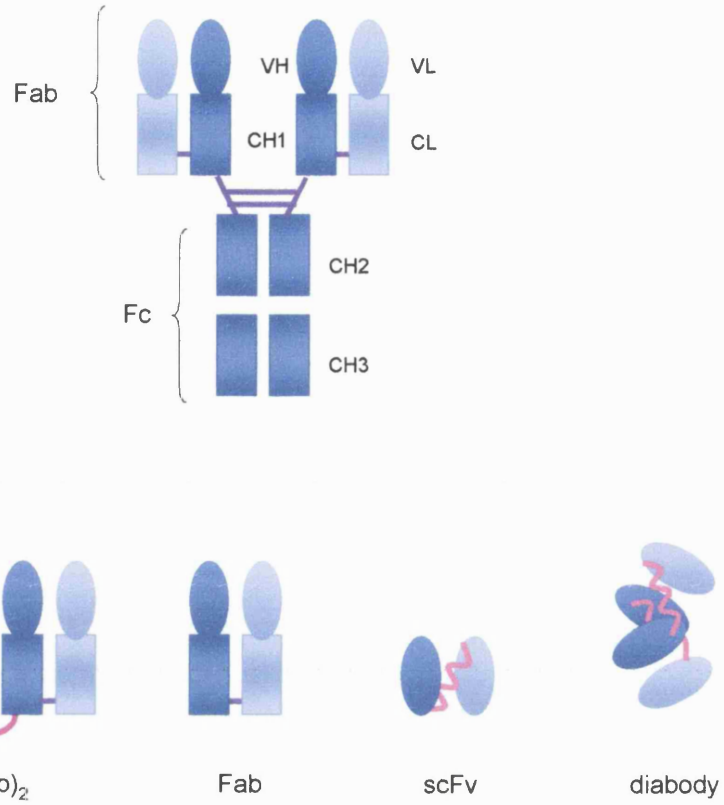


Figure 1.1 IgG and recombinant antibody fragments. VH and VL represent the variable heavy and light regions that together form the antigen binding site. CL represents the light chain constant region, with heavy chain constant region comprising of CH1, CH2 and CH3 domains. The CH2 and CH3 domains form the Fc fragment which is attached to CH1 via the hinge region. Both monomeric and dimeric forms of the engineered Fab and scFv antibody fragments are shown in which heavy and light chains are joined by peptide linkers.

1.1.1 Rescue of V-genes from hybridomas for the construction of antibody fragments.

Advances in antibody engineering have allowed the construction of a number of different antibody fragments. Cloned fragments have been engineered for secretion as functional antibody fragments in different expression systems including *E.coli* (Skerra and Pluckthun,

1988; Better *et al.*, 1988), yeast (Davis *et al.*, 1991), plants (Tavladoraki *et al.*, 1993) and mammalian cells (Dorai *et al.*, 1994).

The Fv (VH VL) [Figure 1.1] is the smallest immunoglobulin fragment containing the complete antibody binding site (Riechmann *et al.*, 1988; Skerra and Pluckthun, 1988). This fragment can be produced in a functional form in the periplasm of *E.coli* but has limited stability due to weak hydrophobic interactions between domains. Fv fragments may be stabilised by covalent linkage either by introduction of a disulphide bond or by linkage via a flexible peptide. Addition of cysteines for the disulphide linked Fv (dsFv) usually results in a dramatic decrease in protein expressed in the periplasm and most often requires the dsFv to be prepared by refolding (Glockshuber *et al.*, 1990; Brinkmann *et al.*, 1993). A further disadvantage of the dsFv is that it is difficult to predict the precise orientation of the VH and VL domains (which varies for different antibodies and can also change on binding to antigen), making it difficult to assign a single successful position for a disulphide bridge. Covalent linkage by addition of flexible peptide linker sequence to give an optimum spatial arrangement between the domains to form a single chain fragment (scFv) (Bird *et al.*, 1988; Huston *et al.*, 1988) [Figure 1.1] offers a means of overcoming such constraints and is the most frequently used format. Alternatively, stability may be significantly improved by inclusion of the first constant domain of the light and heavy chains (CL CH1) within the construct to form a Fab (Better *et al.*, 1988; Neuberger *et al.*, 1984) [Figure 1.1]. However, functional protein yeilds of Fab fragments are often much lower than those of which express well in the periplasm scFvs (Skerra and Pluckthun 1991) but have a tendency to form dimers and higher aggregates (Griffiths *et al.*, 1993; Holliger *et al.*, 1993; Arndt *et al.*, 1998). Clearly different formats have advantages and disadvantages and the format of choice, in the main, will be dependent on the desired end application of the antibody. For example, the smaller size of (monomeric) scFvs means that they are particularly suited for applications such as (tumour) tissue imaging [Table

1], targeted delivery of drugs, toxins or radionuclides due to their lower retention times in non-target tisses, faster blood clearence and better penetration of target tissues (Colcher *et al.*, 1998).

Table 1 Properties of antibody based products (Adams and Schier, 1999)

structure	molecular weight (kDa)	clearance	tumour penetration	tumour retention
IgG	155	+	+	+ + + +
$F(ab')_2$	100	+ +	+ +	+ + +
Diabody	50	+ + +	+ + +	+ +
scFv	25	+ + + +	+ + + +	+

As cloned sequences, antibody domains have the potential to be fused with other proteins. In this way novel molecules with desired effector functions that exploit antibody specificity have been engineered. Antibodies have been fused to proteins such as enzymes, plant and bacterial toxins and cytokines to develop novel therapeutic agents (reviewed Morrison, 1992). Also, fusion of antibody fragments with peptides has been used to produce homodimeric molecules to improve the avidity of binding (Pack and Pluckthun, 1992; Holliger *et al.*, 1993). Similarly heterodimeric molecules with dual specificity can be produced by linking together antibody fragments derived from antibodies binding to different antigens/epitopes (Kostelny *et al.*, 1992). There is of course the potential to produce whole recombinant human antibodies for recruitment of immune effector function mechanisms associated with different antibody isotypes and subclasses such as cell killing by ADCC and complement by the addition of IgG1 and IgA1 domains (Huls *et al.*, 1999a; Huls *et al.*, 1999b).

1.1.2 Phage display of antibody fragments

Filamentous bacteriophage fd are ssDNA phage that infect male *E.coli*. The viral genome is encased in a sheath composed of approximately 2800 copies of the major coat protein (gene VIII protein, gVIIIp). At one end of the virion particle are the minor coat proteins (gene III proteins, gIIIp) of which there are 3-5 copies. Gene III protein has 2 functions, the C-terminal domain is required for virion morphogenesis and the knob-like structures of N-terminal domains for viral infectivity (Crissman and Smith, 1984). Cloned heavy and light chains directed to the periplasm are able to fold correctly and assemble as functional antibody fragments (Better et al., 1988; Skerra and Pluckthun, 1988) and can also be displayed on the surface of bacteriophage fd. This was first demonstrated by McCafferty and co-workers who produced a scFv fragment as a gene III N-terminal fusion protein (McCafferty et al., 1990). Antibody fragments have also been produced fused to the major coat protein gVIIIp (reviewed by Marks et al., 1992). The key feature of this system is linkage of the surface displayed antibody fragment with the antibody genes that are encoded within the phage genome [Figure 1.2].

1.1.3 Selection of antibodies from phage display libraries

Repertoires of antibody fragments generated by the rescue of V-genes from heterogeneous cell populations have been proposed as an alternative to hybridoma technology for the production of antibodies (reviewed by Hoogenboom *et al.*, 1998). As the majority of original H and L chain pairings will be lost this method relies on desired antigen binding specificities arising from the random combination of heavy and light chains. Due to the many different combinations that can arise from a mixed population of B cells, isolation of antibodies from such repertoires requires the use of a powerful selection system. The ability to select phage

displaying antibody fragments from a mixed population according to their binding characteristics was first demonstrated by McCafferty and co-workers in 1990

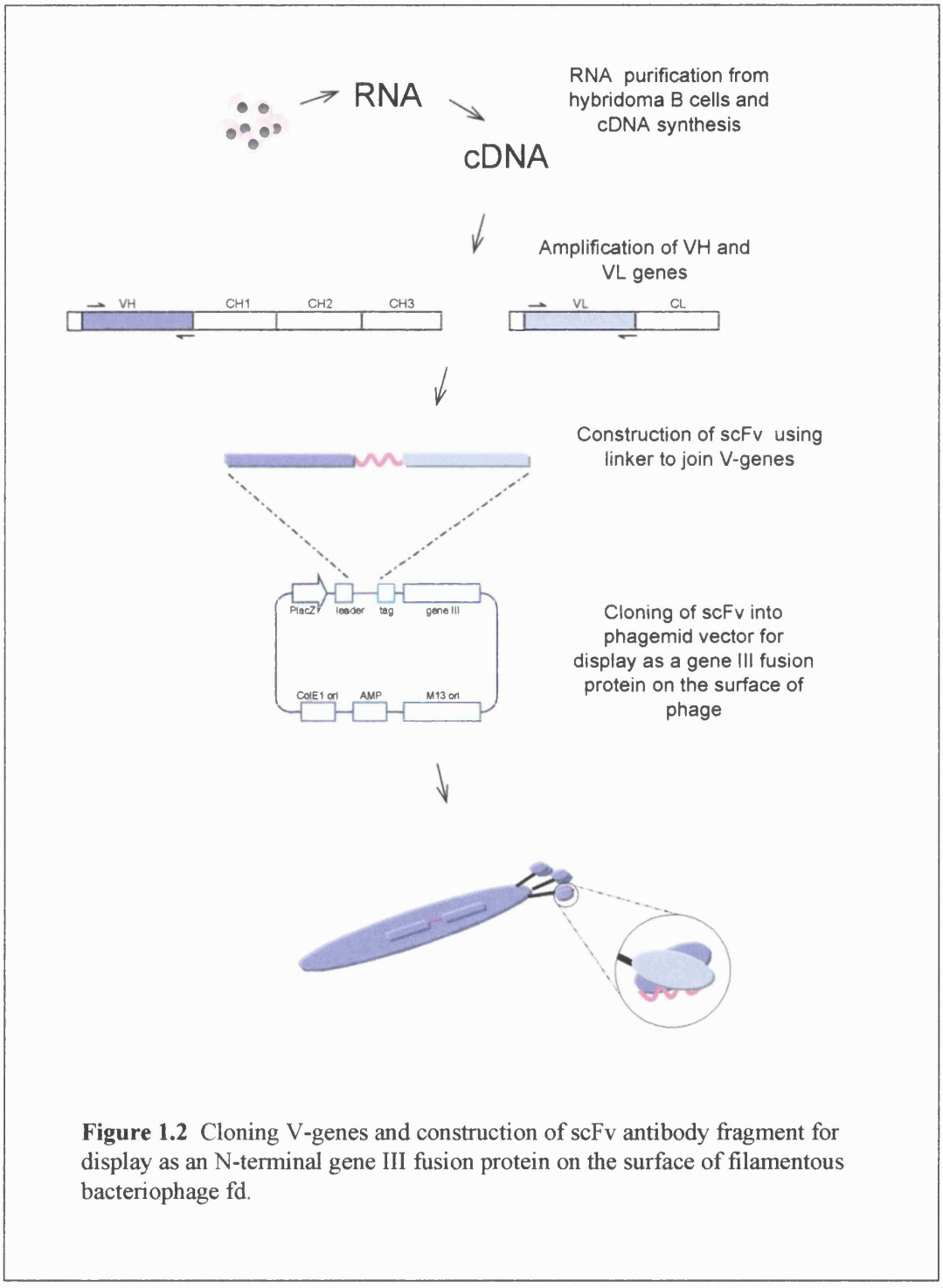


Figure 1.2 Cloning V-genes and construction of scFv antibody fragment for display as an N-terminal gene III fusion protein on the surface of filamentous bacteriophage fd.

(McCafferty *et al.*, 1990). Selection involves the sequential enrichment of specific binding phage from a large excess of non-binding phage [Figure 1.3]. This is achieved by incubation of a phage population with antigen with the removal of non binders by washing followed by elution of bound phage. These phage are then used to infect bacteria to recover specific binding phage that can be used for a further round of selection. In a single round of panning enrichment factors of 20 fold (Marks *et al.*, 1991) and up to 1000 fold (McCafferty *et al.*, 1990) have been observed, with several rounds of selection resulting in the successful isolation of rare binding ($1/10^7$) phage. The first selections of phage antibodies were performed by (bio) panning against immobilised antigen on affinity columns (McCafferty *et al.*, 1990; Clackson *et al.*, 1991) and antigen coated onto plastic (Marks *et al.*, 1991). Later selections have used immobilised antigen on BIAcore sensor chips (Malmborg *et al.*, 1996). With such selection strategies it is important to consider factors that could compromise the conformational integrity of the antigen. Adsorption of antigen onto plastic, for example, can lead to the isolation of antibodies that are unable to recognise the antigen in its native form. Conformational integrity of the antigen can be retained by indirect coating using an antigen specific antibody for capture (Sanna *et al* 1995) [which would necessitate incubation with non-specific Ig of the same species and isotype to prevent isolation of antibodies against the capturing antibody]. The panning method can also be adapted to direct selection towards specific epitopes by using monoclonal antibodies to block immunodominant epitopes (Ditzel *et al.*, 1995; Ping *et al.*, 1996). Alternatively, a competitor antigen highly homologous to the antigen of interest may be added to prevent the isolation of cross-reactive antibodies (Parsons *et al.*, 1996). However, all of these methods are limited in that it is difficult to quantitate the amount of antigen that is used for selection. A more powerful technique that allows selection on the basis of binding affinity, is to perform selections with soluble biotinylated antigen (Hawkins *et al.*, 1992). Phage can be incubated with chosen amounts of antigen and phage

bound to the antigen-biotin complex retrieved using streptavidin coated magnetic beads. The concentration of antigen used for selection may be decreased to favour enrichment of antibodies with higher affinities (Schier *et al.*, 1996). However, despite incorporation of depletion rounds this technique often results in the isolation of streptavidin binders, especially when naïve repertoires are used.

More difficult are those selections performed against impure antigens due to enrichment of phage against non-relevant antigens. Such antigens may be those that cannot be purified from contaminants or cell surface receptors that require incorporation in the cell membrane to retain their conformation/functionality. Selections can be performed against cells grown in culture (either monolayers or suspension) but require incorporation of extensive depletion and/or subtraction steps such as cell sorting by flow cytometry (de Kruif *et al.*, 1995; Kipriyanov *et al.*, 1997) to eliminate non-relevant binders. Sophisticated selections can be designed against antigens of greater complexity such as 'pathfinder selection' for the *in situ* isolation of novel antibodies (Osbourn *et al.*, 1998). Furthermore, *in vivo* selection where phage are injected directly into the blood of mice followed by retrieval of the target organ (thymus) has proved to be successful for the isolation of antibodies against the vascular endothelium (Johns *et al.*, 2000).

1.1.4 Antibody repertoires

The application and affinity of antibody that is required will determine which type of antibody repertoire is chosen. A single non-biased repertoire providing a universal source for the isolation of high affinity antibodies would be the ideal. At present two such 'single pot' repertoires the naïve and synthetic have been described. These repertories (potentially) contain specificities against any antigen however, affinities are often low. Alternatively, an

antigen biased repertoire constructed from immunised B cells can be used. The properties of the different types of repertoires are described.

1.1.4.1 Immune repertoires

An immune repertoire is one that has been derived from a population of B cells that have been enriched for a desired specificity by immunisation. This method relies on the immune response to generate high affinity antibodies. Antibody genes are encoded by a number of different gene segments. The light chain variable region is composed of 2 gene segments, the variable (V) and joining (J) and the heavy chain variable region of 3 gene segments V, diversity (D) and J. Complete variable regions genes are generated by somatic recombination of these gene segments (Tonegawa, 1983). Gene rearrangements occur in the bone marrow during differentiation of hematopoietic precursor stem cells. This random recombination of gene segments generates much of the diversity of antibody genes. Variation is further increased by imprecise joining between gene segments and then by pairing of the heavy and light chains (Berek and Milstein, 1988). These events lead to the production of B cells that display on their surface Ig of single antigen binding specificity. B cells leave the bone marrow and circulate in the periphery as the naïve B cell repertoire.

Antibodies are produced in two stages, the primary antibody response generated by binding of membrane bound antibody to antigen results in clonal expansion of cells from the naïve B-cell repertoire. Antigen binding stimulates the B cell (with T cell help) to proliferate and differentiate into short lived plasma cells that secrete large amounts of soluble antibody or long lived memory cells. Typically antibodies produced by the primary response are of low affinity (10^5-$10^7 M^{-1}$). Affinity maturation in the secondary response produces higher affinities (10^8-$10^9 M^{-1}$) by somatic hypermutation of V-region genes that are then selected for improved

binding specificity to antigen. For immune libraries V-genes are derived from the IgG mRNA of B cells from this secondary response. Immune repertoires have been in the main generated from immunised animals but have also been produced from antigen-biased human B cells (Burton *et al.*, 1991; Barbas and Burton, 1996). Antibodies against a vast array of antigens ranging from simple haptens to 'difficult' antigens such as specific major histocompatibility complex (MHC)/peptide complexes (Anderson *et al.*, 1996, Chames *et al.*, 2000) have been isolated from immune repertoires. Selection from immune repertoires can allow access to more and sometimes better antibodies than those obtained by the hybridoma technique (Chester and Hawkins, 1995). A major disadvantage associated with immune repertoires is that for every new antigen specificity required, a new phage antibody library has to be created. Also, use of these repertoires is limited in that they cannot be used to generate antibodies against self-antigens and toxic molecules. But in some instances, such as the isolation of naturally occurring high affinity human antibodies this route may still prove to be of use. For example, immune repertoires can provide access to antibodies produced after viral infection (Burton *et al.*, 1991) or those generated in patients with autoimmune disease (Graus *et al.*, 1997) or cancer (Cai *et al.*, 1995).

1.1.4.2 Naïve repertoires

Naïve or 'non-immune' repertoires have been constructed as a means of by passing hybridoma technology, the problems associated with immune repertoires and as a means of directly isolating human antibodies. The huge diversity contained within the naïve repertoire, estimated in humans to consist of between 10^{10}-10^{12} different B cells (Winter *et al.*, 1994), has been used as a source of V-genes for the creation of naïve libraries. Such libraries have been produced from the IgM mRNA of B cells of unimmunised human donors isolated from peripheral blood lymphocytes (PBLs), bone marrow and spleen cells (Marks *et al.*, 1991) and

also from animal sources (Gram *et al.*, 1992). The first naïve human library containing 3 x 10^7 clones resulted in the generation of antibodies to over 25 different antigens, including 'foreign' antigens such as turkey egg white lysosome, bovine serum albumin (Marks *et al.*, 1991) and 'self' antigens such as thyroglobulin, mucin and CD4 (Griffiths *et al.*, 1993). The average affinity of these antibodies was like those observed with the primary antibody response (reviewed by de Haard *et al.*, 1998). The recent production of much larger repertoires such as the naïve scFv library of 1.4 x 10^{10} clones, made from PBLs, tonsil B cells and bone marrow B cells from 43 non–immunised individuals, have allowed isolation of antibodies with higher affinities typical of those found in the secondary immune response (Vaughan *et al.*, 1996). So, it appears that neither immunisation or affinity maturation are needed for the generation of human antibodies when naïve repertoires are used. Major advantages of using such 'single pot' repertoires are that: a single library may be used for all antigens; human antibodies can be directly isolated against antigens including self as well as those that are non-immunogenic and toxic; antibodies can be isolated relatively quickly and high affinities isolated when large repertoires are used. Associated disadvantages are the lower affinities that are isolated when smaller repertoires are used and the time and technical expertise required to create repertoires. In addition, the unknown and uncontrollable contents of repertoires such as positive and negative selection, of the immune system which can limit the diversity present in the IgM repertoire as well as the unknown immune history of the B-cell donor will influence the content and quality of the library.

1.1.4.3 Synthetic repertoires

To overcome limitations of naïve repertoires construction of synthetic repertoires, in which contents, local variability and overall diversity are controlled and defined by the *in vitro* assembly of V-gene segments from V-gene building blocks, was devised. The CDR3 loop of

the heavy chain, which is central in forming the antigen binding site, has high variability and as such is the most diverse (CDR) loop in composition and in length [2-25 residues] (Chothia *et al.*, 1989). Therefore, this region has been targeted for the introduction of diversity, using oligonucleotide primers encoding a stretch of randomised amino acids, into sets of cloned germline VH genes (Hoogenboom and Winter, 1992) or rearranged V-genes (Barbas *et al.*, 1992). Several synthetic repertoires have been constructed that have yielded antibodies against many different antigens with micromolar and nanomolar affinities (reviewed by Hoogenboom *et al.*, 1998).

1.1.5 Improving affinities

The affinities of cloned antibodies can be improved by mimicking the natural somatic hypermutation and selection process of affinity maturation. This technique is based on the introduction of mutations into the V-genes followed by selection of higher affinity variants. Diversity may be introduced either randomly or directed to defined residues or regions. Random methods include the use of mutator strain E.coli (Low *et al.*, 1996; Irving *et al.*, 1996); error prone PCR (Hawkins *et al.*, 1992) and chain shuffling (Clackson *et al.*, 1991; Marks *et al.*, 1992). Directed approaches using codon based mutagenesis have targeted mutations to CDR loops and also to framework regions to produce improvements in binding affinity as well as stability and expression (Schier and Marks, 1996; Baca *et al.*, 1997; Kipriyanov *et al.*, 1997; Forsberg *et al.*, 1997).

1.1.6 Creating and engineering antibodies for clinical applications

Selection of antibodies from phage display libraries coupled with the ability to further engineer the antibody's characteristics offers a powerful alternative to hybridoma technology for the generation of antibodies. Desired binding specificities can be isolated within a

relatively short period of time, four rounds of selection may be performed in a week and clones screened for specific binding specificity in the second, allowing access to specific binders from more that 10^9 clones in a few weeks. Once isolated it is possible to tailor properties of the antibody such as affinity, format (size and valency) and effector function that are suitable for the desired application. Furthermore, the rapid growth rates of the bacterial host means that soluble antibody can be easily purified from cultures after induction for 4 hours. Success in generating these molecules is demonstrated by recombinant antibodies and their fragments currently representing more than 30% of all biological proteins undergoing clinical trials (Hudson, 1998).

The small size of antibody fragments is a characteristic that is particularly desirable for *in vivo* tumour imaging . Antibody fragments have proven to yield more rapid tumour penetration, faster plasma clearance [Table 1] and reduced immunogenicity compared to whole antibody (Adams et al., 1998a; Adams and Shier, 1999). Imaging with antibody fragments has been further improved by increasing functional affinity (avidity) using multivalent antibody fragments (Adams et al., 1993; Wu et al., 1996; Adams et al., 1998b).

The favourable characteristics possessed by antibody fragments for imaging as well as the potential to tailor antibody molecules for desired properties meant that antibody engineering technology was chosen for the generation of antibodies for the *in-vivo* diagnosis of inflammatory and malignant disease.

.

1.2 Inflammation

1.2.1 Neutrophils and inflammation

Inflammation is the local reaction of vascularised tissue to injury that is characterised by redness, heat, swelling and pain. These symptoms are brought about by dilation and increased permeability of blood vessels that result in an increase in the local blood flow and leakage that leads to accumulation of fluid, plasma protein and blood cells. The swelling causes tissue tension which results in pain.

Neutrophils are characteristic cells of acute inflammation and infection. These cells can be mobilised rapidly to reach such sites quickly and in large numbers. In the adult, these cells originate from a myeloid stem cell precursor that eventually becomes committed to develop into neutrophils and mononuclear phagocytes. The mature neutrophil or granulocyte is also known as a polymorphonuclear leucocyte (PMN) due to the lobed appearance of the nucleus. It is characterised by cytoplasmic granules and cell surface receptors that are involved in chemotaxis and phagocytosis. A reserve of neutrophils that can be rapidly recruited exists in the bone marrow, the number of which exceeds the total blood neutrophil population. Neutrophils have a short circulation time, possibly only a few hours, many migrate to the gut where they leave the circulation. Due to the inability of these cells to replicate and their limited ability for protein synthesis they have a rapid turnover with 10^{11} cells formed and destroyed each day. Despite this high turnover, neutrophil blood levels are maintained at a constant level in normal individuals, however this can change rapidly in acute inflammation which produces neutrophil leucocytosis (Topley and Wilson, 1973).

Chemotaxis involves the movement of cells along a chemical gradient resulting in the unidirectional locomotion of cells. The movement of neutrophils through tissues towards sites

of inflammation and infection is directed by chemotactic factors such as C5a generated by complement activation (complement binds to the surface of micro-organisms or damaged cells), formylmethionyl peptides (peptides with the *E.coli* start sequence for protein synthesis) and IL-8. These molecules have multiple affects on neutrophil function, low concentrations as encountered on first entering the lesion have mainly a chemotactic action. Higher concentrations such as at the centre of lesions cause stimulation of granule release and a metabolic burst, as well as increased adhesiveness (Topley and Wilson, 1973).

Once at the site of inflammation the neutrophils perform their primary function, which is to destroy antigens and pathogens. Neutrophils are able (as are macrophages) to phagocytose antigens for intracellular destruction in their phagolysosomes. Recognition of target antigens is mediated by binding of antibody to the Fc receptors FcγRII (CD32) and FcγRIII (CD16) or by binding of activated complement components to the CR1 and CR3 receptors.

1.2.2 Monoclonal antibodies as agents for selective radiolabelling of neutrophils

In-vivo labelling using anti-neutrophil monoclonal antibodies has been investigated in an attempt to simplify the current procedure of *in-vitro* labelling of neutrophils. Early studies using ^{99m}Tc-labelled monoclonal antibodies showed that it was possible to use these reagents for selective *in-vivo* radiolabelling of circulating neutrophils (Locher *et al.*, 1986; Seybold *et al.*, 1988). It was demonstrated that these agents could be used for scintographic imaging of inflammation (Becker *et al.*, 1989; Thakur *et al.*, 1988; Thakur, 1990; Becker *et al.*, 1992; Thakur *et al.*, 1996) as labelling of neutrophils does not prevent chemotactic migration to inflammatory or infectious lesions (Thakur *et al.*, 1990).

1.2.3 NCA-95 is a neutrophil specific marker

The non specific cross reactive antigen 95 (NCA-95), also known as CD66b in the CD nomenclature , has a molecular weight of approximately 95 kDa (Buchegger *et al.*, 1984; Arakawa *et al.*, 1990; Kuroki *et al.*, 1990) and is the product of carcinoembryonic antigen (CEA) gene family member 6 (CGM6). NCA-95 belongs to the CEA subgroup of the CEA gene family of molecules. All expressed members of the CEA gene family are characterised by their highly homologous domain structures. The N-terminal domain consists of 108 amino acids and is homologous to the Ig variable (IgV) domain, is followed by up to six domains homologous to the Ig constant (IgC) domain (Williams and Barclay, 1988). There are two IgC like domains, which comprise of 93 and 85 amino acids, known as Type A and Type B domains respectively (Hammarstrom *et al.*, 1998).

CEA and NCA are also members of the CEA subgroup of the CEA gene family and as such share a high degree of sequence identity with NCA-95. For example the IgV-like N domain of NCA-95 is 85% and 70% identical to NCA and CEA respectively (Neumaier *et al.*, 1988; Arakawa *et al.*, 1990; Berling *et al.*, 1990). CEA is the largest of the CEA family members with a molecular weight of approximately 180 kDa. It has seven Ig-like domains in its structure (N-A1-B1-A2-B2-A3-B3) and is attached to the cell membrane via a glycophosphatidylinositol (Gpi) anchor (Hammarstrom *et al.*, 1998).

NCA and NCA-95 have an identical domain organisation containing three Ig-like domains (N-A1-B1), (Berling *et al.*, 1990; Arakawa *et al.*, 1990) and also have Gpi linked membrane attachment (Kuroki *et al.*, 1990; Neumaier *et al.*, 1988) [Figure 1.4]. Like all members of this subgroup NCA-95 is highly glycosylated, NCA-95 has 11 potential N-linked glycosylation

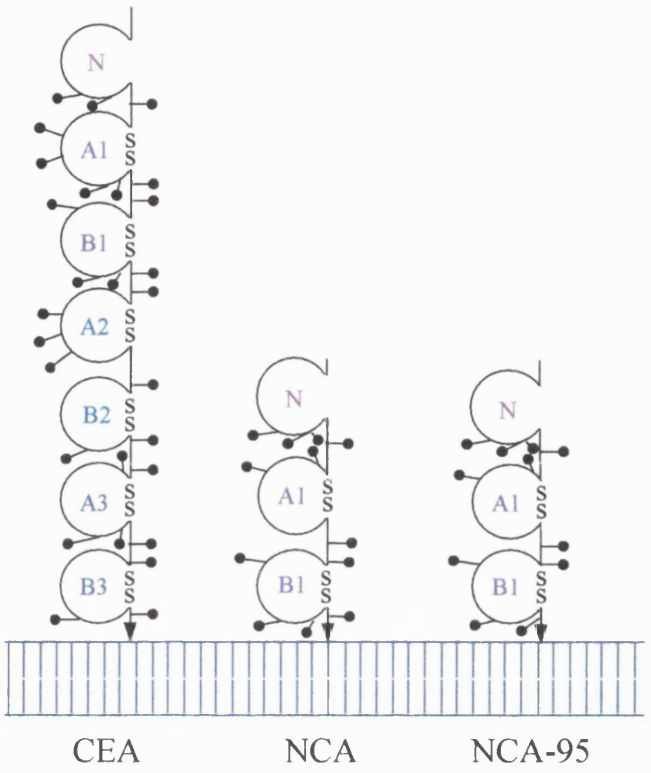


Figure 1.4 Diagrammatic representation of CEA, NCA and NCA-95 based on their cDNA sequence. The circles show segments that form IgV-like (N) domains and IgC-like (A and B) domains. The Gpi-linkage to the cell membrane is shown as ▼. Potential sites for N-linked glycosylation (Asn-X-Thr/Ser) are shown as ♦ (adapted from Hammarström et al 1998).

sites, with NCA having an additional site. Eight of these sites are conserved in CEA (Neumaier *et al.*, 1988). NCA-95 is expressed on the surface of human granulocytes (Buchegger *et al.*, 1984) and is also present on granulocyte bone marrow precursor cells (Wahren *et al.*, 1980; Berling *et al.*, 1990).

1.3 Cancer

1.3.1 Colorectal cancer

1.3.1.1 Incidence and development

Cancer of the colon and rectum is the second commonest cause of cancer related death in the western world and there are more than 30 000 new cases per year in the UK with an average five year survival of 40% (Rhodes, 2000). The incidence of this disease increases from the age of 50 onwards, with an earlier onset observed in high risk cases such as those with an inherited susceptibility or with a long history of inflammatory bowel disease (Lynch *et al.*, 1991).

Dietary and environmental factors can have an influence on the development of colorectal cancer as can both inherited and somatic mutations (Fearon *et al.*, 1994). A diet with a high saturated fat intake is known to be associated with an increased risk that also correlates with red meat intake (Willett, 1989) and a decrease associated with increased dietary fibre (Cummings *et al.*, 1992). The effect of environment and diet on disease incidence has been shown to affect risk in first generation immigrants, resulting from exposure to the typical American diet, that is high in cholesterol and fat and low in fibre (Armstrong and Doll, 1975).

The development of colorectal cancer is generally believed to be a multistage process that involves the transition from normal epithelium sequentially through a hyperproliferative state followed by adenomatous changes with increasing dysplasia and to malignancy with eventual metastisis and death. There is a strong association between adenomas of the colon and development of malignant disease, with removal of adenomatous polyps significantly reducing the incidence of subsequent cancer development (Gilbertsen, 1974; Winawer and Shike, 1992; Murakami *et al.*, 1990). Patients with adenomatous polyposis coli (APC) who develop

hundreds to thousands of adenomatous polyps have their entire colon removed due to the inevitable development of cancer. The changes that are involved in the development of colorectal cancers from pre-existing adenomas are thought to occur over a period of 5-15 years (Morson, 1971; Muto *et al.*, 1975).

Hyperproliferation of the colonic epithelium and failure of cells to enter the normal pathway of differentiation results in the accumulation of immature proliferating cells to form a polyp (Boland, 1993). Polyps can be classified histopathologically into tubular and villous adenomas, as well as a form that is a mixture of the two known as tubulovillous adenomas. The most common are the tubular adenomas which are generally smaller, better differentiated with less cellular atypia than tubulovillous or villous adenomas (Sleisenger and Toribara, 1994). The development of malignancy is associated with increasing size, villous growth pattern and more severe degrees of dysplasia (Dixon *et al.*, 1993).

1.3.1.2 Treatment for colorectal cancer

Colorectal cancer can be classified histologically according to the degree of differentiation ie; confined to the bowel wall, invasion into the bowel wall, lymph node involvement and distant metastasis, known as Dukes A, B, C (Dukes, 1932) and Dukes D (Turnball *et al.*, 1967) respectively. The type of treatment and its success is associated with the stage of cancer, early Dukes stages (A and B) being curable with resection. However, this disease usually presents itself at a late stage when metastasis are present and prognosis is less favourable (current overall 5 year survival at 37%) due to subsequent development of minimal residual disease. So despite surgical advances, adjuvant therapy and screening programs, there has been no appreciable decrease in mortality rate in the last 40 years.

1.3.2 Monoclonal antibodies and cancer

Since the advent of hybridoma technology considerable focus has been placed on the production of anti-tumour antibodies to develop reagents for the clinical management of maligancy. Conjugation with radioactive isotopes, cytotoxic drugs or toxins allows monoclonal antibodies to be used as targeting agents for the delivery of therapeutic molecules. Also activation of effector functions of the native antibody may kill tumour cells by mechanisms such as ADCC and complement mediated lysis (Mellstedt *et al.*, 1991).

The production of cancer specific antibodies requires the identification of markers expressed on malignant cells. The ideal marker should be one that is densely expressed exclusively on tumour cells and is uniformly distributed amongst all tumour cells. However, such tumour specific antigens are rare and from the many cancer binding antibodies that have been produced, it is apparent that truly distinct cancer antigens are not required. The tumour associated antigens CEA (Gold and Freedman, 1965), TAG-72 (Colcher *et al.*, 1981), 17.1A (Herlyn *et al.*, 1979) and mucin (Girling *et al.*, 1989; Gendler *et al.*, 1991; Price *et al.*, 1993) are examples of markers that have a quantitative and/or qualitative difference in expression between normal and tumour cells.

1.3.2.1 Diagnostic and therapeutic applications of monoclonal antibodies in colorectal cancer

The technique of radioimmunodetection (RAID) [also referred to as radioimmunoscintigraphy (RIS)] has been investigated in order to overcome the limitations of traditional colorectal imaging techniques for *in vivo* detection and characterisation of disease foci (Goldenberg *et al.*, 1978). Radiolabelled antibodies have been extensively tested in colorectal cancer for their ability to specifically target primary, recurrent and metastatic disease. In the majority of

studies performed with CEA antibodies and patients with recurrent or metastatic disease, a detection rate of over 80% of known tumour sites has been reported. The use of RAID in the diagnosis of primary colorectal cancer has been successful in confirming known sites of cancer as well as revealing occult lesions (Goldenberg and Larson, 1992). However it is difficult to determine the overall usefulness of RAID due to the difficulty in comparing the results obtained between groups using different methods, antibodies, labels and imaging procedures (Goldenberg and Griffiths, 1992; Stocchi and Nelson, 1998). Radiolabelled antibodies targeted to colorectal cancers have also been used for the accurate detection of tumour extension in radioimmunoguided surgery (RIGS) to aid the surgeon in accomplishing radical resection (reviewed by Martin and Thurston, 1998).

Tumour associated antigens that are released into the circulation have been of use as indicators of malignancy. Serum CEA is commonly used to assess the progress of patients following surgical treatment for colorectal cancer (Minton *et al.*, 1985) and has also been shown to be of use in the detection of residual or recurrent disease (Cooper *et al.*, 1979; Goldenberg, 1979).

1.3.2.2 Immunotherapy for colorectal cancer

Passive immunotherapy for the treatment of colorectal cancer in clinical trials has overall been disappointing with clinical response rates in most cases limited to 5-10%, and there is no evidence to date that radioimmunotherapy of colorectal cancer patients is beneficial (Stocchi and Nelson, 1998). The use of native antibody has had more success in inducing remission in patients with lymphoma or leukemia (Scheinberg, 1991) than in those with solid tumours (Riethmuller and Johnson, 1992). These poor results have been attributed in part to inaccessibility of antibodies to cancer cells growing in solid tumours (Hagan *et al.*, 1986; Jain, 1996).

A more realistic application for immunotherapy may be in the targeting of micrometastases when cancer cells are few and dispersed as individual cells or small clusters (Riethmuller *et al.*, 1993; Riethmuller *et al.*, 1998). Micrometastases have been shown to be present at the time of surgery and their detection in bone marrow of colon cancer patients (Schlimok *et al.*, 1987) is associated with poor prognosis (Lindemann *et al.*, 1992). The prevention of metastases with adjuvant therapy has been shown to be of benefit (Forman, 1994) using chemotherapeutic agents such as 5-fluorouracil (Moertel *et al.*, 1990). Adjuvant immunotherapy may be advantageous over conventional adjuvant therapies in its potential to target micrometastases specifically as well as having a mechanism of action which is independent of the cell cycle with limited toxicity. Of the many clinical trials conducted using immunotherapy the most promising results to date have been those obtained by Riethmuller and colleagues (Riethmuller *et al.*, 1994). They used the murine IgG2a monoclonal B72.3 against epithelial cell surface glycoprotein 17.1A in a randomised trial with 189 patients with Dukes C carcinoma. After a median follow up of 5 years a 30% reduction in the overall death rate and a 27% decrease in the recurrence rate was observed. The results were similar to those obtained with radiochemotherapy but with lower toxicity. These observations support the theory that the use of antibodies is most effective against individual cells rather than cells growing in solid tumours.

1.3.2.3 Antibody therapy and immunogenicity

The majority of antibodies that have been used in patient therapy have been murine monoclonals. However their use is hampered due to their immunogenicity and the production of human anti-mouse antibody (HAMA) responses. Typically up to 50% of patients will produce a HAMA response on first administration of antibody, a figure which increases to more than 90% with subsequent administrations (reviewed by Hand *et al.*, 1994). HAMA

affects the efficiency of binding of readministered antibody due to rapid clearance from the circulation. Additionally adverse reactions associated with murine antibody treatment, such as nausea, vomiting and diarrhoea, have been reported in 3-6 % of patients (Stocchi and Nelson, 1998).

Conversely, HAMA may have an advantageous effect, in terms of anti-cancer activity, through the generation of antibodies against antigenic determinants within the antibody variable regions (idiotype). Some of these anti-idiotypic antibodies may bear an internal image of the tumour antigen, which may continue to be an immune stimulus after clearance of the original antibody (Jerne, 1974). Studies that have reported a more successful clinical outcome in patients that show an anti-idiotypic response (Verrill *et al.*, 1986; Koprowski *et al.*, 1984; Fagerberg *et al.*, 1995) provide evidence for the potential benefit of HAMA suggesting that antibody therapy may also be considered as active specific immunotherapy.

1.3.3 Mucins as markers for malignant disease

1.3.3.1 Aberrant and incomplete glycosylation of mucin is a cancer associated phenomenon

Mucins are large (MW >200kd) glycoproteins that can contain 50-90% carbohydrate. The multiple complex branched oligosaccharide side chains are linked via O-glycosidic bonds to serine and threonine amino acid residues of the polypeptide core (Patton *et al.*, 1995; Hanisch *et al.*, 1996).

The gene encoding MUCIN 1 (MUC1) was the first of the epithelial mucin genes to be cloned (Gendler *et al.*, 1987; Siddiqui *et al.*, 1988). At present 9 different epithelial mucin genes (MUC1-4, 5AC, 5B and 6-8) have been identified (Kim and Gum, 1995) but MUC1 is the only one that has a cell surface transmembrane region (Gendler *et al.*, 1990). The polypeptide

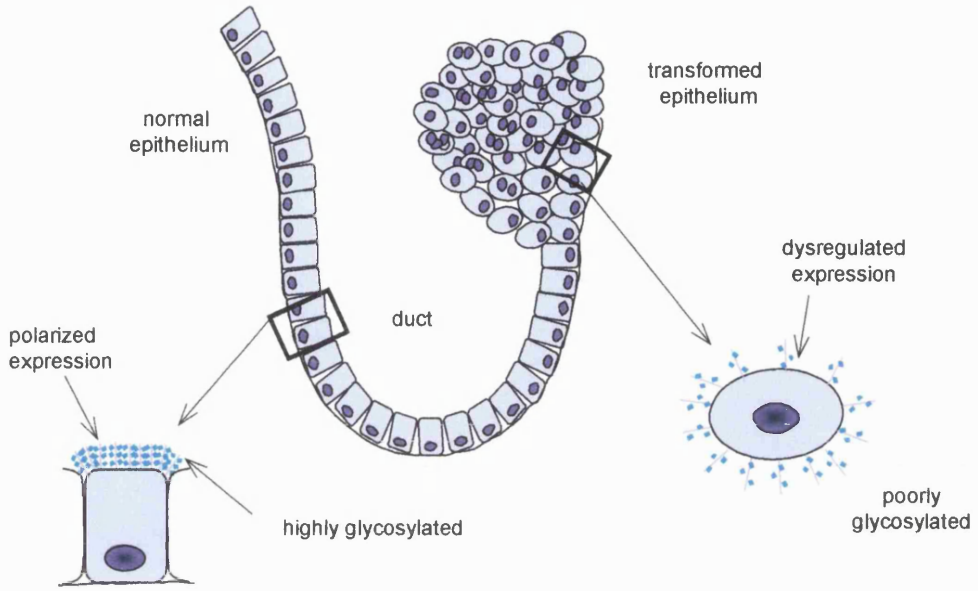


Figure 1.5 Schematic representation of expression of human mucin MUC1 on normal and transformed epithelium. (adapted from Finn et al 1995)

core of MUC1 comprises of 30-100 repeats of the 20 amino acid sequence PDTRPAPGSTAPPAHGVTSA (Gendler *et al.*, 1988). MUC1 mucin is expressed by a variety of normal tissues such as breast, ovary, lung, colon and pancreas by specialised glandular epithelial cells (Devine and McKenzie, 1992) and lactating mammary gland (Taylor Papadimitriou *et al.*, 1986). In malignant cells expression of MUC1 is elevated (Girling *et al.*, 1989), is no longer polarised to the apical cell surface, and is also secreted into the serum (Gendler *et al.*, 1991; Price *et al.*, 1993) [Figure 1.5]. In addition, the mucins produced by normal and malignant tissues differ in their carbohydrate composition (Feizi, 1985). Cancer associated mucin is characterised by incomplete and aberrant glycosylation which results in

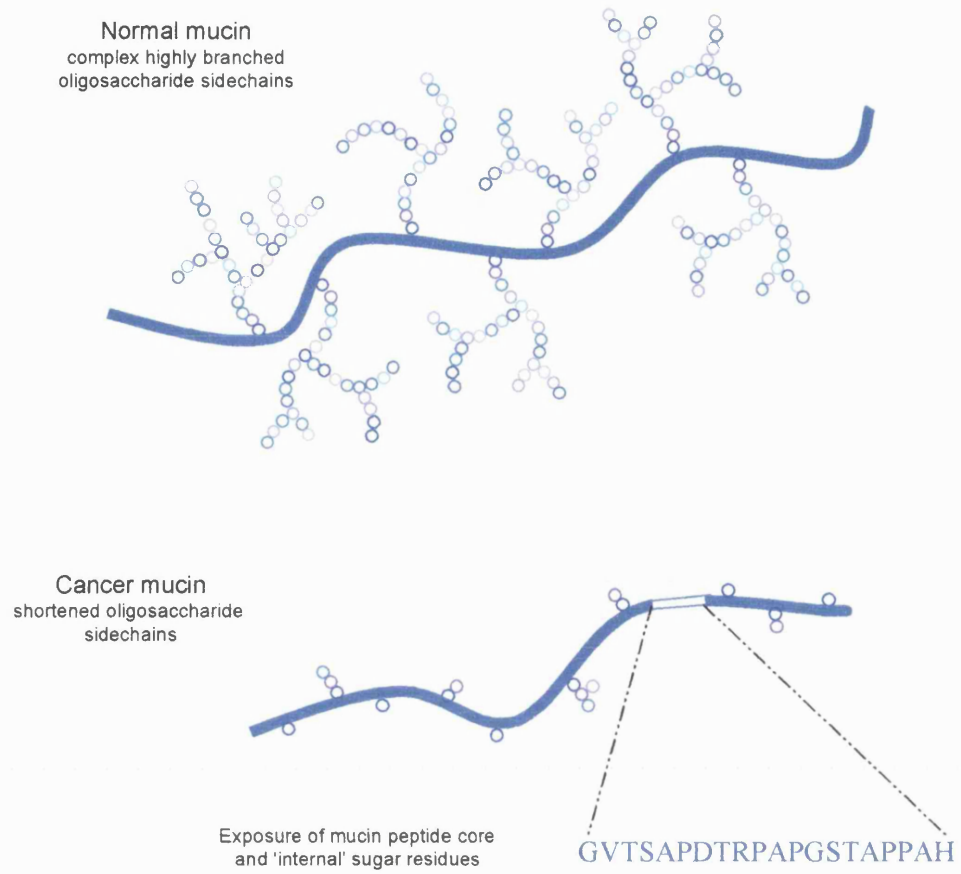


Figure 1.6 Differences in the structure of MUC1 mucin expressed by normal and tumour cells. Normal MUC1 has more complex O-linked oligosaccharide side chains than cancer mucin which has simpler and fewer side chains. The reduction in glycosylation leads to exposure of the tandem repeat of the polypeptide core (adapted from Agrawal et al 1998).

fewer and shorter oligosaccharide side chains linked to the polypeptide core (Boland *et al.*, 1982a). This reduction in glycosylation leads to the exposure of cryptic antigens such as internal sugar units (Kjeldsen *et al.*, 1988) and naked peptide sequence (Burchell *et al.*, 1989) that are normally masked by the elongated and highly branched carbohydrate side chains on mucin [Figure 1.6].

