1 Introduction

1.1 Preface

Cancer is the most frequent cause of death in the industrialised world, necessitating an increase in the search for treatments. The immunotherapeutic strategies for cancer treatment, ranges from the interleukin-2 therapies starting in the 1980s and the monoclonal antibody (mAb) therapies in the early 1990s to the plethora of reagents and methods becoming available in the new millennium as genetic engineering and recombinant expression systems evolve. The detection of tumour markers of high specificity opens up the possibility for immunotherapeutic targeting. Among the well-characterised tumour-specific antigens are the carbohydrate antigens such as gangliosides GM2, GD2 and GD3, as well as glycoproteins with Lewis^{Y}, globo-H, Tn, TF and sTn oligosaccharides. These antigens have been used as targets in both passive and active immunotherapeutic strategies, where the approaches adopted include vaccination with whole or lysed tumour cells, purified or synthetic carbohydrates, immunogenic carbohydrate derivatives or carbohydrates conjugated with immunogenic carriers, and all administrated with immunological adjuvants. Alternatively, antibody-mediated targeting strategies can be adopted if tumour-specific antibodies are available. Clinical treatments with monoclonal antibodies against some of these antigens have been reported, but in this respect, the carbohydrate antigens have not been investigated to the same extent as their protein counterparts (recently reviewed by von Mehren (von Mehren et al., 2003)).

The “magic bullet” concept is probably the simplest and most comprehensive strategy: Find a tumour specific antigen, generate specific antibodies, arm antibodies with toxins and shoot. The strategy has been thoroughly investigated with full size antibodies, but smaller recombinant antibody-toxins (immunotoxins) such as fusions with *Pseudomonas* exotoxin A (Pastan, 2003) have also investigated. The FDA approval of Gentuzumab ozogamicin (Mylotarg®), a humanised IgG4 that targets CD33 and delivers calicheamicin into leukemic cells, where it induces DNA strand breaks and apoptosis, is a nice proof of principle for this strategy (FDA-approved antibodies can be found at http://www.fda.gov/cber/efoi/approve.htm).

Antibodies themselves can also be applied directly if they are capable of mediating an immune response such as antibody-dependent cellular cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC). Examples of these are the FDA-approved humanised IgG1’s Trastuzumab (Herceptin®) and Alemtuzumab (Campath®), as well as the chimeric IgG1 Rituximab (Rituxan®).

Targeting the cancer cells with either naked or armed antibodies has the drawback that only the cells that are hit directly will be eliminated. Cancer cells that do not display the targeted antigen will not be

hit, and may proliferate and establish a new population that may not be targeted using the same treatment. This problem is further underlined by the general lach of homogeneity of expression of tumour antigens, especially in solid tumours.

Therapeutic strategies that include bystander effects could potentially solve the escape problem because all cells in the vicinity of the tumour marker may be targeted by the therapy. This will of course not eliminate single tumour cells in the circulation that do not display the targeted antigen, but seems potent in the treatment of small solid tumours, micrometasatsis, metastasis and minimal residual tumours. Therapeutic strategies with bystander effects include radioimmunotherapy and therapy with some prodrugs. The last of the FDA approved monoclonal antibodies, the mouse IgG1 Ibritumomab tiuxetan (Zevalin^{TM}), an ^{90}Y conjugate and Tositumomab (BEXXAR^{TM}), an ^{131}I conjugate are excellent examples of the radioimmunotherapeutic strategy. The advancing role of radiolabelled antibodies in cancer therapy has recently been reviewed in (Goldenberg, 2003).

The main focus of this thesis is the development of an antibody-mediated strategy for cancer treatment based on the tumour specific Thomsen-Friedenreich disaccharide (TF).

The first primary concern in such a development is the quality of the antigen. What is the tumour specificity of the antigen? What is the frequency of expression on different tumour types? What is the level of expression on tumour cells and what is the extend of the homogeneity of expression? Is the antigen shed, and if so how fast and to what extent? To address some of these questions, chapter 1.2 will provide a thorough review of the biology and tumour relevance of TF.

The second milestone is the generation of specific and high affinity monoclonal antibodies. Is the affinity of the antibody adequate? Is it stable enough for therapeutic applications? Is it available in the amounts needed? It may often be an advantage to work with recombinant antibodies, because it enables subsequent optimisation.