The changes in mucin expression produce differences between normal and malignant tissues that are immunologically distinct (Gold and Miller, 1978). The majority of antibodies that have been used to define such tumour associated changes have been generated against MUC1 mucin. The specificity of these different antibodies has been shown to be directed mainly against the immunodominant MUC1 mucin protein core. With the hydrophilic sequence PDTRPAP (Girling *et al.*, 1989) of the 20 amino acid tandem contributing in full or part to each epitope (Price *et al.*, 1998), some antibodies also show the involvement of carbohydrate residues (Liu *et al.*, 1995; Hanisch *et al.*, 1995; Spencer *et al.*, 1996). Other antibodies have specificities that are directed solely against carbohydrate epitopes (Galanina *et al.*, 1998).

Other mucin genes appear to have distinct patterns of expression within mucin producing tissues. MUC2 is expressed in normal human intestine and colon, and in colonic tumours, as is MUC3 but with its own distinct pattern (Chang *et al.*, 1994). MUC2 expression in colorectal adenomas and carcinomas of different histological types has also been shown (Blank *et al.*, 1994). An aberrant expression of MUC5AC has been observed in rectosigmoid villous adenomas which account for 10% of all colorectal tumours (Buisine *et al.*, 1996). However the expression of mucin -associated carbohydrate antigens is independent of the individual mucin gene or group of mucin genes that is expressed (Itzkowitz *et al.*, 1992).

1.3.3.2 STn is a tumour associated antigen

1.3.3.2 (i) The simple mucin-type carbohydrate antigens Tn, T and STn

The initial step in the synthesis of mucin O-linked oligosaccharides is the addition of N-acetylgalactosamine (GalNAc) to Ser or Thr to form the Tn antigen (GalNAcα1-O-Ser/Thr) (Strous and Dekker, 1992). The other simple mucin-type carbohydrate antigens are synthesised from this Tn antigen [Figure 1.7]. Addition of galactose to Tn gives rise to the T antigen (Galβ1-3GalNacα1-O-Ser/Thr) whereas if alpha 2-6 sialylation of Tn occurs the STn

antigen (NeuAcα2-6GalNAcα1-O-Ser/Thr) is formed. These antigens form the core region structures of the oligosaccharide side chains of mucin. The backbone and peripheral carbohydrate domains of the oligosaccharide side chains (Feizi *et al.*, 1984) are built by further glycosylation of these core region structures, with the exception of STn which acts as an inhibitor of further chain elongation (Kurosaka *et al.*, 1983; Itzkowitz *et al.*, 1989).

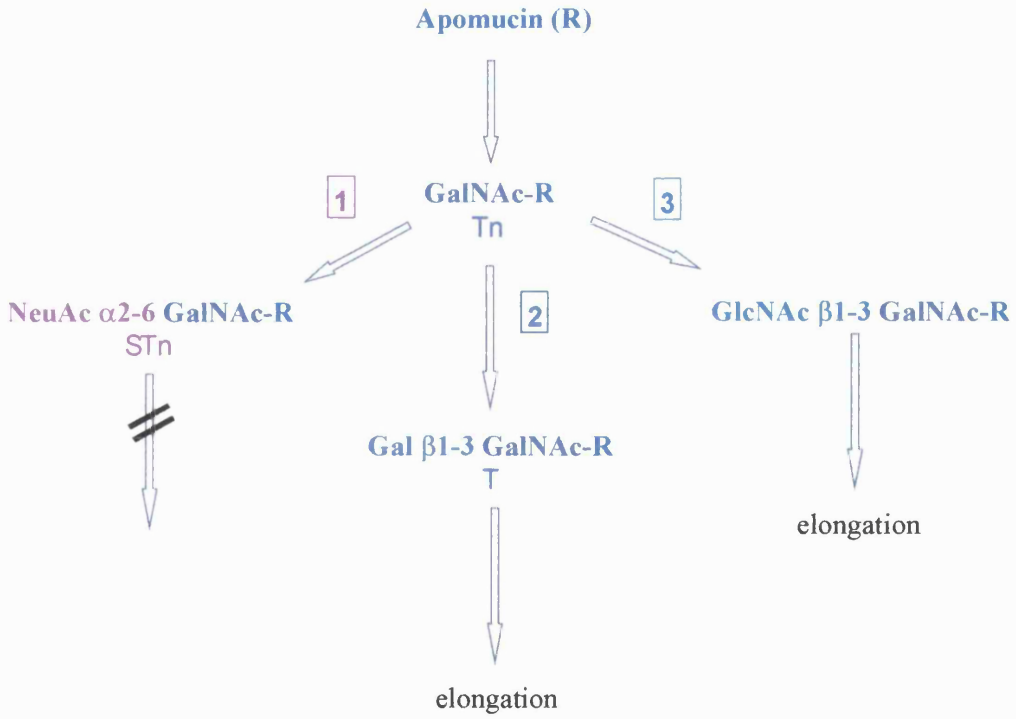


Figure 1.7 Simplified scheme of the biosynthesis of O-linked oligosaccharide side chains of mucin. The three most well characterised pathways are indicated after the formation of Tn by polypetidyl GalNac transferase. Pathway 1 is catalysed by α2-6 sialyltransferase, pathway 2 by β1-3 galactosyltransferase and pathway 3 by β1-3 N-acetylglucoaminyltrasferase. Synthesis along pathways 2 and 3 may proceed to further oligosaccaride elongation wheras pathway 1 is a terminal step (adapted from Itzkowitz et al 1989).

During oncogenic transformation a combination of blocked synthesis and a reduction in the O-acetylation of STn (Ogata *et al.*, 1995) results in the incomplete synthesis of the carbohydrate side chains. This leads to the exposure of novel carbohydrate epitopes such as STn (Hakomori, 1985) [Figure 1.8].

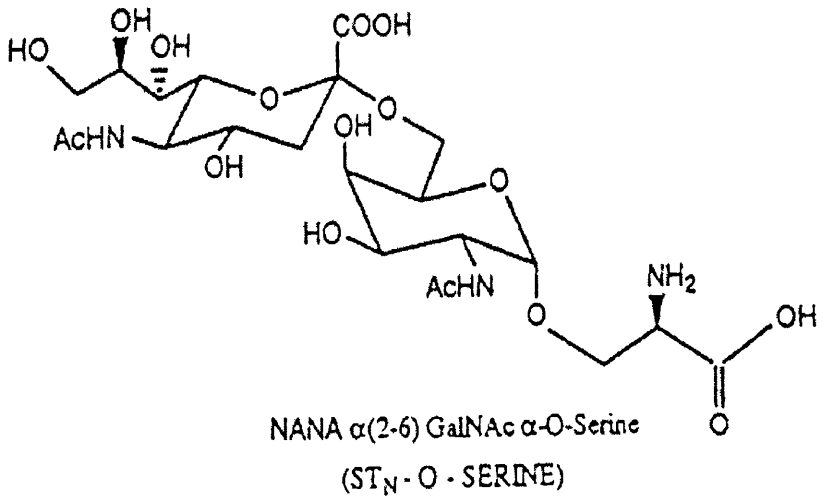


Figure 1.8 The carbohydrate antigen STn -O-serine (NeuAc α2-6 GAlNAc α1-0-serine)

1.3.3.2 (ii) Expression of STn in malignancy

The presence of the simple mucin-type antigens on tumours has been studied using a variety of monoclonal antibodies and lectins. Changes in Tn and T expression have a strong association with cancer (Springer *et al.*, 1975) and expression of STn is one of the most specific of carbohydrate changes observed between a wide range of normal and tumour tissues (Nakada *et al.*, 1994).

STn has been detected at low levels in limited areas of normal bronchus, uterus, salivary gland, palatine tonsil, testis, stomach, duodenum, capillary endothelium, small intestine, appendix, colorectum, gall bladder, urinary bladder, skin, and eosophagus (Yonezawa *et al.*, 1992). Goblet cell mucin from fetal colonic epithelium also shows expression of STn (Itzkowitz *et al.*, 1989).

The mouse monoclonal antibody B72.3 (Colcher *et al.*, 1981) binds specifically to tumour tissues which have the ability to produce mucin including; lung, stomach, colon, pancreas, gall bladder, ovary and breast (Nuti *et al.*, 1982; Thor *et al.*, 1986). Epitope analysis has revealed that B72.3 is directed against STn (Kjeldsen *et al.*, 1988; O'Boyle *et al.*, 1996). Comparative studies with the anti-STn monoclonal antibody TKH2 (Kjeldsen *et al.*, 1988) show similar binding between the 2 antibodies (Yonezawa *et al.*, 1992). Such binding has also been shown by others with anti-STn monoclonal antibodies (Devine *et al.*, 1994; Siddiki *et al.*, 1993).

STn expression has also been shown in salivary gland carcinomas (Therkildsen *et al.*, 1993), gastric carcinomas (Boland *et al.*, 1982b; David *et al.*, 1992) and lymph node metastases as well as normal looking mucosa adjacent to gastric carcinomas (David *et al.*, 1992).

1.3.3.2 (iii) A sensitive and specific marker for colorectal cancer

Colon cancer shows a nearly ubiquitous expression of STn (Orntoft *et al.*, 1990; Jass *et al.*, 1994; Jass *et al.*, 1995; Itzkowitz *et al.*, 1989; Itzkowitz *et al.*, 1990a; David *et al.*, 1992; Yonezawa *et al.*, 1992; Ohshio *et al.*, 1994; Giuffre *et al.*, 1996; Nakada *et al.*, 1994), Itzkowitz and coworkers have shown that this antigen is expressed by up to 96% of colon cancers (Itzkowitz *et al.*, 1989). STn expression is observed in the majority of poorly

differentiated adenocarcinomas and mucinous cancers (Itzkowitz *et al.*, 1989) as well as tumours with different Dukes staging (Itzkowitz *et al.*, 1990b; Yonezawa *et al.*, 1992).

1.3.3.2 (iv) Detection during the early stages of malignant transformation

Altered glycosylation is apparent even during the early stages of malignant transformation as shown by the detection of STn in transitional mucosa, that is tissues with colonocytes adjacent to histologically malignant tissues (Itzkowitz *et al.*, 1989; MacLean and Longenecker, 1991; Yonezawa *et al.*, 1992). STn has also been shown to be specifically expressed by adenomatous colorectal polyps (Itzkowitz *et al.*, 1992).

1.3.3.2 (v) STn is a useful prognostic indicator

Tissue expression of STn by colorectal tumours has a strong association with poor prognosis, with survival at 5 years for STn negative tumours being 100% compared to 73% for STn positive tumours. The expression of STn is independent of factors such as; age, gender, tumour location, Dukes staging, depth of invasion and ploidy status (Itzkowitz *et al.*, 1990a).

Similarly STn expression has also been shown to be an independent predictor of outcome of patients with gastric cancer (Victorzon *et al.*, 1996) with expression correlating with an adverse outcome (Maeda *et al.*, 1994; Werther *et al.*, 1994; Terashima *et al.*, 1998).

The detection of STn on circulating mucins in patient sera has also been demonstrated to be of prognostic value. Serum STn in ovarian cancer patients is a predictor of poor prognosis that is independent of tumour grade, stage or histological subtype. Survival at 5 years is approximately 85% in STn serum negative cancer patients compared to 10% in STn positive patients (Kobayashi *et al.*, 1992). Serum STn has also been of use as a prognostic indicator in gynaecologic cancers (Ryuko *et al.*, 1992) and gastric cancer (Takahashi *et al.*, 1994).

AIM

To apply antibody engineering techniques to isolate antibody fragments with potential for clinical use for *in-vivo* imaging of inflammatory and malignant disease.

1. To generate antibody fragments for imaging sites of inflammation by:
   (i) Rescue of V-genes from a mouse monoclonal antibody directed against the neutrophil specific marker NCA-95.
   (ii) Selection of NCA-95 antigen binding specificities from an immune antibody repertoire.

2. Selection from immune and naïve antibody repertoires for specificities directed against the known tumour-associated carbohydrate antigen STn.

3. Rescue of V-genes for construction of a scFv antibody fragment from an undefined carcinoma selective, human monoclonal antibody.

Chapter 2

Materials and Methods

2.1 Reagents and buffers

Reagents and buffers were purchased from BDH Chemicals Ltd, Poole and Sigma Chemicals, Poole unless otherwise stated.

2.1.1 Growth media for E.coli

LB

10 g bacto-tryptone (Difco Laboratories Ltd, Surrey), 5 g bacto-yeast extract (Difco), 10 g NaCl

Dissolved in 900 ml distilled water (dH_2O), pH adjusted to 7.5 with 2M NaOH and volume made up to 1 L, autoclaved. Immediately before use ampicillin was added to give a final concentration of 100 μg/ml.

2xTY

16 g bacto-tryptone, 10 g bacto-yeast extract, 5 g NaCl

Dissolved in 1 L dH_2O and autoclaved. Immediately before use sterile 20% (w/v) glucose was added to give a final concentration of 2% (v/v) together with ampicillin to a final concentration of 100 μg/ml (referred to as 2TYAG). For phage rescue media was prepared with ampicillin and with kanamycin at a final concentration of 25 μg/ml (referred to as 2TYAK).

LB agar and 2xTY agar

15 g bacto-agar (Difco) was added to 1 L of medium before autoclaving. For selective plates, LB agar / 2xTY agar was cooled to approximately 45°C before adding ampicillin (and glucose).

Minimal media plates

M9 salts 5X

64 g $Na_2HPO_4.H_2O$, 15 g KH_2PO_4, 5 g NH_4Cl, 2.5 g NaCl

Dissolved in 1 L dH_2O, pH adjusted to 7.4 and autoclaved in 200 ml aliquots.

20% $MgCl_2$

10 g $MgCl_2$ dissolved in 50ml dH_2O, sterilized by autoclaving.

Thiamine-Hydrochloride

100 mg thiamine-HCl dissolved in 10 ml H_2O, sterilised by filtration.

To prepare minimal agar 7.5 g of agar was dissolved in 375 ml dH_2O and autoclaved and allowed to cool to approximately 45°C before adding 100 ml of 5X M9 salts, 10ml 20% (w/v) glucose, 0.5 ml 20% $MgCl_2$ and 0.25 ml Thiamine-Hydrochloride.

2.1.2 Oligonucleotides

Murine V-gene primers

Oligonucleotide primers used for the amplification of murine Fd fragment and complete kappa light chain (Kettleborough *et al.*, 1993), secondary primers used to add linker sequences (Orum *et al.*, 1993) and the 5' -*SfiI* site into VK chain are listed in Table 2.1. Murine VH and VL genes were amplified and assembled as a scFv using new forward consensus primers designed to anneal to the end of framework 4. The forward light chain primers also included the $(Gly_4Ser)_3$ linker sequence as did the reverse heavy chain primer [Table 2.1]. Primers were obtained from Oswell, Edinburgh.

Table 2.1 Oligonucleotides used for amplification of V-genes and assembly of antibody fragments

Primers for amplification of Fd fragment and kappa chain for construction of Fab fragments

Fd fragment primers

VHa	GCC ATG GGC GAC GTC MAG CTT CAG GAG TCR GGA CC
VHb	GCC ATG GGC GAC GTC CAG CTG AAG SAS TCA GGA CC
VHc	GCC ATG GCC GAC GTC MWG SQG GTG GAG TCT GGG GGA
VHd	GCC ATG GCC GAC GTC ARS STG GWG GAA TCT GGA GGA
VHe	GCC ATG GCC GAC GTC ARG STG RTS GAG TCT GGA GG
VHf	GCC ATG GCC GAC GTC CAR SWG CAG CAR WCT GGG
VHg	GCC ATG GCC GAC GTC CAG WTG SWG CAQ TCT GGA
VHh	GCC ATG GCC GAC GTC CAG CTG CAG CAG TCW GTG
VHi	GCC ATG GCC GAC GTC MAS WTG SWG GWG WCT GGA GG
VHj	GCC ATG GCC GAC GTC CAG MTS CAG CAG WCT GG
γ-ASN	TTT TGT GCG GCC GCC QPG GTS PTG CTG GCP GGG TG

kappa chain primers

Vka	ATA TCC ATG GCA GAC RTC MAG ATR AWC CAG WCT MCA
Vkb	ATA TCC ATG GCA RAM ATT QTG CTG ACW CAR TWT CC
Vkc	ATA TCC ATG GCA GAT RWT QTG ATG ACC CAA ACT CCA
Vkd	ATA TCC ATG GCA SRA AWT STT CTC WWM CAG TCT CC
Vke	ATA TCC ATG GCA RRC RTT SWG ATG AWC ACA GTC QCC A
Vkf	ATA TCC ATG GCA GAT ATT GTG ATR ACQ CAG GMT RMA
Vkg	ATA TCC ATG GCA RRW ATT GTG ATG ACC CAR WCW C
κ-ASN	TAT TCA CGA GTT AAC ACT CAT TCC TGT TGA AGC

Secondary primers

PEL-HIg	AAA GGA GGT AAC CCA TGA AAT ACC TAT TGC CTA CGG CAG CCG CTG GAT TGT TAT TAC TCG CTG GCC AAC CAG CCA TGG GCG ACG TC
3'SD	TTT CAT GGG TTA CCT CCT TTA ACT CGA GTT AAC ACT CAT TCC
VkSfiI-SN	CCA GGC CCC CTT TAG GCC CAG CCG GCC ATG GCA

Primers for amplification of VH and VL and construction of scFv fragments

VH primers

VH-1BSc-5'	CGG TAG TGG TAA GAG CTC TGA AGG TAA AGG TGA CGT CCA GCT GAA GSA STC AGG ACC
VHSc-ASN-A	GAG TTT TTG TTC TGC GGC CGC TGC AGA GAC AGT GAC CAG AGT CC
VHSc-ASN-B	GAG TTT TTG TTC TGC GGC CGC TGA GGA GAC QGA GAG AGT GGT QC

VL primers

Vkfr1d	ATA TCC ATG GCA SRA AWT STT CTC WWM CAG TCT CC
VkSc-3'A	CAG AGC TCT TAC CAC TAC CAG GAG TAG AGC CTT TQA TTT CCA TQG TSC C
VkSc-3'B	CAG AGC TCT TAC CAC TAC CGG AAG TAG AGC CTT TCA GCT CCA GCT TGG TCC C

ambiguity codes M = A or C W = A or T R = A or G S = G or C Q = G or T

Human V-gene primers

Primers (a kind gift from R. E. Hawkins) used for the amplification and assembly of human V-genes as scFv fragments (Hawkins *et al.*, 1994) are listed in Table 2.2. Secondary primers introduce a 5' -*SfiI/NcoI* restriction site to the heavy chain and a 3' -*NotI* site to the light chain and assembly primers allow addition of the $(Gly_4Ser)_3$ linker.

Table 2.2 Primers used for the amplification of human V genes and assembly as scFv fragments

Primers for the primary amplification of VH		**Secondary and Assembly Primers**	
Human VH BACK			
HVH1BACK	CAG GTG CAG CTG GTG CAG TCT G	HVH1Sfi	TCG CGG CCC AAC CGG CCA TGG CCC AGG TGC AGC TGG TGC AG
HVH2BACK	CAG GTG AAG TTA AGG CAG TCT G	HVH2Sfi	TCG CGG CCC AAC CGG CCA TGG CCC AGG TCA ACT TAA GGG AG
HVH3BACK	GAG GTG CAG CTG GTG GAG TCT G	HVH3Sfi	TCG CGG CCC AAC CGG CCA TGG CCG AGG TGC AGC TGG TGG AG
HVH4BACK	CAG GTG CAG CTG CAG GAG TCG G	HVH4Sfi	TCG CGG CCC AAC CGG CCA TGG CCC AGG TGC AGC TGC AGG AG
HVH5BACK	GAG GTG CAG CTG CTG CAG TCT G	HVH5Sfi	TCG CGG CCC AAC CGG CCA TGG CCG AGG TGC AGC TGC TGC AG
HVH6BACK	CAG GTA CAG CTG CAG CAG TCA G	HVH6Sfi	TCG CGG CCC AAC CGG CCA TGG CCC AGG TAC AGC TGC AGC AC
Human JH			
HJ12FOR	TGA GGA GAC GGT GAC CAG GGT GCC	SCHJ12FOR	AGA GCC ACC TCC GCC TGA ACC GCC TCC ACC TGA GGA GAC GGT GAC CAG GG
HJ45FOR	TGA GGA GAC GGT GAC CAG GGT TCC	SCHJ45FOR	AGA GCC ACC TCC GCC TGA ACC GCC TCC ACC TGA GGA GAC GGT GAC CAG GG
HJ3FOR	TGA AGA GAC GGT GAC CAT TGT CCC	SCHJ3FOR	AGA GCC ACC TCC GCC TGA ACC GCC TCC ACC TGA AGA GAC GGT GAC CAT TG
HJ6FOR	TGA GGA GAC GGT GAC CGT GGT CCC	SCHJ6FOR	AGA GCC ACC TCC GCC TGA ACC GCC TCC ACC TGA GGA GAC GGT GAC CGT GG
Primers for the primary amplification of VL			
Human κ BACK			
HK14BACK	GAC ATC CAG ATG ACC CAG TCT CC	SCK14BACK	GGC GGA GGT GGC TCT GGC GGT GGC GGA TCG GAC ATC CAG ATG ACC CAG
HK26BACK	GAT ATT GTG ATG ACT CAG TCT CC	SCK26BACK	GGC GGA GGT GGC TCT GGC GGT GGC GGA TCG GAC ATT GTG ATG ACT CAG T
HK30BACK	GAA ATT GTG TTG ACG CAG TCT CC	SCK30BACK	GGC GGA GGT GGC TCT GGC GGT GGC GGA TCG GAA ATT GTG TTG ACG CAG T
HK50BACK	GAA ACG ACA CTC ACG CAG TCT CC	SCK30BACK	GGC GGA GGT GGC TCT GGC GGT GGC GGA TCG GAA ATT GTG TTG ACG CAG T
Human Jκ			
HJ124FOR	ACG TTT GAT CTC CAC CTT GGT CCC	HJK124NOT	GAT ATG AGA TAC TGC GGC CGC ACG TTT GAT CTC CAC CTT GG
HJK30FOR	ACG TTT GAT ATC CAC TTT GGT CCC	HJK30NOT	GAT ATG AGA TAC TGC GGC CGC ACG TTT GAT ATC CAC TTG GG
HJK50FOR	ACG TTT AAT CTC CAG TCG TGT CCC	HJK50NOT	GAT ATG AGA TAC TGC GGC CGC ACG TTT AAT CTC CAG TCG TG
Human λ BACK			
HL10BACK	CAG TCT GTG TTG ACG CAG CCG CCC TC	SCHL1BACK	GGC GGA GGT GGC TCT GGC GGT GGC GGA TCG CAG TCT GTG TTG ACG CAG
HL20BACK	CAG TCT GCC CTG ACT CAG CCT GCC TC	SCHL2BACK	GGC GGA GGT GGC TCT GGC GGT GGC GGA TCG CAG TCT GCC CTG ACT CAG
HL30BACK	TCC TAT GAG CTG ACT CAG CCA CVC TC	SCHL3BACK	GGC GGA GGT GGC TCT GGC GGT GGC GGA TCG TCC TAT GAG CTG ACT CAG
HL40BACK	CAC GTT ATA CTG ACT CAA CCG CCC TC	SCHL4BACK	GGC GGA GGT GGC TCT GGC GGT GGC GGA TCG GAG GTT ATA CTG ACT CAA C
Human Jλ			
HJL1FOR	ACC TAG GAC GGT GAC CTT GGT CCC	HJL10NOT	GAT ATG AGA TAC TGC GGC CGC ACC TAG GAC GGT GAC CTT GG
HJL23FOR	ACC TAG GAC GGT CAG CTT GGT CCC	HJL23NOT	GAT ATG AGA TAC TGC GGC CGC ACC TAG GAC GGT GAC CTT GG
HJL7FOR	ACC GAG GAC GGT CAG CTG GGT GCC	HJL70NOT	GAT ATG AGA TAC TGC GGC CGC ACC GAG GAC GGT CAG CTG GG

Sequencing primers

The T3-FWD (5'-ATT AAC CCT CAC TAA AGG GA-3') and T3-REV (5'-TAA TAC GAC TCA CTA TAG GG-3') primers were used for sequencing of cloned VK genes in *pBluescript*. pUC-FOR (5'-CGA CGT TGT AA ACG ACG GCC AGT-3') and pUC-REV (5'-CAG GAA ACA GCT ATG AC-3') primers were used for sequencing of cloned scFv fragments in *pUC119-polyHIS6myc*. Primers were obtained from Oswell, Edinburgh.

2.1.3 Plasmids

pFAB5cHis (a kind gift from J. Engberg) is a derivative of *pBluescript KS+* and *pHEN*. This phagemid vector carries 5' -*SfiI* and 3' -*NotI* sites that allow for fragments to be inserted in frame with an upstream LacZ promoter and pelB signal sequence and downstream truncated bacteriophage gene III and six histidine residues [Figure 2.1].

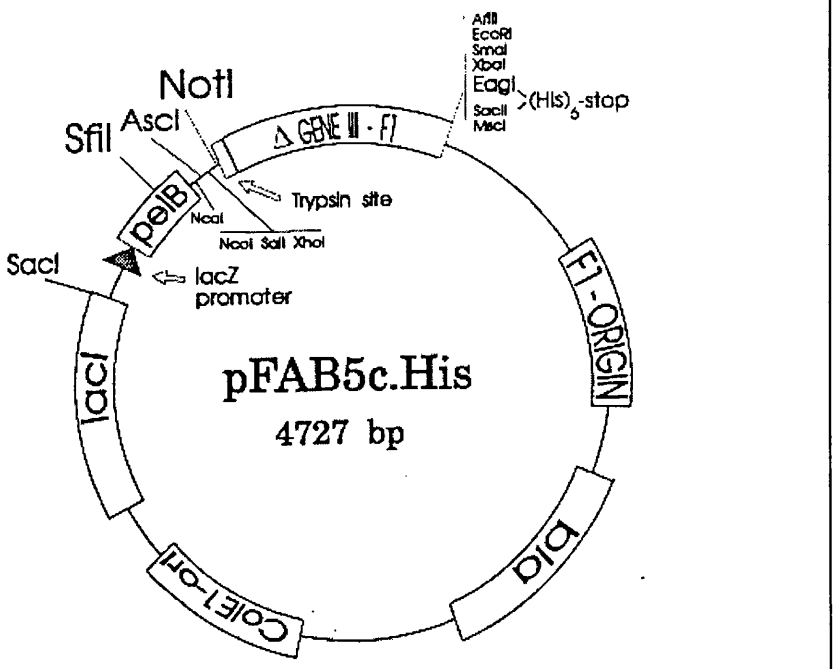


Figure 2.1 Phagemid vector pFAB5cHis

Plasmid *pUC119-polyHIS6myc* (MRC, Cambridge) is a derivative of *pUC119* and carries 5' - *SfiI/NcoI* and 3' -*NotI* cloning sites. Fragments are cloned in frame with an upstream LacZ promoter and pelB signal sequence and has a downstream cassette of c-myc-derived sequence and six histidine residues [Figure 2.2].

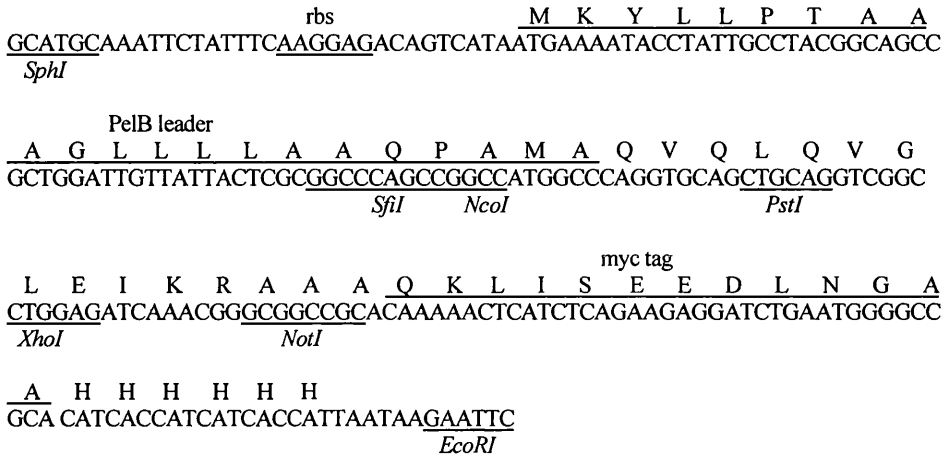


Figure 2.2 Plasmid vector pUC119-polyHIS6myc

2.2 Tissue culture techniques

Thawing of cells, growth and maintenance of cell cultures

The hybridoma cell line S2.5 and fusion partner HMy were removed from liquid nitrogen and placed in a 37°C water bath. The thawed cells were transferred to a 20 ml universal container and resuspended in 4 ml of Dulbecco's Modified Eagle Medium (DMEM) [Gibco Life Tecnologies, Paisley, Scotland] supplemented with 10% (v/v) Foetal calf serum (Gibco), 50 units/ ml penicillin, 50 μg/ ml streptomycin and 2 mM glutamine. Cells were then pelleted by centrifugation 700 g for 5 minutes at room temperature and resuspended in 10 ml of supplemented DMEM. Cells were transferred into a 25 cm^2 flask and grown in a humidified 37°C incubator with 5% CO_2. When a cell density of approximately 3 x 10^6 was reached

cultures were transferred to 80 cm^2 flasks. Cells were grown to exhaustion in order to maximise the concentration of antibody in the culture supernatant.

Freezing down and storage of cells

Cells were pelleted by centrifugation at 700 g for 5 minutes at 4°C, resuspended at 1 x 10^6 cells/ ml in cold freezing medium [10% (v/v) DMSO in foetal calf serum] and aliquoted into cryotubes. These were then placed overnight at -70° C before being transferred for storage to a liquid nitrogen tank.

2.3 E.coli techniques

2.3.1 Growth of liquid cultures

A single well-defined colony, grown on an agar plate (LB or 2xTY), was used to innoculate LB or 2xTY medium. Cultures were grown in 20 ml universal tubes (for culture volumes of 3-5 ml), 50 ml Falcon tubes (for culture volumes of 10 ml) or autoclaved baffled conical flasks (for culture volumes of 50 ml - 1 litre) at 37°C with shaking at 180 r.p.m. in an orbital incubator (Gallenkamp).

2.3.2 Preparation and heat shock transformation of competent E.coli cells

Competent cells for heat shock transformation were prepared using a modified $CaCl_2$ method (T. Klonisch personal communication). A single colony from a freshly streaked plate (LB) was used to inoculate 3 ml of LB and grown at 37°C overnight with shaking. A 0.5 ml aliquot of the overnight culture was then used to inoculate 19.5 ml of LB in a conical flask and incubated at 37°C for 65 minutes with shaking. Before pelleting cells by centrifuging at 9000 g for 10 minutes the cells were cooled by placing the flask on ice for 30 minutes. The cell pellet was resuspended in 10 ml of ice cold 50 mM $CaCl_2$, incubated on ice for 2 hours and then centrifuged at 9000 g for 10 minutes. The pellet was resuspended in 2 ml ice cold 50 mM

$CaCl_2$. The cells were either used immediately for transformation or stored in 200 µl aliquots at -70°C.

Transformation by heat shock

Frozen competent cells were thawed on ice, DNA was mixed with the cells that were then incubated on ice for 60 minutes. The cells were heat shocked by incubation at 42°C for 2 minutes and then placed on ice for 5 minutes. An 800 µl aliquot of LB was added and bacteria incubated at 37°C for 30 minutes with shaking before plating cells on LB agar plates with ampicillin (100 µg/ml). Plates were incubated overnight in a 37°C oven.

2.3.2. Preparation and electroporation of competent E.coli cells

A 1/100 dilution of overnight culture was used to innoculate 500 ml LB which was grown with shaking at 37°C until an approximate OD A_{600} 0.5 was reached. The flask was chilled on ice for 15-30 minutes and cells pelleted by centrifugation at 4000 g for 15 minutes at 4°C. The cells were gently resuspended in ice cold water and pelleted as before. The cells were resuspended in 10 ml of ice cold 10% (v/v) glycerol and pelleted again. Cells were finally resuspended in 1 ml of ice cold 10% (v/v) glycerol. Cells were stored in 50 µl aliquots at -70°C for a maximum of 6 months.

Electroporation

DNA was mixed with a thawed aliquot of cells incubated on ice for 1 minute before being transferred to a pre-chilled 0.2 cm electroporation cuvette (Bio-Rad Laboratories, Herfordshire). The electroporator (Gene Pulser Plus, BioRad) was pre-set at: voltage 2.5 kV, capacitance 25 µF, resistance 200 Ohm. The cuvette was placed in the electroporation chamber and pulsed once with a time constant of 4-4.5 milli sececonds. The cuvette was removed and the cells immediately resuspended in 1 ml of 2TY with 2% (v/v) glucose and

then incubated with shaking at 37°C for 30 minutes. Dilutions of the culture were made and 100 µl plated out on 2TYAG agar plates and incubated overnight in a 37°C oven. To determine efficiency of transformation controls with 1 ng vector DNA were used.

2.4 RNA isolation and purification from tissue culture cells

Total RNA was isolated from S2.5 hybridoma cells using the Qiagen RNAeasy Kit (Qiagen Ltd, Dorking) according to the manufacturer's instructions. S2.5 cells were harvested from tissue culture medium by centrifugation 700 g for 5 minutes. Pelleted cells were washed using sterile PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 pH 7.4) before addition of 350 µl lysis buffer RLT to $1x10^7$ cells. Cell lysates were homogenised by passing the lysate 3-4 times through a sterile plastic syringe fitted with a 19G needle. The lysate was centrifuged at 15 000 g for 3 minutes in microcentrifuge (Biofuge 15, Heraeus) before application to a RNAeasy spin column. The spin column was centrifuged at 15 000 g for 15 seconds and 700 µl wash buffer RW1 applied to the spin column followed by two 500 µl aliquots of RPE buffer. The column was dried by centrifugation at 15 000 g for 2 minutes. RNA was eluted by pipetting 50 µl diethyl pyrocarbonate (DEPC) treated water directly onto the spin column membrane and centrifuging at 15 000 g for 1 minute.

DNAse treatment

Isolated RNA was treated with DNase to eliminate DNA from the sample. Two units of DNAse (Boehringer Mannheim, UK) were added to the eluted RNA and incubated at 37°C for 30 minutes. Treated RNA was then purified on a RNAeasy column. The RNA was mixed with 50 µl of DEPC treated H_2O, 350 µl of lysis buffer RLT and 250 µl ethanol (96-100%).

The RNA solution was then applied to the column, which was washed and eluted as above. The RNA was used immediately for cDNA synthesis or stored at -70°C.

2.5 cDNA synthesis

The First Strand cDNA synthesis kit (Pharmacia Biotech, Hertfordshire) was used to synthesise cDNA from NCA-102 RNA (from Dr J Saccavini, CIS BIO International, France) and from S2.5 RNA. A 20 µl aliquot of 1-5 µg of total RNA was denatured by incubation at 65°C for 10 minutes and then chilled on ice. A 11 µl aliquot of the bulk first strand cDNA reaction mix (M-MLVRT, 80 units RNAguard, RNase, DNAse free BSA, 25 mM dATP, dCTP, dGTP and dTTP) was then added to RNA along with 1 µl 200 mM dithiothreitol (DTT). Synthesis was primed using 1.5 µg of either the oligo $(dT)_{18}$ primer (5'-AAC TGG AAG AAT TCG CGG CCG CAG GAA T_{18}-3') or chain specific primers. Reactions were incubated at 37°C for 1 hour and cDNA amplified directly by PCR, after denaturing RNA:DNA heteroduplex by heating to 95°C.

Pharmacia 'You Prime First Strand Ready-To-Go-Beads' were used to synthesise cDNA from S2.5 RNA. A 20 µl aliquot containing 5 µg total RNA was used to resuspend the beads to give a reaction mix containing: 50 mM Tris-HCl (pH 8.3); 8 mM $MgCl_2$; 10 mM DTT; 0.4 mM oligo(dT); 0.9 mM dNTPs, and 50 units of M-MLVRT). Reactions were incubated at 37°C for 90 minutes.

2.6 DNA isolation and purification

Different methods were used for the preparation of plasmid DNA, depending on the yield and quality of DNA that was required. The Mini-prep method was used in earlier work (Chapter 3) to isolate 10 - 30 µg of DNA for restriction digests, transformations and sequencing. Later

work used the Wizard DNA mini-prep Kit (Promega, Southampton, UK), which produced better quality DNA with slightly higher yields (10 - 50 μg) and was more convenient to use. The Maxi-prep CsCl method was used for the isolation of plasmid DNA suitable for use as a cloning vector. The midi-prep method with the Qiagen plasmid purification kit was used to isolate DNA that was suitable for automated sequencing. ssDNA prepared from M13 bacteriophage was used for T tracking (sequencing terminated with ddTTP only).

2.6.1 Mini-prep

Cells were pelleted from a 5 ml overnight culture by centrifugation at 3000 g for 15 minutes at 4°C. The pellet was resuspended in 300 μl of Maxi buffer (50 mM glucose; 25 mM Tris-HCl pH 7.5; 10 mM EDTA) and transferred to a 1.5 ml tube. Cells were lysed with 600 μl of lysis buffer [0.2M NaOH; 1% (w/v) SDS] and neutralised with 300 μl of 3 M potassium acetate. The tube was inverted gently to obtain a cleared lysate, before centrifugation at 15 000 g in a microfuge for 6 minutes at room temperature. The cleared supernatant was transferred to a clean tube, RNaseA added to a final concentration of 50 μg/ml and incubated at 37°C for 90 minutes. Protein was removed by standard phenol/chloroform extraction and DNA precipitated using 3 M sodium acetate with ethanol as described below (2.6.7). DNA was pelleted by centrifugation at 15 000 g for 10 minutes at room temperature and washed in 70% (v/v) ethanol before resuspension in 50 μl of TE buffer.

2.6.2 Promega Wizard Mini-prep

DNA was prepared from a 3 ml of overnight culture according to manufacturer's instructions. Cells were pelleted by centrifugation at 3000 g for 10 minutes at 4°C and cells resuspended in 200 μl resuspension buffer (50 mM Tris-HCl, pH 7.5; 10 mM EDTA; 100 μg/ml RNase A). The cell suspension was transferred to a clean 1.5 ml tube and 200 μl of lysis buffer was added, the tube was inverted to produce a clear lysate and then mixed gently with 200 μl 1.32

M potassium acetate, pH 4.8. The lysate was centrifuged at 15 000 g for 6 minutes at room temperature and the clear supernatant transferred to a fresh tube. A 1 ml aliquot of Wizard mini-prep DNA purification resin was added to the supernatant and mixed by inversion of the tube. The resin/DNA mix was passed through a Wizard mini-column using a syringe. The column was washed by passing 2 ml wash buffer (83 mM NaCl; 8.3 mM Tris-HCl, pH 7.5; 2.08 mM EDTA; 58% ethanol) through the column. The resin was dried by centrifugation at 15 000 g for 20 seconds and the mini-column transferred to a clean 1.5 ml tube. To elute the DNA 50 µl of TE was applied to column and incubated for 1 minute at room temperature before spinning the mini-column at 15 000 g for 20 seconds.

2.6.3 Midi Prep

Approximately 100 µg of plasmid DNA was prepared using the Qiagen-tip 100 according to the manufacturer's instructions. A 1/500 dilution of a culture grown for 8 hours at 37°C was used to innoculate 25 ml of LB with ampicillin (100 µg/ml) which was then grown at 37°C for 12-16 hours. The bacterial cells were pelleted by centrifugation at 6000 g (JA10 rotor, Beckman), the supernatant discarded and the centrifuge tube inverted over a paper towel to drain off any remaining medium. The bacterial pellet was gently resuspended in 4 mls of buffer P1 (50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 100 µg/ ml RNase A). An equal volume of buffer P2 [200 mM NaOH; 1% (w/v) SDS] was added to the cell suspension, the sample was mixed gently and incubated at room temperature for 5 minutes. A 4 ml aliquot of pre-chilled buffer P3 (3 M potassium acetate, pH 5.5) was added and the lysate mixed immediately by inverting the tube 4-6 times. The sample was incubated on ice for 15 minutes before centrifuging at 20 000 g (SS-34 rotor, Sorvall Superspeed) for 30 minutes at 4°C. The cleared supernatant was removed and re-centrifuged at 20 000 g for 15 minutes at 4°C to pellet any suspended or particulate material before application of the sample to the Qiagen tip. The Qiagen tip 100 was equilibrated before use by applying 4 ml of buffer QBT [750 mM NaCl;

50 mM MOPS, pH 7.0; 15% (v/v) ethanol; 0.15% (v/v) Triton X-100] and allowing the column to empty by gravity flow. The cleared supernatant was then applied to the column and allowed to enter the resin by gravity flow. The column was washed twice with 10 ml of buffer QC [1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% (v/v) ethanol] again by allowing the buffer to move through the column by gravity flow. DNA was eluted from the column with 5 ml of buffer QF [1.25 M NaCl; 50 mM Tris-HCl, pH 8.5; 15% (v/v) ethanol] and collected into a 10 ml tube. DNA was precipitated from the eluate by mixing with 0.7 volumes (3.5 ml) isopropanol, and pelleted by centrifugation at 15 000 g for 30 minutes at 4°C. After centrifugation the outside of the centrifugation tube was marked to locate the pellet before carefully decanting the supernatant. The DNA pellet was washed twice using 2 ml of 70% (v/v) ethanol. After centrifuging at 15 000 g for 10 minutes at 4°C the supernatant was carefully decanted and the pellet air dried for 5-10 minutes. Finally, the DNA was dissolved in 1 ml of TE buffer.

2.6.4 Maxi-Prep CsCl Method

A 1 L culture was innoculated using 5 ml of an over day culture (grown with shaking at 37°C for 8 hours) and grown at 37°C for 12-16 hours with shaking at 37°C. The bacterial cells were pelleted by centrifugation at 6000 g for 30 minutes, the supernatant discarded. The bacterial pellet was gently resuspended in 40 ml of Maxi buffer and the cells lysed by mixing with 80 ml of lysis buffer. After mixing with 40 ml 3 M potassium acetate the lysate was spun at 3000 g for 20 minutes at 4°C. The supernatant was decanted and filtered through nylon into a clean centrifuge tube and mixed briefly with 0.6 vol (100 ml) of isopropanol. DNA was pelleted by centrifugation at 15 000 g for 30 minutes at 4°C (SS-34 rotor, Sorval Superspeed centrifuge). The pellet was washed with 70% (v/v) ethanol and resuspended in approximately 8 ml of TE buffer and transferred to a 20 ml universal container. A 0.5 ml aliquot of ethidium bromide

(10 mg/ml) was added followed by 10 g CsCl. The volume was adjusted to 10 ml with TE buffer before transfer to an ultracentrifuge tube (Beckman quick-seal tube). The tube was sealed and centrifuged at 85 000 g (Ti 70 rotor, Beckman ultracentrifuge L7-65) for 48 hours at 4°C. Supercoiled plasmid DNA (the lower of 2 bands) was visualised in UV light and removed using a hypodermic needle inserted into the side of the tube into a universal containing sterile distilled H_2O. DNA was pelleted by centrifugation at 3000 g for 30 minutes 4°C, after the addition of 2.5 volumes of ethanol. The pellet was washed with 70% (v/v) ethanol, air dried and resuspended in 800 µl TE buffer. The DNA sample was extracted with phenol/chloroform until a clear interface was obtained. The DNA was then precipitated with 1/10 volume 3 M sodium acetate and 0.6 volume isopropanol, and spun at 15 000g for 10 minutes at room temperature. The pellet was washed with 70% (v/v) ethanol and resuspended into 360 µl TE buffer. Finally an ethanol precipitation was performed as described above and the DNA resuspended into 1 ml of TE buffer.

2.6.6 Preparation of ssDNA for sequencing from bacteriophage M13

Polyethylene glycol (PEG) precipitated phage resuspended in TE buffer prepared from phagemid clones (2.12.1) was used for the isolation of ssDNA. Phenol/chloroform extractions were performed followed by a chloroform extraction and DNA ethanol precipitated as described below. DNA was pelleted by centrifugation at 15 000 g, for 10 minutes at room temperature and the ssDNA was resuspended in 20 µl TE buffer.

2.6.7 Phenol/chloroform extraction

Phenol/chloroform extraction was used for the removal of proteins from DNA preparations and from DNA treated with enzyme. The DNA sample was mixed with an equal volume of phenol/chloroform [25:24:1 (v/v) phenol: chloroform : isoamyl-alcohol, saturated with TE pH 8.0] by vortexing (for fragments, 10Kb) or by inversion of the tube for larger fragments (10-

30Kb). The sample was then centrifuged at 15 000 g for 15 minutes at room temperature. The upper aqueous layer was removed and transferred to a clean tube and the interface and lower organic phase discarded. This was repeated until a clear interface was obtained after which a final extraction with chloroform was performed. DNA was recovered by ethanol or isopropanol precipitation.

Ethanol/isopropanol precipitation

The volume of sample was estimated and a 1/10 volume of 3 M sodium acetetate pH 5.5 added and mixed. DNA was precipitated by addition of 2 volumes of ethanol and pelleted by centrifugation at 15 000 g for 10 minutes at room temperature. The pellet was briefly air dried and dissolved in the required volume of TE buffer (10 mM Tris pH 7.5, 1 mM EDTA). Isopropanol precipitation used when a minimal volume was required. DNA was precipitated by the addition of 0.6 volumes of isopropanol and after centrifugation the pellet washed with 70% (v/v) ethanol before dissolving in buffer.

2.6.8 DNA Quantitation

The concentration of DNA and RNA was determined by measurement of absorbance at 260 nm and 280 nm and the concentration calculated according to an OD of 1 corresponding to approximately 50 μg/ml for dsDNA and 40 μg/ml for ssDNA and RNA. The purity of the preparation was determined using the ratio between the readings at 260 nm and 280 nm (OD_{260}/ OD_{280}), pure preparations of DNA and RNA having OD_{260}/ OD_{280} of 1.8 and 2.0 respectively. When the sample size was too small for spectrophotometric quantitation, ethidium bromide flourescent quantitation of dsDNA was used to estimate the concentration of DNA. The DNA concentration was estimated by direct comparison of fluorescence in sample DNA with a known concentration of marker DNA which had been separated on an agarose gel containing ethidium bromide.

2.6.9 Purification of PCR products

The Wizard PCR purification kit (Promega) was used for the isolation of PCR products from excised gel bands obtained after agarose gel electrophoresis. The agarose gel slice was melted by incubation at 70°C for 3 minutes and 1 ml of PCR purification resin added. The resin and DNA were mixed by vortexing briefly and then passed through a wizard mini-column. The mini-column was washed by pushing through 2 ml 80% (v/v) isopropanol, and the column dried by centrifugation at 15 000 g for 20 seconds. After transfer of the mini-column to a clean 1.5 ml tube 50 µl of H_2O was applied to the column and incubated for 1 minute. DNA was eluted by centrifugation at 15 000 g for 20 seconds.

2.7 Cloning

2.7.1 Vector preparation

10 µg of purified DNA was digested with 10-20 units of required restriction endonucleases in a volume of 100 µl in the buffers and under conditions recommended by the supplier. The 5' phosphate groups were removed, to prevent re-ligation of the vector, by addition of 9 units of calf intestinal alkaline phosphatase (CIP) [Boehringer Mannheim, Lewes] and 11 µl of CIP buffer (50 mM NaCl; 10 mM Tris-Cl, pH 7.9; 10 mM $MgCl_2$) to the reaction and continuing the incubation at 37°C for a further 30 minutes. CIP was inactivated at 75°C for 10 minutes following addition of EGTA to 10 mM. The digested and phosphatased DNA was purified by phenol extraction and ethanol precipitation or gel purified.

T Vector for cloning of PCR products

EcoRV linearised *pBluescript KS*$^-$ (Stratagene, UK) was incubated with 10 units of Taq polymerase (Pharmacia) and 2 mM dTTP at 70°C for 2 hours before phenol/chloroform extraction and precipitation. The resulting T vector which contained a single overhanging T

on each 3' end was digested with *KpnI* followed by phenol/chloroform extraction and ethanol precipitation, to generate a directional vector for the PCR product.

2.7.2 Ligations

Vector and insert DNA were ligated using an approximate molar ratio of 1:3 (vector: insert) and incubated in a final volume of 25 µl with ligase buffer (50 mM Tris-Cl, pH 7.8; 10 mM $MgCl_2$; 10 mM DTT; 1 mM ATP; 25 µg/ ml BSA) and 2 units of T4 DNA ligase overnight at 16°C. Prior to the ligation of PCR products into the T vector phosphate groups were added to the 5' OH of PCR products using T4 Polynucleotide Kinase (Boehringer Mannheim). DNA was incubated in phosphorylation buffer (70 mM Tris-Cl, pH 7.6; 10 mM $MgCl_2$, 5 mM DTT) with 1 mM ATP and 5 units of enzyme at 37°C for 30 minutes. The enzyme was then heat inactivated at 70°C for 15 minutes.

Control ligation reactions were always included (i) without insert (as an indicator of the level of self-ligation of the vector) and (ii) without insert and without ligase (to determine the presence of any undigested vector).

The ligation product and controls were purified using the GENECLEAN II kit (Anachem Ltd, Bedfordshire, UK) according to manufacturer's instructions. Three volumes of 6 M iodide solution was added to the DNA together with 5 µl of GLASSMILK solution (approx 5 µl/5 µg DNA) which was vortexed and then incubated at room temperature for 5 minutes. The mixture was centrifuged for 5 seconds at 15 000 g and the supernatant removed. The pellet was washed 3 times using 500 µl of NEW WASH solution. Finally the pellet was resuspended in 20 µl of H_2O and incubated at 55°C for 3 minutes. Supernatant containing eluted DNA was removed after centrifugation at 15 000 g for 30 seconds.

2.8 DNA sequencing

2.8.1 Manual sequencing of double stranded DNA

Manual sequencing was performed using the Sequenase Kit Version 2.0 (USB, Ohio, USA) according to the manufacturer's instructions. Approximately 3-5 µg of double stranded DNA was mixed with 2 µl 2 M NaOH in a final volume of 10 µl and heated at 65°C for 5 minutes. The template was cooled at room temperature for 10 minutes and then 3 pmol primer and 3 µl 3 M sodium acetate added. Sterile dH_20 (sd H_20) was added to give a volume of 30 µl followed by 75 µl ethanol and the mix incubated in a dry ice/ethanol bath for 20 minutes. The precipitated DNA was centrifuged at 15 000 g for 10 minutes at room temperature. The pellet was washed with 70% (v/v) ethanol and resuspended in 7.5 µl sdH_2O. The labelling solution was made by adding 2 µl of Sequenase 5 x reaction buffer (200 mM Tris-HCl pH 7.5; 100 mM $MgCl_2$; 250 mM NaCl), 1 µl 0.1M DTT, 2 µl diluted labelling mix (7.5 µM dGTP; 7.5 µM dCTP; 7.5 µM dTTP), 0.5 µl (0.185MBq) S^{35} dATP [Deoxyadenosine 5'-(α-Thio) triphosphate, (^{35}S) NEN DuPont, Dreiech, Germany] and 2 µl (1.2 units) diluted Sequenase to the DNA followed by incubation at room temperature for 5 minutes. A 3.5 µl aliquot of the labelling mix was added to a tube containing 2.5 µl of termination mix for each of the four dideoxynucleotides (dNTPs) (8 µM of dideoxy and 80 µM of four dNTPs that had been incubated at 37°C. The four mixes were incubated at 37°C and after 5 minutes the reaction stopped by adding 4 µl of Stop solution [95% (v/v) formamide; 20 mM EDTA; 0.05% (w/v) bromophenol blue; 0.05% (w/v) xylene cyanol].

6 % (w/v) polyacrylamide gels were cast in 33 x 42 cm glass plates with 0.4 mm spacers. The gels were pre-run overnight at 300 V. Sequence reaction products were heated to 75°C for 2 minutes and 3 µl aliquots were loaded on the gel. The DNA was electrophoresed at a constant 1 500 V for various durations depending on the primer position relative to the sequence to be

read. Gels were fixed in 10% (v/v) acetic acid, 10% (v/v) methanol before being vacuum dried for 15 minutes at 75°C and exposed to X-ray film (Fuji RX) at room temperature overnight.

2.8.2 Single stranded DNA sequencing reactions

This method was used for 'T-tracking' to enable identification of different clones that had been selected using NCA-95 with the CEA library. Approximately 3 μg of single stranded DNA was mixed with 2 μl of Sequenase 5 x reaction buffer and 3 pmol primer in a final volume of 10 μl. The DNA mix was heated to 65°C for 2 minutes and then cooled for 10 minutes at room temperature. A 1 μl aliquot of 0.1 M DTT, 2 μl of the diluted labelling mix, 0.5 μl S^{35} dATP and 2 μl of the diluted Sequenase enzyme were added to the DNA mix and incubated at room temperature for 5 minutes. A 3.5 μl aliquot of this labelling mix was added to a tube containing 2.5 μl of termination mix for T only (8 μM of ddTTP and 80 μM dATP, dGTP, dCTP) that had been incubated at 37°C. The termination reaction was incubated at 37°C and the reaction stopped after 5 minutes, by adding 4 μl of Stop solution.

2.8.3 Automated sequencing

Automated DNA sequencing was performed by The Advanced Biotechnology Centre (Imperial College, London), using the Applied Biosystems 373A Automated DNA sequencer. The sequence was analysed using Chromas 1.41 (http//:trishul.sci.gu.edu.au/~conor/chromas.html) and VH and VL genes were assigned to germline segments using the International Immunogenetics database (IMGT) [http://imgt.cnusc.fr:8104/textes/Mppage.html#5] and V-BASE (http: www.mrc-cpe.cam.ac.uk, Tomlinson *et al.*, MRC Centre for Protein Engineering).

2.9 Polymerase Chain Reaction

PCR (Saiki *et al.*, 1985) was used for the amplification of V-genes, extension of V-genes by the incorporation of restriction enzyme sites and linker sequences, assembly and amplification of antibody fragments and amplification of fragments from bacterial clones. Reaction conditions were optimised for individual applications but were based on the general reaction conditions described. Typically reactions comprised of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 0.2 mM dNTPs, 20 nmol of each primer, 2.5 units of Taq DNA polymerase and 10-500 ng of template DNA, in a final volume of 50 µl. The reaction mixture was overlaid with light mineral oil. Reactions were amplified after incubation at 94°C for 4 minutes using typically 30 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes. PCR amplification was checked using a 2 µl sample on a 2% (w/v) agarose gel.

2.9.1 PCR amplification of antibody fragments from individual bacterial clones.

This method was used to screen transformants for recombinant clones and for the amplification of fragments to enable assessment of diversity by *BstNI* analysis.

Reactions were performed under standard PCR conditions in a total volume of 20 µl. A sterile toothpick was used to touch a single colony and then carefully swivelled in the PCR reaction mix. Reactions were amplified using 25 cycles at 94°C for 45 seconds, 55°C for 45 seconds and 72°C for 90 seconds.

2.9.1.1 BstNI digestion of amplified antibody fragments

After PCR a *BstNI* digestion mix of 0.2 µl BSA (10 mg/ml), 4 µl NEB2 buffer (10X), 15.5 µl H_2O with 0.5 µl (5 units) *BstNI* was added directly to the completed PCR mix. Samples were incubated at 60°C for 3 hours and analysed on 2% (w/v) agarose.

2.10 Agarose gel electrophoresis

Size analysis of DNA fragments was performed by running DNA samples alongside appropriate DNA size markers in agarose (FMC Bio Products, Rockland, USA) gels. The 1xTBE buffer (100 mM Tris; 100 mM Boric acid: 1.25 mM EDTA) was used in the gel as running buffer, for general analysis. Ethidium bromide was added to the gel at a final concentration of 0.5 μg/ ml. Typically 500 ng DNA size marker (Pharmacia Biotech) such as λ DNA *HindIII/EcoR1*, would be applied with 20% (w/v) Ficoll/Orange G loading dye. Where DNA was to be recovered from the gel, 1xTAE buffer (40 mM Tris, 1 mM EDTA, pH 8.0 adjusted with glacial acetic acid) was used. Gels were run in a midi horizontal electrophoresis unit (Flowgen) until sufficient separation was observed. Bands were visualised using an UV transilluminator (Genetic Research Instrumentation Ltd, Essex) and gels photographed using a DS34 polaroid direct screen instant camera (Genetic Research Instrumentation) using Polaroid film type 667.

Bands corresponding to amplified products that were to be recovered were carefully excised using a fresh sterile scalpel blade for each band, and transferred to separate 1.5 ml tubes. DNA was purified from agarose using the Wizard PCR prep DNA purification system (2.6.9)

2.11 Protein analysis

2.11.1 SDS polyacrylamide gel electrophoresis (SDS PAGE)

Polyacrylamide gels were made using Protogel solution [30% (w/v) acrylamide, crosslinked with 2.7% (w/v) bisacrylamide (37.5:1 ratio), National Diagnostics, Hull]. A separating gel of 10% (w/v) acrylamide 0.375 M Tris-HCl pH 8.8; 0.1% (w/v) SDS was used with a stacking gel of 5% (w/v) acrylamide, 0.126 M Tris-HCl pH 6.8, 0.1% (w/v) SDS with running buffer [25 mM Tris; 192 mM glycine; 0.2% (w/v) SDS]. Samples were diluted to appropriate

concentration, mixed with 2 x loading buffer [1.25 mM Tris-HCl, pH 6.8; 20% (v/v) glycerol ; 2% (v/v) β-mercaptoethanol ; 0.1% (w/v) bromophenol blue 0.1% (w/v) SDS] and incubated (as was protein size marker) at 100°C for 5 minutes before loading. Gels were run until the dye front was at the bottom and then used for Western blotting.

2.11.2 Western Blots

The protein gel was equilibrated in transfer buffer [24.7 mM Tris, 246 mM NaCl, 2% (v/v) methanol] for half an hour before being transferred by electrophoresis onto nitrocellulose membrane (TransBlot Transfer Medium, Biorad). The membrane was blocked in 5% (w/v) Marvel (MAR) skimmed milk powder (Premier Beverages, Stafford) in phosphate buffered saline (PBS) [MAR/PBS] for 1 hour at room temperature followed by a further 1 hour incubation at room temperature after the addition of primary antibody. Three 15 minute washes were then performed using PBS with 0.05% (v/v) Tween-20. The membrane was then incubated with horseradish peroxidase (HRP) labelled antibody diluted in 1% (w/v) MAR/PBS for 1 hour at room temperature. After washing the membrane as described, proteins were visualised using DAB substrate (0.25 mg/ml diaminobenzidine tetrahydrochloride, 0.5 µl H_2O_2 in PBS), colour development was stopped by rinsing the membrane in PBS.

Dot Blot analysis was performed on culture supernatants to check for the production of soluble scFv fragments. A 2 µl aliquot of culture supernatant was pipetted onto the nitrocellulose membrane and allowed to dry. Soluble scFv fragments were detected with anti-myc tag antibody 9E10 (ICRF, London) and labelled anti-mouse antibody with blocking and incubation conditions as those used for Western blots.

2.11.3 ELISA

In general approximately 3-5 μg/ml of antigen was coated onto ELISA plates [Falcon 3192 flexible Immnoassay plates (Becton Dickinson, Oxford), Maxisorp C Immunoassay plates, Immunolon 4 Dynatech (Nunc, Denmark)] carbonate/bicarbonate (BIC) buffer at 4°C overnight or at 37°C for 1 hour. The wells were washed three times in PBS and blocked using 200 μl per well of blocking solution of 3% (w/v) MAR/PBS or 3% (w/v) bovine serum albumin/PBS (BSA/PBS) at 37°C for 1 hour. Wells were then washed three times with PBS and incubated with the primary antibody (diluted in 1% (w/v) blocking solution) for 1 hour at room temperature. Wells were washed as before and then incubated with diluted HRP labelled secondary antibody for 1 hour at room temperature. After washing, 100 μl of substrate [3,3', 5,5' tetramethylbenzidine 5 mg / 500 μl in dimethylsulphoxide (DMSO) added to 50 ml citrate buffer (0.5 M sodium acetate adjusted to pH 6.0 with 0.5 M citric acid) to which 37 μl of H_2O_2 was added before use] was used. The reaction was stopped with the addition of 50 μl of 12% (v/v) H_2SO_4 and A_{490nm} measured (Dynatech MR 5000 plate reader).

2.12 Growth and Expression of phage antibodies

2.12.1 Rescue of phage

Typically 50 μl of phagemid bacterial glycerol stock, was used to inoculate 50 ml 2xTY with 100 μg/ml ampicillin and 2% (w/v) glucose (2TYAG) and grown in a 250 ml sterile flask with shaking (250 r.p.m.) for approximately 2 hours at 37°C. For rescue of phage from selections the OD_{600nm} of glycerol stocks was measured to calculate the volume of innoculum required to represent at least 10 of each clone based on an OD_{600nm} of 1.00 = $3x10^8$ cells. When an OD_{600nm} of 0.5 was reached, 5 ml of bacteria (2×10^9 cells) were transferred to a 50 ml falcon tube and M13-KO7 helper phage (Stratagene) added to give a multiplicity of infection (m.o.i.) of 20:1 of phage:bacteria, followed by incubation at 37°C for 30 minutes. Bacteria were

pelleted by centrifugation at 9000 g for 10 minutes, resuspended in 25 ml 2TY with 100 μg/ml ampicillin and 25 μg/ml kanamycin (2TYAK), transferred to a 250 ml flask and grown overnight with shaking (300 rpm) at 30°C. The next day bacteria were pelleted by centrifugation at 9000 g for 20 minutes and the supernatant transferred to a fresh tube.

Phage were PEG precipitated by adding 1/5th volume of PEG/NaCl and incubating on ice for 1hour. Phage were pelleted by centrifugation at 9000 g for 15 minutes at 4°C and the supernatant discarded. The phage pellet was resuspended in 1 ml sterile PBS using a filter tip and transferred to an eppendorf tube and centrifuged at 15 000 g for 2 minutes to remove any remaining bacteria. The supernatant was transferred to a new tube and a second PEG precipitation performed by adding 200 μl PEG/NaCl and incubating on ice for 20 minutes. Phage were pelleted by centrifuging at 15 000 g for 5 minutes and the supernatant removed. The pellet was carefully washed to remove any remaining PEG before resuspension in 1 ml of sterile PBS and bacteria removed by centrifugation as before. PEG precipitated phage were stored on ice until required.

2.12.2 Growth and rescue of phage particles in 96-well microtitre plates

This method was used for screening large numbers of clones to identify antigen binding clones. A master plate was prepared by inoculating 100 μl 2TYAG in a sterile 96 well flat bottomed plate (Costar, Bucks) with individual colonies using sterile toothpicks, and allowed to grow with shaking overnight at 30°C. The next day the master plate used to innoculate 100 μl 2TYAG in a sterile 96 well round bottomed plate (Costar). This plate was incubated at 37°C with shaking until an OD_{600} 0.5 was reached (approx. 2.5 hours). To each well of the master plate 30 μl of 60% (v/v) glycerol was added, before storage at -70°C. Helper phage were added at a ratio of 20:1 (20 μl/well of helper phage stock in 2TYAG) and incubated at

37°C for 30 minutes. The plates were then spun at 1000 g for 10 minutes and the supernatant removed using a multi-channel pipette. The bacterial pellet was resuspended in 150 µl 2TYAK and cultures grown overnight at 30°C with shaking. The next day the plate was spun at 1000 g for 10 minutes and 50 µl of supernatant used for each phage ELISA.

2.12.3 Induction of soluble protein expression

A 10 ml 2TYAG culture was inoculated using 10 µl of an overnight culture and grown with shaking until an OD_{600nm} of 0.5 was reached. The cells were pelleted by centrifugation and resuspended in 2xTY with 100 µl ampicillin and IPTG added to a final concentration of 1 mM. The culture was grown overnight at 30°C and the cleared supernatant stored at 4°C.

2.13 Selection of phage antibodies from phage display libraries

2.13.1 Panning

Antigen was coated onto immunotubes using 1 ml of 10-50 µg/ml of antigen [NCA (CIS-BIO International, France); STn-HSA conjugate (Biomera, Canada); STn-polyacrylamide conjugates (Syntesome, Russia)] diluted in PBS or BIC coating buffer (depending on the antigen) at 4°C overnight. After coating the tube was rinsed twice with PBS and blocked for a minimum of 1 hour with blocking agent [2% (w/v) MAR, BSA, human serum albumin (HSA) or gelatin]. Phage were blocked with an equal volume of blocking solution (2 x concentration used to block tubes) for 1 hour. The tube was washed twice with PBS, blocked phage added to the tube and incubated for 30 minutes on an end-over-end rotator and then left stationary for 2 hours.

The tube was washed 20 times with PBS-Tween [PBS, 0.1% (v/v) Tween 20] by filling the tube using a wash bottle and immediately tipping out and then a further 20 times with PBS.

Bound phage were eluted by adding 1 ml of freshly prepared 100 mM triethylamine (700 µl triethylamine in 50 ml H_2O) and incubating at room temperature for 10 minutes. The eluted phage were transferred to a 1.5 ml tube containing 0.5 ml 1M Tris-HCl pH 7.4 and either kept on ice or stored at 4°C.

2.13.2 Biotinylated antigen selection

A 500 µl aliquot of phage (10^{12}-10^{13}) was mixed with an equal volume of 4% (w/v) blocking agent in a 1.5 ml tube and incubated at room temperature on a rotator for 1 hour. Streptavidin beads (Dynal, Oslo) [100 µl per selection] were blocked by resuspension in blocking agent and incubation on the rotator for a minimum of 1 hour. Biotinylated antigen (50-500 nM) was added directly to the blocked phage and incubated on the rotator for 1 hour. The beads were resuspended in 250 µl of blocking agent and then added to the phage-antigen mix and incubated on the rotator at room temperature for 15 minutes. The tube was then placed in a magnetic rack for 1 minute and the supernatant carefully removed. The beads were washed 5 times with 1 ml blocking agent and 5 times with 1 ml PBS-Tween 20 and finally resuspended in 100 µl of PBS. Beads were used directly to infect log phase *E.coli.* TG1 cells as described.

2.13.3 Infection

Preparation TG1 E.coli bearing F' pillus

A stock of *E.coli* TG1 (TG1 Δ(lac-pro) supE thi hsdD5/Fɔ traD36 $proA^+B^+$ $lacI^q$ lacZΔM15) cells were prepared to provide a starter culture for growth of TG1 cells expressing the F' pillus required for phage infection. TG1 cells were plated out from frozen stocks onto a minimal media plate and grown overnight at 37°C. A single colony was used to start a 5 ml 2xTY culture which was grown for 8 hours at 37°C and then used to innoculate 50 ml 2xTY for

overnight growth at 37°C. The overnight culture was stored at 4°C and streaked out onto 2TYAG plates to check for ampicillin resistance.

Infection and titration

A 0.5 ml aliquot of stock *E.coli* TG1 cells was used to innoculate 50 ml 2xTY and the culture grown with shaking at 37°C until an OD_{600nm} 0.5 was reached. The neutralised eluted phage were diluted (1 ml of phage with 4 ml 2xTY) and added to 5 ml of the mid-log phase TG1 culture in a 50 ml falcon tube. The culture was then incubated for 30 minutes in a 37°C waterbath. Bacteria were pelleted by centrifugation at 9000 g for 5 minutes and resupended in 1 ml 2xTY. Bacteria were plated out onto two large 2TYAG plates and incubated at 37°C overnight. After overnight growth the bacterial colonies were resuspended in 2TYAG using a glass spreader, the suspension from the 2 plates was pooled and aliquoted into 1.5 ml tubes. Sterile glycerol was added to a final concentration of 15% (v/v) , cells were stored at -70°C.

Selected phage were titrated (output titre) by taking a 50 µl aliquot from the resuspended infected TG1 cells before plating out. A 1/10 dilution series (typically from 10^{-1} to 10^{-4}) was set up and 100 µl of each dilution, alongside an undiluted aliquot plated out onto 2TYAG plates which were incubated at 37°C overnight. The titre of phage stock used for selections (input titre) was also determined. Serial 1/100 dilutions input phage were set up in a final volume of 500 µl. An equal volume of mid-log phase TG1 cells was added to the 10^{-10} and 10^{-12} dilutions. The cells were incubated for 30 minutes 37°C and then 100 µl plated out onto 2TYAG plates.

2.13.4 ELISA screening of selected phage

Phage were rescued in 96 well microtitre plates as described (2.12.2). A Falcon 3192 flexible Immunoassay plate was coated using 100 µl of antigen (5 µg/ml - 20 µg/ml in PBS or BIC buffer) at 4°C overnight. After overnight coating the plate was washed twice with PBS and 150 µl/well of 2% (w/v) blocking agent (MAR, BSA, HSA or gelatin in PBS) added to each well, and the plate blocked for 1 hour at room temperature. The plate was washed then twice with PBS-Tween and 50 µl of 4% (w/v) blocking agent aliquoted into each well. An equal volume of phage supernatant, was added to the appropriate wells and mixed by pipetting. After incubation for 1.5 hours at room temperature the plates were washed 3 times with PBS-Tween. HRP labelled anti-fd antibody (Pharmacia Biotech) was diluted 1/1000 in 1% (w/v) blocking solution, 100 µl added per well and incubated for 1 hour at room temperature. Detection of bound antibody by addition of substrate was as detailed in the ELISA method.

2.14 Immunohistochemistry

2.14.1 Staining of neutrophils

Frozen neutrophil slides (prepared by G Cambridge) were defrosted for 15 minutes at room temperature and then unwrapped and air dried for 5 minutes at room temperature. Slides were fixed in cold ethanol for 10 minutes. Phage supernatants were incubated for 30 minutes and the slides washed twice with PBS. Sheep anti-fd antibody (1/1000) was incubated for 30 minutes, the slides washed and incubated with FITC conjugated goat anti-sheep IgG antibody (DAKO Corporation, Denmark).

2.14.2 Tissue sections

Cryostat sections (5 micron) of LS174T primary human tumour xenografts grown in Balb/c nude mice and human normal and tumour sections were cut and mounted on 3-aminopropyl triethoxysilane glass sides (prepared by G. M. Boxer). Air dried slides were fixed in acetone

for 10 minutes and rinse briefly in tap water. Slides were washed and flooded using Tris buffered saline (TBS) [20 mM Tris, 137 mM NaCl pH 7.6]. Sections were blocked for 20 minutes using a 1/30 dilution of horse serum and then incubated with supernatants for 45 minutes. The sections were then wash 3 times over a 10 minute period using TBS. Binding of the human monoclonal antibody S2.5 was detected using anti-human IgM mouse monoclonal antibody (DAKO). For detection of cmyc-tagged scFv binding the mouse monoclonal antibody 9E10 diluted was used at a concentration of 20 μg/ml. Antibody was incubated for 45 minutes and then the slides washed as before. The biotinylated anti-mouse antibody diluted 1/200 from the ABC kit (Vector Laboratories) was then applied to the sections and incubated for 45 minutes. After washing, diluted avidin-biotin peroxidase complex (2 drops of reagent A to 7.5 mls of TBS followed by 2 drops of reagent B) was incubated on the slides for 1 hour and 45 minutes. The slides were washed and then incubated with DAB substrate [1mg/ml diaminobenzidine tetrahydrochloride with 0.03% (v/v) H_2O_2 added before use] for 5 minutes. The reaction was stopped by washing the slides in tap water. The slides were counterstained using Harris Haematoxylin for 2 minutes and then washed in tap water for 2 minutes. Sections were dehydrated by immersion for 30 seconds in 70%, 90%, 100% (v/v) ethanol and finally in inhibisol (perchloroethylene) before mounting using DPX. Sections were photographed using a Ziess Axiophot microscope with Fuji chrome daylight film using an 80B blue filter.

Chapter 3

Phage display of antibody fragments for the isolation of NCA-95 antigen binding specificities

3.1 Introduction

This chapter describes the two approaches that were used for the isolation of engineered antibody fragments against the neurophil specific marker NCA-95. This antigen has proved to be an informative marker when used as a target for imaging sites of inflammation and infection. The first aproach was to rescue the binding specificity of the NCA-95 binding mouse hybridoma NCA-102 by cloning of V-genes and construction of antibody fragments. As an alternative to the hybridoma derived antibody fragments, selections were performed with NCA-95 against a murine antibody repertoire displayed on phage.

3.1.1 Immunoscintigraphy with monoclonal antibodies against NCA-95

Immunoscintigraphy with ^{99m}Tc-BW 250/183, an anti-NCA-95 monoclonal antibody, has been successfully used to diagnose muscoskeletal infection (Reuland *et al.*, 1991), inflammatory bowel disease (Segarra *et al.*, 1991; Almers *et al.*, 1996) and infective endocarditis (Morguet *et al.*, 1994). More recently immunoscintigraphy with this antibody has been demonstrated to be of clinical value in patients with prolonged undiagnosed fever or fever of unknown origin (FUO). Imaging of neutrophils allowed the diagnosis and elimination of pyogenic causes of FUO and the diagnosis of metastatic malignant disease, high-grade spondylodiskitis and endocarditis (Meller *et al.*, 1998). Bone marrow immunoscintigraphy with ^{99m}Tc-labelled antibodies against NCA-95 has been used to demonstrate the extent of disease as well as patient response to treatment in malignant osteopetrosis (Thelen *et al.*, 1998).

3.1.2 The mouse monoclonal antibody NCA-102

The mouse IgG1 monoclonal antibody NCA-102 was isolated from fusions of NSI/Ag4 plasmocytoma cells with spleen cells from mice immunised with purified CEA (Collet *et al.*, 1991). In vitro cytofluorometry was used to screen for binding to the neutrophil specific

marker, NCA-95. The specificity of NCA-102 was confirmed by immunocytological staining which also showed binding to neutrophil bone marrow precursors. NCA-102 reacts with rat, rabbit, pig, cat and dog peripheral blood as determined by immunoperoxidase staining (Collet *et al.*, 1993).

The suitability of this antibody as an agent for *in vivo* labelling of neutrophils for the scintigraphic detection of inflammatory foci was investigated by Collet and co-workers. The $F(ab')_2$ fragment of NCA-102, determined to have an affinity constant (Ka) of 1.2×10^8 l/mol, was used in order to eliminate Fc mediated clearance and complement mediated toxicity, and possibly reduce any human anti-mouse antibody (HAMA) response. Initial results with ^{111}In-$F(ab')_2$ show that NCA-102 could be used to image foci of infection in patients (Collet *et al.*, 1993).

The low affinity of this antibody however, means that binding and therefore *in-vivo* labelling will be compromised. The affinity of anti-neutrophil antibodies on the efficiency of labelling in humans appears to be of importance with higher affinity antibodies showing a greater proportion of *in-vivo* binding (Becker *et al.*, 1989; Thakur *et al.*, 1996).

Rescuing the V-genes of the murine monoclonal antibody NCA-102 offers the possibility of engineering characteristics of this antibody for optimal imaging by improving affinity using *in-vitro* maturation.

3.2 Cloning the V-genes from NCA-102

3.2.1 Amplification of Fd and K genes for construction of a Fab fragment

Amplification of the heavy chain Fd fragment (VH and CH1 domains) and the complete kappa light chain of NCA-102, for expression of a Fab fragment, was performed using the panel of primers described in section 2.1.2 (Table 2.1). NCA-102 total RNA (prepared by CIS BIO International, France) was used for the synthesis of cDNA using the specific K-ASN and γ-ASN primers and the random oligo dT primer. The quality of the different cDNA templates was compared in the subsequent PCR amplification of heavy and light chain genes. A single heavy chain product of approximately 600 bp with primer mVHb was obtained with both γ-ASN and random primed cDNA [Figure 3.1 (a)].

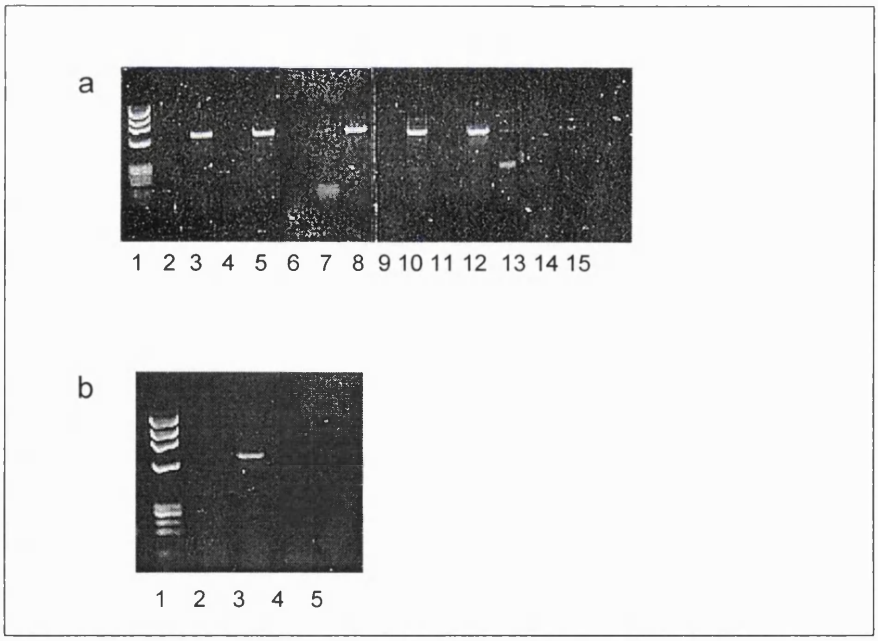


Figure 3.1 **(a)** PCR amplification of random primed and κ-ASN primed cDNA from NCA 102 using primers to amplify the kappa chain (VLCL). Lane 1: φX174 *HaeIII* DNA marker (1353, 1078, 872, 603, 310, 281, 271, 234, 194 bp) Lanes 2-8: random primed cDNA template with primers Vkfr1a-g, Lanes 9-15: light chain cDNA template with primers Vkfr1a-g. **(b)** PCR amplification of random primed cDNA from NCA 102 using primers to amplify the Fd fragment (VHCH1). Lane 1: φX174 *HaeIII* DNA marker, Lanes 2-5: PCR reactions with primers mVH1a-d.

Three light chain products, of approximately 600 bp, were obtained from random primed cDNA with the Vkb, Vkd and Vkg primers. Only 2 products, primed by Vkb andVkd, were obtained with the K-ASN primed cDNA [Figure 3.1 (b)].

The light chain PCR products (from random primed cDNA) were sequenced in order to identify the light chains derived from the NSI/Ag4 fusion partner which is non-secretory but produces light chain mRNA (Kohler *et al.*, 1976). Comparison of light chain sequences with the NSI/Ag4 sequence, revealed that both Vkb and Vkg primed light chains were derived from the NS1 fusion partner. Therefore the Vkd light chain product was used in subsequent amplifications. Sequence analysis of the cloned NCA-102 light chain was performed with DNAplot from the International Immunogenetics database (IMGT) [http://imgt.cnusc.fr:8104/textes/Mppage.html#5]. The sequence alignment is shown to the nearest germline sequences with numbering and CDRs according to IMGT [Figure 3.2]. NCA-102 uses the IGKV4S4 germline sequence from which there are a total of 16 mutations that result in amino acid changes. These include a change of Ser to Thr at codon 30 of CDR1 and Asp to Ala at codon 56 of CDR2.

Secondary PCRs were performed on the heavy and light chains and the pooled PCR products for each chain purified from low melting point agarose and used in the assembly and amplification reactions. Assembly of the Fab fragment was attempted a number of times, varying the template concentration, $MgCl_2$ concentration, annealing time and temperature but did not result in the successful production of a Fab fragment. In order to optimise annealing between the heavy and light chains, the overlapping sequence between the 2 chains was increased from 20 bp to 42 bp by further extension of the light chain using the 5'SD primer. However, variation of annealing conditions failed to produce a Fab fragment. Finally *XhoI*

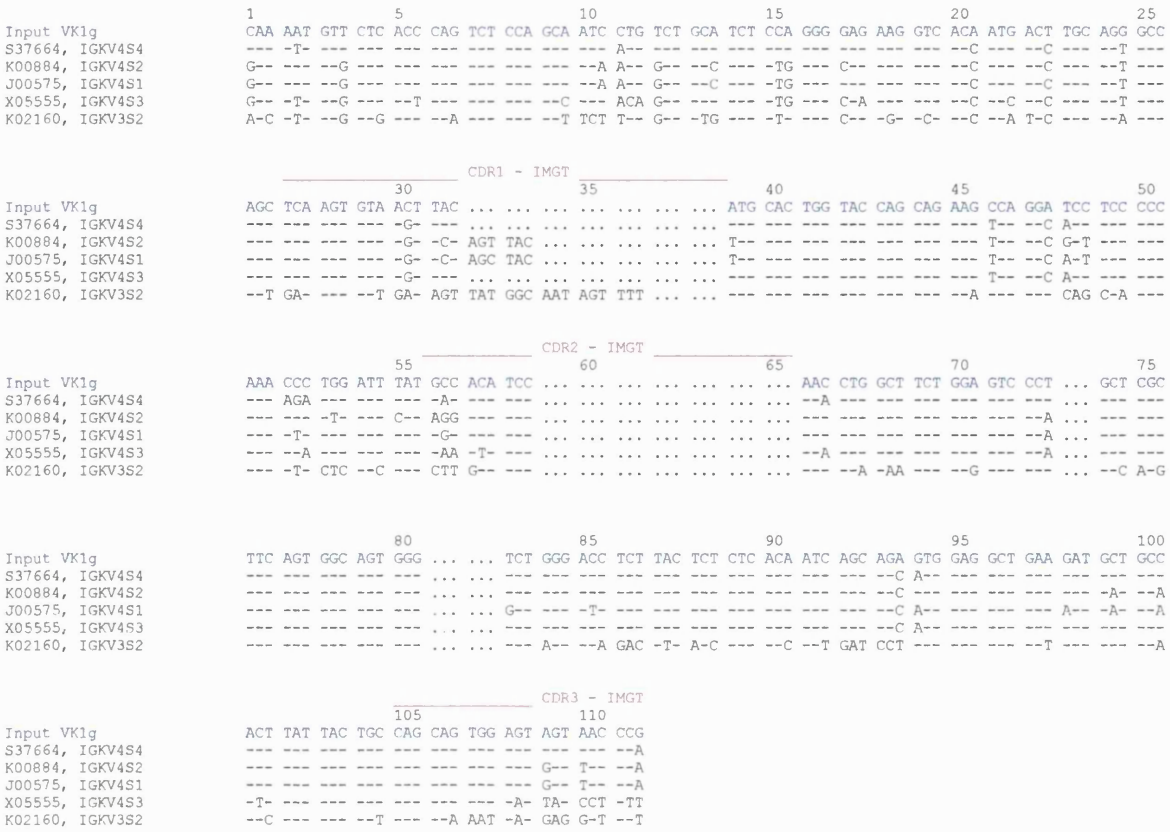


Figure 3.2 Sequence alignment of cloned VK gene from the murine monoclonal antibody NCA-102 (numbering and CDRs according to IMGT).

digestion of the extended heavy and light chains was performed followed by ligation, the ligation mix was used for amplification of the Fab using the appropriate primers but was also unsuccessful in generating a Fab fragment. Therefore, as an alternative, the expression of NCA-102 as a scFv was attempted.