The third critical point is the biodistribution and biokinetics of the antibody. What is the tumour targeting efficiency? How well does the antibody penetrate tumours, and what about the tumour retention? What is the serum-clearance rate of the antibody? Recombinant antibodies in different formats (size, valency, pI and protein sequence) can be evaluated and compared. Furthermore, chimeric or humanised versions of the antibody will often elicit less immunogenicity, and are therefore the preferred choice when strategies with dose fractionation are needed. To address the aspects of recombinant antibody, some of the advances in this technology are summarised in chapter 1.3, with the focus on antibody fragments and their generation by phage display technology.

The outcome of the evaluations summarised above will affect the final decisions with respect to the following questions. What is the most potent therapeutic strategy? Will the naked antibody function directly? Does the biodistribution and biokinetics favour radioimmunotherapy, and if so, which radionuclide is best suited? Or do the data favour immunotoxin strategies or prodrug strategies?

1.2 History and Facts on the Thomsen-Friedenreich Antigen

The Thomsen-Friedenreich antigen (TF) was discovered more than 70 years ago after contamination of a blood sample with neuraminidase-producing bacteria. Thomsen and Friedenreich discovered TF as a result of investigating the observation that red blood cells became pan-agglutinable upon neuraminidase treatment (Friedenreich, 1930). Klenk and Uhlenbruck solved the chemical structure of TF in 1960 (Klenk & Uhlenbruch, 1960), and the establishment of TF as a tumour antigen followed in 1975 as a result of the pioneering work of Springer (Springer et al., 1975). The extensive work by Karsten and co-workers has established TF as a risk factor and shown an involvement of TF in the formation of liver metastasis.

1.2.1 Structure and synthesis of TF

TF is a disaccharide composed of D-galactose coupled to N-acetyl-D-galactosamine (Galβ1-3GalNAc). It is found in the α-form *O*-linked to proteins at Ser or Thr residues (generally referred to as TF, TFα or TFβα, the later only introduced to destinguised between the 4 anomers of Gal-GalNAc. Sometimes it has been referred to as T antigen). The β-form of TF is found *O*-linked to glycosphingolipids or gangliosides (referred to as TFβ or TFββ). TFαα (Galα1-3GalNAcα) and TFαβ (Galα1-3GalNAcα) do not occur naturally, but have been synthesised.

In contrast to *N*-glycosylations which are initiated in the Golgi apparatus by addition of large branched structures *en bloc* to Asn residues, followed by extensive trimming by glycosidases and extension by glycosyltransferases, *O*-glycosylations target Ser and Thr residues and are initiated by the addition of N-acetyl-galactosamine (GalNAc) by polypeptide α-GalNAc-transferase. The *O*-linked oligosaccharides are further extended sequentially in the Golgi apparatus by a complex panel of glycosyltransferases. Nearly each step is performed by a family of glycosyltransferases with slightly different specificities and substrate requirements. The initial steps in the pathways of *O*-glycosylation that affect the synthesis of TF are briefly outlined in **Figure 1**.
In normal tissue, *O*-glycan structures with the Core-2 motif (see **Figure 1**) are further elongated with up to ~20 monosaccharides. The long chains contain structures such as polylactosamines, i antigens, L-fucose and/or sialic acids. Moreover, they can be terminated by blood groups (ABO), tissue antigens (Lewis antigens), sulfate or sialic acids (for review see (Brockhausen, 2000; Fukuda, 2002)).