3.2.2 Cloning of NCA-102 as a scFv

Modified primers were designed for the amplification of variable region sequences (VH and VL) together with primers enabling the incorporation of the $(Gly_4Ser)_3$ linker (Bird *et al.*, 1988; Huston *et al.*, 1988) [Table 2.1]. PCR products of approximately 350 bp, were obtained

from random primed cDNA, using VH1Bsc-5' with VHsc-ASN-A for the heavy chain and Vkd with Vksc-3'B for the light chain [Figure 3.3]. Approximately 5 ng of each purified

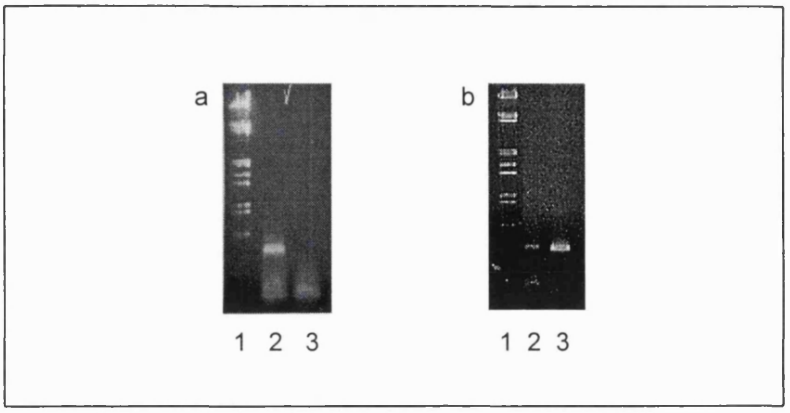


Figure 3.3 **(a)** PCR amplification of the VH gene from NCA 102 cDNA, Lane 1: λ *HindIII/EcoRI* DNA marker (21 227, 5148, 4973, 4268, 3530, 2027, 1094, 1584, 1375, 974, 831, 564, 125bp) Lane 2: reaction primed with VH-sc-ASN-A, Lane 3: reaction primed with VH-sc-ASN-B. **(b)** PCR amplification of the VL gene from NCA 102 cDNA, Lane 1: λ *HindIII/EcoRI* DNA marker, Lane 2: reaction primed with VKsc-3'A, Lane 3: reaction primed with VKsc-3'B.

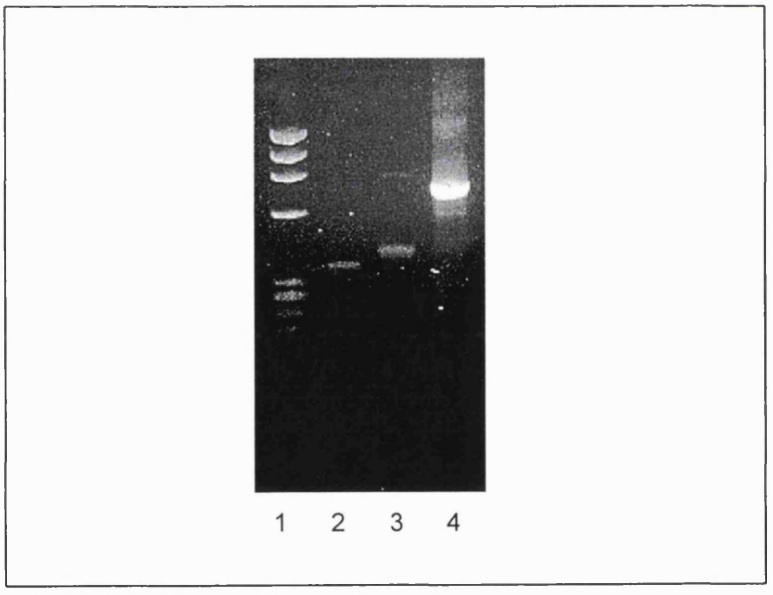


Figure 3.4 PCR assembly of scFv gene derived from NCA 102, Lane 1: φX174 *HaeIII* DNA marker (1353, 1078, 872, 603, 310, 281, 271, 234, 194 bp), Lane 2: VKsc-3'B primed light chain PCR product, Lane 3: VH-sc-ASN-A primed heavy chain PCR product, Lane 4: scFv assembled from heavy and light chain PCR products.

heavy and light chain was used in the assembly PCR and the scFv fragment amplified with Vkd and VHsc-ASN-A primers [Figure 3.4]. The scFv was then cloned into the phagemid vector *pFAB5cHis* for expression as a gene III fusion protein and chemically competent *E.coli* cells transformed. PEG precipitated phage were tested for binding to neutrophils by immunohistochemistry and to NCA-95 by ELISA. However, despite the production of phage, functional binding of the NCA-102 derived scFv could not be demonstrated.

3.3 Selecting for NCA-95 binding clones from an immune CEA scFv repertoire

Selection from a phage display library for NCA-95 binding clones was investigated as an alternative to the construction and expression of NCA-102 derived Fab or scFv fragments. The murine scFv library of 2.8 x 10^5 clones, derived from the spleen of a mouse immunised with CEA (Chester *et al.*, 1994), was used. Selection from an immune repertoire offered the possibility of isolating antibody fragments with a range of binding specificities as well as clones with a higher affinity than that of the monoclonal antibody NCA-102.

Three rounds of selections were performed by panning against NCA-95 coated immunotubes together with control selections using CEA. Marvel was used as a blocking agent. A 31 fold enrichment was observed after the 2nd round of selection with NCA-95 and a 83 fold enrichment after the 3rd round of selection with CEA [Table 3.1].

Table 3.1 Selection performed with the CEA library

selection round	Enrichment[a] NCA (CEA)	binding[b] clones (%)
1	1	nd[c]
2	31 (6)	nd[c]
3	37 (83)	14/29 (48)

[a] output round n/input round n divided by output round 1 /input round 1, [b] as determined by screening for binding to NCA-95, [c] nd not determined

3.4.1 Screening of selections identifies NCA-95 binding phage

Individual clones from the 3rd round of selection were screened for binding to NCA-95 by phage ELISA. Forty eight percent of the clones tested showed binding to NCA-95 [Table 3.2]. Phage that showed binding to NCA-95 were also cross-reactive with CEA. Sequence analysis, by T-tracking, of ten clones identified 4 different sequences.

3.4.2 Soluble scFvs bind to NCA-95 and CEA

Each of the four different scFv clones were subcloned from *pHEN* into *puc119polyhis* for soluble scFv expression. Bacterial supernatants were used directly in ELISA, after overnight induction with IPTG, to test the binding of myc-tagged scFvs. The results with soluble scFvs confirmed that clones binding to NCA-95 also bound to CEA [Figure 3.6].

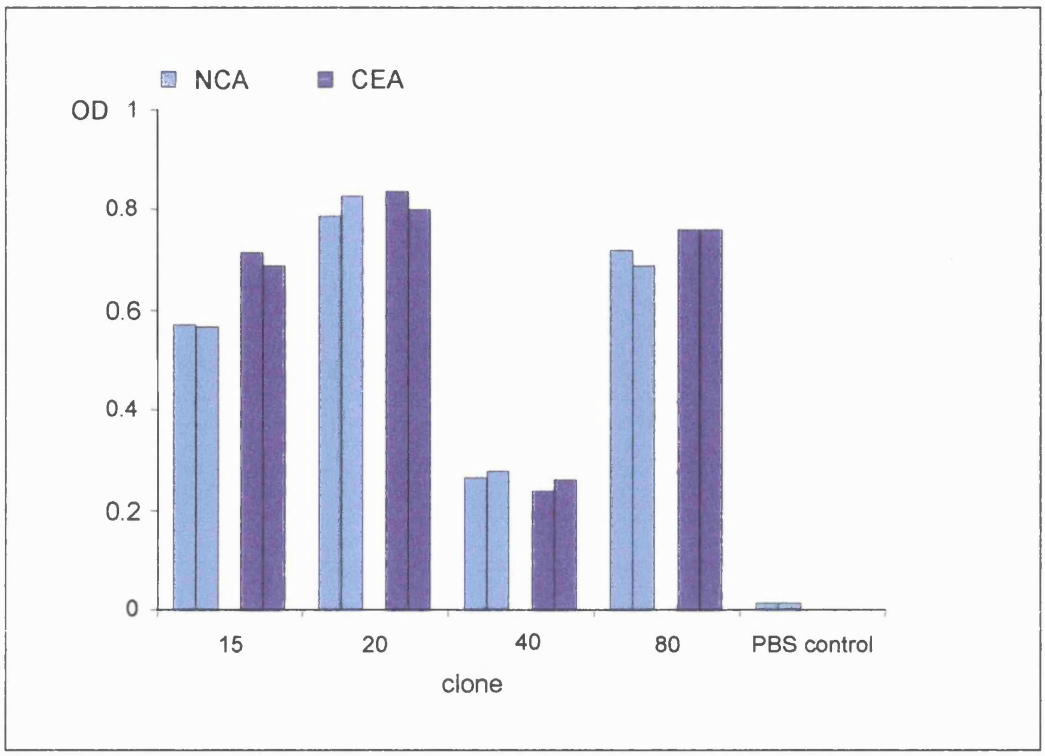


Figure 3.5 Binding of soluble scFv antibody fragments to NCA and CEA. Clones 15, 20, 40 and 40 were induced overnight with IPTG for soluble antibody expression. Culture supernatants were tested by ELISA for functional binding to NCA-95 and CEA which was detected using the anti-myc antibody 9E10.

3.4 Discussion

In this chapter 2 different approaches for isolating binding specificities against the neutrophil specific marker NCA-95 were investigated. V-genes were rescued from a hybridoma for construction of a functional antibody fragment and selections performed with an immune antibody repertoire using phage display in order to isolate desired binding specificities.

Firstly, rescue of NCA-95 binding specificity was attempted by cloning of V-genes from the murine hybridoma NCA-102. This monoclonal antibody had demonstrated neutrophil specific binding (Collet *et al.*, 1993) and therefore had potential use as an imaging agent for sites of inflammation and infection. So, despite the many known difficulties associated with rescuing

V-genes from hybridomas this method was used as it provided a means of rescuing an important binding specificity and also the possibility of improving affinity.

Many of the obstacles associated with engineering antibody fragments from hybridomas were encountered with cloning of NCA-102. Firstly, amplification of the light chain (VLCL) was complicated by additional V-gene products derived from the Ig mRNA of the myeloma NSI/Ag4 fusion partner. The presence of such additional rearranged antibody sequences is commonly observed when rescuing V-genes from hybridomas (de Haard *et al.*, 1998). Sequencing was therefore required to identify the hybridoma derived light chain.

Despite many efforts to optimise both annealing and PCR conditions, extension of overlapping sequence as well as ligation of heavy and light chains, a Fab fragment could not be constructed. The inability to link heavy and light chains in the overlapping reaction is often due to a difference in the relative amounts of heavy and light chain PCR products. However, titration of heavy and light chain PCR products against one another for the assembly PCRs failed to generate a product of the correct size.

It is possible that secondary structure formation in the constant regions of the amplified heavy and light chains may have prevented annealing of the heavy and light chains (Orum *et al.*, 1993). Formation of such structures are indicated by the required optimisation of PCR conditions for amplification of the heavy chain as well as difficulties encountered during sequencing of the constant region of the light chain. This means that annealing of the heavy and light chains in the assembly reaction would not have been possible, as the low temperatures used would have favoured secondary structures.

In order to minimise the influence of secondary structures, the alternative scFv format was chosen for cloning. This fragment could be constructed by elimination the sequences encoding the constant region domains (CL and CH1). Amplified heavy and light chain V-region genes were joined with a $(Gly_4Ser)_3$ linker to form a scFv. This was achieved without extensive optimisation of PCR or annealing conditions.

The scFv was cloned into the phagemid vector *pFAB5cHis* for expression as a gene III fusion protein. Despite several attempts, functional binding of phage could not be demonstrated to either neutrophils by immunohistochemistry or NCA-95 by ELISA. Others have also encountered difficulties in demonstration of functional binding when rescuing hybridomas (Roovers *et al.*, 1998), reporting typical frequencies of binding clones of 11/90 clones tested (Clackson *et al.*, 1991). And, where many non-binding clones are generated phage display has even been used for retrieval of desired binding specificities (Krebber *et al.*, 1997, Roovers *et al.*, 1998). Non-binding fragments obtained after cloning of hybridomas as antibody fragments have been estimated to comprise of 50-95% clones that are generated (Bradbury *et al.*, 1995). Such clones may arise through the introduction of errors during PCR, these can result in frame shifts, the introduction of stop codons due to miscalling of bases or the addition/deletion of bases. It is quite possible therefore, that a functionally binding scFv fragment was present within the population of generated fragments which could have been isolated if extensive screening had been employed. Prior to screening however, *BstN1* analysis and sequencing of V-genes amplified from independent PCRs should be have been performed in order to determine the level of diversity present within the V-gene population as well as identify the presence/extent of PCR introduced errors.

Another factor that requires consideration is that format that was chosen to screen for binding reactivity. Antibody fragments displayed on the surface of phage is the most commonly used format for screening for functional binding antibodies. An advantage of using this format is that multivalent display may allow identification of clones with weak binding due to an increase in functional binding. However, the amount of antibody displayed cannot be determined which means that if the antibody is poorly displayed, either because of inefficient rescue or due to cleavage of the fragment from gene III protein, it will be difficult to determine functional binding reactivity. For this reason screening should also have been performed using induced soluble antibody, purified and quantitated from different clones.

As an alternative to rescuing the binding specificity of NCA-102, selection of NCA-95 binding scFv fragments from an immune repertoire using phage display was attempted. Panning with NCA-95 was performed alongside CEA and gave different enrichment values (rounds 2 and 3) for each antigen indicating that selections were antigen dependent. Selections resulted in the isolation of 4 different clones, the binding of these clones to both NCA and CEA was demonstrated with soluble scFv fragments and scFv fragments expressed on the surface of phage.

Selections were performed using the NCA-95 antigen with the CEA immune repertoire on the basis that specificities against NCA-95 should be present due to the high degree of homology that exists between these two molecules. The results of the selection, and preliminary characterisation of binding of individual clones, show that it is possible to select from an immune repertoire using an antigen that is closely related to the antigen used for immunisation. Thus, demonstrating a broader application for the CEA immune repertoire. Although NCA-95 specific antibodies would be desirable, it was isolation of clones able to

recognise NCA-95 in the first instance that was of most importance. Indeed, cross-reactivity with CEA need not necessarily prevent use of such antibodies for the detection of inflammatory foci, as demonstrated for the CEA cross-reactive hybridoma NCA-102 (Collet *et al.*, 1993).

Summary

Rescue of the hybridoma NCA-102 by cloning of V-genes for the construction of functional antibody fragments was unsuccessful. Cloned Heavy (VH CHI) and Light (VL CL) chain genes could not be used to construct a Fab fragment. The construction of a scFv from cloned V-genes proved to be successful however, functional binding could not be demonstrated. It is possible that the NCA-95 binding specificity may have been retrieved if the generated clones had been screened more extensively.

The selections performed with the CEA immune repertoires resulted in the isolation of 4 different clones that demonstrated binding to both NCA-95 and CEA. These results show that it is possible to select for specificities against an antigen that is related to the immunogen used to generate the immune repertoire. The affinity/specificity of the selected clones have the potential to be fine tuned to create antibody fragments with desired characteristics.

Chapter 4

Selection from phage display libraries to identify antibodies binding to STn, a tumour associated mucin carbohydrate antigen

4.1 Introduction

In this chapter selection of antibody fragments from human and murine libraries with specificities against the tumour associated antigen STn is described. The rare expression of this simple mucin-type carbohydrate antigen in normal tissues and its wide expression in a variety of carcinomas has meant that antibodies directed against STn can be used to provide diagnostic (Sakahara *et al.*, 1995) as well as prognostic information (Itzkowitz *et al.*, 1990a; Maeda *et al.*, 1994; Werther *et al.*, 1994; Terashima *et al.*, 1998). Anti-STn antibodies have also been used as diagnostic and/or prognostic tools for the detection of STn in patient sera in colorectal (Imada *et al.*, 1999a; Sato *et al.*, 1999) gastric (Takahashi *et al.*, 1994; Imada *et al.*, 1999b), breast (Albuquerque *et al.*, 1995), ovarian (Kobayashi *et al.*, 1992; Kudoh *et al.*, 1999) and gynaecological cancers (Ryuko *et al.*, 1992). Selection of STn binding specificities from antibody repertoires displayed on phage offers an alternative, potentially faster (compared to hybridoma technology) route for the isolation of such antibodies. Furthermore, the use of human antibody repertoires allows direct isolation of human antibodies.

4.1.1 Selection for STn binding antibodies from phage display libraries

Selections for STn binding antibody fragments were performed with three different repertoires. Two human naïve repertoires, a scFv library of 1.4 x 10^{10} different clones (Vaughan *et al.*, 1996) and a Fab library of 3.7 x 10^{10} different clones (de Haard *et al.*, 1999), and an immune scFv repertoire of 1.4 x 10^{7} clones prepared from a mouse immunised with the MUC1 producing breast cancer cell lines T47D, MCF7 and ZR75 [Table 4.1].

Table 4.1 Antibody repertoires, antigens and blocking agents used for selections

repertoire	source of B cells	antigen[a]	blocking agent[b]
Naïve human scFv, 1.4 x 10^{10} clones	tonsil, PBLs and bone marrow from 43 non-immunised human donors (Vaughan *et al.*, 1996).	STn-HSA, STn-PAA	BSA, HSA, MAR
Immune murine scFv 1.4 x 10^{7} clones	spleen from mouse immunised with MUC1 producing breast cancer cell lines T47D, MCF7 and ZR75 (K. A. Chester, personal communication)	STn-PAA, MUC1 peptide	Gelatin
Naïve human Fab, 3.7 x 10^{10} clones	PBLs from 4 healthy donors and part of tumour-free spleen from a patient with gastric carcinoma (de Haard *et al.*, 1999)	STn-PAA, Gal-PAA	MAR

[a] antigen conjugated to human serum albumin (HSA) and poly[N-C2-hydroxyethyl] acrylamide (PAA).
[b] bovine serum albumin (BSA), skimmed milk powder marvel (MAR)

4.1.2. Other antigens used for selections

4.1.2.1 The α-galactosyl antigen

The carbohydrate antigen Galα1-3Galβ1-4GlcNAc-R (α-galactosyl antigen) produces a polyclonal response that constitutes approximately 1% of human IgG (Galili *et al.*, 1984). It has been estimated that as many as 1% of circulating B cells are capable of producing this antibody (Galili *et al.*, 1993). Since the immune response this antigen generates is seen in all four IgG subclasses (Ravindran *et al.*, 1988) as well as IgM and IgA isotypes (Collins *et al.*, 1995; Hamadeh *et al.*, 1995), this specificity should be represented in a naïve antibody library. Selections were therefore performed against the naïve human scFv and Fab library using a polyacrylamide conjugate of the α-galactosyl antigen (Gal-PAA) as a 'positive' control for the selection of carbohydrate binding phage antibodies. Any clones selected by this method would themselves be of interest as they would be useful in a variety of clinical situations including hyperacute graft rejection, tumour immunology and autoimmunity.

4.1.2.2 MUC1 peptide

A MUC1 60-mer peptide (a trimer of the MUC1 tandem repeat) was used for selections performed with the murine MUC1 scFv library. This peptide was used to compare the selection results obtained using peptide and carbohydrate antigens.

4.2 Testing binding of STn

4.2.1 Determination of an optimum STn-HSA concentration for coating immunotubes

Immunotubes were coated using 5 μg/ml, 10 μg/ml, 20 μg/ml and 40 μg/ml of STn-HSA and HSA which was used as a negative control. The antigen was detected using anti-STn mouse monoclonal antibody (DAKO) with 3% BSA as a blocking agent. Maximum binding was observed with 20 μg/ml of STn-HSA. This concentration was then used to determine the best blocking agent for detection of this antigen and to determine binding of STn-HSA to different ELISA plates [Figure 4.1].

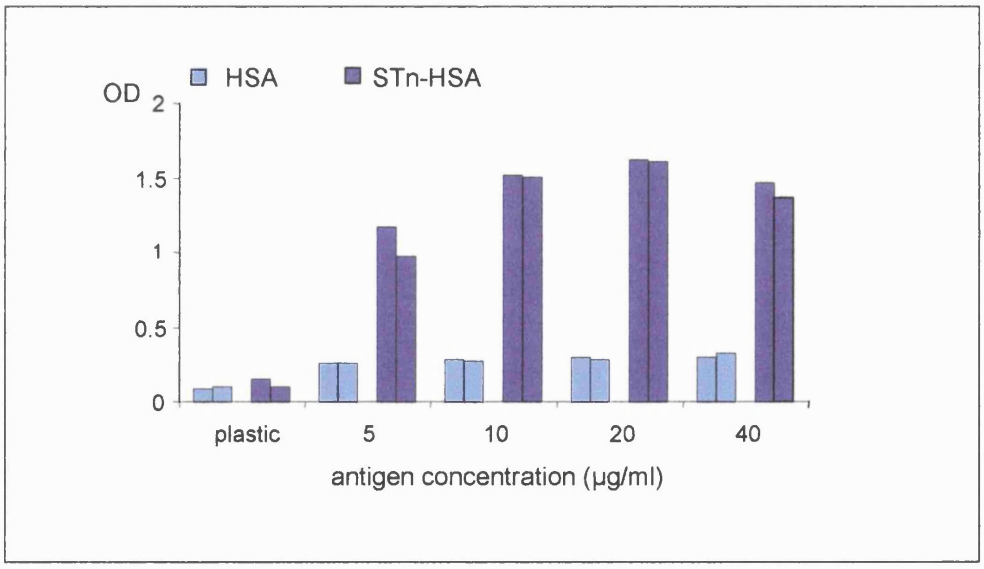


Figure 4.1 Determination of optimum STn-HSA concentration for coating immunotubes. Immunotubes were coated using 5 μg/ml, 10 μg/ml, 20 μg/ml and 40 μg/ml of STn-HSA and HSA which was used as a negative control. The antigen was detected using an anti-STn mouse monoclonal antibody.

4.2.2. Comparison of different blocking agents for the detection of STn coated onto immunotubes

Three tests were performed in parallel to compare the detection of coated STn-HSA (20 μg/ml) using 3% Marvel skimmed milk powder (MAR), 3% BSA and 3% HSA as blocking agents. HSA and BIC buffer coated tubes were used as negative controls. The test performed using BSA as a blocking agent gave the highest signal to noise ratios [Figure 4.2] STn-PAA was coated onto immunotubes at 20 μg/ml. Tests were performed to compare 3% BSA and 3% MAR as blocking agents. Glc-PAA and BIC buffer coated tubes were used as negative controls. The 2 blocking agents BSA and MAR gave comparable signals for the detection of STn-PAA [Figure 4.3]

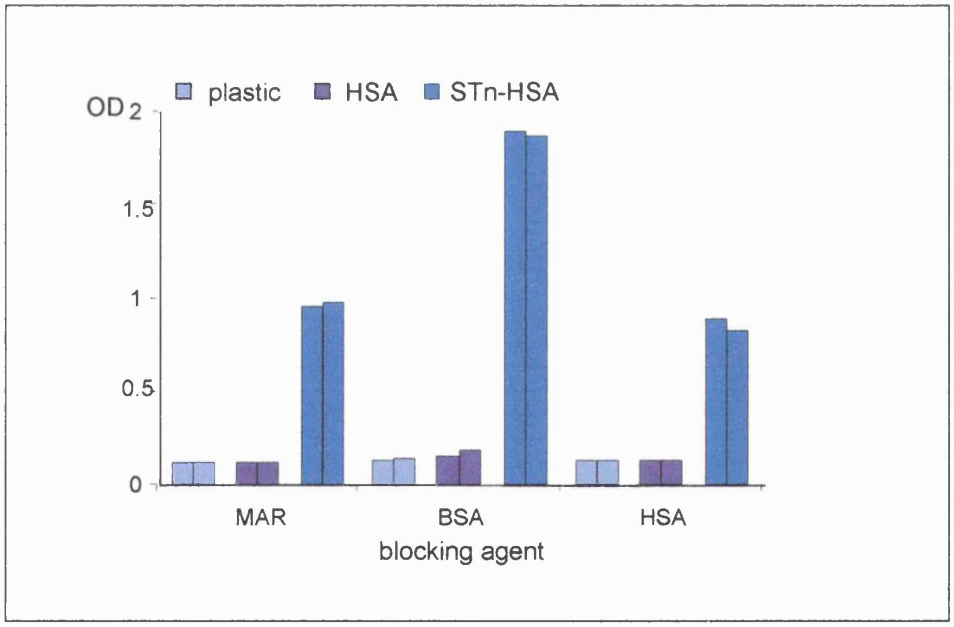


Figure 4.2 Comparison of different blocking agents for the detection of STn-HSA on immunotubes. STn-HSA coated at onto immunotubes at 20 μg/ml and detected using the anti-STn monoclonal antibody with three different blocking agents: 3% Marvel skimmed milk powder (MAR), 3% BSA and 3% HSA. HSA and BIC buffer coated tubes were used as negative controls.

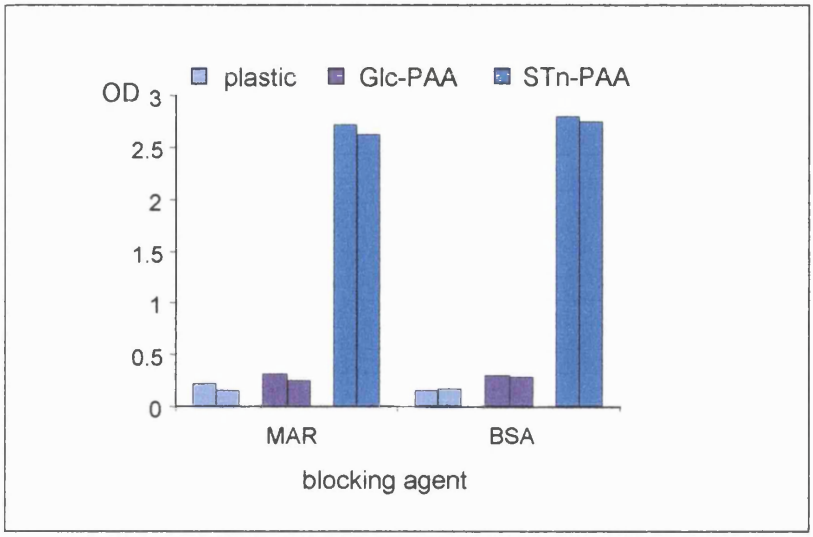


Figure 4.3 Comparison of blocking agents for the detection of STn-PAA on immunotubes. STn-PAA was coated onto immunotubes at 20 μg/ml and detected using the anti-STn monoclonal antibody with 3% BSA and 3% MAR as blocking agents. Glc-PAA and BIC buffer coated tubes were used as negative controls.

4.2.3 Testing binding of STn to ELISA plates

4.2.3.1 Comparison of different ELISA plates for the coating of STn

Three different plates, Nunc maxisorp, Falcon 3912 and Immunolon 4 Dynatech strips were compared for maximal binding of STn-HSA. Antigen was coated at 5, 10 and 20 μg/ml. The anti-STn monoclonal antibody was titered (1/50, 1/100, 1/300) along with the alkaline phosphatase labelled rabbit anti-mouse antibody (1/500, 1/1000) in order to determine optimal concentrations required for detection of coated antigen. The highest signal to noise ratio was obtained with the Falcon 3912 plate with STn-HSA coated at 20 μg/ml, using 1/50 anti-STn Mab and 1/1000 alkaline phosphatase labelled rabbit anti-mouse Mab [Figure 4.4].

4.2.3.2 Comparison of 2 hour and overnight coating of STn

STn-HSA and STn-PAA were coated at 5 μg/ml and 10 μg/ml for 2 hours or overnight, wells coated with HSA (10 μg/ml) and with coating buffer were used as negative controls. STn was detected with 1/10 and 1/50 dilutions of anti-STn monoclonal antibody using 3% BSA to

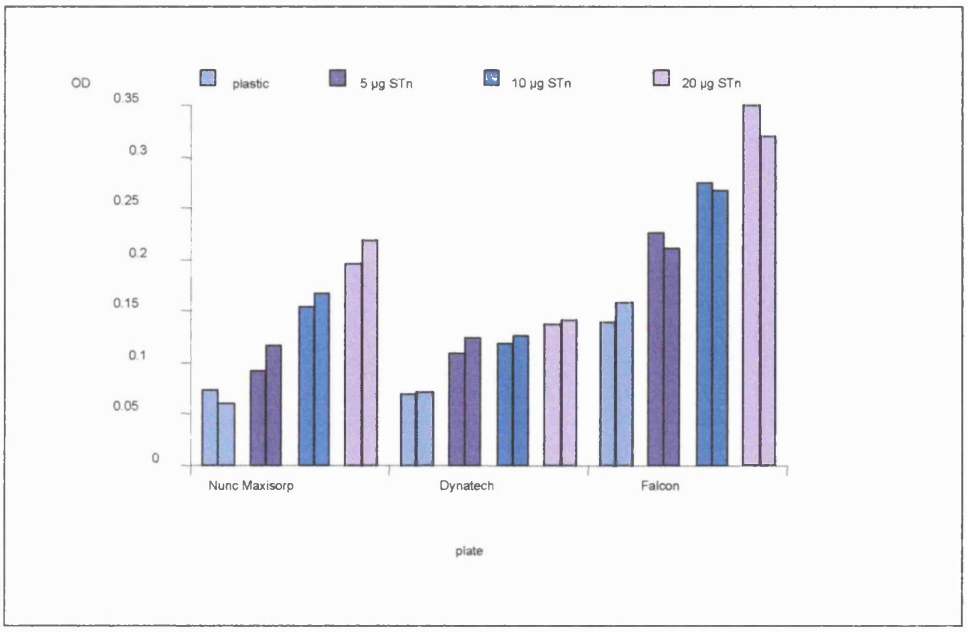


Figure 4.4 Comparison of different ELISA plates for coating of STn. Nunc maxisorp, Falcon 3912 and Immunolon 4 Dynatech strips were compared for maximal binding of STn-HSA. Antigen was coated at 5, 10 and 20 µg/ml and detected using a 1/50 dilution of anti-STn monoclonal and a 1/1000 dilution of alkaline phosphatase labelled rabbit anti-mouse antibody.

block. The observations were made with both antibody dilutions, the only difference being a decrease in signal intensity with the higher dilution. STn-HSA that had been coated for 2 hours gave a good signal at both coating concentrations. However, STn-PAA could not be detected after coating for 2 hours. Overnight coating of the antigens did not increase the signal observed with STn-HSA any further, whereas STn-PAA could only be detected on the plate after overnight coating [Figure 4.5].

4.2.3.4 Comparison of Marvel and Tween 20 as blocking agents

The skimmed milk powder Marvel that is used as a blocking agent contains a high proportion (approximately 52%) of carbohydrate. The small O-linked sugars and many different free sugars that are present in bovine milk are known to contain sialic acid (NeuAc) and GalNAc residues (Glockner, 1976; Hvarregaard, 1996). In order to determine if any of the sugars

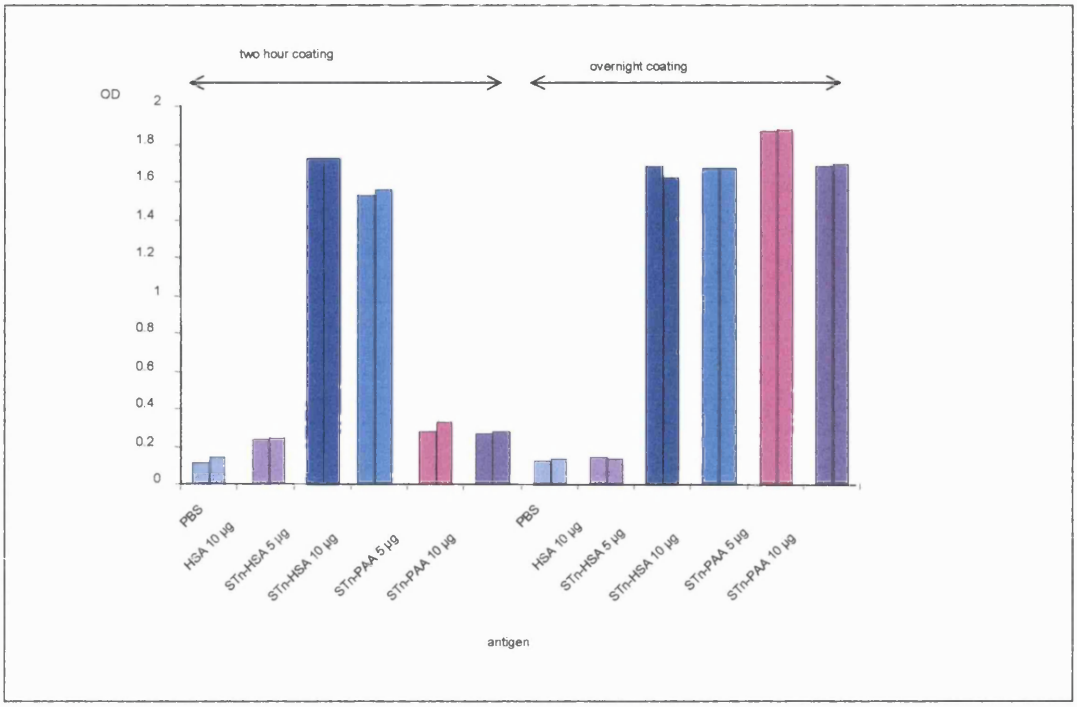


Figure 4.5 Comparison of 2 hour and overnight coating of STn. STn-HSA and STn-PAA were coated at 5 µg/ml and 10 µg/ml for 2 hours or overnight, HSA (10 µg/ml) coated wells and coating buffer were used as negative controls. STn was detected with a 1/50 dilutions of anti-STn monoclonal antibody using 3% BSA to block.

in Marvel contributed to a loss of signal in the detection of STn, the blocking agents MAR and Tween 20 were compared. The antigens were coated at 10 µg/ml with HSA, Glc-PAA, NeuAc-PAA and BIC used as negative controls. Specific signals were obtained for both forms of antigen with both MAR and Tween 20 but differences were seen between the antigens and the different blocks. STn-HSA when blocked with Tween 20 gave an OD that was 1 OD unit higher than that obtained when MAR was used. With STn-PAA a slightly higher signal (0.5 OD unit greater) was obtained with MAR compared to Tween 20 [Figure 4.6].

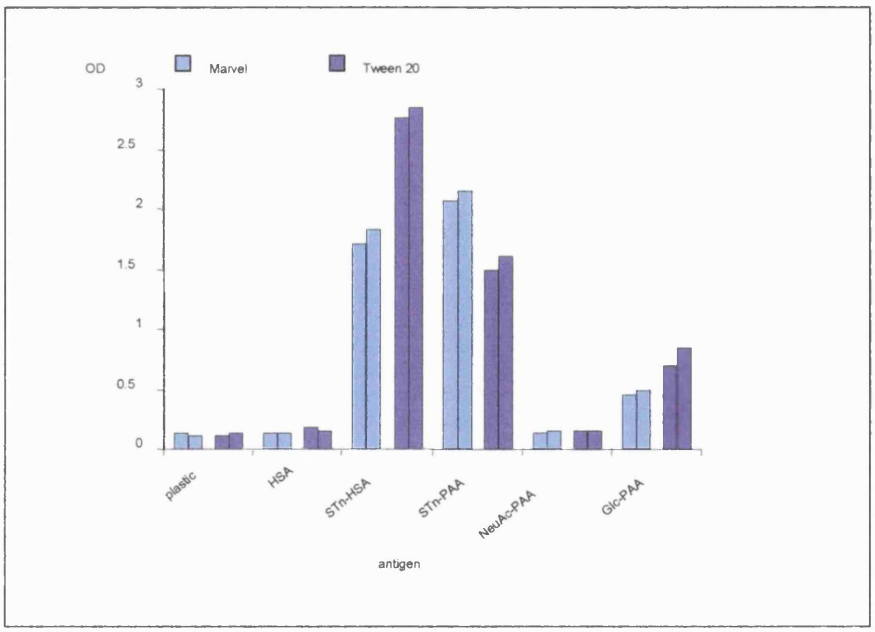


Figure 4.6 Comparison of Marvel and Tween 20 blocking agents for detection of STn. STn-HSA and STN-PAA were coated at 10 μg/ml with HSA, Glc-PAA, NeuAc-PAA and BIC used as negative controls. Wells were blocked with either MAR and Tween 20 and STn detected as before

4.2.4 Lectin detection of STn

The elderberry bark lectin (*Sambucus Nigra*) was used as an alternative to antibody detection to confirm that this antigen could be coated onto plastic without affecting its conformation. STn-HSA, STn-PAA and Neu-PAA were coated at 20 μg/ml onto a Falcon 3912 plate. STn was detected with a 1/100 dilution of biotinylated lectin and 1/1000 dilution of horseradish peroxidase labeled streptavidin using 2% BSA to block. The elderberry bark lectin showed specific binding to both forms of STn [Figure 4.7].

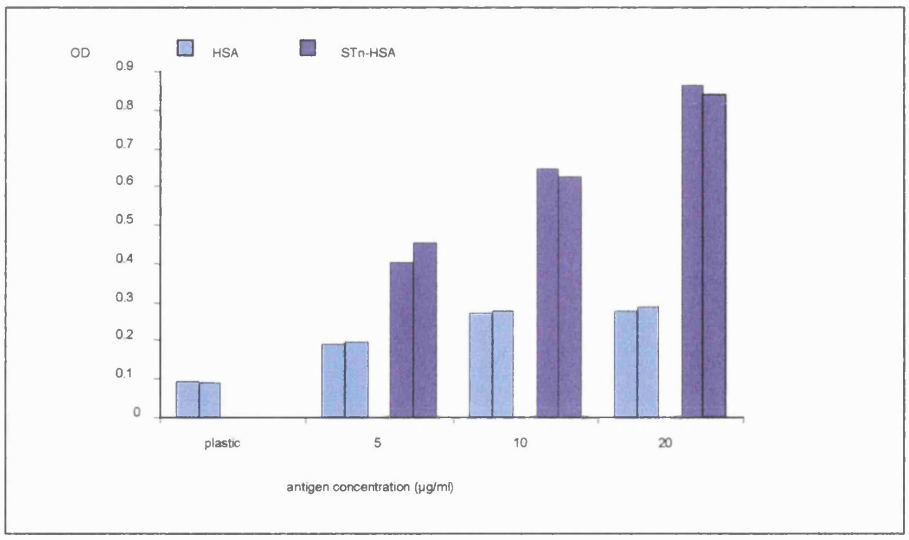


Figure 4.7 Elderberry bark lectin (*Sambucus Nigra*) detection of STn-HSA. STn-HSA, STn-PAA and Neu-PAA were coated at 20 µg/ml and detected with a 1/100 dilution of biotinylated lectin and a 1/1000 dilution of horseradish peroxidase labeled streptavidin using 2% BSA to block.

4.3 Naïve scFv repertoire selections

The first selection was performed by panning against an STn-HSA coated immunotube blocked with 3% BSA. For the first round, only 10 washes were performed before elution of bound phage. Selection strategies with different STn conjugates and blocking agents applied to the 9.2 x 10^4 clones obtained after this first round of selection are outlined in Figure 4.8.

4.3.1 Selections by panning on STn coated immunotubes

4.3.1.1 STn-HSA selections

The first selection strategy involved panning against STn-HSA for 4 rounds. After the first round of selection, 2% HSA was used to block in order to eliminate any phage that might bind HSA. An 8000 fold enrichment was obtained after 3 rounds of selection which did not increase after the fourth round [Table 4.2]. The third round of selection was then repeated with STn-HSA, 2 selections were performed in parallel to compare the results obtained using the blocking agents 2% BSA and 2% MAR. A significant difference in enrichment was

obtained between round 3 selections that had been blocked with BSA, MAR and HSA. A 3 fold and 25 fold enrichment was obtained with BSA and MAR respectively, compared with the 8000 fold enrichment when HSA was used [Table 4.2].

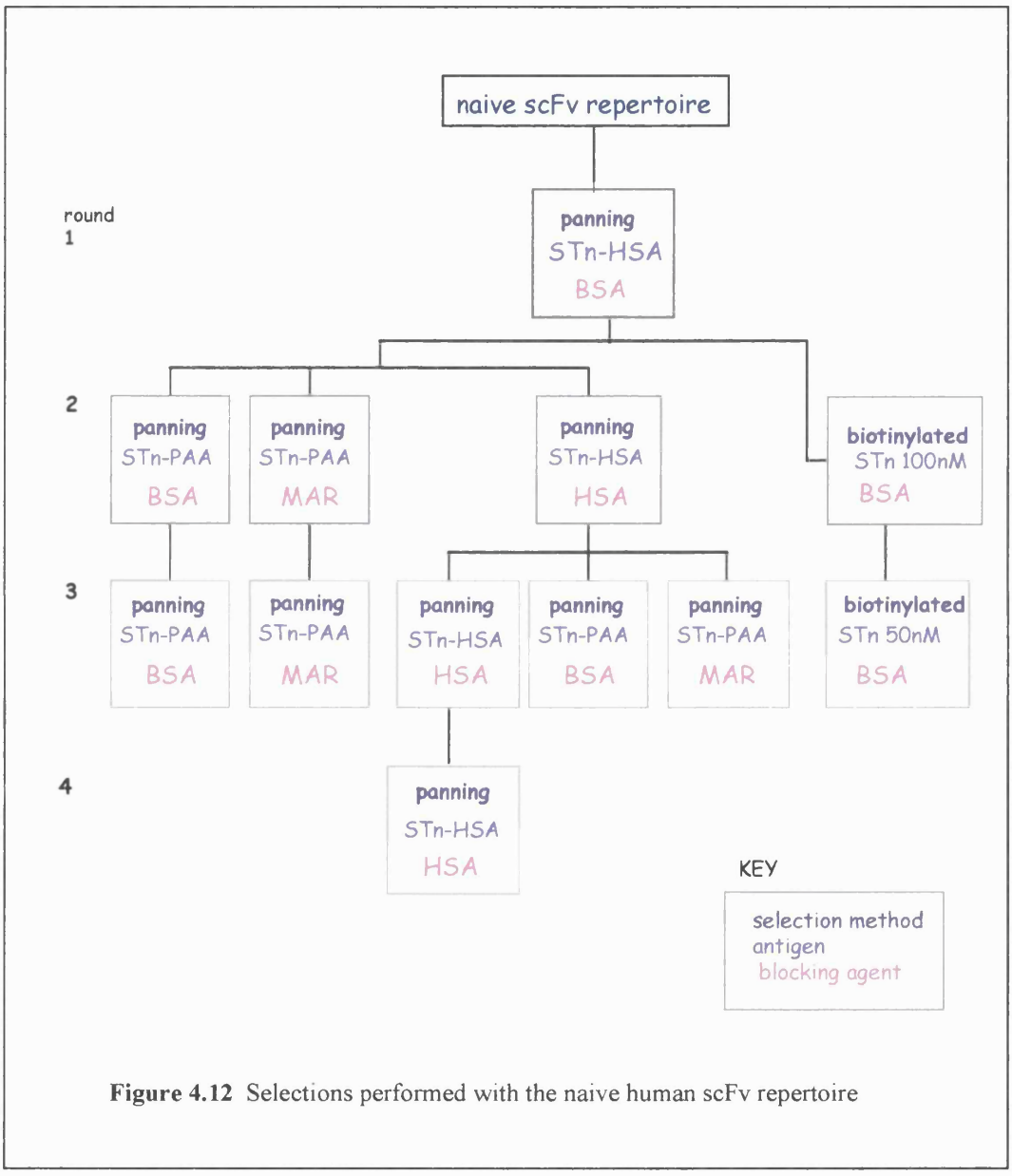


Figure 4.12 Selections performed with the naive human scFv repertoire

Table 4.2 Selection by panning against coated STn-HSA

selection round	antigen concentration	blocking agent	enrichment[a]	specific binding (%)	non-specific binding (%)
1	STn-HSA 20 μg/ml	BSA	1	0/32	1/32 (3)
2	STn-HSA 20 μg/ml	HSA	3	0/24	6/24 (17)
3(i)[b]	STn-HSA 20 μg/ml	HSA	8000	0/24	4/24 (17)
4	STn-HSA 20 μg/ml	HSA	4000	0/32	23/32 (72)
3(ii)[b]	STn-HSA 20 μg/ml	BSA	3	0/94	22/94 (23)
3(iii)[b]	STn-HSA 20 μg/ml	MAR	25	0/94	30/94 (32)

[a] output round n/input round n divided by output round 1/input round 1 [b] Three different round 3 selections

4.3.1.2 STn-HSA and STn-PAA selections

After the first round of selection using STn-HSA the second and third round selections were performed using STn-PAA. Separate second and third round selections were performed using 2% BSA and 2% MAR to block. An enrichment of 7600 fold was obtained when the selections were blocked with BSA and only 14 fold when MAR was used [Table 4.3].

Table 4.3 Selection by panning against coated STn-HSA and STn-PAA

selection round	antigen concentration	blocking agent	enrichment[a]	specific binding (%)	non-specific binding (%)
1	STn-HSA 20 μg/ml	BSA	1	0/32	1/32 (3)
2(i)	STn-PAA 20 μg/ml	BSA	0.1	0/94	22/94 (23)
3(i)	STn-PAA 20 μg/ml	BSA	7600	0/94	32/94 (34)
2(ii)	STn-PAA 20 μg/ml	MAR	5	0/94	29/94 (30)
3(iii)	STn-PAA 20 μg/ml	MAR	14	0/94	37/94 (40)

[a] output round n/input round n divided by output round 1/input round 1

4.3.1.3 Selections performed by panning and with biotinylated antigen

The final approach used with the naïve scFv library was to combine panning with solution phase selection using biotinylated antigen. After the first round of selection by panning against coated STn-HSA, second and third round selections were performed with 100nM and 50nM of biotinylated STn-PAA (biot-STn) respectively, 2% BSA was used to block. Control selections were performed with 100nM and 50nM of the biotinylated Glc-PAA and NeuAc-PAA carbohydrate antigens. The biotinylated STn selections gave enrichment of 2×10^5 after the 3rd round of selection, similar enrichment was observed with the control selections [Table 4.4].

Table 4.4 Selection by panning against coated STn and with biotinylated STn

selection round	antigen concentration	enrichment[c]	specific binding (%)	non-specific binding (%)
1	STn-HSA[a] 20 μg/ml	1	0/32	1/32 (3)
2	biot-STn[b] 100 nM	435	0/80	26/80 (30)
3	biot-STn[b] 50 nM	2×10^5	0/80	48/80 (60)

[a] BSA used to block selection, [b] MAR used to block selections, [c] output round n/input round n divided by output round 1/input round 1

4.3.2 Screening for specific clones from the naïve scFv library selections

Individual clones from selection rounds were screened for specific binding using the phage ELISA method. The numbers of clones screened from the individual rounds of each selection strategy are shown in Tables 4.2, 4.3 and 4.4. ELISA screening of individual clones failed to identify specific binders in any of the selection rounds. However after the 2nd, 3rd and 4th round selections, for all of the selection strategies, a high proportion of non-specific binding clones that gave strong ELISA signals were present. The enrichment factors observed with each different blocking agent used in the panning selections were for 'selection' of non-specific binding clones. The selections performed with biotinylated-STn also failed to select for specific binders and after the 3rd round, 60% of the clones tested gave non-specific binding.

4.4 Immune MUC1 murine scFv library selections

The library was selected against STn antigen and the MUC1 60 mer peptide [Figure 4.10]. For determination of the optimum coating concentration of MUC1 peptide onto immunotubes MUC1 peptide was coated at; 5, 10 and 20 μg/ml and detected using monoclonal antibody VU-4-H5 (Price *et al.*, 1998). Tests were performed in parallel with 2% MAR and 2% BSA to block. There was little difference in the signals that were observed between the 3 concentrations used and the different blocking agents [Figure 4.9]. It was therefore decided to perform selections using peptide coated at 10 μg/ml with BSA as the blocking agent.

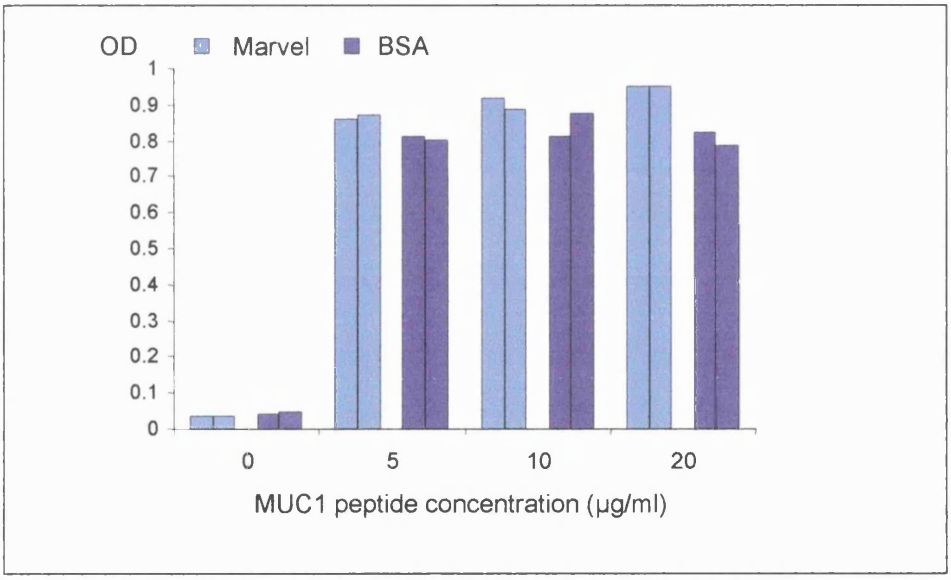


Figure 4.9 Testing coating of immunotubes with MUC1 peptide. Optimum coating concentration was determined by coating MUC1 peptide at 5, 10 and 20 μg/ml onto immunotubes. Peptide was detected with the anti-MUC1 monoclonal antibody VU-4-H5 using either 2% MAR or 2% BSA to block.

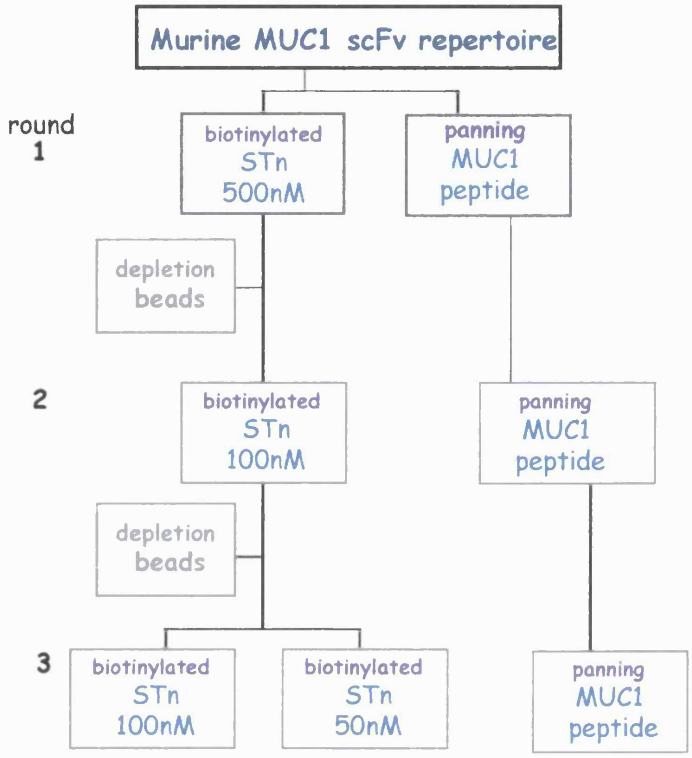


Figure 4.13 Selections performed with the immune MUC1 scFv repertoire

4.4.1 Selections using biotinylated-STn

Selections were performed with decreasing amounts of biotinylated-STn with 500 nM and 100 nM for rounds 1 and 2 respectively, 2% gelatin was used to block [Figure 4.10]. Two selections were performed in parallel for round 3 with 100 nM and 50 nM of antigen. Phage were incubated with beads (200 µl) before the 2nd and 3rd rounds of selection in order to eliminate streptavidin binding phage. Enrichment factors of 120 and 10 000 fold were obtained after the 3rd round [Table 4.5].

Table 4.5 Selection using biotinylated STn

selection round	antigen concentration[a]	enrichment[b]	specific binding (%)	non-specific binding (%)
1	STn-PAA 500 nM	1	nd[c]	
2	STn-PAA 100 nM	10	0/47	7/47 (15)
3(i)	STn-PAA 100 nM	120	0/47	10/47 (21)
3(ii)	STn-PAA 50 nM	10 000	0/47	23/47 (49)

[a] Gelatin used to block selections, [b] output round n/input round n divided by output round 1/input round 1, [c] nd not determined

4.4.1.1 Screening for STn binding phage

Individual clones were screened for binding to biotinylated-STn by coating of antigen onto streptavidin coated ELISA plates. Clones were screened from the 2nd and each of the 3rd rounds of selection. Specific binders were not identified in any of these selection rounds. Clones screened from the 3rd round of selection performed with 50 nM of antigen contained a high proportion (49%) of streptavidin binders (clones did not show binding to uncoated wells) [Table 4.5].

4.4.2 Selections by panning against MUC1 60 mer

Three rounds of selection were performed by panning against antigen coated immunotubes using 2% BSA as a blocking agent [Figure 4.10]. After 3 rounds of selection only a 6 fold

enrichment was observed. Individual clones were screened for binding specificity to the MUC1 60 mer peptide using phage ELISA but no specific clones were identified [Table 4.6].

Table 4.6 Selection by panning[a] against coated MUC1 60mer[b]

selection round	enrichment[c]	specific binding (%)	non-specific binding (%)
1	1	nd[d]	
2	10	nd[d]	
3	6	0/47	0/47

[a] Marvel used to block selections, [b] MUC1 peptide coated at 10 μg/ml, [c] output round n/input round n divided by output round 1/input round 1, [d] nd not determined

4.5 Selections performed with the naïve human Fab repertoire

The different selection strategies applied to the naïve human Fab library are outlined in Figure 4.11.

4.5.1 STn selections

The first round of selection was performed by panning against STn-PAA coated at a concentration of 50 μg/ml. A high concentration of antigen was used in order to maximise the density of antigen on the immunotube. Marvel was used to block the selections as no difference had been seen in any of previous selections, with regard to titre values and numbers of non-specific binding clones, when other blocking agents had been used. The output phage

titre for the first round of selection was very low, 800 clones were recovered from an input of 5×10^{12}, that is only 1.6×10^{-8} % of the phage used for selection.

4.5.1.1 Selections by panning on STn coated immunotubes

Further rounds of selection were performed by panning against STn-PAA coated at 40 μg/ml.

At the 3rd round of selection, in an attempt to deplete any phage that may have been binding

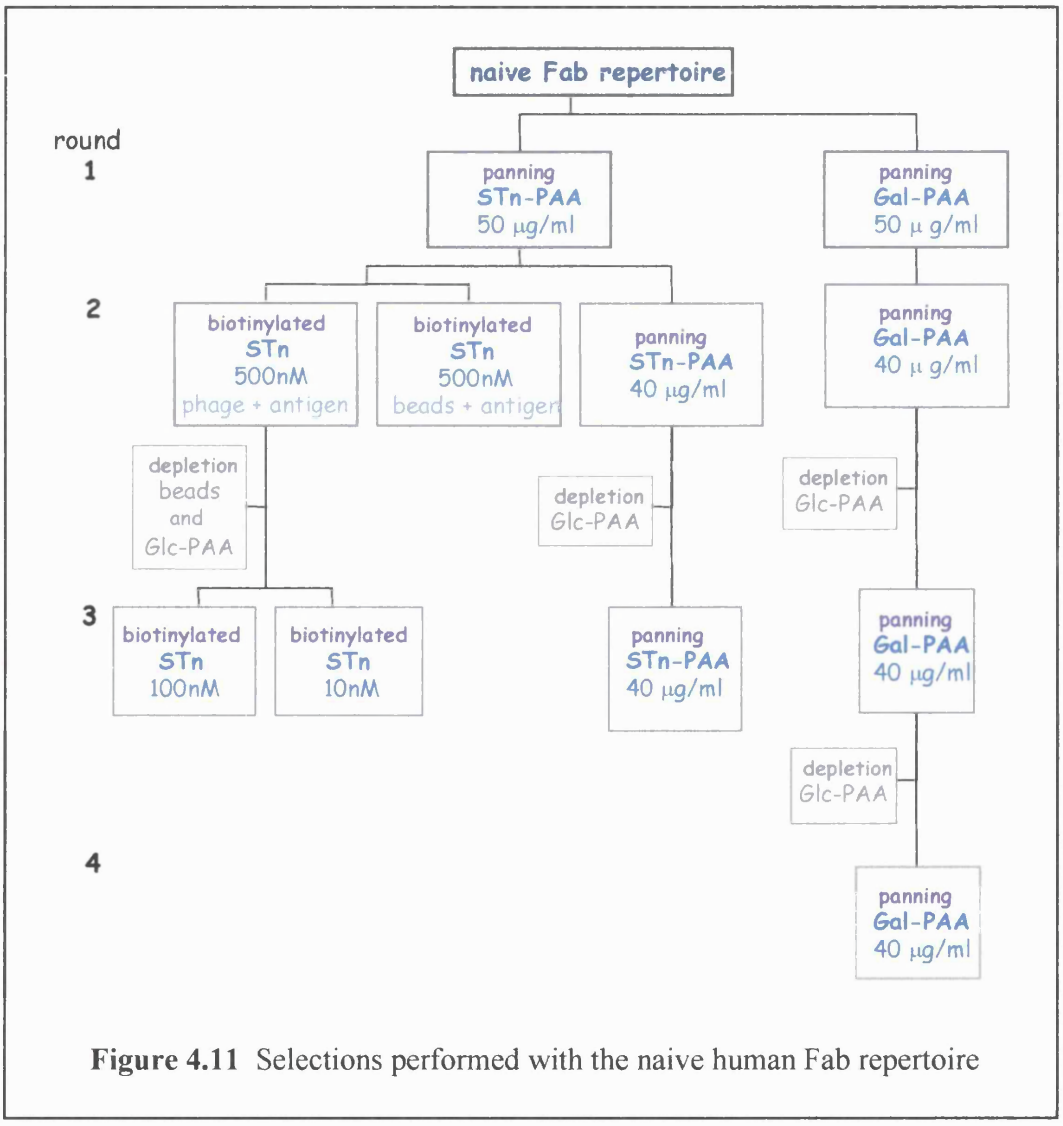


Figure 4.11 Selections performed with the naive human Fab repertoire

to the PAA carrier, Glc-PAA at 100 μg/ml was incubated with blocked phage before panning. A huge enrichment of 2 x 10^5 was observed after the 2nd round of selection which increased to 3 x 10^6 fold after the final round of selection [Table 4.7].

Table 4.7 Selection from the naïve Fab repertoire by panning against coated STn

selection round	antigen concentration[a]	enrichment[b]	specific binding (%)	non-specific binding (%)
1	STn-PAA 50 μg/ml	1	0/94	30/94 (32)
2	STn-PAA 40 μg/ml	2 x 10^5	1/47 (2)	36/47 (76)
3	STn-PAA 40 μg/ml	3 x 10^6	0/47	47/47 (100)

[a]MAR used to block selections [b]output round n/input round n divided by output round 1/input round 1

4.5.1.2 Selections with biotinylated STn

Two selections were performed in parallel for the second round of selection using 500nM biotinylated-STn [Figure 4.11]. The standard method of selection, incubation of phage with biotinylated antigen then capture of antigen binding phage with streptavidin coated magnetic beads, was compared with capture of antigen with the beads before incubation with phage. There was no difference observed in the number of phage recovered after the 2 different methods of selection [Table 4.8].

Table 4.8 Selection by panning against coated STn and biotinylated-STn

selection round	antigen concentration[a]	enrichment[b]	specific binding (%)	non-specific binding (%)
1	STn-PAA 50 µg/ml	1	0/94	30/94 (32)
2(i)[c]	biot-STn 500 nM	5×10^6	0/46	20/46 (43)
2(ii)[d]	biot-STn 500 nM	4×10^6	0/46	18/46(39)
3(i)[c]	biot-STn 100 nM	1×10^6	nd[f]	
3(ii)[c]	biot-STn 10 nM	2×10^5	nd[f]	

[a] marvel used to block selections, [b] output round n/input round n divided by output round 1/input round 1 [c] selections performed by mixing phage with antigen, [d] selection performed by capturing antigen with beads before incubation with phage, [f] nd not determined

The third round of selection was performed from the 2nd round clones that had been obtained using the standard method of selection. Two selections were performed in parallel using 100 nM and 10 nM of biotinylated-STn, with phage that had first been incubated with 200 µl of beads and 1 mM Glc-PAA to eliminate streptavidin binding and PAA binding clones.

The enrichment factors obtained after the 2nd round of selection were equally high (10^6 fold) for the two different methods. A small decrease in enrichment was seen after the 3rd round of selection both with 100 nM antigen (5 fold) and 10 nM antigen (25 fold) [Table 4.8].

4.5.2 Screening for STn binding specificities from the naïve Fab library selections

Individual clones were screened for specific binding by phage ELISA. The results from selection rounds performed by panning are summarised in Table 4.7 and with biotinylated antigen in Table 4.8.

A single clone, E2, from the 2nd round of panning gave specific binding to STn. The 2nd and 3rd rounds contained a high proportion of non-specific binding clones, 76% and 100% respectively. The clones tested from round 2 of the biotinylated antigen selections did not contain any specific binders, approximately 40% of clones gave non-specific binding.

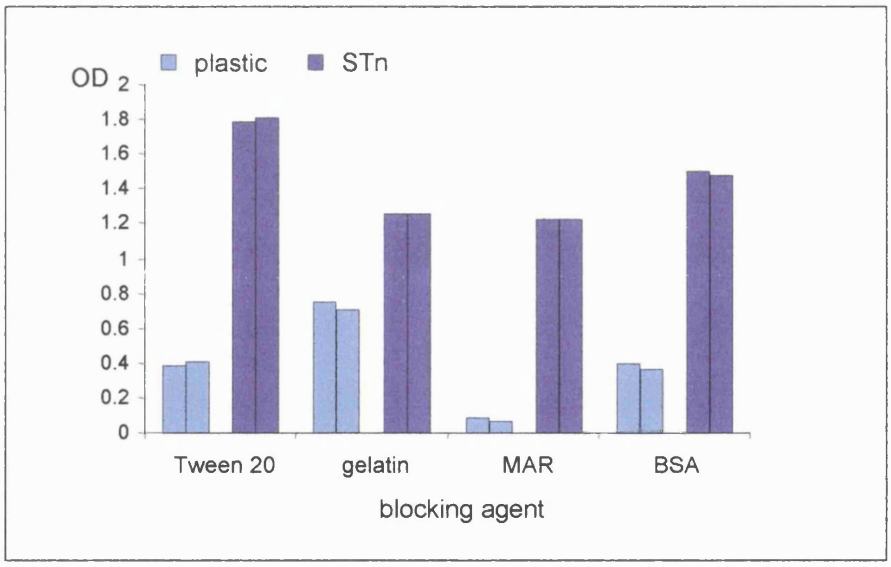


Figure 4.12 Testing binding of Fab clone E2. Detection of specific binding of PEG precipitated phage-Fab E2 to STn-PAA by phage ELISA was tested using the blocking agents: 0.1% Tween 20, 1% gelatin, 2% MAR and 2% BSA.

4.5.2.1 Testing the binding specificity of clone E2

PEG precipitated phage was prepared from a 50 ml culture of clone E2 to further test its binding specificity. Detection of specific binding using phage ELISA was tested using different blocking agents, 0.1% Tween 20 (Tw20), 1% gelatin (Gel), 2% MAR and 2% BSA. E2 showed binding to STn with all of the blocking agents, signals between Tween 20, MAR and BSA were comparable and gelatin gave the highest background [Figure 4.12].

Screening against different carbohydrate-PAA conjugates

Clone E2 was tested for binding against different PAA carbohydrate conjugates; NeuAc-GalNAc (STn), GalNAc (Tn), Neu5Ac (sialic acid), Glc and α-Gal (Table 4.9). The results using neat, 1/10 and 1/100 diluted PEG precipitated phage are shown in [Figure 4.13]. Undiluted (neat) phage showed binding to all of the antigens, the highest signals were with STn, Tn and Glc. However when E2 was used at 1/100 dilution a different pattern of binding to the antigens was observed. The highest binding signal was obtained with Tn and the lowest with α-Gal, with binding to antigens in the order Tn > Glc > NeuNAc > STn > Gal.

Table 4.9 Carbohydrate antigens used to test specificity of clone E2

carbohydrate antigen[1]	abreviation	chemical name
Glc β1-	Glc	β-D-glucose
Neu5Ac α2-	NeuAc	α-N-acetyl neuraminic acid (sialic acid)
GalNAc α1-	Tn	α-N-acetyl-D-galactosamine
Neu5Ac α2-6 GalNAc α1-	STn	
Gal α1-3 Gal β1-4 GlcNAc	αGal	

[1] carbohydrate antigen-PAA (poly[N-C2-hydroxyethyl] acrylamide)

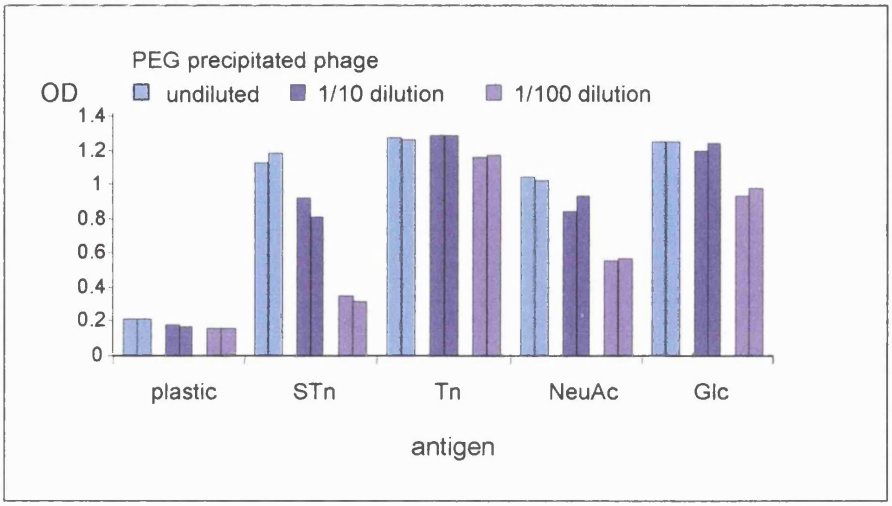


Figure 4.13 Testing the specificity of Fab clone E2. Binding of phage-Fab was tested using either undiluted, 1/10 and 1/100 dilutions of PEG precipitated phage against different carbohydrate antigens; NeuAc-GalNAc (STn), GalNAc (Tn), Neu5Ac (sialic acid), Glc and α-Gal.

4.5.3 α-galactosyl (Gal-PAA) antigen selections

Four rounds of selection were performed by panning against coated Gal-PAA, 2% MAR was used to block [Table 4.10]. The number of phage recovered after the first round of selection was higher 8.2×10^{5} (1.6×10^{-5} %) than that obtained by panning with STn (1.6×10^{-8} %). The values for enrichment therefore were significantly different for selections with Gal and STn. Screening of individual clones, by phage ELISA, did not result in the isolation of any specific clones. Selection with this 'control' carbohydrate antigen resulted, like the other selections, in high numbers of non-specific binding clones being present in the selection rounds, 63% in round 3 and 98% in round 4.

Table 4.10 Selection by panning against α-Galactosyl antigen

selection round	antigen concentration[a]	enrichment[b]	specific binding (%)	non-specific binding (%)
1	Gal-PAA 50 μg/ml	1	nd	
2	Gal-PAA 40 μg/ml	2	nd	
3	Gal-PAA 40 μg/ml	18	0/48	30/47 (63)
4	Gal-PAA 40 μg/ml	100 000	0/50	46/47 (98)

[a] MAR used to block selections, [b] output round n/input round n divided by output round 1/input round 1

4.6 Discussion

This chapter investigated selection from antibody repertoires using phage display as a means of generating antibodies of with desired binding specificities. Selections were performed, with naïve human scFv and Fab repertoires and an immune murine scFv repertoire, for the isolation of binding specificities against the tumour associated carbohydrate antigen STn.

Before starting the selections it was necessary to consider how results might be affected by the use of skimmed milk powder Marvel (MAR) as a blocking agent for selection and ELISA. This is because MAR contains free sugars some of which are composed of the NeuNAc and GalNAc residues that could possibly compete for and /or inhibit binding of STn specific phage. To determine if these free sugars had any affect on the observed signals different

blocking agents (BSA, MAR and HSA) were compared for detection of coated STn with the anti-STn monoclonal antibody. The best signal to noise ratio was given with BSA for the detection of STn-HSA, with little difference observed between different blocking agents for the detection of STn-PAA. These results indicated that the free sugars present in MAR might affect selections against STn-HSA, so MAR, BSA and HSA were compared in selections performed with the naïve scFv repertoire. Despite the use of different blocking agents selections performed both by panning and with biotinylated antigen in solution did not result in the isolation of any STn binding specificities from the naïve scFv repertoire. Furthermore, neither BSA, HSA nor MAR were effective in eliminating or reducing non-specific/polyreactive clones that were recovered after each round of selection. Screening by phage ELISA showed that observed enrichment values, for selections, corresponded to the enrichment of polyreactive clones. Clearly use of BSA and HSA offered no advantage over MAR for the selection of STn specific phage. These alternative blocking agents were used due to the presence of lower levels of protein glycosylation compared with skimmed milk powder. However, despite the use of different blocking conditions favouring selection of polyreactive clones was favoured over those for STn binding phage. An explanation for these findings is that the carbohydates present on BSA and HSA interacted with STn binding phage and inhibited specific binding to antigen, thereby making it impossible to select for STn binders.

The disappointing results obtained with the naïve repertoire compared to the positive results obtained with the immune repertoire selections from Chapter 3, lead to the use of the immune MUC1 repertoire. This murine scFv repertoire had been generated by immunisation with MUC1 expressing cells and such potentially contained specificities directed STn (MUC1 associated antigen). However, selections performed using biotinylated antigen failed again to

isolated any STn binding specificities. The enrichment values observed corresponded to an enrichment of streptavidin binding clones despite depletion with streptavidin beads before each round of selection.

Control selections with MUC1 peptide were also performed with the immune MUC1 repertoire. Selections with coated peptide failed to isolate MUC1 binding clones. These results imply that MUC1 binding specificities (and therefore STn binding specificities) were not contained in this repertoire. However, this is unlikely as this repertoire was prepared from mice showing a measured immune response to MUC1 peptide after immunisation with a MUC1 producing breast cancer cell line (K A Chester personal communication). Difficulty in selecting for MUC1 binding specificities has been reported by Henderikx and co-workers (Henderikx *et al.*, 1998). They compared solid phase and solution phase selections with peptides of different lengths as well as cell selection for the isolation of MUC1 binding scFv fragments from a naïve human repertoire. These methods were used alone and in combination but binding clones were only isolated from selections performed with biotinylated MUC1 100 mer peptide. In light of these findings it is probable that the inability to select for MUC1 binding specificities from the immune repertoire was due to the selection strategies employed.

Next, as an alternative 'single pot' resource the larger naïve human Fab repertoire was used for selections with STn-PAA. These selections used higher concentrations of antigen than previously so as to give maximal coating of the immunotube. Marvel was chosen to block the selections as little difference had been observed with alternative blocking agents for the naïve scFv repertoire selections. The number of phage recovered from the Fab repertoire after the first round of selection with STn was surprisingly low. The titres obtained from selections with protein antigens performed in parallel indicated that this was an antigen specific

observation. Low titres after the first round of selection have previously been observed with this repertoire (H.R. Hoogenboom personal communication). Further rounds of selection were performed incorporating steps to deplete phage with specificities against the polyacrylamide (PAA) carrier molecule by pre-incubation of phage with Glc-PAA [which presented the PAA carrier in a conformation identical to that of the antigen used for selection (N. V. Bovin personal communication)]. The huge enrichment values obtained with subsequent selections were to be expected considering the low titre obtained after the first round of selection. Amongst the many non-specific binding clones obtained after screening of different selections a single clone was identified from phage recovered after the 2^{nd} round of panning that did not show binding to plastic. The binding specificity of this clone was tested using PEG precipitated phage against different carbohydrate-PAA conjugates. The results obtained were surprising in that higher binding was observed to the related carbohydrate antigen Tn (GalNAc-PAA) compared to STn (NeuAc-GalNAc-PAA). It is possible that the Tn epitope was exposed on the STn molecule due to loss of the sialic acid residues perhaps due to instability of the STn reagent. However, this does not explain the ability of this clone to bind to NeuNAc or the high signal that was obtained with glucose. This cross reactivity may be due to a low affinity of binding or due to the epitope being a conformational one that is common to both Tn and Glc. There is, of course, the possibility that binding is directed against the PAA carrier molecule.

Control carbohydrate antigen selections were also attempted with naïve Fab repertoire. The Gal antigen (Gal α1-3 Gal β1-4 GlcNAc-R) was chosen as Gal-binding specificities were expected to be present in the naïve repertoire due to the high abundance of anti-Gal antibodies in human sera (Galili *et al.*, 1993). Again, selections failed to generate anything but polyspecific clones. Polyreactive clones are known to be present in naïve repertoires (H.R.

Hoogenboom personal communication), but normally (with protein antigens) do not present a problem. These polyreactive clones reflect polyreactive antibodies found in the normal circulation that are produced without any stimulus by exogenous antigens. Such antibodies are derived from unmutated or slightly mutated germ-line genes (Roggenbuck *et al.*, 1994) generally belong to the IgM isotype and demonstrate low affinity binding to different antigens (Avrameas and Ternynck, 1993). The selection strategies employed to isolate carbohydrate binding specificities from the naïve repertories clearly favoured isolation of these polyreactive antibodies.

Difficulty in isolating antibodies from repertoires by panning against carbohydrate antigens has been reported by others (Marks *et al.*, 1993; Deng *et al.*, 1994; Dinh *et al.*, 1996; Wang *et al.*, 1997). Wang and colleagues constructed naïve gene III and gene VIII Fab antibody repertoires from human PBLs as a means of characterising the polyclonal immune response against the Gal antigen. Selections performed with these 'enriched' repertoires only resulted in the isolation of 2 clones after selection with biotinylated Gal-BSA (Wang *et al.*, 1997). Indicating, as results in this chapter have, that carbohydrate antigen selection cannot be performed using conditions normally used for protein antigens. The lower intrinsic binding affinity of anti-carbohydrate antibodies [10^5 - 10^6 M^{-1} compared to 10^8 - 10^9 M^{-1} for peptides (Wang *et al.*, 1995)] needs to be considered when designing selection strategies. The use of biotinylated antigen enables more phage to interact and also subjects binding phage to decreased shearing forces during repeated washes (when compared to panning). Indeed, biotinylated antigen was used to isolate anti-Gal (Wang *et al.*, 1997) and anti-Le^x antigen (Dinh *et al.*, 1996) binding specificities. Selections performed in this chapter against biotinylated STn with phage recovered after panning against coated antigen did not result in

the isolation of desired binding specificities probably because the desired binding specificities were lost in the first selection round.

Selection of antibodies using phage display typically results in the retrieval of 5-20 clones from repertoires containing up to 10 billion different clones. A recent comparison of selection strategies demonstrated that panning with different antigen concentrations and use of fewer washes resulted in the recovery of different phage populations (Lou *et al.*, 2001). From these observations it was concluded that in order to fully exploit the diversity present in naïve repertoires several selection strategies should be employed for each antigen. Others have also suggested this as a means of preventing loss of desired specificities and/or domination of selection by unwanted polyspecific clones (Bender et al., 1994; Llorente et al., 1999).

Another factor that may affect selection is how antibodies are displayed on phage. Multivalent display of antibody fragments has been demonstrated, by comparison of phage and phagemid vectors, to be preferable for the isolation of carbohydrate binding specificities (MacKenzie and To, 1998). Recently, 'hyper phage' which lack functional gene III protein have been used to increase the percentage of phage carrying antibody due to phagemid encoded gene III antibody fusions being the only source of gene III in phage assembly (Rondot *et al.*, 2001). Selections with 'hyper phage' gave enrichment values of greater than 50% at round 2 compared with 3% obtained with conventional phage. This system obviously has advantages for selection of antibodies with low intrinsic affinity and where antibodies are expected to be limited.

Summary

The isolation of antibodies against the tumour associated mucin carbohydrate antigen STn by selection from antibody repertoires using phage display proved to be disappointing. Selections

were performed with the naïve human scFv and Fab repertoires and the immune MUC1 repertoire failed to generate desired the desired binding specificity and were dominated by polyreactive clones. These findings correspond observations made by others when trying to isolate carbohydrate binding specificities. To retrieve STn binding specificities by repertoire selection, strategies need to be designed that take into consideration the low intrinsic affinities of anti-carbohydrate antibodies. Use of the new 'hyper phage' with the naïve repertoires may allow isolation of these binding specificities due to increased (as well as multivalent) expression of clones within these repertoires.

Chapter 5

Construction of a human scFv antibody fragment with reactivity against adenocarcinomas

5.1 Introduction

This chapter describes rescue of V-genes from the tumour reactive human hybridoma S2.5 for construction of a functional antibody fragment.

5.1.1 Human monoclonal antibodies for cancer therapy

The use of human monoclonal antibodies would circumvent problems of immunogenicity encountered with rodent antibodies. They also offer the possibility of generating antibodies against subtle tumour antigens on tumour cells that may not be immunogenic in an animal system due to masking by strong species specific antigens.

However, the development and subsequent use of human monoclonal antibodies has been limited when compared with rodent antibodies. This is due to a number of factors, primarily by the limited availability of ideal fusion partners and the lack of effective immunisation protocols to enrich for tumour antigen sensitised B cells. Once isolated, human hybridomas often show a lack of stability of immunoglobulin production and low levels of antibody secretion with the predominance of IgM isotype production.

Despite these difficulties lymphocytes from cancer patients have been used to successfully isolate anti-cancer antibodies. Regional tumour draining lymph nodes of patients with metastatic disease have proved to be a reliable source of B lymphocytes sensitised against tumour associated antigens (Schlom *et al.*, 1980; Sikora and Wright, 1981; Cote *et al.*, 1983; Haspel *et al.*, 1985; Hirohashi *et al.*, 1986; Borup Christensen *et al.*, 1986; Glassy, 1987; Imam *et al.*, 1991; Chang *et al.*, 1993), which can be enriched using by in-vitro immunisation (Koda and Glassy, 1990).

5.1.2 The human monoclonal antibody S2.5

Lymphocytes secreting the human IgM monoclonal antibody S2.5 were isolated from the lymph node of a patient with a colonic polyp showing signs of malignant behaviour (Yiu *et al.*, 1991). This monoclonal antibody was produced by fusion of lymphocytes with the human lymphoblastoid cell line LICR-LON-HMy2 (HMy) derived from the ARH-77 human plasma cell leukaemia (Edwards *et al.*, 1982). Fusions were screened for reactivity against crude membrane extract (CME), prepared from fresh tumour tissue from several colon cancers. S2.5 was one of a number of fusions that was selected for further characterisation.

Initial characterisation of S2.5 by immunohistochemical staining of primary tumour xenografts showed reactivity with moderately differentiated and moderately well differentiated adenocarcinomas derived from LS174T and HT29 colon carcinoma cell lines, respectively, as well as the OVCA cell line derived from an ovarian adenocarcinoma (Yiu unpublished results).

The observed reactivity of S2.5 with tumour xenografts warranted further investigation of the binding characteristics of this antibody. This however was not possible due to low levels of secretion (1-10 μg/ml). Furthermore, immunohistochemical screening of human tissues would be compromised due to the presence of endogenous immunoglobulins.

Construction of a scFv, derived from VH and VL region genes of S2.5 followed by cloning into a vector allowing bacterial expression of the soluble antibody fragment would provide a means to overcome these problems. The introduction of a myc-tag onto the scFv would enable detection using a high affinity mouse anti-myc monoclonal and so enable screening of human tissues in further characterisation studies.

5.2 Cloning of S2.5 as a scFv

Before cloning of the human monoclonal antibody S2.5, the class of antibody was confirmed as IgM and the concentration determined to be 1-10 μg/ml by dot blot and Western analysis. Secretion of reactive antibody was confirmed by testing the tissue culture supernatant for

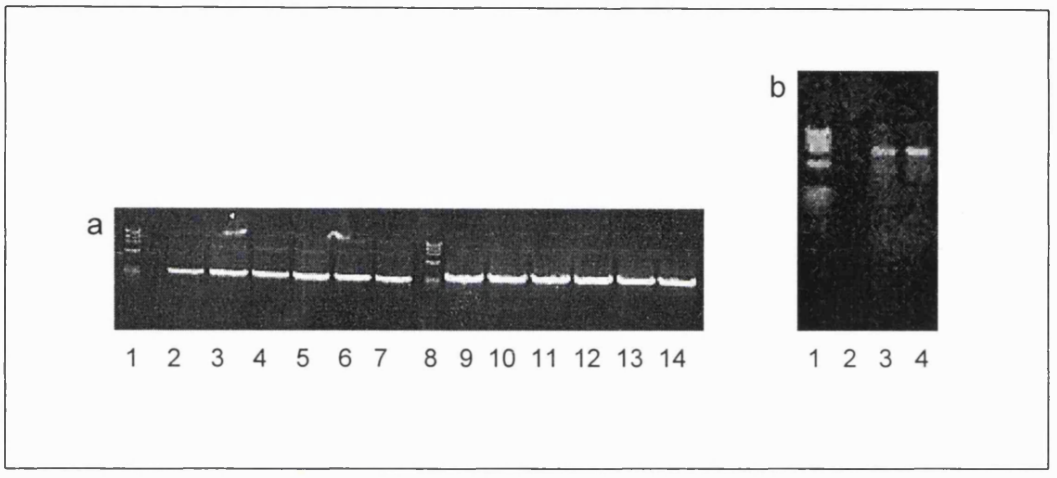


Figure 5.1 **(a)** PCR amplification of V-genes from human monoclonal antibody S2.5 total RNA. Lanes 1 and 8: φX174 *HaeIII* DNA marker (1353, 1078, 872, 603, 310, 281, 271, 234, 194 bp); Lanes 2-7: Light chain PCR products amplified with pooled Human κ Back and Human Jκ Forward primers; Lanes 9-14: Heavy chain PCR products amplified with pooled Human VH4,5,6 Back and Human JH Forward primers. **(b)** PCR assembly of scFv from extended heavy and light chain V-gene PCR products. Lane 1: φX174 *HaeIII* DNA marker; Lane 2: negative control; Lanes 2 and 3: scFv PCR product.

binding to mouse LS174T tumour xenografts using immunohistochemical staining [Figure 5.3]. RNA extracted from antibody secreting cells was used for oligo dT primed synthesis of cDNA. Equimolar mixes of 3-4 primary PCR primers were used for heavy and light chain V-gene amplification [Table 2.2]. Light chain products were obtained with the human K BACK primer mix and the human JK forward primer mix. For the heavy chain, products were obtained with HVH4BACK, HVH5BACK and HVH6BACK and the JH forward primer mix [Figure 5.1(a)]. A secondary amplification was performed with purified V_H and V_L products in order to incorporate restriction endonuclease sites and linker sequences. An *NcoI* restriction

endonuclease site was added to the 5' end of VH and a *NotI* site added to the 3' end of VL. The $(Gly_4Ser)_3$ linker sequence was added to join the 3' end of heavy to the 5' end of the light chain. Assembly and amplification of scFv was performed with pooled 5'-HVHSfi and 3'-HJKNOT primers using approximately equal quantities of purified extended heavy and light chain products [Figure 5.1(b)]. For bacterial expression *NcoI/NotI* digested scFv was cloned into the plasmid *pUC119-polyHIS6myc* and competent TG1 cells transformed by electroporation. Positive recombinants were identified by PCR amplification of scFv fragments from individual clones and the diversity analysed by *BstN1* restriction endonuclease digestion [Figure 5.2]. The observed patterns could be grouped into 9 distinct profiles.

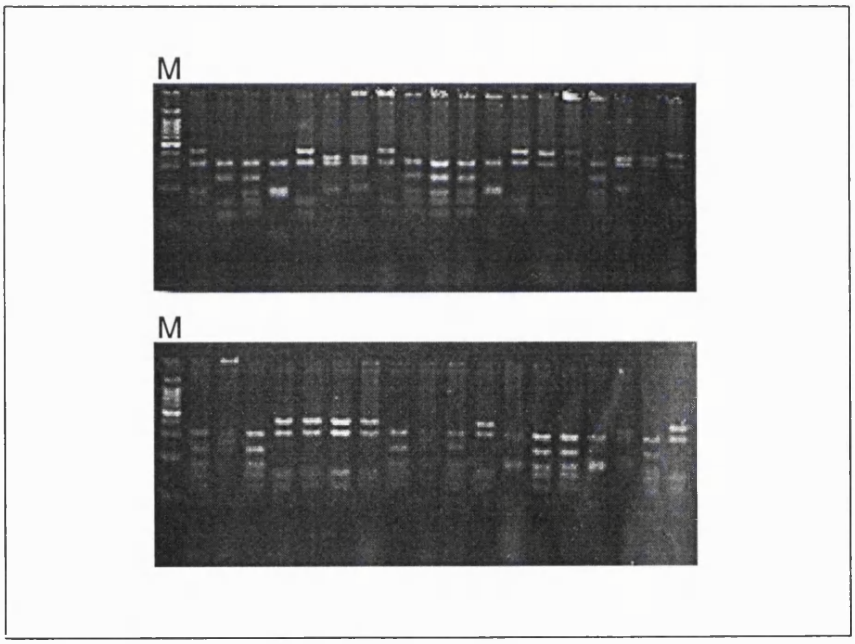


Figure 5.2 Diversity of scFvs derived from the human monoclonal antibody S2.5 as demonstrated by *Bst*NI fingerprinting, M is the 123 base pair ladder DNA marker (123, 246, 369, 492, 615, 738, 861, 984, 1107, 1230bp).

Clones representative of each restriction enzyme profile were induced for soluble scFv expression. Bacterial supernatants when checked for scFv expression by dot blot with the anti-myc tag monoclonal antibody 9E10 showed variable levels of secretion between the

different clones. Bacterial supernatants were tested, alongside S2.5 as a positive control, for binding to LS174T tumour xenografts.

5.3 Immunohistochemistry

5.3.1 Immunohistochemical staining of LS174T tumour xenografts

Binding to LS174T by S2.5 shows heterogeneous reactivity with malignant cells, immunohistochemical reactions are mainly cytoplasmic with some lesser membrane reactivity [Figure 5.3, Figure 5.4]. Binding to LS174T of supernatant from the S2.5 secretory fusion partner HMy was absent, confirming that the binding observed with the S2.5 culture supernatant was due to antibody produced by the hybridoma [Figure 5.5].

As the S2.5 scFv clones were expressed with a myc-tag, the myc tagged anti-CEA scFv MFE-23 was used as a positive control for detection. MFE-23-myc gave characteristic CEA uniform positivity of glandular structures with LS174T [Figure 5.6, Figure 5.8]. Bacterial supernatants that were positive for scFv expression were screened against LS174T for binding reactivity, clone C9 and clone C8 gave diffuse reactivity with malignant glandular structures. Both clones had the same *BstNI* profile but clone C9 gave slightly more intense reactions [Figure 5.7] and so was selected for screening of human tissue.

5.3.2 Immunohistochemical reactivity of scFv C9 S2.5 with normal human tissue

A panel of various normal human tissues, summarised in Table 5.1, were used to determine binding of scFv C9 S2.5. This scFv clone gave uniform reactivity with basal epithelium of the cervix and with the duodenum. It gave a distinctive pattern of positive reactivity in the large bowel with antibody binding localised at the basal aspect of the crypt mucosa [Figure 5.16].

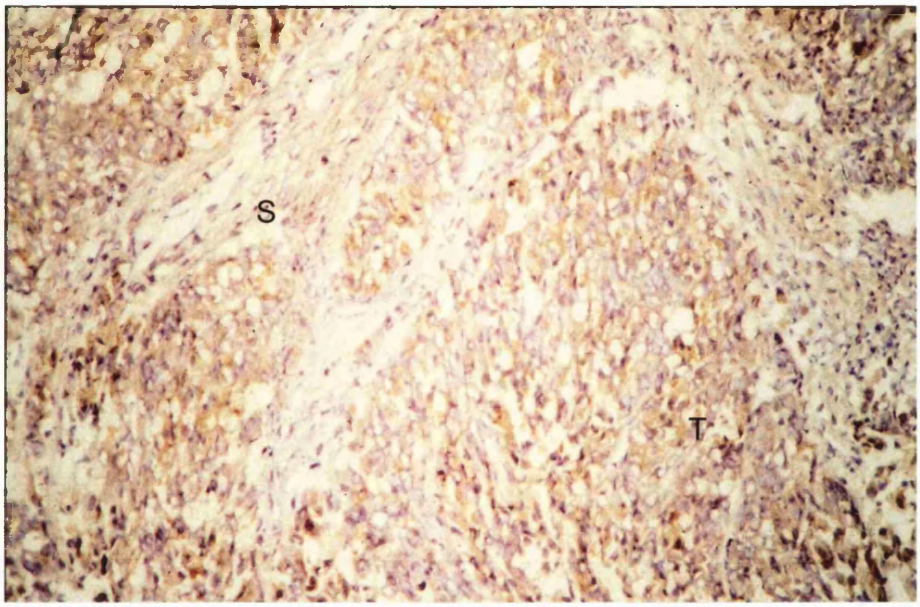


Figure 5.3 LS174T tumour xenograft with human monoclonal antibody S2.5 showing reactivity with malignant cells with mainly cytoplasmic and some membrane reactivity, magnification x 200 [tumour tissue (T) and stromal tissue (S) indicated].

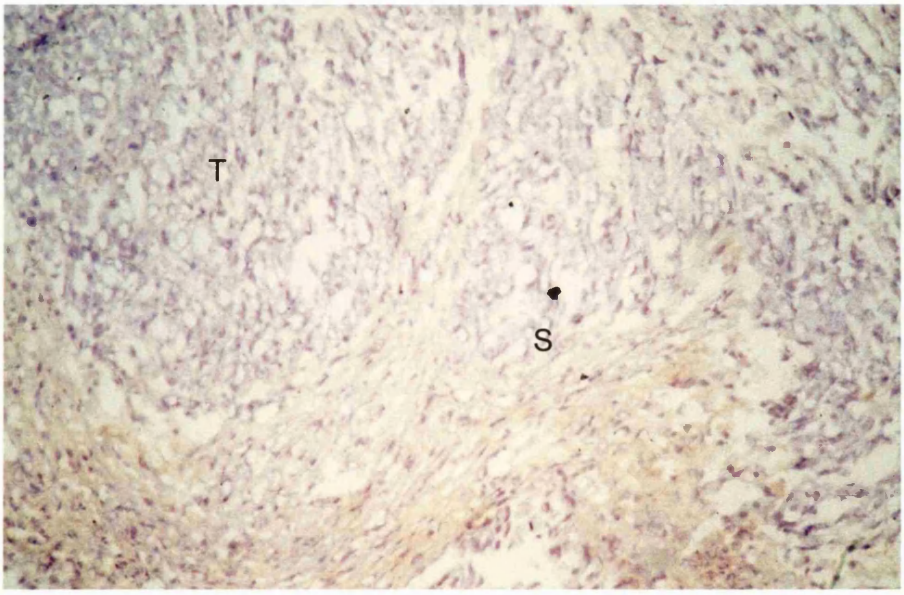


Figure 5.4 LS174T tumour xenograft, omission control for S2.5 (absence of reactivity in tumour cells), magnification x 200 [tumour tissue (T) and stromal tissue (S) indicated].

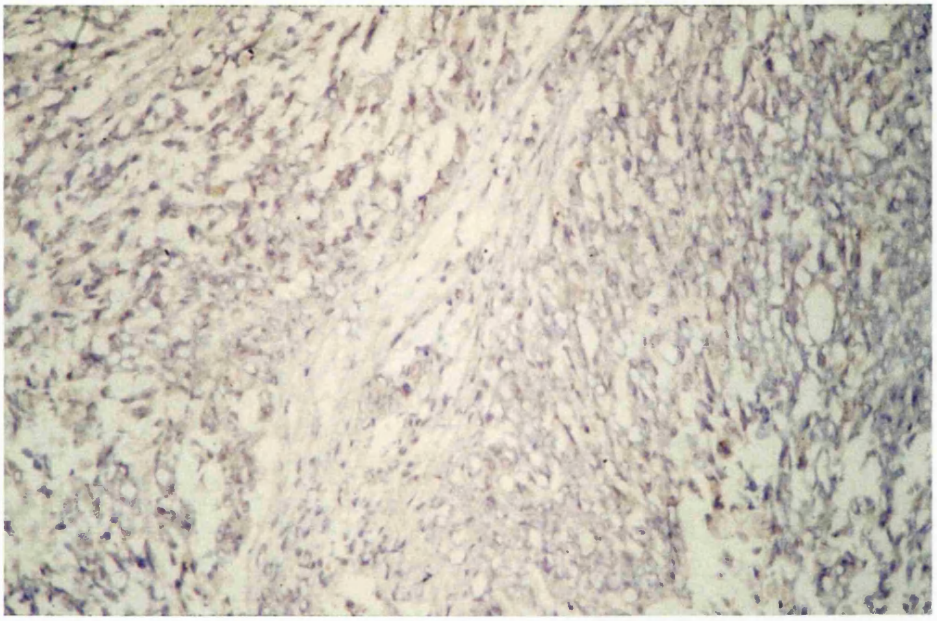


Figure 5.5 LS174T tumour xenograft staining with fusion partner HMy, negative control for S2.5 showing absence of reactivity with malignant cells, magnification x 200

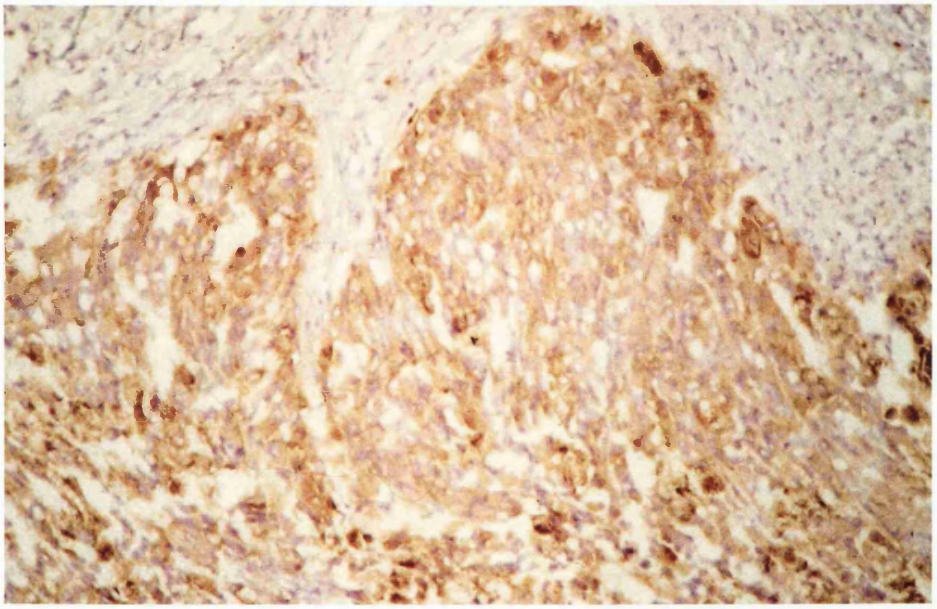


Figure 5.6 LS174T tumour xenograft with anti-CEA scFv MFE-23-myc, malignant glandular structures are uniformly positive for CEA, magnification x 200.

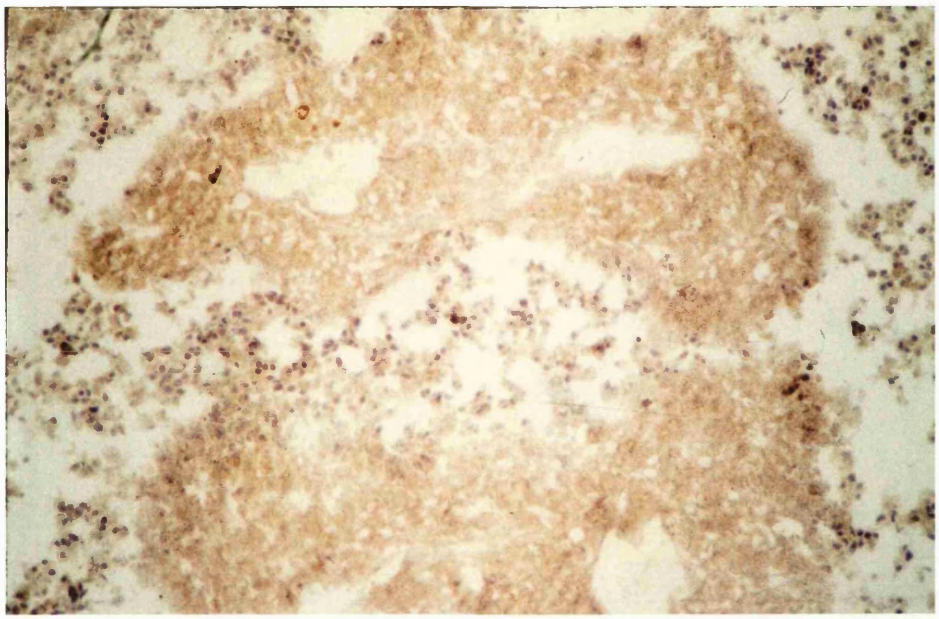


Figure 5.7 LS174T tumour xenograft with scFv C9 showing diffuse reactivity with malignant glandular structures, magnification x 200.

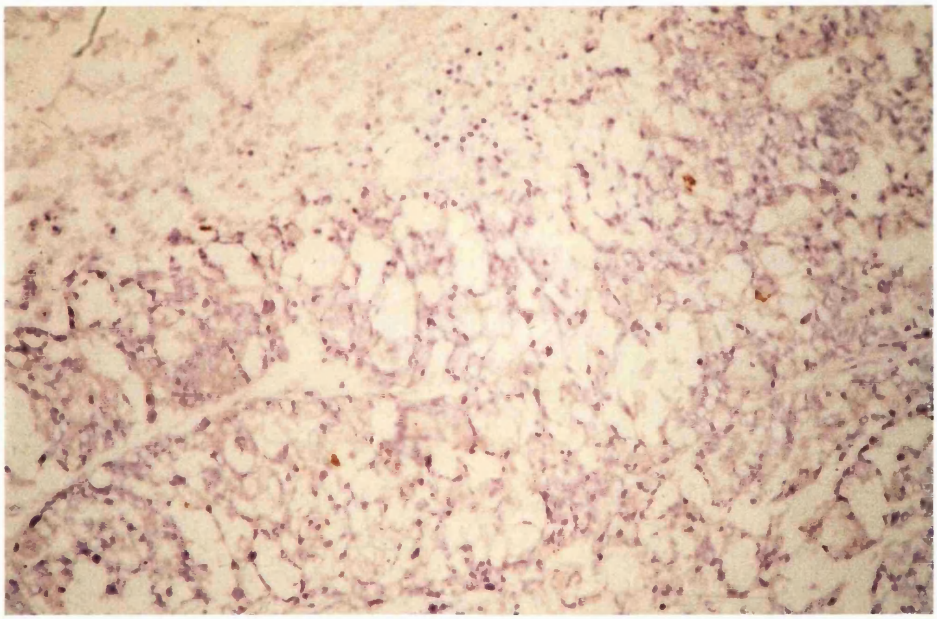


Figure 5.8 LS174T tumour xenograft, negative omission control for scFv detection magnification x 200.

Table 5.1 Immunohistochemical reactivity of scFv C9 S2.5 with normal human tissue

Adrenal (n = 2)	–	Lung (n = 1)	
		Bronchiolar epithelium	+
Cervix (n = 2)		Alveolar epithelium	
Basal epithelium	++	Ovary (n = 1)	+
Colon (n = 1)	–	Pituitary (n = 2)	+/++
Basal goblet cells in crypt (n = 5)	++		
		Prostate (n = 1)	
Duodenum (n = 2)		glands	++
epithelium	++		
		Spleen (n = 1)	
Endometrium (n = 2)	–	Red pulp and vessels	++
Gallbladder (n = 2)		Testis (n = 1)	+
Mucosa	+		
		Thyroid (n= 1)	+
Hypothalamus (n = 1)	+		
Kidney (n = 2)	–	Placenta	
		1st trimester (n = 1)	–
Liver (n = 1)	–	full term (n= 3)	–

– = negative, + = weak positivity, ++ = strongly positive

There was some reactivity with the gall bladder mucosa that was accompanied by a low level of non-specific reactivity with the muscularis. Neural elements were negative but some diffuse reactivity was observed with the hypothalamus. In lung, bronchiolar epithelium was negative but C9 bound specifically to alveolar epithelium (pneumocytes). C9 gave weak reactivity with both the epithelium and stroma of the ovary and was also positive with the pituitary and prostate. Specific binding to the red pulp and of vessels in the spleen was observed, in the testis C9 reactivity was extracellular with limited cellular positivity and in the thyroid the cells lining the ducts were positive. C9 did not react with adrenal tissue, endometrium, kidney, liver, early or late placental tissue.

5.3.3 Immunohistochemical reactivity of scFv C9 with human neoplastic tissue

The reactivity of C9 with various tumour tissues is summarised in Table 5.2. Reactions were variable and were observed at the cell membrane, cytoplasmically, and luminally epithelial glandular structures. C9 binding of breast carcinoma and metastases showed overall weak binding of cytoplasm and cell membrane with greater reactivity observed with ductal invasive breast carcinoma [Figure 5.9, Figure 5.10]. Heterogeneous reactions were observed in bladder carcinoma [Figure 5.11] and one bladder transitional cell carcinoma. Squamous cell carcinoma of the cervix showed cytoplasmic staining which varied in intensity in areas with keratinising epithelium showing the most positivity [Figure 5.12, Figure 5.13, Figure 5.14]. With colorectal adenocarcinomas C9 showed binding to malignant glandular structures and some luminal surfaces were highlighted [Figure 5.15]. Binding was quite distinct from that observed in normal colon with C9 [Figure 5.16] and the observed reactivity of the positive CEA control MFE-23 with colorectal adenocarcinoma [Figure 5.17]. Two examples of hepatic colorectal adenocarcinoma metastasis were negative for C9 reactivity. C9 binding to adenocarcinoma of the duodenum was weak but uniform in malignant cells and an example of leiomyosarcoma, smooth muscle tumour was also positive. Lung adenocarcinoma stained positively as did one squamous carcinoma which demonstrated greater reactivity in keratinising cells [Figure 5.18]. C9 showed uniform binding reactivity with both papillary serous [Figure 4.19, Figure 4.20] and mucoid ovarian adenocarcinomas, serous carcinomas showed strong luminal binding as well as cytoplasmic reactivity. Adenocarcinomas of the stomach and the pancreas showed heterogeneous reactivity with malignant cells. In addition to the tumour tissues mentioned above C9 was screened for reactivity against; an adrenal Conn's tumour, endometrial carcinoma, lung alveolar soft part sarcoma, lung large cell undifferentiated adenocarcinoma and germ cell tumour, all were negative for C9 staining.

Table 5.2 Immunohistochemical reactivity of scFv C9 S2.5 with human tumour tissue

Tissue section (n)[a]	Degree of staining[b]		
	–	+	++
Adrenal Conns tumour (1)	1		
Breast carcinoma (1)		1	
Ductal invasive (1)			1
Metastatic adenocarcinoma (1)		1	
Bladder carcinoma (1)			1
Bladder transitional cell carcinoma (2)	1		1
Cervix squamous cell carcinoma (1)		1	
Colorectal adenocarcinoma (12)		4	8
Hepatic metastasis (2)	2		
Duodenal adenocarcinoma (1)			1
Endometrial carcinoma (1)	1		
Leiomyosarcoma (1)		1	
Lung adenocarcinoma (1)		1	
Lung alveolar soft part sarcoma (1)	1		
Lung carcinoma (1)		1	
Squamous cell (2)			2
Large cell undifferentiated (1)	1		
Ovarian adenocarcinoma (10)	3	4	3
Mucoid (2)		2	
Serous papillary (4)	1	3	
Pancreatic adenocarcinoma (2)			2
Stomach adenocarcinoma (3)	1	1	1
Mucoid (1)		1	
Teratoma			
Ovarian (1)	c		
Testicular (2)	2		

[a] n = number of cases

[b] – = less than 5%, + = weakly positive 5% to 50%, ++ = more than 50%

c = equivocal

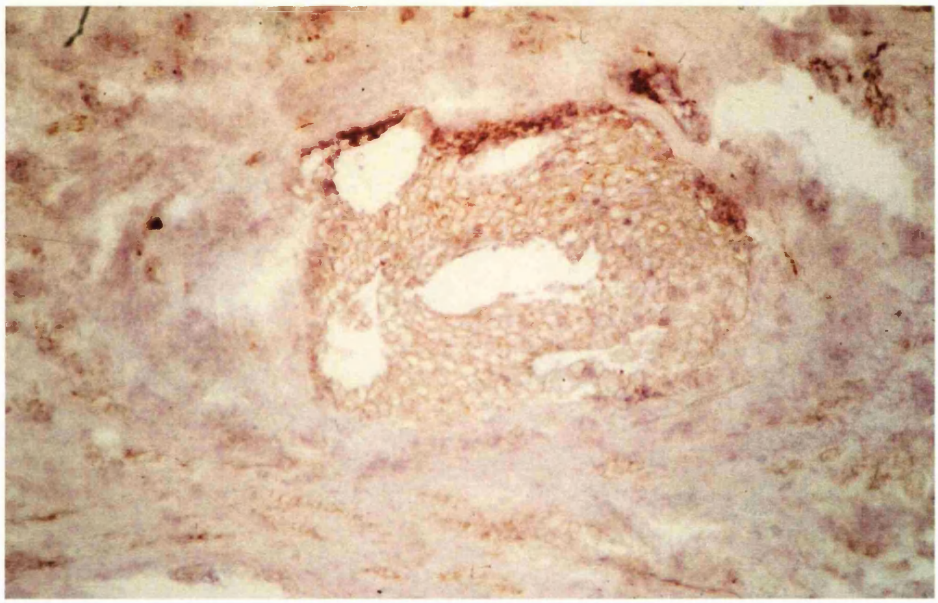


Figure 5.9 Human breast carcinoma with scFv C9, cytoplasmic and membrane reactivity magnification x 200.

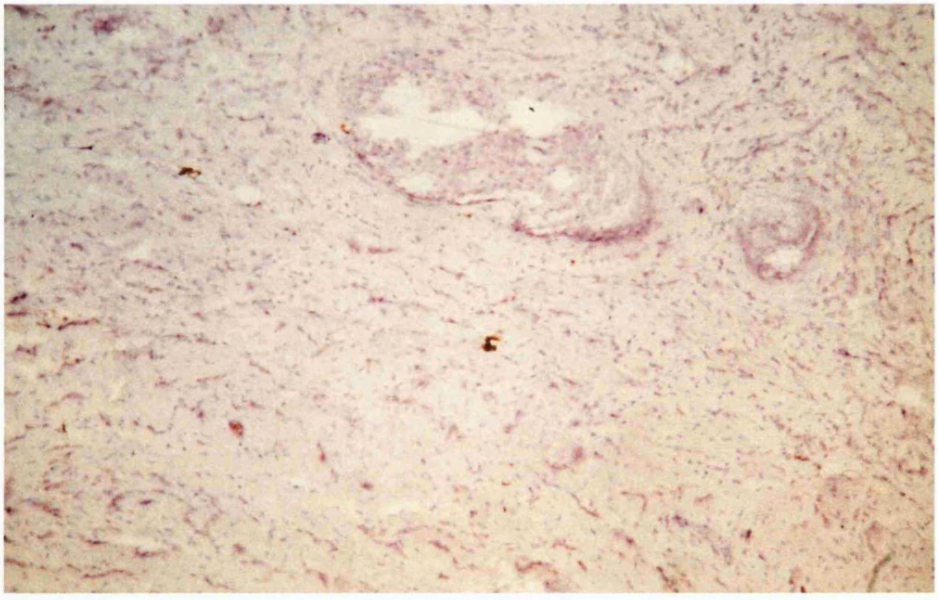


Figure 5.10 Human breast carcinoma, negative omission control for scFv detection, magnification x 100.

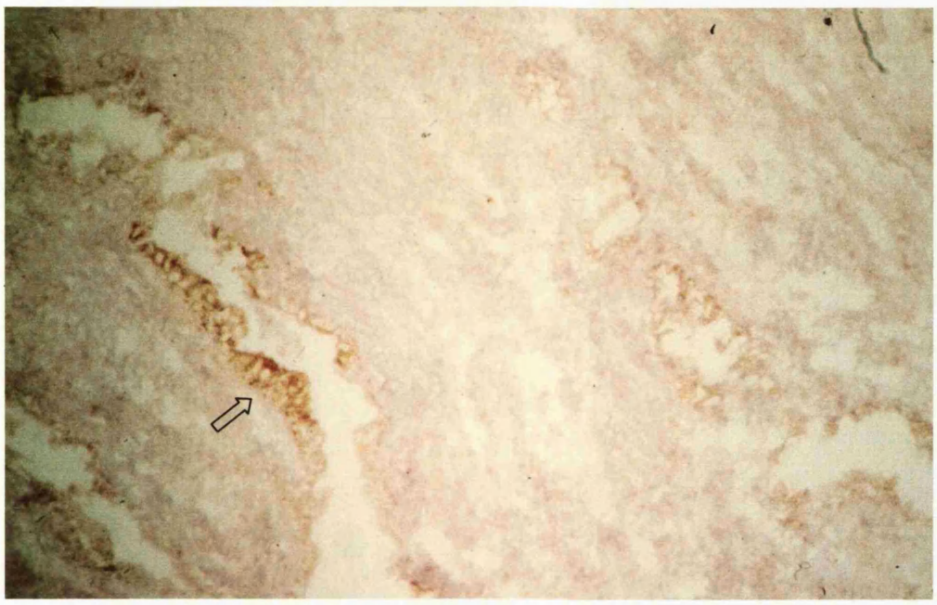


Figure 5.11 Human carcinoma of the bladder with scFv C9 showing heterogeneous reactivity with malignant epithelium (arrowed), magnification x 200.

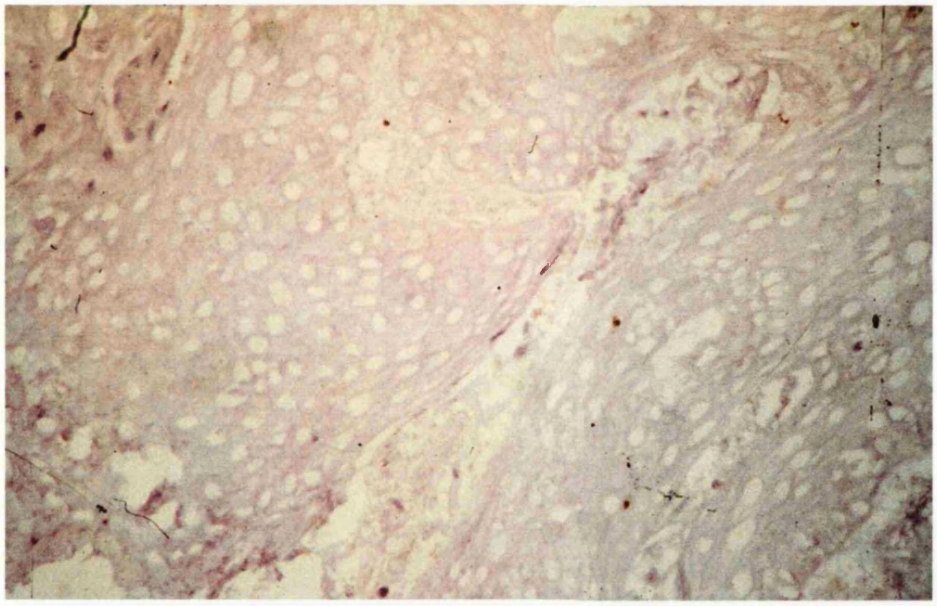


Figure 5.12 Squamous cell carcinoma of the cervix, negative omission control, magnification x 400.

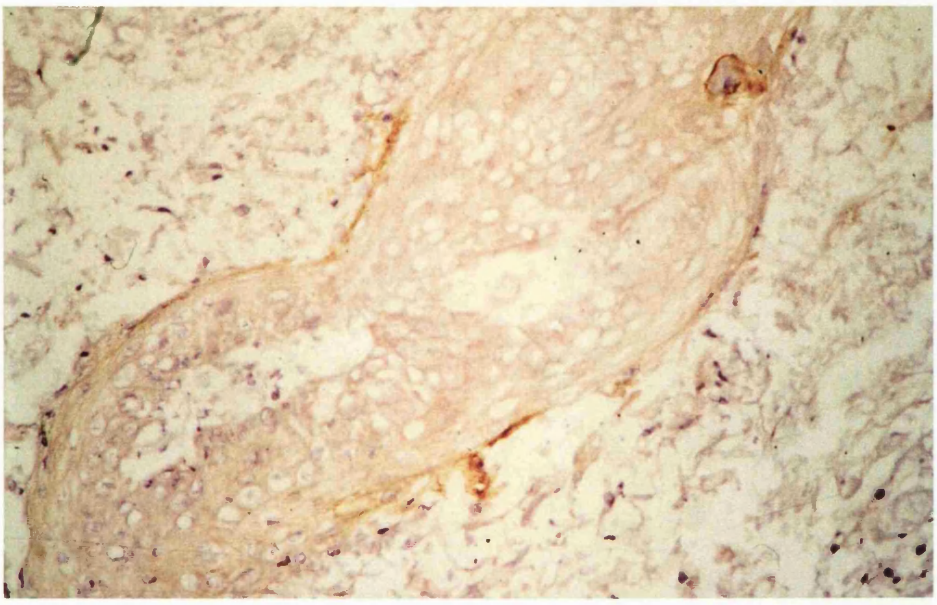


Figure 5.13 Squamous cell carcinoma of the cervix with scFv C9, diffuse cytoplasmic reactivity and with more intense reactivity with keratinising epithelium magnification x 400.

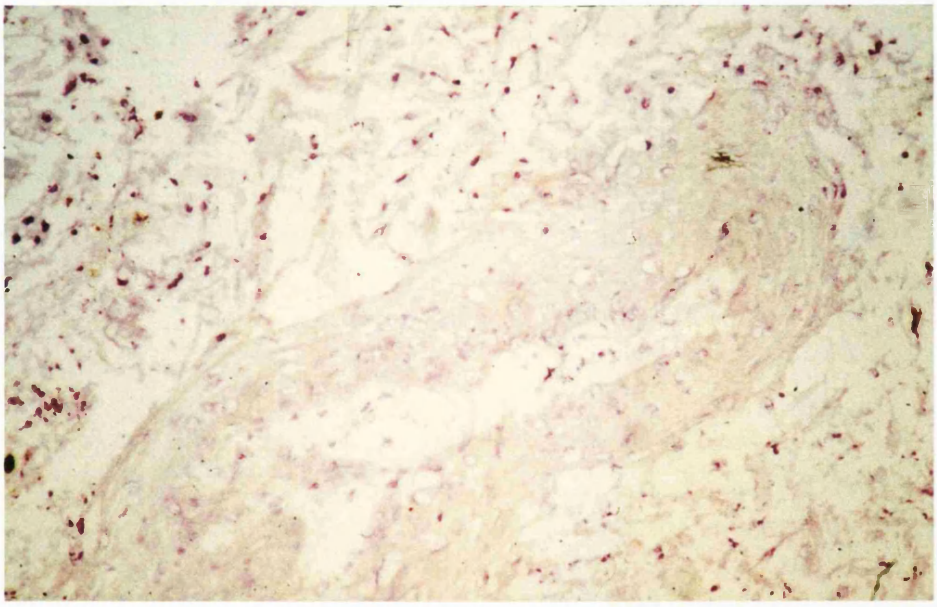


Figure 5.14 Human squamous cell carcinoma of the cervix with scFv C8, magnification x 400.

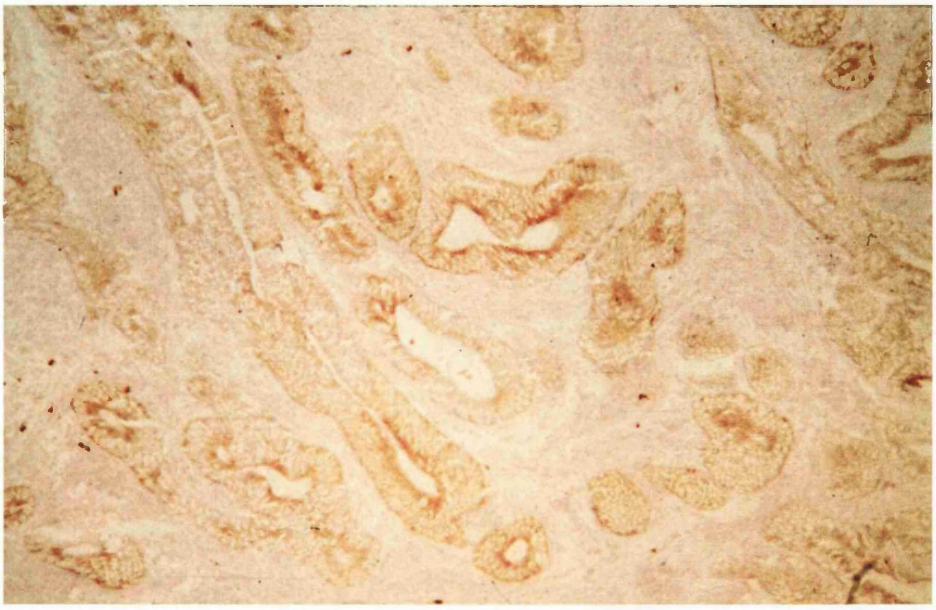


Figure 5.15 Human colorectal adenocarcinoma with scFv C9 showing binding to malignant glandular structures, some luminal surfaces are highlighted, magnification x 100.

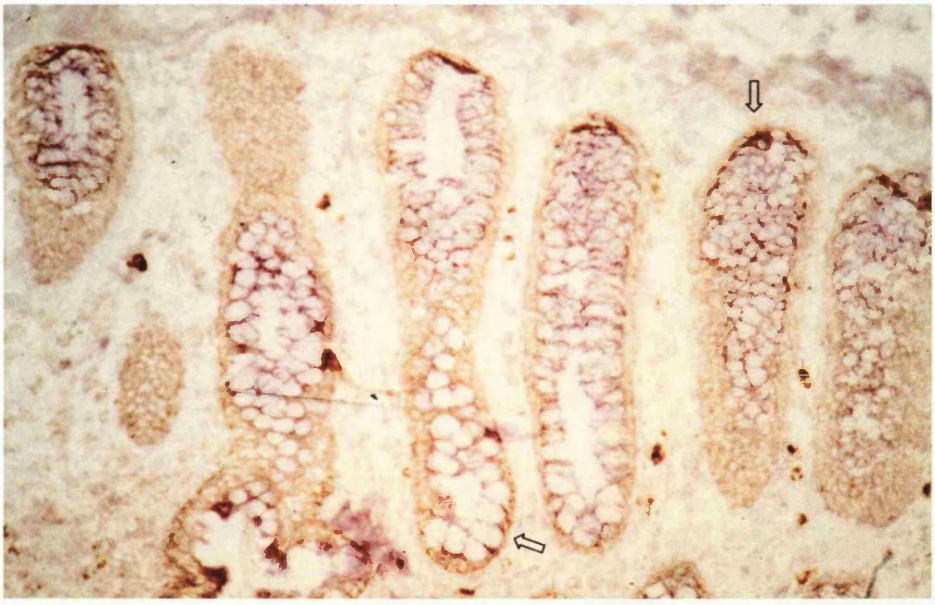


Figure 5.16 Normal human colonic mucosa with scFv C9 showing reactivity at basal aspect of crypt epithelium (arrowed), magnification x 200.

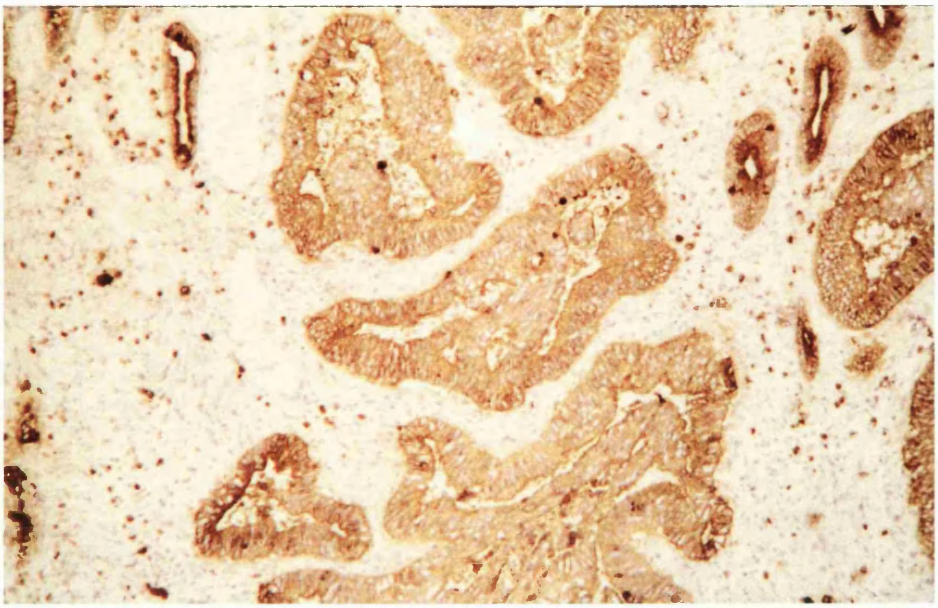


Figure 5.17 Human colorectal adenocarcinoma with scFv MFE-23, malignant glandular structures are uniformly positive for CEA, magnification x 100.

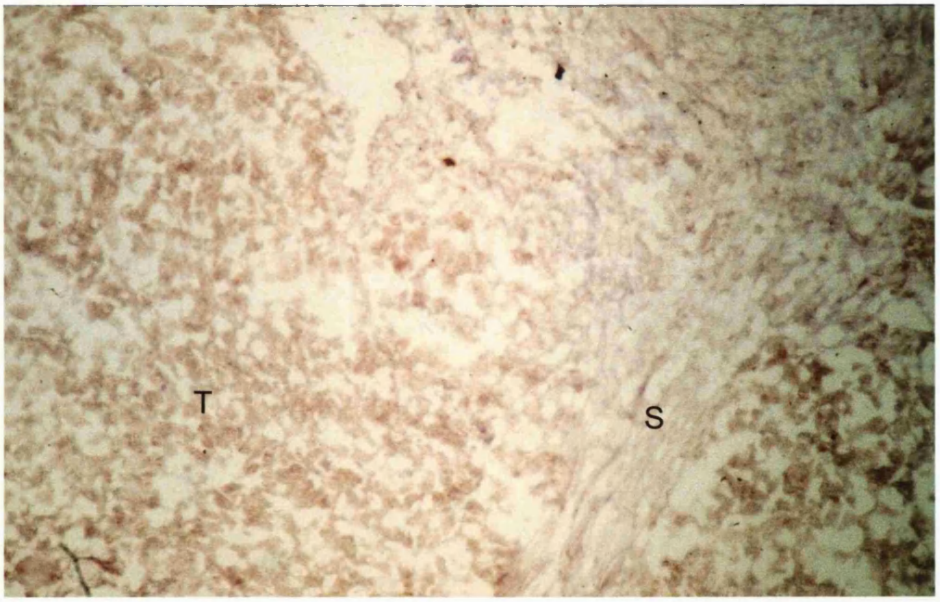


Figure 5.18 Human lung squamous cell carcinoma with scFv C9 showing reactivity with malignant cells, magnification x 400 (tumour tissue (T) and stromal tissue (S) indicated.

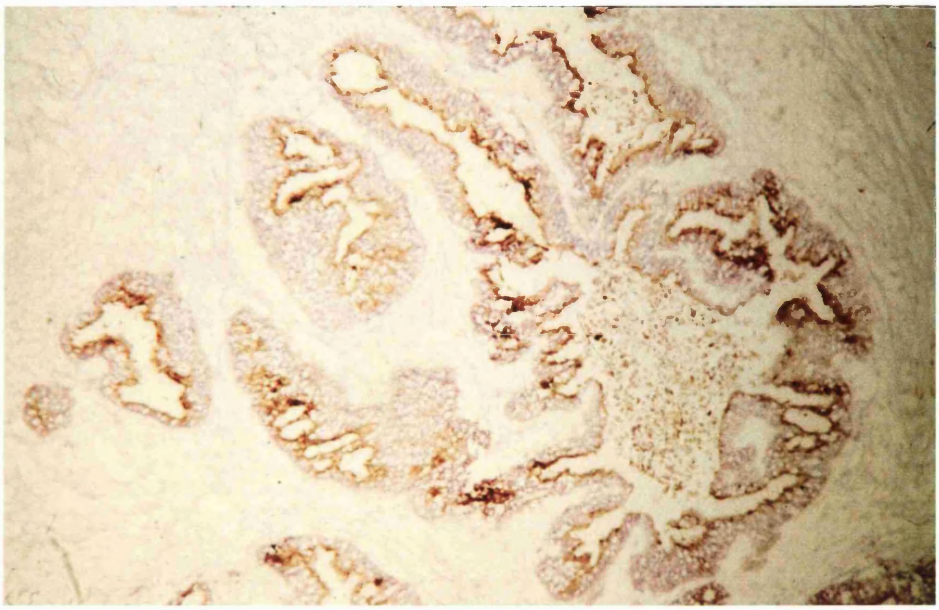


Figure 5.19 Human papillary serous adenocarcinoma of the ovary with scFv C9 showing reactivity with malignant glands, strong luminal staining with some cytoplasmic reactivity, magnification x 200.

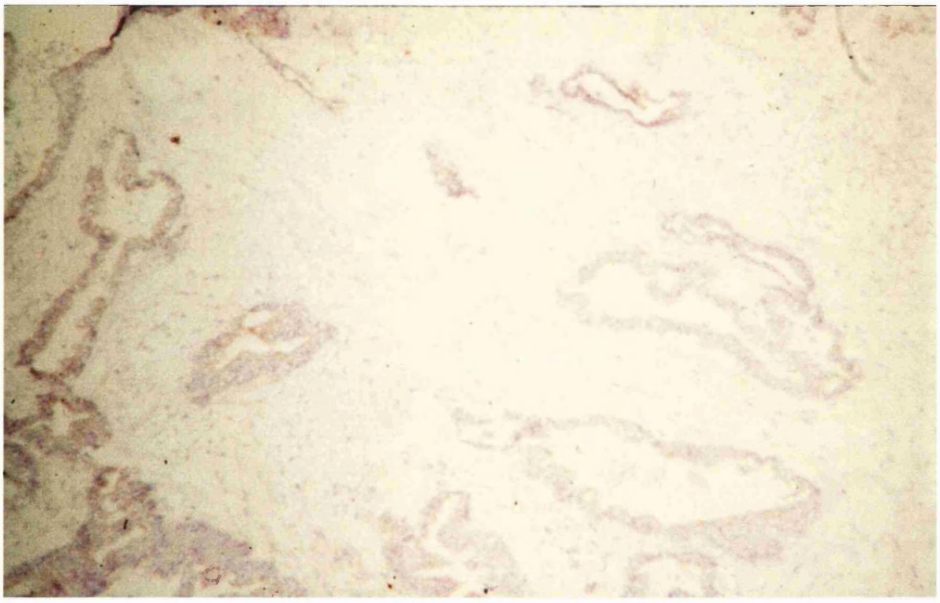


Figure 5.20 Human papillary serous adenocarcinoma of the ovary, negative omission control, magnification x 100.

5.4 Determination of S2.5 V-gene usage

The cloned S2.5 VH and VL genes were sequenced and sequence analysis performed using V-BASE (http: www.mrc-cpe.cam.ac.uk). Sequence alignments are shown [Figure 5.21 and Figure 5.22] to the nearest germ line sequences, numbering and CDRs are according to Kabat (Kabat and Wu, 1991). Except for CDR1 of both VH and VK regions where alignments are according to Chothia and Tomlinson (Chothia *et al.*, 1992), (Tomlinson *et al.*, 1995).

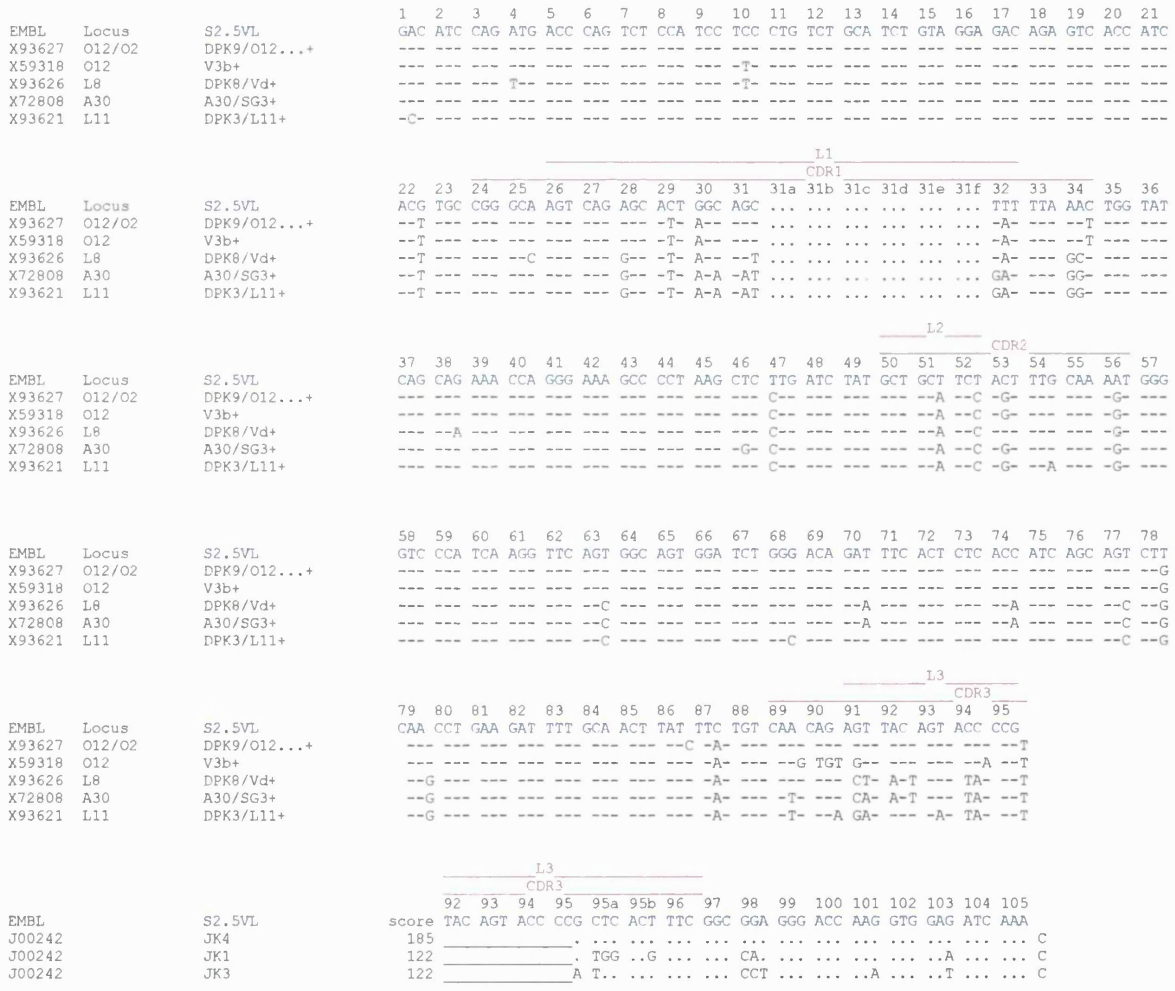


Figure 5.21 Sequence alignment using V-BASE of VK gene from scFv clone C9, derived from the human monoclonal antibody S2.5, to nearest germ line sequences. Numbering and CDRs are according to Kabat (Kabat and Wu, 1991).

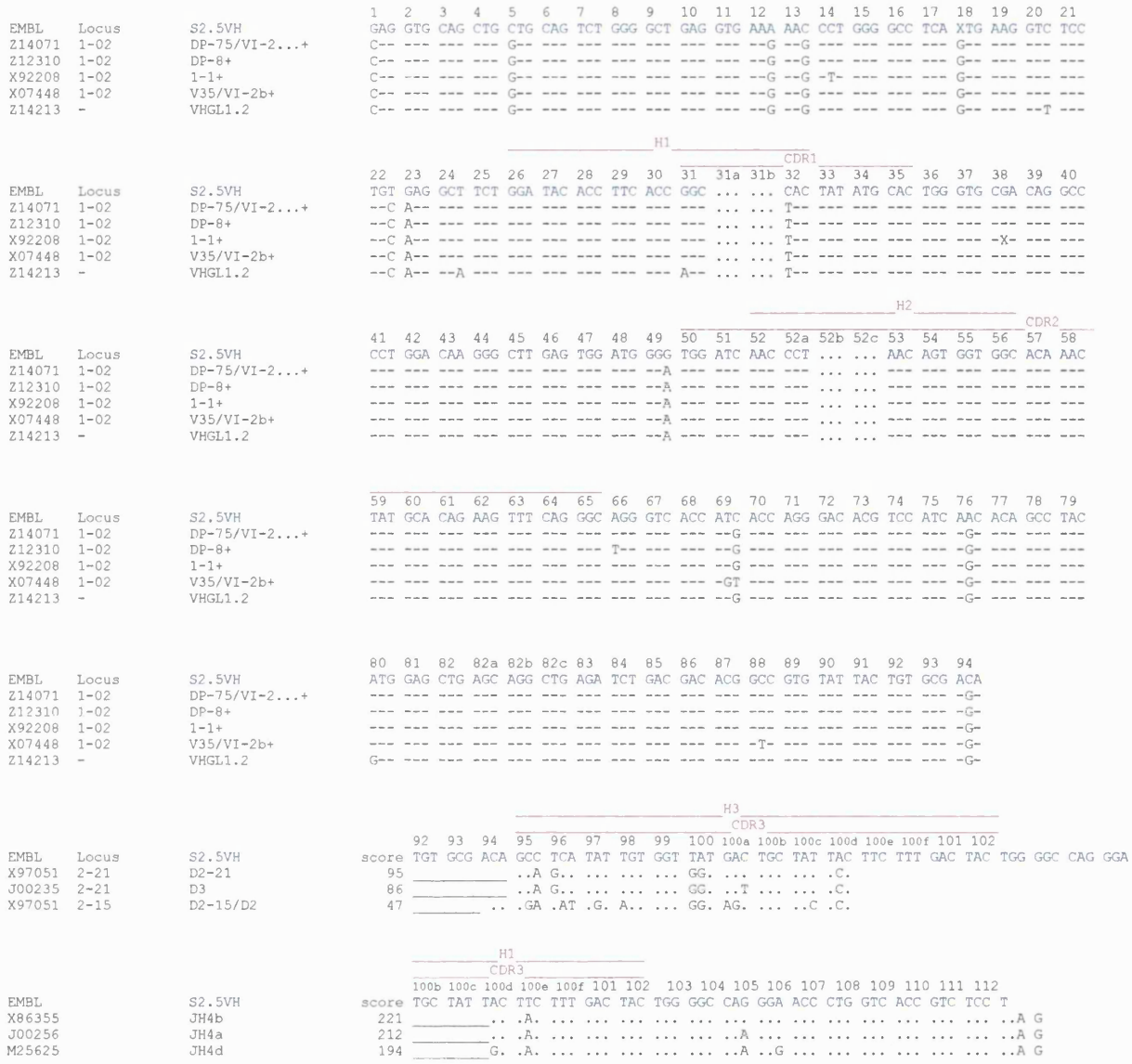


Figure 5.22 Sequence alignment using V-BASE of VH gene from scFv clone C9, derived from the human monoclonal antibody S2.5, to nearest germ line sequences. Numbering and CDRs are according to Kabat (Kabat and Wu, 1991).

The light chain (VJ) of C9 scFv uses the VL segment DPK9, and the J segment JK4. There are a total of 15 mutations, 8 of which result in amino acid changes. Three of these changes occur in CDR1 and two in CDR2. The CDR1 mutations result changes at; codon 29 Ile to Thr, codon 30 Ser to Gly and at codon 32 Tyr to Phe. The mutations in CDR2 result in changes at codon 53 of Ser to Thr and codon 56 of Ser to Asn.

Thr, codon 30 Ser to Gly and at codon 32 Tyr to Phe. The mutations in CDR2 result in changes at codon 53 of Ser to Thr and codon 56 of Ser to Asn.

The heavy chain (VDJ) sequence of clone 9 uses the VH segment DP75 and the D (diversity) segment D2-21 with the J (joining) segment JH4b. There are a total of 12 mutations, 7 of which result in amino acid changes, one of which is located in CDR1 which results in a change of Tyr to His at codon 32.

5.5 Discussion

In this chapter antibody engineering techniques were used to rescue the binding specificity of a rare tumour reactive human IgM monoclonal antibody. V-genes were cloned for the construction of a scFv antibody fragment to provide the S2.5 antibody in a format suitable for further characterisation.

V-genes were amplified from mRNA isolated from hybridoma cells secreting LS174T tumour xenograft reactive antibody. Multiple V-gene products were obtained for the heavy and light chains from mRNA derived from both S2.5 and the secretory human fusion partner HMy. This meant that the only way to identify the S2.5 derived V-genes was by screening for functional binding activity after construction and expression of scFvs. Amplification of more than one V-gene product and screening to identify original binding specificity when rescuing V-genes from hybridomas has been reported by others (Clackson *et al.*, 1991, Bradbury *et al.*, 1995, de Haard *et al.*, 1998, Roovers *et al.*, 1999).

Diversity of cloned V-gene sequences was assessed before screening by *BstN1* digestion, nine different *BstN*1 profiles were generated. As only a small number of clones had been generated screening for functional binding using phage display (Krebber *et al.*, 1997, Roovers *et al.*,

1998) was not necessary. Instead, soluble antibody was induced and culture supernatants tested directly for binding reactivity against LS174T tumour xenografts by immunohistochemistry.

Soluble scFv production as detected by dot blot analysis of induced culture supernatants was identified in five of the nine representative clones. Immunohistochemical staining of LS174T tumour xenografts with these supernatants revealed that clones C8 and C9 (with the same *BstN1* profile) showed reactivity with malignant cells like that of the parent S2.5 IgM antibody. The greater intensity of staining observed with C9 compared to C8 was probably due to differences in concentration of soluble scFv in the culture supernatant.

As greater intensity of staining had been observed with C9 this clone was used to test binding against a range of normal and tumour human tissues. The anti-carboxypeptidase G_2 (CPG_2) scFv was used as a negative control (results not shown). Reactivity of C9 with normal tissues was limited and mainly directed against epithelial cells, apart from the reactivity observed with vesicular epithelium in the spleen. C9 reacted specifically with malignant cells from a range of tissues including breast carcinoma and metastasis, pancreatic and stomach adenocarcinomas. Reactivity in carcinoma of the cervix was quite distinct, localising to keratinising epithelium compared to the basal reactivity observed in normal cervix. Differences were also observed in the reactivity with normal colon and colorectal adenocarcinoma. In normal colon C9 positivity occurred at the basal aspects of crypts which changed to binding to malignant glandular cells in colorectal adenocarcinoma with no reactivity observed in hepatic metastases. C9 showed consistent reactivity with ovarian adenocarcinoma.

So, rescue of S2.5 binding specificity was successful despite switching from the multivalent IgM to the monovalent scFv fragment. How is it possible that binding of the scFv fragment was detected when there is an obvious decrease in the functional binding affinity (avidity)? As well as the monomeric form, scFv fragments are known to exist as dimers (50-60 kDa) and trimers (90 kDa) which would, of course, show an avidity advantage over the monomeric form (Holliger *et al.*, 1993). To determine if such species were responsible for the observed binding reactivities, soluble antibody should have been analysed by non-denaturing gel electrophoresis and purified by gel filtration.

Summary

Antibody engineering techinques were successfully used to allow characterisation of a human IgM monoclonal antibody. Rescue of V-genes for the construction of a scFv fragment was necessary to overcome problems assocaited with the hybridoma, such as low levels of antibody, that prevented further characterisation. The S2.5 derived scFv demonstrated binding reactivity against a range of human adenocarcinomas including breast, ovarian, cervix and colon. These findings show that the S2.5 antibody has a binding specificity that could potentially be of use for the detection of malignant disease.

Chapter 6

General Discussion

Discussion

Advances in recombinant antibody technology combined with the power of selection using phage display have had a revolutionary impact on the generation of antibodies. The ability to construct tailor-made reagents in terms of valency, multispecificity as well as effector function has led to the development of novel antibody based molecules. The viability of antibody engineering techniques as a means of generating antibodies of therapeutic interest is evident from the hundreds of antibody based molecules that are currently undergoing clinical trials (Glennie and Johnson, 2000). The aim of this thesis was to investigate if antibody engineering techniques could be used to isolate antibodies for the detection of inflammatory and malignant disease. A number of different routes can be taken when trying to isolate recombinant antibodies each having associated advantages and disadvantages. For example, cloning of V-genes from hybridomas may be desirable for rescue of well characterised binding specificities for the construction novel antibody based molecules or for improvement of binding affinity. However, problems are often encountered during cloning when rescuing binding specificity (see below) which can also result in a reduction of binding affinity. Another disadvantage, due to difficulties associated with generating human hybridomas, is that this approach relies on animal immunisation to generate antigen specific B-cells for subsequent immortalisation. It is possible to by-pass hybridoma technology by selecting binding specificities from antibody repertoires using phage display. High affinity antibodies can be isolated from repertoires constructed from V-genes isolated from immune B-cells however, this technique requires new immunisations and repertoires to be built for each new antigen. The non-human nature of antibodies isolated from such repertoires (and those cloned from murine hybridomas) means that humanisation will be required to overcome problems of immunogenicity, if these antibodies are to be developed for therapy. Naïve antibody repertoires made from V-gene pools of B-cells of non-immunised individuals provide a means of eliminating the requirement

of animal immunisation as well as allowing the direct isolation of human antibodies (Vaughan *et al.*, 1996; de Haard *et al.*, 1999). Once isolated antibody V-genes can be fine tuned *in-vitro* for binding to their antigen using methods that mimic the *in-vivo* affinity maturation process (Marks *et al*, 1992; Hoogenboom, 1997; Chowdhury and Pastan, 1999; Hoogenboom and Chames, 2000). Alternatively affinity maturation may be by-passed by the construction of multivalent molecules (Holliger and Winter, 1997; Hudson and Kortt, 1999),

Thus antibody engineering techniques provide many alternative routes for isolating and improving antibodies with desired binding characteristics. This thesis explored some of these different methods. V-genes were rescued from murine and human hybridomas for the construction of functional antibody fragments and the direct selection of desired specificities from immune and naïve antibody repertoires using phage display was investigated.

Rescuing V-genes from Hybridomas

Cloning of V-genes provides a means of rescuing binding specificities of hybridomas in a format that allows tailoring of these molecules for desired applications. This technique, however has many associated difficulties some of which were experienced when cloning the neutrophil specific murine monoclonal antibody NCA-102. Problems were encountered during all key stages of cloning from amplification of individual V-genes, to assembly of antibody fragment genes and expression. Functional binding of the NCA-102 derived scFv antibody fragment could not be demonstrated. Inability to show functional binding when attempting to clone monoclonal antibodies has been reported by others (Bradbury *et al.*, 1995; Kipriyanov *et al.*, 1996; Krebber *et al.*, 1997). Many cloning problems are related to not finding the correct antigen binding V-gene pair due to 50-95% of generated clones comprising of non-binding or non-functional antibody fragments (Hoogenboom *et al.*, 1990).

Transcription of high levels of aberrant H and L chain mRNAs in the hybridoma, derived either from unsuccessful V-gene recombinations in the B-cell or due to the hybridoma being a fusion product of different B-cells, results in a high frequency of non-antigen binding antibody fragments after cloning (Carroll *et al.*, 1988; Hoogenboom *et al.*, 1990). Thus, screening of clones for binding specificity, as required for cloning of the human hybridoma S2.5, is often necessary and where large numbers of scFvs are generated may require the use of phage display and selection to retrieve binding specificities (Krebber *et al.*, 1997; Roovers *et al.*, 1998). Other factors that may contribute (alone or in combination) to the generation of non-binding clones are the introduction of errors during PCR and cloning artifacts such as deletions, recombinations, insertions or frameshifts (reviewed by Bradbury *et al.*, 1995). For example, V-gene mutations acquired during PCR that produce oligonucleotide changes in N or C terminal regions have been demonstrated to result in the loss (partial) of antigen binding (de Haard *et al.*, 1998). De Haard and co-workers established that alteration of antigen binding of scFvs derived from several murine monoclonal antibodies, were due to mutations acquired within VH FR1 area that affected folding of the antibody fragments. Mutation of V-region sequences may also affect antibody fragment gene expression by modulation of periplasmic export/transport across the bacterial membrane (Ayala *et al.*, 1995).

Selection from antibody repertoires using phage display

Display of antibody repertoires on the surface of phage coupled with selection against antigen has been demonstrated to be a powerful system for the rapid isolation of antigen specific antibody fragments (Hoogenboom and Chames 2000). Selection of antibodies from repertoires is based on 2 principles; firstly that it is possible to enrich specific binders from a background of irrelevant clones for a range of different affinities and secondly that the system permits the relative enrichment of clones encoding high affinity binders over low affinity

clones. The feasiblity of selecting from an immune repertoire was clearly demonstrated with the selections performed with the NCA-95 antigen. However selection of antibodies against the carbohydrate antigen STn from naïve repertoires proved to be more difficult. Inability to select for carbohydrate binding specificities from phage antibody repertoires has been observed by others (Marks *et al.*, 1993; Deng *et al.*, 1994; Dinh *et al.*, 1996; Wang *et al.*, 1997; P. Hollinger and G. Winter personal communication). This difficulty is also reflected by the limited number of publications in this field, those reported include antibodies against blood group antigens H and B (Marks *et al.*, 1993) and the Lewis antigen Le^x (Dinh *et al.*, 1996). Dinh and colleagues isolated 2 high affinity anti-Le^x antigen binding clones (that also showed cross-reactivity to the related antigen SLe^x) from 2 repertoires enriched for carbohydrate binding specificities. These repertoires, displayed on gene VIII, were generated using VH domains belonging to gene families that demonstrated carbohydrate binding and were selected using biotinylated antigen.

Future work

Further development of clones selected against the NCA-95 antigen

If the NCA-95 antigen binding clones can be demonstrated to bind specifically to neutrophils there is potential for these clones to be used as imaging agents for the early recognition and diagnosis of inflammatory disease. Such a reagent would be particularly desirable in rheumatoid arthritis to assess any subclinical involvement of joints as well as for the detection of more widespread disease, thereby facilitating early therapeutic intervention for more effective treatment (D. Isenberg personnel communication). Any cross-reactivity of clones with CEA would not limit their application as imaging agents for sites of inflammation and or infection (Locher *et al.*, 1986; Seybold *et al.*, 1988) as normal CEA expression is restricted to

the luminal surface of gut epithelium. If however neutrophil specificity cannot be demonstrated the ability to recognise CEA could be exploited to develop reagents for the detection of malignant disease such as carcinoma of the breast, lung and ovary.

Isolation of anti-STn antibodies

Carbohydrate binding recombinant antibodies have been successfully isolated from enriched repertoires displayed as gene VIII fusions to increase avidity of binding (Dinh *et al.*, 1996; Wang *et al.*, 1997). If anti-STn antibodies were to be selected from a phage display library then a geneVIII immune repertoire should be used. Such a repertoire could be generated from an immunised mouse but a repertoire made from human V-genes would obviously be preferable. STn-KLH (with adjuvant) has been successfully used to induce a humoral immune response in breast (MacLean *et al.*, 1993; Longenecker *et al.*, 1993; Longenecker *et al.*, 1994; MacLean *et al.*, 1996; Miles *et al.*, 1996), ovarian (MacLean *et al.*, 1996) and colorectal cancer patients (MacLean *et al.*, 1996), PBLs isolated from these patients would provide a source of V-genes enriched for STn binding specificities for construction of a gene VIII immune human repertoire.

The conformation of the carbohydrate antigens may also be of importance. Zhang and co-workers have demonstrated that STn exists in 2 quite distinct configurations on the cell surface both as clustered and unclustered arrays (Zhang *et al.*, 1995). Testing of B72.3, an anti-STn monoclonal antibody, showed that it bound exclusively to the clustered form. Other antibodies TKH2 and CC49 showed stronger binding to clustered STn when compared with unclustered STn (Zhang *et al.*, 1995). So, when reselecting for STn binding specificities, STn should be presented in both configurations. Selections should also be performed using the STn positive cell line LS-C with depletion against the STn negative LS-B cell line (Ogata *et*

al., 1994). Or alternatively selection strategies using ovine submaxilliary mucin (OSM) which has STn in the clustered form (Colcher *et al.*, 1981) together with biotinylated synthetic clustered and unclustered forms could be employed.

Due to the multivalent nature of interaction of antibody fragments from a gene VIII library any positive binding clones would more than likely be of a low affinity. However once isolated, V-genes could be subjected to *in-vitro* maturation (Marks *et al.*, 1992; Chowdhury and Pastan, 1999) to provide the characteristics required for imaging (Adams *et al.*, 1998). This method has been demonstrated to be successful for improving the binding affinity of an anti-carbohydrate binding antibody selected by panning (Deng *et al.*, 1994). Alternatively the functional affinity could increased by multimerisation of the antibody fragments as diabodies and triabodies (reviewed by Hudson, 1998) which have been demonstrated to have superior imaging characteristics of solid tumours than their monovalent forms (Adams *et al.*, 1993; Tai *et al.*, 1995; Adams and Schier, 1999).

Further characterisation of binding specificity of rescued human V-genes

The results obtained from the preliminary characterisation of S2.5 derived scFv antibody fragment are encouraging and warrant further analysis of its binding specificity. It is possible that the specificity of this antibody is directed against a novel tumour antigen as the parent hybridoma was isolated by screening against crude tumour membrane extract. For further characterisation studies large scale production and purification the scFv is required to obtain a known concentration of monomeric scFv. The purified scFv could be used on an affinity column to isolate the antigen from a crude tumour membrane extract and/or from LS174T tumour membrane extract, for Western blot analysis of extracts separated by PAGE (reducing

and non-reducing) and for immunoprecipitation. The bio-distribution of this scFv could also be investigated by *in-vivo* tumour targeting studies in mice.

Conclusion

Antibody engineering offers exciting alternatives to the hybridoma technique for the generation of monoclonal antibodies. This new methodology can be applied to overcome some on the limitations and difficulties associated with traditional methods and allows tailoring of antibodies that can then be used for the generation of novel antibody based molecules. However, as demonstrated by the work carried out in this thesis, these new techniques have their own associated problems. Although generalisations may be made on the applicability of individual approaches, success is dependent on many factors including properties of the target antigen as well individual antibody genes. The work presented in this thesis has demonstrated the feasibility of using selection from phage displayed antibody repertoires and rescue of V-genes from hybridomas for the isolation of antibody fragments, against markers of inflammation and malignancy.

References

Adams, G.P., McCartney, J.E., Tai, M.S., Oppermann, H., Huston, J.S., Stafford, W.F.d., Bookman, M.A., Fand, I., Houston, L.L. and Weiner, L.M. (1993) Highly specific in vivo tumor targeting by monovalent and divalent forms of 741F8 anti-c-erbB-2 single-chain Fv. Cancer Res 53, 4026-34.

Adams, G.P. and Schier, R. (1999) Generating improved single-chain Fv molecules for tumor targeting. J Immunol Methods 231, 249-60.

Adams, G.P., Schier, R., Marshall, K., Wolf, E.J., McCall, A.M., Marks, J.D. and Weiner, L.M. (1998a) Increased affinity leads to improved selective tumor delivery of single-chain Fv antibodies. Cancer Res 58, 485-90.

Adams, G.P., Schier, R., McCall, A.M., Crawford, R.S., Wolf, E.J., Weiner, L.M. and Marks, J.D. (1998b) Prolonged in vivo tumour retention of a human diabody targeting the extracellular domain of human HER2/neu. Br J Cancer 77, 1405-12.

Albuquerque, K.V., Price, M.R., Badley, R.A., Jonrup, I., Pearson, D., Blamey, R.W. and Robertson, J.F. (1995) Pre-treatment serum levels of tumour markers in metastatic breast cancer: a prospective assessment of their role in predicting response to therapy and survival. Eur J Surg Oncol 21, 504-9.

Almers, S., Granerus, G., Franzen, L. and Strom, M. (1996) Technetium-99m scintigraphy: more accurate assessment of ulcerative colitis with exametazime-labelled leucocytes than with antigranulocyte antibodies. Eur J Nucl Med 23, 247-55.

Arakawa, F., Kuroki, M., Misumi, Y., Oikawa, S., Nakazato, H. and Matsuoka, Y. (1990) Characterization of a cDNA clone encoding a new species of the nonspecific cross-reacting antigen (NCA), a member of the CEA gene family. Biochem Biophys Res Commun 166, 1063-71.

Arevalo, J.H., Taussig, M.J. and Wilson, I.A. (1993) Molecular basis of crossreactivity and the limits of antibody-antigen complementarity. Nature 365, 859-63.

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, 617-31.

Avrameas, S. and Ternynck, T. (1993) The natural autoantibodies system: between hypotheses and facts. Mol Immunol 30, 1133-42.

Ayala, M., Balint, R.F., Fernandez-de-Cossio, L., Canaan-Haden, J.W., Larrick, J.W. and Gavilondo, J.V. (1995) Variable region sequence modulates periplasmic export of a single-chain Fv antibody fragment in Escherichia coli. Biotechniques 18, 832, 835-8, 840-2.

Baca, M., Presta, L.G., O'Connor, S.J. and Wells, J.A. (1997) Antibody humanization using monovalent phage display. J Biol Chem 272, 10678-84.

Barbas, C.F., 3rd and Burton, D.R. (1996) Selection and evolution of high-affinity human anti-viral antibodies. Trends Biotechnol 14, 230-4.

Barbas, C.F.d., Bain, J.D., Hoekstra, D.M. and Lerner, R.A. (1992) Semisynthetic combinatorial antibody libraries: a chemical solution to the diversity problem. Proc Natl Acad Sci U S A 89, 4457-61.

Becker, W., Borst, U., Fischbach, W., Pasurka, B., Schafer, R. and Borner, W. (1989) Kinetic data of in-vivo labeled granulocytes in humans with a murine Tc-99m-labelled monoclonal antibody. Eur J Nucl Med 15, 361-6.

Becker, W.S., Saptogino, A. and Wolf, F.G. (1992) The single late (99)Tc(m) granulocyte antibody scan in inflammatory diseases. Nucl Med Commun 13, 186-192.

Bender, E., Pilkington, G.R. and Burton, D.R. (1994) Human monoclonal Fab fragments from a combinatorial library prepared from an individual with a low serum titer to a virus. Hum Antibodies Hybridomas 5, 3-8.

Berek, C. and Milstein, C. (1988) The dynamic nature of the antibody repertoire. Immunol Rev 105, 5-26.

Berling, B., Kolbinger, F., Grunert, F., Thompson, J.A., Brombacher, F., Buchegger, F., Von Kleist, S. and Zimmermann, W. (1990) Cloning of a carcinoembryonic antigen gene family member expressed in leukocytes of chronic myeloid leukemia patients and bone marrow. Cancer Res 50, 6534-6539.

Better, M., Chang, C.P., Robinson, R.R. and Horwitz, A.H. (1988) Escherichia coli secretion of an active chimeric antibody fragment. Science 240, 1041-3.

Bird, R.E., Hardman, K.D., Jacobson, J.W., Johnson, S., Kaufman, B.M., Lee, S.M., Lee, T., Pope, S.H., Riordan, G.S. and Whitlow, M. (1988) Single-chain antigen-binding proteins [published erratum appears in Science 1989 Apr 28;244 (4903):409]. Science 242, 423-6.

Blank, M., Klussmann, E., Kruger Krasagakes, S., Schmitt Graff, A., Stolte, M., Bornhoeft, G., Stein, H., Xing, P.X., McKenzie, I.F., Verstijnen, C.P. and *et al.* (1994) Expression of MUC2-mucin in colorectal adenomas and carcinomas of different histological types. Int J Cancer 59, 301-6.

Boland, C.R. (1993) The biology of colorectal cancer. Implications for pretreatment and follow-up management. Cancer 71, 4180-6.

Boland, C.R., Montgomery, C.K. and Kim, Y.S. (1982a) Alterations in human colonic mucin occurring with cellular differentiation and malignant transformation. Proc Natl Acad Sci U S A 79, 2051-5.

Boland, C.R., Montgomery, C.K. and Kim, Y.S. (1982b) A cancer-associated mucin alteration in benign colonic polyps. Gastroenterology 82, 664-72.

Borup Christensen, P., Erb, K., Jensenius, J.C., Nielsen, B. and Svehag, S.E. (1986) Human-human hybridomas for the study of anti-tumor immune response in patients with colorectal cancer. Int J Cancer 37, 683-8.

Boulianne, G.L., Hozumi, N. and Shulman, M.J. (1984) Production of functional chimaeric mouse/human antibody. Nature 312, 643-6.

Bradbury, A., Ruberti, F., Werge, T., Amati, V., Di Luzio, A., Gontoloni, S., Hoogenboom, H., F., Piccioli, P., Biocca, S. and Cattaneo, A. (1995) The cloning of hybridoma V regions for their ectopic expression in intracellular and intercellular immunization. In: C. Borrebaeck, A, K. (Ed) Antibody Engineering. Oxford University Press, New York, p. 253-362.

Brinkmann, U., Reiter, Y., Jung, S.H., Lee, B. and Pastan, I. (1993) A recombinant immunotoxin containing a disulfide-stabilized Fv fragment. Proc Natl Acad Sci U S A 90, 7538-42.

Buchegger, F., Schreyer, M., Carrel, S. and Mach, J.P. (1984) Monoclonal antibodies identify a CEA crossreacting antigen of 95 kD (NCA-95) distinct in antigenicity and tissue distribution from the previously described NCA of 55 kD. Int J Cancer 33, 643-649.

Buisine, M.P., Janin, A., Maunoury, V., Audie, J.P., Delescaut, M.P., Copin, M.C., Colombel, J.F., Degand, P., Aubert, J.P. and Porchet, N. (1996) Aberrant expression of a human mucin gene (MUC5AC) in rectosigmoid villous adenoma. Gastroenterology 110, 84-91.

Burchell, J., Taylor Papadimitriou, J., Boshell, M., Gendler, S. and Duhig, T. (1989) A short sequence, within the amino acid tandem repeat of a cancer-associated mucin, contains immunodominant epitopes. Int J Cancer 44, 691-6.

Burton, D.R., Barbas, C.F.d., Persson, M.A., Koenig, S., Chanock, R.M. and Lerner, R.A. (1991) A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. Proc Natl Acad Sci U S A 88, 10134-7.

Carroll, W.L., Mendel, E. and Levy, S. (1988) Hybridoma fusion cell lines contain an aberrant kappa transcript. Mol Immunol 25, 991-5.

Chames, P., Hufton, S.E., Coulie, P.G., Uchanska-Ziegler, B. and Hoogenboom, H.R. (2000) Direct selection of a human antibody fragment directed against the tumor T-cell epitope HLA-A1-MAGE-A1 from a nonimmunized phage-Fab library. Proc Natl Acad Sci U S A 97, 7969-74.

Chang, H.R., Koda, K., Chang, S. and Baird, S. (1993) AgSK1, a novel carcinoma associated antigen. Cancer Res 53, 1122-7.

Chang, S.K., Dohrman, A.F., Basbaum, C.B., Ho, S.B., Tsuda, T., Toribara, N.W., Gum, J.R. and Kim, Y.S. (1994) Localization of mucin (MUC2 and MUC3) messenger RNA and peptide expression in human normal intestine and colon cancer. Gastroenterology 107, 28-36.

Chester, K.A., Begent, R.H., Robson, L., Keep, P., Pedley, R.B., Boden, J.A., Boxer, G., Green, A., Winter, G., Cochet, O. and *et al.* (1994) Phage libraries for generation of clinically useful antibodies. Lancet 343, 455-6.

Chester, K.A. and Hawkins, R.E. (1995) Clinical issues in antibody design. Trends Biotechnol 13, 294-300.

Chothia, C., Lesk, A.M., Gherardi, E., Tomlinson, I.M., Walter, G., Marks, J.D., Llewelyn, M.B. and Winter, G. (1992) Structural repertoire of the human VH segments. J Mol Biol 227, 799-817.

Chothia, C., Lesk, A.M., Tramontano, A., Levitt, M., Smith Gill, S.J., Air, G., Sheriff, S., Padlan, E.A., Davies, D., Tulip, W.R. and *et al.* (1989) Conformations of immunoglobulin hypervariable regions. Nature 342, 877-83.

Chowdhury, P.S. and Pastan, I. (1999) Improving antibody affinity by mimicking somatic hypermutation in vitro. Nat Biotechnol 17, 568-72.

Clackson, T., Hoogenboom, H.R., Griffiths, A.D. and Winter, G. (1991) Making antibody fragments using phage display libraries. Nature 352, 624-8.

Colcher, D., Hand, P.H., Nuti, M. and Schlom, J. (1981) A spectrum of monoclonal antibodies reactive with human mammary tumor cells. Proc Natl Acad Sci U S A 78, 3199-203.

Colcher, D. (1998) Centralized radiolabeling of antibodies for radioimmunotherapy. J Nucl Med 39, 11S-13S.

Collet, B., Maros, S., Moisan, A., Le Cloirec, J., Moinereau, M., Aumaitre, E., Toujas, L. and Bourguet, P. (1993) 111Indium-F(ab)'2-NCA 102 monoclonal antibody: in vitro study of a specific agent for the detection of inflammatory foci. Nucl Med Biol 20, 175-82.

Collet, B., Moisan, A., Maros, S., Moinnereau, M., Le Cloirec, J., Saccavini, J., C. and Bourguet, P. (1991) A new In 111-labeled FAB'2 antigranulocyte antibody for imaging focal sites of infection: in vitro studies and preliminary results. J Nucl Med 325, 1051.

Collins, B.H., Cotterell, A.H., McCurry, K.R., Alvarado, C.G., Magee, J.C., Parker, W. and Platt, J.L. (1995) Cardiac xenografts between primate species provide evidence for the importance of the alpha-galactosyl determinant in hyperacute rejection. J Immunol 154, 5500-10.

Cooper, M.J., Mackie, C.R., Skinner, D.B. and Moossa, A.R. (1979) A reappraisal of the value of carcinoembryonic antigen in the management of patients with various neoplasms. Br J Surg 66, 120-3.

Cote, R.J., Morrissey, D.M., Houghton, A.N., Beattie, E.J., Jr., Oettgen, H.F. and Old, L.J. (1983) Generation of human monoclonal antibodies reactive with cellular antigens. Proc Natl Acad Sci U S A 80, 2026-30.

Crissman, J.W. and Smith, G.P. (1984) Gene-III protein of filamentous phages: evidence for a carboxyl-terminal domain with a role in morphogenesis. Virology 132, 445-55.

Cummings, J.H., Bingham, S.A., Heaton, K.W. and Eastwood, M.A. (1992) Fecal weight, colon cancer risk, and dietary intake of nonstarch polysaccharides (dietary fiber) [see comments]. Gastroenterology 103, 1783-9.

David, L., Nesland, J.M., Clausen, H., Carneiro, F. and Sobrinho Simoes, M. (1992) Simple mucin-type carbohydrate antigens (Tn, sialosyl-Tn and T) in gastric mucosa, carcinomas and metastases. APMIS Suppl 27, 162-72.

Davis, G.T., Bedzyk, W.D., Voss, E.W. and Jacobs, T.W. (1991) Single chain antibody (SCA) encoding genes: one-step construction and expression in eukaryotic cells. Biotechnology N Y 9, 165-9.

de Haard, H.J., Kazemier, B., van der Bent, A., Oudshoorn, P., Boender, P., van Gemen, B., Arends, J.W. and Hoogenboom, H.R. (1998) Absolute conservation of residue 6 of immunoglobulin heavy chain variable regions of class IIA is required for correct folding. Protein Eng 11, 1267-76.

de Haard, H.J., van Neer, N., Reurs, A., Hufton, S.E., Roovers, R.C., Henderikx, P., de Bruine, A.P., Arends, J.W. and Hoogenboom, H.R. (1999) A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. J Biol Chem 274, 18218-30.

de Kruif, J., Terstappen, L., Boel, E. and Logtenberg, T. (1995) Rapid selection of cell subpopulation-specific human monoclonal antibodies from a synthetic phage antibody library. Proc Natl Acad Sci U S A 92, 3938-42.

Deng, S.J., MacKenzie, C.R., Sadowska, J., Michniewicz, J., Young, N.M., Bundle, D.R. and Narang, S.A. (1994) Selection of antibody single-chain variable fragments with improved carbohydrate binding by phage display. J Biol Chem 269, 9533-9538.

Devine, P.L., Birrell, G.W., Golder, J.P., Marsh, M.N., Morgan, S., Chang, G., Gillis, D., Hobson, P., Robertson, P., Whybin, R. and *et al.* (1994) Screening and monitoring coeliac disease: multicentre trial of a new serum antibody test kit. Dis Markers 12, 71-80.

Devine, P.L. and McKenzie, I.F. (1992) Mucins: structure, function, and associations with malignancy. Bioessays 14, 619-25.

Dinh, Q., Weng, N.P., Kiso, M., Ishida, H., Hasegawa, A. and Marcus, D.M. (1996) High affinity antibodies against Lex and sialyl Lex from a phage display library. J Immunol 157, 732-8.

Ditzel, H.J., Binley, J.M., Moore, J.P., Sodroski, J., Sullivan, N., Sawyer, L.S., Hendry, R.M., Yang, W.P., Barbas, C.F., 3rd and Burton, D.R. (1995) Neutralizing recombinant human antibodies to a conformational V2- and CD4-binding site-sensitive epitope of HIV-1 gp120 isolated by using an epitope-masking procedure. J Immunol 154, 893-906.

Dixon, A.R., Price, M.R., Hand, C.W., Sibley, P.E., Selby, C. and Blamey, R.W. (1993) Epithelial mucin core antigen (EMCA) in assessing therapeutic response in advanced breast cancer--a comparison with CA15.3. Br J Cancer 68, 947-9.

Dorai, H., McCartney, J.E., Hudziak, R.M., Tai, M.S., Laminet, A.A., Houston, L.L., Huston, J.S. and Oppermann, H. (1994) Mammalian cell expression of single-chain Fv (sFv) antibody proteins and their C-terminal fusions with interleukin-2 and other effector domains. Biotechnology N Y 12, 890-7.

Dukes, C., E. (1932) The classification of cancer of the rectum. J Pathol Bacteriol 35, 323-332.

Edwards, P.A., Smith, C.M., Neville, A.M. and O'Hare, M.J. (1982) A human-hybridoma system based on a fast-growing mutant of the ARH-77 plasma cell leukemia-derived line. Eur J Immunol 12, 641-8.

Erlich, P. (1957) The collated papers of Paul Erlich. In: Himmelweit (Ed) Immunology and Cancer Research. Pergammon Press, London, p. 442-495.

Fagerberg, J., Hjelm, A.L., Ragnhammar, P., Frodin, J.E., Wigzell, H. and Mellstedt, H. (1995) Tumor regression in monoclonal antibody-treated patients correlates with the presence of anti-idiotype-reactive T lymphocytes. Cancer Res 55, 1824-1827.

Fearon, E.R., Ekstrand, B.C., Hu, G., Pierceall, W.E., Reale, M.A. and Bigner, S.H. (1994) Studies of the deleted in colorectal cancer gene in normal and neoplastic tissues. Cold Spring Harb Symp Quant Biol 59, 637-43.

Feizi, T. (1985) Demonstration by monoclonal antibodies that carbohydrate structures of glycoproteins and glycolipids are onco-developmental antigens. Nature 314, 53-7.

Feizi, T., Gooi, H.C., Childs, R.A., Picard, J.K., Uemura, K., Loomes, L.M., Thorpe, S.J. and Hounsell, E.F. (1984) Tumour-associated and differentiation antigens on the carbohydrate moieties of mucin-type glycoproteins. Biochem Soc Trans 12, 591-6.

Forman, W.B. (1994) The role of chemotherapy and adjuvant therapy in the management of colorectal cancer. Cancer 74, 2151-3.

Forsberg, G., Forsgren, M., Jaki, M., Norin, M., Sterky, C., Enhorning, A., Larsson, K., Ericsson, M. and Bjork, P. (1997) Identification of framework residues in a secreted recombinant antibody fragment that control production level and localization in Escherichia coli. J Biol Chem 272, 12430-6.

Galanina, O.E., Simeoni, L.A., Ter Grigoryan, A.G., Belyanchikov, I.M. and Bovin, N.V. (1998) Determination of carbohydrate specificity of monoclonal antibodies against MUC1. Tumour Biol 19 Suppl 1, 79-87.

Galili, U., Anaraki, F., Thall, A., Hill Black, C. and Radic, M. (1993) One percent of human circulating B lymphocytes are capable of producing the natural anti-Gal antibody. Blood 82, 2485-93.

Galili, U., Rachmilewitz, E.A., Peleg, A. and Flechner, I. (1984) A unique natural human IgG antibody with anti-alpha-galactosyl specificity. J Exp Med 160, 1519-31.

Gendler, S., Taylor Papadimitriou, J., Duhig, T., Rothbard, J. and Burchell, J. (1988) A highly immunogenic region of a human polymorphic epithelial mucin expressed by carcinomas is made up of tandem repeats. J Biol Chem 263, 12820-3.

Gendler, S.J., Burchell, J.M., Duhig, T., Lamport, D., White, R., Parker, M. and Taylor Papadimitriou, J. (1987) Cloning of partial cDNA encoding differentiation and tumor-associated mucin glycoproteins expressed by human mammary epithelium. Proc Natl Acad Sci U S A 84, 6060-4.

Gendler, S.J., Lancaster, C.A., Taylor Papadimitriou, J., Duhig, T., Peat, N., Burchell, J., Pemberton, L., Lalani, E.N. and Wilson, D. (1990) Molecular cloning and expression of human tumor-associated polymorphic epithelial mucin. J Biol Chem 265, 15286-93.

Gendler, S.J., Spicer, A.P., Lalani, E.N., Duhig, T., Peat, N., Burchell, J., Pemberton, L., Boshell, M. and Taylor Papadimitriou, J. (1991) Structure and biology of a carcinoma-associated mucin, MUC1. Am Rev Respir Dis 144, S42-7.

Gilbertsen, V.A. (1974) Proctosigmoidoscopy and polypectomy in reducing the incidence of rectal cancer. Cancer 34, suppl:936-9.

Girling, A., Bartkova, J., Burchell, J., Gendler, S., Gillett, C. and Taylor Papadimitriou, J. (1989) A core protein epitope of the polymorphic epithelial mucin detected by the

monoclonal antibody SM-3 is selectively exposed in a range of primary carcinomas. Int J Cancer 43, 1072-6.

Giuffre, G., Vitarelli, E., Tuccari, G., Ponz de Leon, M. and Barresi, G. (1996) Detection of Tn, sialosyl-Tn and T antigens in hereditary nonpolyposis colorectal cancer. Virchows Arch 429, 345-52.

Glaser Wuttke, G., Keppner, J. and Rasched, I. (1989) Pore-forming properties of the adsorption protein of filamentous phage fd. Biochim Biophys Acta 985, 239-47.

Glassy, M.C. (1987) Immortalization of human lymphocytes from a tumor-involved lymph node. Cancer Res 47, 5181-8.

Glennie, M.J. and Johnson, P.W. (2000) Clinical trials of antibody therapy. Immunol Today 21, 403-10.

Glockner, W.N., RA. Dahr, W. Uhlenbruck, G. (1976) Alkali-labile oligosaccharides from glycoproteins of different erythrocyte and milk fat globule membranes. *Biochim Biophys Acta* 443, 402-13.

Glockshuber, R., Malia, M., Pfitzinger, I. and Pluckthun, A. (1990) A comparison of strategies to stabilize immunoglobulin Fv-fragments. Biochemistry 29, 1362-7.

Gold, D.V. and Miller, F. (1978) Comparison of human colonic mucoprotein antigen from normal and neoplastic mucosa. Cancer Res 38, 3204-11.

Gold, P. and Freedman, S.O. (1965) Specific carcinoembryonic antigens of the human digestive system. J Exp Med 122, 467-81.

Goldenberg, D.M. (1979) Carcinoembryonic antigen in the management of colorectal cancer [editorial]. Acta Hepatogastroenterol Stuttg 26, 1-3.

Goldenberg, D.M., DeLand, F., Kim, E., Bennett, S., Primus, F.J., van Nagell, J.R., Jr., Estes, N., DeSimone, P. and Rayburn, P. (1978) Use of radiolabeled antibodies to

carcinoembryonic antigen for the detection and localization of diverse cancers by external photoscanning. N Engl J Med 298, 1384-6.

Goldenberg, D.M. and Griffiths, G.L. (1992) Radioimmunotherapy of cancer: arming the missiles. J Nucl Med 33, 1110-2.

Goldenberg, D.M. and Larson, S.M. (1992) Radioimmunodetection in cancer identification. J Nucl Med 33, 803-14.

Gorman, S.D., Clark, M.R., Routledge, E.G., Cobbold, S.P. and Waldmann, H. (1991) Reshaping a therapeutic CD4 antibody. Proc Natl Acad Sci U S A 88, 4181-5.

Gram, H., Marconi, L.A., Barbas, C.F.d., Collet, T.A., Lerner, R.A. and Kang, A.S. (1992) In vitro selection and affinity maturation of antibodies from a naïve combinatorial immunoglobulin library. Proc Natl Acad Sci U S A 89, 3576-80.

Griffiths, A.D. and Duncan, A.R. (1998) Strategies for selection of antibodies by phage display. Curr Opin Biotechnol 9, 102-8.

Griffiths, A.D., Malmqvist, M., Marks, J.D., Bye, J.M., Embleton, M.J., McCafferty, J., Baier, M., Holliger, K.P., Gorick, B.D., Hughes Jones, N.C. and *et al.* (1993) Human anti-self antibodies with high specificity from phage display libraries. EMBO J 12, 725-34.

Hagan, P.L., Halpern, S.E., Dillman, R.O., Shawler, D.L., Johnson, D.E., Chen, A., Krishnan, L., Frincke, J., Bartholomew, R.M., David, G.S. and *et al.* (1986) Tumor size: effect on monoclonal antibody uptake in tumor models. J Nucl Med 27, 422-7.

Hakomori, S. (1985) Aberrant glycosylation in cancer cell membranes as focused on glycolipids: overview and perspectives. Cancer Res 45, 2405-14.

Hamadeh, R.M., Galili, U., Zhou, P. and Griffiss, J.M. (1995) Anti-alpha-galactosyl immunoglobulin A (IgA), IgG, and IgM in human secretions. Clin Diagn Lab Immunol 2, 125-31.

Hammarstrom, S., Olsen, A., Teglund, S. and Baranov, V. (1998) The nature and expression of the human CEA family. In: C.P. Stanners (Ed) Cell Adhesion and Communication Mediated by the CEA family Basic and Clinical Perspectives. Harwood Academic Publishers, p. 1-30.

Hand, P., H., Kashmiri, S., V, S. and Schlom, J. (1994) Potential for recombinant immunoglobulin constructs in the management of carcinoma. Cancer 73, 1105-1113.

Hanisch, F.G., Stadie, T. and Bosslet, K. (1995) Monoclonal antibody BW835 defines a site-specific Thomsen-Friedenreich disaccharide linked to threonine within the VTSA motif of MUC1 tandem repeats. Cancer Res 55, 4036-40.

Hanisch, F.G., Stadie, T.R., Deutzmann, F. and Peter Katalinic, J. (1996) MUC1 glycoforms in breast cancer--cell line T47D as a model for carcinoma-associated alterations of 0-glycosylation. Eur J Biochem 236, 318-27.

Haspel, M.V., McCabe, R.P., Pomato, N., Janesch, N.J., Knowlton, J.V., Peters, L.C., Hoover, H.C., Jr. and Hanna, M.G., Jr. (1985) Generation of tumor cell-reactive human monoclonal antibodies using peripheral blood lymphocytes from actively immunized colorectal carcinoma patients. Cancer Res 45, 3951-61.

Hawkins, R.E., Llewelyn, M.B. and Russell, S.J. (1992) Adapting antibodies for clinical use. BMJ 305, 1348-52.

Hawkins, R.E., Zhu, D., Ovecka, M., Winter, G., Hamblin, T.J., Long, A. and Stevenson, F.K. (1994) Idiotypic vaccination against human B-cell lymphoma. Rescue of variable region gene sequences from biopsy material for assembly as single-chain Fv personal vaccines. Blood 83, 3279-88.

Henderikx, P., Kandilogiannaki, M., Petrarca, C., von Mensdorff Pouilly, S., Hilgers, J.H., Krambovitis, E., Arends, J.W. and Hoogenboom, H.R. (1998) Human single-chain Fv

antibodies to MUC1 core peptide selected from phage display libraries recognize unique epitopes and predominantly bind adenocarcinoma. Cancer Res 58, 4324-32.

Herlyn, M., Steplewski, Z., Herlyn, D. and Koprowski, H. (1979) Colorectal carcinoma-specific antigen: detection by means of monoclonal antibodies. Proc Natl Acad Sci U S A 76, 1438-52.

Hirohashi, S., Clausen, H., Nudelman, E., Inoue, H., Shimosato, Y. and Hakomori, S. (1986) A human monoclonal antibody directed to blood group i antigen: heterohybridoma between human lymphocytes from regional lymph nodes of a lung cancer patient and mouse myeloma. J Immunol 136, 4163-8.

Holliger, P., Prospero, T. and Winter, G. (1993) "Diabodies": small bivalent and bispecific antibody fragments. Proc Natl Acad Sci U S A 90, 6444-8.

Holliger, P. and Winter, G. (1997) Diabodies: small bispecific antibody fragments. Cancer Immunol Immunother 45, 128-30.

Holliger, P. and Hoogenboom, H. (1998) Antibodies come back from the brink. Nat Biotechnol 16, 1015-6.

Hoogenboom, H.R. (1997) Designing and optimizing library selection strategies for generating high-affinity antibodies. Trends Biotechnol 15, 62-70.

Hoogenboom, H.R. and Chames, P. (2000) Natural and designer binding sites made by phage display technology. Immunol Today 21 (8), 371-378.

Hoogenboom, H.R., de Bruine, A.P., Hufton, S.E., Hoet, R.M., Arends, J.W. and Roovers, R.C. (1998) Antibody phage display technology and its applications. Immunotechnology 4, 1-20.

Hoogenboom, H.R., Raus, J.C. and Volckaert, G. (1990) Cloning and expression of a chimeric antibody directed against the human transferrin receptor. J Immunol 144, 3211-7.

Hoogenboom, H.R. and Winter, G. (1992) By-passing immunisation. Human antibodies from synthetic repertoires of germline VH gene segments rearranged in vitro. J Mol Biol 227, 381-8.

Houlston, R.S., Murday, V., Harocopos, C., Williams, C.B. and Slack, J. (1990) Screening and genetic counselling for relatives of patients with colorectal cancer in a family cancer clinic. BMJ 1990 Sep 1;301(6749):446.

Hudson, P.J. (1998) Recombinant antibody fragments. Curr Opin Biotechnol 9, 395-402.

Hudson, P.J. and Kortt, A.A. (1999) High avidity scFv multimers; diabodies and triabodies. J Immunol Methods 231, 177-89.

Huston, J.S., Levinson, D., Mudgett Hunter, M., Tai, M.S., Novotny, J., Margolies, M.N., Ridge, R.J., Bruccoleri, R.E., Haber, E., Crea, R. and *et al.* (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in Escherichia coli. Proc Natl Acad Sci U S A 85, 5879-83.

Huls, G., Heijnen, I.A., Cuomo, E., van der Linden, J., Boel, E., van de Winkel, J.G. and Logtenberg, T. (1999a) Antitumor immune effector mechanisms recruited by phage display-derived fully human IgG1 and IgA1 monoclonal antibodies. Cancer Res 59, 5778-84.

Huls, G.A., Heijnen, I.A., Cuomo, M.E., Koningsberger, J.C., Wiegman, L., Boel, E., van der Vuurst de Vries, A.R., Loyson, S.A., Helfrich, W., van Berge Henegouwen, G.P., van Meijer, M., de Kruif, J. and Logtenberg, T. (1999b) A recombinant, fully human monoclonal antibody with antitumor activity constructed from phage-displayed antibody fragments. Nat Biotechnol 17, 276-81.

Hvarregaard, J.A., MH. Berglund, L. Rasmussen, JT. Petersen, TE. (1996) Characterization of glycoprotein PAS-6/7 from membranes of bovine milk fat globules. *Eur J Biochem* 240, 628-36.

Imada, T., Rino, Y., Hatori, S., Takahashi, M., Amano, T., Kondo, J. and Suda, T. (1999a) Sialyl Tn antigen expression is associated with the prognosis of patients with advanced colorectal cancer. Hepatogastroenterology 46, 208-14.

Imada, T., Rino, Y., Takahashi, M., Hatori, S., Shiozawa, M., Amano, T. and Kondo, J. (1999b) Serum CA 19-9, SLX, STN and CEA levels of the peripheral and the draining venous blood in gastric cancer. Hepatogastroenterology 46, 2086-90.

Imam, S.A., Mills, L.A. and Taylor, C.R. (1991) Detection by a human monoclonal antibody of a glycoprotein associated with malignant proliferation of mammary epithelial cells. Br J Cancer 64, 1001-10.

Irving, R.A., Kortt, A.A. and Hudson, P.J. (1996) Affinity maturation of recombinant antibodies using E. coli mutator cells. Immunotechnology 2, 127-43.

Itzkowitz, S.H., Bloom, E.J., Kokal, W.A., Modin, G., Hakomori, S. and Kim, Y.S. (1990a) Sialosyl-Tn. A novel mucin antigen associated with prognosis in colorectal cancer patients. Cancer 66, 1960-6.

Itzkowitz, S.H., Bloom, E.J., Lau, T.S. and Kim, Y.S. (1992) Mucin associated Tn and sialosyl-Tn antigen expression in colorectal polyps. Gut 33, 518-23.

Itzkowitz, S.H., Dahiya, R., Byrd, J.C. and Kim, Y.S. (1990b) Blood group antigen synthesis and degradation in normal and cancerous colonic tissues. Gastroenterology 99, 431-42.

Itzkowitz, S.H., Yuan, M., Montgomery, C.K., Kjeldsen, T., Takahashi, H.K., Bigbee, W.L. and Kim, Y.S. (1989) Expression of Tn, sialosyl-Tn, and T antigens in human colon cancer. Cancer Res 49, 197-204.

Jain, R.K. (1996) Delivery of molecular medicine to solid tumors. Science 271, 1079-80.

Jass, J.R., Allison, L.J. and Edgar, S.G. (1995) Distribution of sialosyl Tn and Tn antigens within normal and malignant colorectal epithelium. J Pathol 176, 143-9.

Jass, J.R., Allison, L.M. and Edgar, S. (1994) Monoclonal antibody TKH2 to the cancer-associated epitope sialosyl Tn shows cross-reactivity with variants of normal colorectal goblet cell mucin. Pathology 26, 418-22.

Jerne, N.K. (1974) Towards a network theory of the immune system. Ann Immunol Paris 125c, 373-89.

Johns, M., George, A.J. and Ritter, M.A. (2000) In vivo selection of sFv from phage display libraries. J Immunol Methods 239, 137-51.

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, 522-5.

Kabat, E.A. and Wu, T.T. (1991) Identical V region amino acid sequences and segments of sequences in antibodies of different specificities. Relative contributions of VH and VL genes, minigenes, and complementarity-determining regions to binding of antibody-combining sites. J Immunol 147, 1709-19.

Kettleborough, C.A., Saldanha, J., Ansell, K.H. and Bendig, M.M. (1993) Optimization of primers for cloning libraries of mouse immunoglobulin genes using the polymerase chain reaction. Eur J Immunol 23, 206-11.

Kim, Y.S. and Gum, J.R., Jr. (1995) Diversity of mucin genes, structure, function, and expression [editorial] [see comments]. Gastroenterology 109, 999-1001.

Kipriyanov, S.M., Kupriyanova, O.A., Little, M. and Moldenhauer, G. (1996) Rapid detection of recombinant antibody fragments directed against cell-surface antigens by flow cytometry. J Immunol Methods 196, 51-62.

Kipriyanov, S.M., Moldenhauer, G., Martin, A.C., Kupriyanova, O.A. and Little, M. (1997) Two amino acid mutations in an anti-human CD3 single chain Fv antibody fragment that affect the yield on bacterial secretion but not the affinity. Protein Eng 10, 445-53.

Kjeldsen, T., Clausen, H., Hirohashi, S., Ogawa, T., Iijima, H. and Hakomori, S. (1988) Preparation and characterization of monoclonal antibodies directed to the tumor-associated O-linked sialosyl-2----6 alpha-N-acetylgalactosaminyl (sialosyl-Tn) epitope. Cancer Res 48, 2214-20.

Kobayashi, H., Terao, T. and Kawashima, Y. (1992) Serum sialyl Tn as an independent predictor of poor prognosis in patients with epithelial ovarian cancer. J Clin Oncol 10, 95-101.

Koda, K. and Glassy, M.C. (1990) In vitro immunization for the production of human monoclonal antibody. Hum Antibodies Hybridomas 1, 15-22.

Kohler, G., Howe, S.C. and Milstein, C. (1976) Fusion between immunoglobulin-secreting and nonsecreting myeloma cell lines. Eur J Immunol 6, 292-5.

Kohler, G. and Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256, 495-7.

Koprowski, H., Herlyn, D., Lubeck, M., DeFreitas, E. and Sears, H.F. (1984) Human anti-idiotype antibodies in cancer patients: Is the modulation of the immune response beneficial for the patient? Proc Natl Acad Sci U S A 81, 216-9.

Kostelny, S.A., Cole, M.S. and Tso, J.Y. (1992) Formation of a bispecific antibody by the use of leucine zippers. J Immunol 148, 1547-53.

Krebber, A., Bornhauser, S., Burmester, J., Honegger, A., Willuda, J., Bosshard, H.R. and Pluckthun, A. (1997) Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a reengineered phage display system. J Immunol Methods 201, 35-55.

Kudoh, K., Kikuchi, Y., Kita, T., Tode, T., Takano, M., Hirata, J., Mano, Y., Yamamoto, K. and Nagata, I. (1999) Preoperative determination of several serum tumor markers in patients with primary epithelial ovarian carcinoma. Gynecol Obstet Invest 47, 52-7.

Kuroki, M., Matsuo, Y. and Matsuoka, Y. (1990) Nonspecific cross-reacting antigen (NCA) expressed by human granulocytes: six species with different peptide sizes and membrane anchoring forms. Biochem Biophys Res Commun 166, 701-8.

Kurosaka, A., Nakajima, H., Funakoshi, I., Matsuyama, M., Nagayo, T. and Yamashina, I. (1983) Structures of the major oligosaccharides from a human rectal adenocarcinoma glycoprotein. J Biol Chem 258, 11594-8.

Lindemann, F., Schlimok, G., Dirschedl, P., Witte, J. and Riethmuller, G. (1992) Prognostic significance of micrometastatic tumour cells in bone marrow of colorectal cancer patients. Lancet 340, 685-9.

Liu, X., Sejbal, J., Kotovych, G., Koganty, R.R., Reddish, M.A., Jackson, L., Gandhi, S.S., Mendonca, A.J. and Longenecker, B.M. (1995) Structurally defined synthetic cancer vaccines: analysis of structure, glycosylation and recognition of cancer associated mucin, MUC-1 derived peptides. Glycoconj J 12, 607-17.

Llorente, M., Sanchez-Palomino, S., Manes, S., Lucas, P., Kremer, L., De Alboran, I.M., Toran, J.L., Alcami, J., Del Real, G. and Martinez, A.C. (1999) Natural human antibodies retrieved by phage display libraries from healthy donors: polyreactivity and recognition of human immunodeficiency virus type 1gp120 epitopes. Scand J Immunol 50, 270-9.

Locher, J.T., Seybold, K., Andres, R.Y., Schubiger, P.A., Mach, J.P. and Buchegger, F. (1986) Imaging of inflammatory and infectious lesions after injection of radioiodinated monoclonal anti-granulocytes antibodies. Nucl Med Commun 7, 659-70.

Longenecker, B.M., Reddish, M., Koganty, R. and MacLean, G.D. (1993) Immune responses of mice and human breast cancer patients following immunization with synthetic sialyl-Tn conjugated to KLH plus detox adjuvant. Ann N Y Acad Sci 690, 276-91.

Longenecker, B.M., Reddish, M., Koganty, R. and MacLean, G.D. (1994) Specificity of the IgG response in mice and human breast cancer patients following immunization against synthetic sialyl-Tn, an epitope with possible functional significance in metastasis. Adv Exp Med Biol 353, 105-24.

Lou, J., Marzari, R., Verzillo, V., Ferrero, F., Pak, D., Sheng, M., Yang, C., Sblattero, D. and Bradbury, A. (2001) Antibodies in haystacks: how selection strategy influences the outcome of selection from molecular diversity libraries. J Immunol Methods 253, 233-42.

Low, N.M., Holliger, P.H. and Winter, G. (1996) Mimicking somatic hypermutation: affinity maturation of antibodies displayed on bacteriophage using a bacterial mutator strain. J Mol Biol 260, 359-68.

Lynch, H.T., Smyrk, T., Watson, P., Lanspa, S.J., Boman, B.M., Lynch, P.M., Lynch, J.F. and Cavalieri, J. (1991) Hereditary colorectal cancer. Semin Oncol 18, 337-66.

MacKenzie, R. and To, R. (1998) The role of valency in the selection of anti-carbohydrate single-chain Fvs from phage display libraries. J Immunol Methods 220, 39-49.

MacLean, G.D. and Longenecker, B.M. (1991) Clinical significance of the Thomsen-Friedenreich antigen. Semin Cancer Biol 2, 433-9.

MacLean, G.D., Reddish, M., Koganty, R.R., Wong, T., Gandhi, S., Smolenski, M., Samuel, J., Nabholtz, J.M. and Longenecker, B.M. (1993) Immunization of breast cancer patients using a synthetic sialyl-Tn glycoconjugate plus Detox adjuvant. Cancer Immunol Immunother 36, 215-22.

MacLean, G.D., Reddish, M.A., Koganty, R.R. and Longenecker, B.M. (1996) Antibodies against mucin-associated sialyl-Tn epitopes correlate with survival of metastatic adenocarcinoma patients undergoing active specific immunotherapy with synthetic STn vaccine. J Immunother Emphasis Tumor Immunol 19, 59-68.

Maeda, M., Shoji, M., Kawagoshi, T., Futatsuya, R., Honda, T. and Brady, L.W. (1994) Distribution of 111In- and 125I-labeled monoclonal antibody 17-1A in mice bearing xenografts of human pancreatic carcinoma HuP-T4. Cancer 73, 800-7.

Malmborg, A.C., Duenas, M., Ohlin, M., Soderlind, E. and Borrebaeck, C.A. (1996) Selection of binders from phage displayed antibody libraries using the BIAcore biosensor. J Immunol Methods 198, 51-7.

Marks, J.D., Hoogenboom, H.R., Bonnert, T.P., McCafferty, J., Griffiths, A.D. and Winter, G. (1991) By-passing immunization. Human antibodies from V-gene libraries displayed on phage. J Mol Biol 222, 581-97.

Marks, J.D., Hoogenboom, H.R., Griffiths, A.D. and Winter, G. (1992) Molecular evolution of proteins on filamentous phage. Mimicking the strategy of the immune system. J Biol Chem 267, 16007-10.

Marks, J.D., Ouwehand, W.H., Bye, J.M., Finnern, R., Gorick, B.D., Voak, D., Thorpe, S.J., Hughes-Jones, N.C. and Winter, G. (1993) Human antibody fragments specific for human blood group antigens from a phage display library. Biotechnology (N Y) 11, 1145-9.

Martin, E.W., Jr. and Thurston, M.O. (1998) Intraoperative radioimmunodetection. Semin Surg Oncol 15, 205-8.

McCafferty. (1996) Factors affecting panning efficiency. In: B. Kay, G. Winter and McCafferty (Eds), Pahge display of peptides and proteins. Academic Press.

McCafferty, J., Griffiths, A.D., Winter, G. and Chiswell, D.J. (1990) Phage antibodies: filamentous phage displaying antibody variable domains. Nature 348, 552-4.

Meller, J., Ivancevic, V., Conrad, M., Gratz, S., Munz, D.L. and Becker, W. (1998) Clinical value of immunoscintigraphy in patients with fever of unknown origin. J Nucl Med 39, 1248-53.

Mellstedt, H., Frodin, J.E., Masucci, G., Ragnhammar, P., Fagerberg, J., Hjelm, A.L., Shetye, J., Wersall, P. and Osterborg, A. (1991) The therapeutic use of monoclonal antibodies in colorectal carcinoma. Semin Oncol 18, 462-77.

Miles, D.W., Towlson, K.E., Graham, R., Reddish, M., Longenecker, B.M., Taylor-Papadimitriou, J. and Rubens, R.D. (1996) A randomised phase II study of sialyl-Tn and DETOX-B adjuvant with or without cyclophosphamide pretreatment for the active specific immunotherapy of breast cancer. Br J Cancer 74, 1292-6.

Minton, J.P., Hoehn, J.L., Gerber, D.M., Horsley, J.S., Connolly, D.P., Salwan, F., Fletcher, W.S., Cruz, A.B., Jr., Gatchell, F.G., Oviedo, M. and *et al.* (1985) Results of a 400-patient carcinoembryonic antigen second-look colorectal cancer study. Cancer 55, 1284-90.

Moertel, C.G., Fleming, T.R., Macdonald, J.S., Haller, D.G., Laurie, J.A., Goodman, P.J., Ungerleider, J.S., Emerson, W.A., Tormey, D.C., Glick, J.H. and *et al.* (1990) Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma [see comments]. N Engl J Med 322, 352-8.

Morguet, A.J., Munz, D.L., Ivancevic, V., Werner, G.S., Sandrock, D., Bokemeier, M. and Kreuzer, H. (1994) Immunoscintigraphy using technetium-99m-labeled anti-NCA-95 antigranulocyte antibodies as an adjunct to echocardiography in subacute infective endocarditis. J Am Coll Cardiol 23, 1171-8.

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, 6851-5.

Morrison, S.L. (1992) In vitro antibodies: strategies for production and application. Annu Rev Immunol 10, 239-65.

Morson, B.C. (1971) Precancerous conditions of the large bowel. Proc R Soc Med 64, 959-62.

Murakami, R., Tsukuma, H., Kanamori, S., Imanishi, K., Otani, T., Nakanishi, K., Fujimoto, I. and Oshima, A. (1990) Natural history of colorectal polyps and the effect of polypectomy on occurrence of subsequent cancer. Int J Cancer 46, 159-64.

Muto, T., Bussey, H.J. and Morson, B.C. (1975) The evolution of cancer of the colon and rectum. Cancer 36, 2251-70.

Nakada, H., Inoue, M., Tanaka, N. and Yamashina, I. (1994) Coexpression of cancer-associated carbohydrate antigens, Tn and sialyl Tn. Glycoconj J 11, 262-5.

Neuberger, M.S., Williams, G.T. and Fox, R.O. (1984) Recombinant antibodies possessing novel effector functions. Nature 312, 604-8.

Neumaier, M., Zimmermann, W., Shively, L., Hinoda, Y., Riggs, A.D. and Shively, J.E. (1988) Characterization of a cDNA clone for the nonspecific cross-reacting antigen (NCA) and a comparison of NCA and carcinoembryonic antigen. J Biol Chem 263, 3202-7.

Nuti, M., Teramoto, Y.A., Mariani Costantini, R., Hand, P.H., Colcher, D. and Schlom, J. (1982) A monoclonal antibody (B72.3) defines patterns of distribution of a novel tumor-associated antigen in human mammary carcinoma cell populations. Int J Cancer 29, 539-45.

O'Boyle, K.P., Markowitz, A.L., Khorshidi, M., Lalezari, P., Longenecker, B.M., Lloyd, K.O., Welt, S. and Wright, K.E. (1996) Specificity analysis of murine monoclonal antibodies reactive with Tn, sialylated Tn, T, and monosialylated (2-->6) T antigens. Hybridoma 15, 401-8.

Ogata, S., Chen, A. and Itzkowitz, S.H. (1994) Use of model cell lines to study the biosynthesis and biological role of cancer-associated sialosyl-Tn antigen. Cancer Res 54, 4036-44.

Ogata, S., Ho, I., Chen, A., Dubois, D., Maklansky, J., Singhal, A., Hakomori, S. and Itzkowitz, S.H. (1995) Tumor-associated sialylated antigens are constitutively expressed in normal human colonic mucosa. Cancer Res 55, 1869-74.

Ohshio, G., Yoshioka, H., Manabe, T., Sakahara, H., Yamabe, H., Imamura, M., Inoue, M., Tanaka, N., Nakada, H. and Yamashina, I. (1994) Expression of sialosyl-Tn antigen (monoclonal antibody MLS102 reactive) in normal tissues and malignant tumors of the digestive tract. J Cancer Res Clin Oncol 120, 325-30.

Orntoft, T.F., Harving, N. and Langkilde, N.C. (1990) O-linked mucin-type glycoproteins in normal and malignant colon mucosa: lack of T-antigen expression and accumulation of Tn and sialosyl-Tn antigens in carcinomas. Int J Cancer 45, 666-72.

Orum, H., Andersen, P.S., Oster, A., Johansen, L.K., Riise, E., Bjornvad, M., Svendsen, I. and Engberg, J. (1993) Efficient method for constructing comprehensive murine Fab antibody libraries displayed on phage. Nucleic Acids Res 21, 4491-8.

Osbourn, J.K., Derbyshire, E.J., Vaughan, T.J., Field, A.W. and Johnson, K.S. (1998) Pathfinder selection: in situ isolation of novel antibodies. Immunotechnology 3, 293-302.

Pack, P. and Pluckthun, A. (1992) Miniantibodies: use of amphipathic helices to produce functional, flexibly linked dimeric FV fragments with high avidity in Escherichia coli. Biochemistry 31, 1579-84.

Parsons, H.L., Earnshaw, J.C., Wilton, J., Johnson, K.S., Schueler, P.A., Mahoney, W. and McCafferty, J. (1996) Directing phage selections towards specific epitopes. Protein Eng 9, 1043-9.

Patton, S., Gendler, S.J. and Spicer, A.P. (1995) The epithelial mucin, MUC1, of milk, mammary gland and other tissues. Biochim Biophys Acta 1241, 407-23.

Ping, T., Tornetta, M.A., Ames, R.S., Bankosky, B.C., Griego, S., Silverman, C., Porter, T., Moore, G. and Sweet, R.W. (1996) Isolation of a neutralizing human RSV antibody from a dominant, non-neutralizing immune repertoire by epitope-blocked panning. J Immunol 157, 772-80.

Price, M.R., Rye, P.D., Petrakou, E., Murray, A., Brady, K., Imai, S., Haga, S., Kiyozuka, Y., Schol, D., Meulenbroek, M.F., Snijdewint, F.G., von Mensdorff Pouilly, S., Verstraeten, R.A., Kenemans, P., Blockzjil, A., Nilsson, K., Nilsson, O., Reddish, M., Suresh, M.R., Koganty, R.R., Fortier, S., Baronic, L., Berg, A., Longenecker, M.B., Hilgers, J. and *et al.* (1998) Summary report on the ISOBM TD-4 Workshop: analysis of 56 monoclonal antibodies against the MUC1 mucin. San Diego, Calif., November 17-23, 1996. Tumour Biol 19 Suppl 1, 1-20.

Price, M.R., Sekowski, M., Ladanyi, A., Uray, K., Ma, Y., Durrant, L. and Tendler, S.J. (1993) Immune recognition of human colonic-tumour-associated MUC-2 mucins using an anti-peptide antibody. Int J Cancer 55, 753-9.

Queen, C., Schneider, W.P., Selick, H.E., Payne, P.W., Landolfi, N.F., Duncan, J.F., Avdalovic, N.M., Levitt, M., Junghans, R.P. and Waldmann, T.A. (1989) A humanized antibody that binds to the interleukin 2 receptor. Proc Natl Acad Sci U S A 86, 10029-33.

Ravindran, B., Satapathy, A.K. and Das, M.K. (1988) Naturally-occurring anti-alpha-galactosyl antibodies in human Plasmodium falciparum infections--a possible role for autoantibodies in malaria. Immunol Lett 19, 137-41.

Reuland, P., Winker, K.H., Heuchert, T., Ruck, P., Muller Schauenburg, W., Weller, S. and Feine, U. (1991) Detection of infection in postoperative orthopedic patients with technetium-99m-labeled monoclonal antibodies against granulocytes. J Nucl Med 32, 2209-14.

Rhodes, J.M. (2000) Colorectal cancer screening in the UK: Joint Position Statement by the British Society of Gastroenterology, The Royal College of Physicians, and The Association of Coloproctology of Great Britain and Ireland. Gut 46, 746-8.

Riechmann, L., Clark, M., Waldmann, H. and Winter, G. (1988) Reshaping human antibodies for therapy. Nature 332, 323-7.

Riethmuller, G., Holz, E., Schlimok, G., Schmiegel, W., Raab, R., Hoffken, K., Gruber, R., Funke, I., Pichlmaier, H., Hirche, H., Buggisch, P., Witte, J. and Pichlmayr, R. (1998) Monoclonal antibody therapy for resected Dukes' C colorectal cancer: seven-year outcome of a multicenter randomized trial. J Clin Oncol 16, 1788-94.

Riethmuller, G. and Johnson, J.P. (1992) Monoclonal antibodies in the detection and therapy of micrometastatic epithelial cancers. Curr Opin Immunol 4, 647-55.

Riethmuller, G., Schneider Gadicke, E. and Johnson, J.P. (1993) Monoclonal antibodies in cancer therapy. Curr Opin Immunol 5, 732-9.

Riethmuller, G., Schneider Gadicke, E., Schlimok, G., Schmiegel, W., Raab, R., Hoffken, K., Gruber, R., Pichlmaier, H., Hirche, H., Pichlmayr, R. and *et al.* (1994) Randomised trial of monoclonal antibody for adjuvant therapy of resected Dukes' C colorectal carcinoma. German Cancer Aid 17-1A Study Group [see comments]. Lancet 343, 1177-83.

Roggenbuck, D., Konig, H., Niemann, B., Schoenherr, G., Jahn, S. and Porstmann, T. (1994) Real-time biospecific interaction analysis of a natural human polyreactive monoclonal IgM antibody and its Fab and scFv fragments with several antigens. Scand J Immunol 40, 64-70.

Rondot, S., Koch, J., Breitling, F. and Dubel, S. (2001) A helper phage to improve single-chain antibody presentation in phage display. Nat Biotechnol 19, 75-8.

Roovers, R.C., Henderikx, P., Helfrich, W., van der Linden, E., Reurs, A., de Bruine, A.P., Arends, J.W., de Leij, L. and Hoogenboom, H.R. (1998) High-affinity recombinant phage antibodies to the pan-carcinoma marker epithelial glycoprotein-2 for tumour targeting. Br J Cancer 78, 1407-16.

Ryuko, K., Iwanari, O., Nakayama, S., Iida, K. and Kitao, M. (1992) Clinical evaluation of serum sialosyl-Tn antigen levels in comparison with CA 125 levels in gynecologic cancers. Cancer 69, 2368-78.

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. and Arnheim, N. (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230, 1350-4.

Sakahara, H., Onodera, H., Shirato, M., Yao, Z., Ohshio, G., Kobayashi, H., Hosono, M., Yano, S., Nakada, H., Imamura, M. and *et al.* (1995) Immunoscintigraphy of colorectal cancer using 111In-labeled monoclonal antibody to mucin. Cancer Immunol Immunother 41, 157-61.

Sanna, P.P., Williamson, R.A., De Logu, A., Bloom, F.E. and Burton, D.R. (1995) Directed selection of recombinant human monoclonal antibodies to herpes simplex virus glycoproteins from phage display libraries. Proc Natl Acad Sci U S A 92, 6439-43

Sato, T., Nishimura, G., Nonomura, A., Miwa, K. and Miyazaki, I. (1999) Serological studies on CEA, CA 19-9, STn and SLX in colorectal cancer. Hepatogastroenterology 46, 914-9.

Scheinberg, D.A. (1991) Current applications of monoclonal antibodies for the therapy of hematopoietic cancers. Curr Opin Immunol 3, 679-84.

Schier, R. and Marks, J.D. (1996) Efficient in vitro affinity maturation of phage antibodies using BIAcore guided selections. Hum Antibodies Hybridomas 7, 97-105.

Schlimok, G., Funke, I., Holzmann, B., Gottlinger, G., Schmidt, G., Hauser, H., Swierkot, S., Warnecke, H.H., Schneider, B., Koprowski, H. and *et al.* (1987) Micrometastatic cancer cells in bone marrow: in vitro detection with anti-cytokeratin and in vivo labeling with anti-17-1A monoclonal antibodies. Proc Natl Acad Sci U S A 84, 8672-6.

Schlom, J., Wunderlich, D. and Teramoto, Y.A. (1980) Generation of human monoclonal antibodies reactive with human mammary carcinoma cells. Proc Natl Acad Sci U S A 77, 6841-5.

Segarra, I., Roca, M., Baliellas, C., Vilar, L., Ricart, Y., Mora, J., Puchal, R. and Martin Comin, J. (1991) Granulocyte-specific monoclonal antibody technetium-99m-BW 250/183 and indium-111 oxine-labelled leucocyte scintigraphy in inflammatory bowel disease. Eur J Nucl Med 18, 715-9.

Seybold, K., Locher, J.T., Coosemans, C., Andres, R.Y., Schubiger, P.A. and Blauenstein, P. (1988) Immunoscintigraphic localization of inflammatory lesions: clinical experience. Eur J Nucl Med 13, 587-93.

Siddiki, B., Ho, J.J., Huang, J., Byrd, J.C., Lau, E., Yuan, M. and Kim, Y.S. (1993) Monoclonal antibody directed against colon cancer mucin has high specificity for malignancy. Int J Cancer 54, 467-74.

Siddiqui, J., Abe, M., Hayes, D., Shani, E., Yunis, E. and Kufe, D. (1988) Isolation and sequencing of a cDNA coding for the human DF3 breast carcinoma-associated antigen. Proc Natl Acad Sci U S A 85, 2320-3.

Sikora, K. and Wright, R. (1981) Human monoclonal antibodies to lung-cancer antigens. Br J Cancer 43, 696-700.

Skerra, A. and Pluckthun, A. (1988) Assembly of a functional immunoglobulin Fv fragment in Escherichia coli. Science 240, 1038-41.

Sleisenger, M., H. and Toribara, N., W. (1994) Colorectal Cancer: The adenoma-carcinoma sequence. Proc R Coll Physicians Edinb 24, 315-335.

Spencer, D.I., Price, M.R., Tendler, S.J., De Matteis, C.I., Stadie, T. and Hanisch, F.G. (1996) Effect of glycosylation of a synthetic MUC1 mucin-core-related peptide on recognition by anti-mucin antibodies. Cancer Lett 100, 11-5.

Springer, G.F., Desai, P.R. and Banatwala, I. (1975) Blood group MN antigens and precursors in normal and malignant human breast glandular tissue. J Natl Cancer Inst 54, 335-9.

Stocchi, L. and Nelson, H. (1998) Diagnostic and therapeutic applications of monoclonal antibodies in colorectal cancer. Dis Colon Rectum 41, 232-50.

Strous, G.J. and Dekker, J. (1992) Mucin-type glycoproteins. Crit Rev Biochem Mol Biol 27, 57-92.

Tai, M.S., McCartney, J.E., Adams, G.P., Jin, D., Hudziak, R.M., Oppermann, H., Laminet, A.A., Bookman, M.A., Wolf, E.J., Liu, S. and *et al.* (1995) Targeting c-erbB-2 expressing tumors using single-chain Fv monomers and dimers. Cancer Res 55, 5983s-5989s.

Takahashi, I., Maehara, Y., Kusumoto, T., Kohnoe, S., Kakeji, Y., Baba, H. and Sugimachi, K. (1994) Combined evaluation of preoperative serum sialyl-Tn antigen and carcinoembryonic antigen levels is prognostic for gastric cancer patients. Br J Cancer 69, 163-6.

Tavladoraki, P., Benvenuto, E., Trinca, S., De Martinis, D., Cattaneo, A. and Galeffi, P. (1993) Transgenic plants expressing a functional single-chain Fv antibody are specifically protected from virus attack. Nature 366, 469-72.

Taylor Papadimitriou, J., Millis, R., Burchell, J., Nash, R., Pang, L. and Gilbert, J. (1986) Patterns of reaction of monoclonal antibodies HMFG-1 and -2 with benign breast tissues and breast carcinomas. J Exp Pathol 2, 247-60.

Terashima, S., Takano, Y., Ohori, T., Kanno, T., Kimura, T., Motoki, R. and Kawaguchi, T. (1998) Sialyl-Tn antigen as a useful predictor of poor prognosis in patients with advanced stomach cancer. Surg Today 28, 682-6.

Thakur, M.L. (1990) Immunoscintigraphic imaging of inflammatory lesions: preliminary findings and future possibilities. Semin Nucl Med 20, 92-8.

Thakur, M.L., Lee, J., Defulvio, J., Richard, M.D. and Park, C.H. (1990) Human neutrophils: evaluation of adherence, chemotaxis and phagocytosis, following interaction with radiolabeled antibodies. Nucl Med Commun 11, 37-43.

Thakur, M.L., Marcus, C.S., Henneman, P., Butler, J., Sinow, R., Diggles, L., Minami, C., Mason, G., Klein, S. and Rhodes, B. (1996) Imaging inflammatory diseases with neutrophil-specific technetium-99m-labeled monoclonal antibody anti-SSEA-1. J Nucl Med 37, 1789-95.

Thakur, M.L., Richard, M.D. and White, F.W.d. (1988) Monoclonal antibodies as agents for selective radiolabeling of human neutrophils. J Nucl Med 29, 1817-25.

Thelen, M.H., Eschmann, S.M., Moll Kotowski, M., Dopfer, R. and Bares, R. (1998) Bone marrow scintigraphy with technetium-99m anti-NCA-95 to monitor therapy in malignant osteopetrosis. J Nucl Med 39, 1033-5.

Therkildsen, M.H., Mandel, U., Christensen, M. and Dabelsteen, E. (1993) Simple mucin-type Tn and sialosyl-Tn carbohydrate antigens in salivary gland carcinomas. Cancer 72, 1147-54.

Thor, A., Ohuchi, N., Szpak, C.A., Johnston, W.W. and Schlom, J. (1986) Distribution of oncofetal antigen tumor-associated glycoprotein-72 defined by monoclonal antibody B72.3. Cancer Res 46, 3118-24.

Tomlinson, I.M., Cox, J.P., Gherardi, E., Lesk, A.M. and Chothia, C. (1995) The structural repertoire of the human V kappa domain. EMBO J 14, 4628-38.

Tonegawa, S. (1983) Somatic generation of antibody diversity. Nature 302, 575-81.

Topley and Wilson. (1973) The Normal Immune Response. In: Linton and Pick (Eds), Principles of Bacteriology , Virology and Immunity, p. 358-362.

Turnball, R., B., Kyle, K., Watson, F., R. and Spratt, J. (1967) Cancer of the colon: the influence of no-touch isolation technique on survival rates. Ann Surg 166, 420-427.

Vaughan, T.J., Williams, A.J., Pritchard, K., Osbourn, J.K., Pope, A.R., Earnshaw, J.C., McCafferty, J., Hodits, R.A., Wilton, J. and Johnson, K.S. (1996) Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library [see comments]. Nat Biotechnol 14, 309-14.

Vaughan, T.J., Osbourn, J.K. and Tempest, P.R. (1998) Human antibodies by design. Nat Biotechnol 16, 535-9.

Verrill, H., Goldberg, M., Rosenbaum, R., Abbott, R., Simunovic, L., Steplewski, Z. and Koprowski, H. (1986) Clinical trial of Wistar Institute 17-1A monoclonal antibody in patients with advanced gastrointestinal adenocarcinoma: a preliminary report. Hybridoma 5 Suppl 1, S175-83.

Victorzon, M., Nordling, S., Nilsson, O., Roberts, P.J. and Haglund, C. (1996) Sialyl Tn antigen is an independent predictor of outcome in patients with gastric cancer. Int J Cancer 65, 295-300.

Wahren, B., Gahrton, G. and Hammarstrom, S. (1980) Nonspecific cross-reacting antigen in normal and leukemic myeloid cells and serum of leukemic patients. Cancer Res 40, 2039-44.

Wang, L., Anaraki, F., Henion, T.R. and Galili, U. (1995) Variations in activity of the human natural anti-Gal antibody in young and elderly populations. J Gerontol A Biol Sci Med Sci 50, M227-33.

Wang, L., Radic, M.Z., Siegel, D., Chang, T., Bracy, J. and Galili, U. (1997) Cloning of anti-Gal Fabs from combinatorial phage display libraries: structural analysis and comparison of Fab expression in pComb3H and pComb8 phage. Mol Immunol 34, 609-18.

Werther, J.L., Rivera MacMurray, S., Bruckner, H., Tatematsu, M. and Itzkowitz, S.H. (1994) Mucin-associated sialosyl-Tn antigen expression in gastric cancer correlates with an adverse outcome. Br J Cancer 69, 613-6.

Willett, W. (1989) The search for the causes of breast and colon cancer. Nature 338, 389-94.

Williams, A.F. and Barclay, A.N. (1988) The immunoglobulin superfamily--domains for cell surface recognition. Annu Rev Immunol 6, 381-405.

Winawer, S.J. and Shike, M. (1992) Dietary factors in colorectal cancer and their possible effects on earlier stages of hyperproliferation and adenoma formation [editorial; comment]. J Natl Cancer Inst 84, 74-5.

Winter, G., Griffiths, A.D., Hawkins, R.E. and Hoogenboom, H.R. (1994) Making antibodies by phage display technology. Annu Rev Immunol 12, 433-55.

Wu, A.M., Chen, W., Raubitschek, A., Williams, L.E., Neumaier, M., Fischer, R., Hu, S.Z., Odom Maryon, T., Wong, J.Y. and Shively, J.E. (1996) Tumor localization of anti-CEA single-chain Fvs: improved targeting by non-covalent dimers. Immunotechnology 2, 21-36.

Yiu, C.Y., Baker, L.A. and Boulos, P.B. (1991) Anti-epithelial membrane antigen monoclonal antibodies and radioimmunolocalization of colorectal cancer. Br J Surg 78, 1212-5.

Yonezawa, S., Tachikawa, T., Shin, S. and Sato, E. (1992) Sialosyl-Tn antigen. Its distribution in normal human tissues and expression in adenocarcinomas. Am J Clin Pathol 98, 167-74.

Zhang, S., Walberg, L.A., Ogata, S., Itzkowitz, S.H., Koganty, R.R., Reddish, M., Gandhi, S.S., Longenecker, B.M., Lloyd, K.O. and Livingston, P.O. (1995) Immune sera and monoclonal antibodies define two configurations for the sialyl Tn tumor antigen. Cancer Res 55, 3364-8.

Zhu, Z., Presta, L.G., Zapata, G. and Carter, P. (1997) Remodeling domain interfaces to enhance heterodimer formation. Protein Sci 6, 781-8.