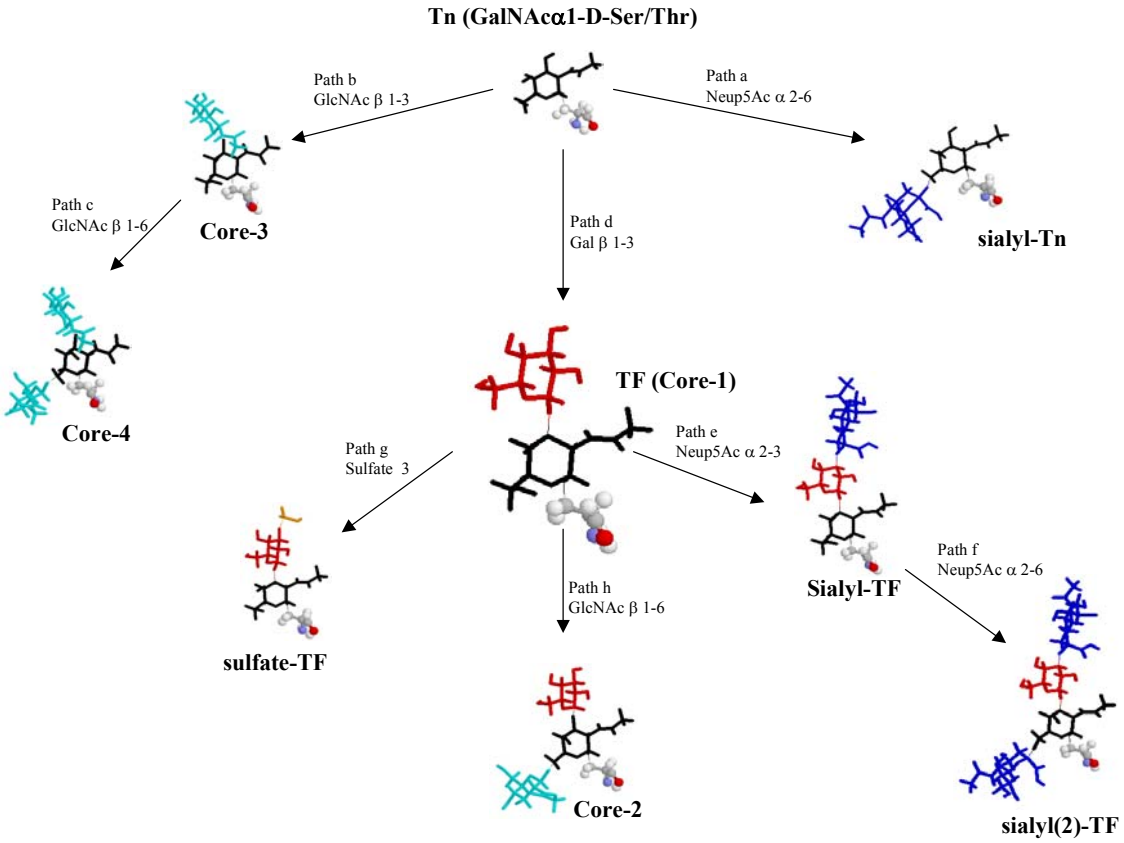

Figure 1 Pathways in the synthesis of TF

The *O*-linked GalNAc, also known as Tn, may undergo 3 different modifications. Tn can be sialylated by α2-6-sialyltransferases to form the terminal structure sialyl-Tn (Path a). Tn can be modified by core-3 β1-3-GlcNAc-transferase to form core 3 (Path b), which may be further modified to core-4 by core-4 β1-6-GlcNAc-transferase (Path c). Finally, Tn can be modified by β1-3-Gal-transferase to form core-1, also known as TF (Path d).
TF itself is not a terminal structure, but is the substrate for 3 different pathways. TF can be modified by addition of sialic acids. The first sialic acid is added by α2-3-sialyltransferase (Path e) to form 3'-sialyl-TF (Neu5Acα2-3Galβ1-3GalNAcα-), and the second by α2-6-sialyltransferase to form 3',6'-disialyl-TF (Neu5Acα2-3Galβ1-3[Neu5Acα2-6]GalNAcα-) (Path f). The 3',6'-disialyl-TF can be further sialylated (2-8) on one of the two sialic acids (not shown). TF can also be modified by Gal 3-sulfotransferase to form 3'-su-TF (Path g). Both sialylated and sulfated TF are terminal structures. In most cases TF is modified by core-2 β1-6-GlcNAc-transferase to form core-2 (Path h).

The aberrant exposure of TF may have several potential explanations at the molecular level. Observing the flowchart of pathways in **Figure 1**, it is plausible to assume that the exposure of TF on the cell surface is caused by either a defect in the processing of TF (Path e-h), an increase in the synthesis of TF (Path d) or a defect in the alternative pathways for Tn-processing (Path a & b/c).
Mutations in enzymes or altered activity levels of enzymes may cause these changes as observed with the decreased activity of core-3 β3-GlcNAc transferase in CaCo-2 cells, and the inactivation of core-2 β6GlcNAc transferase as demonstrated for breast cancer cells by Brockhausen and co-workers (Brockhausen et al., 1991; Brockhausen et al., 1995).

Analogous, changes on the substrate level have been reported to alter certain pathways. For example, TF exposure was increased due to an up-regulation of the UDP-galactose transporter activity in colon cancer tissue, and in colon cancer cell lines following transfections with UDP-galactose transporter constructs (Kumamoto et al., 2001). Recently, a defect in the UDP-N-acetylglucosamine 2-epimerase was shown to minimise the sialylation of TF (manuscript in preparation U. Schöber, personal communication).

1.2.2 Detection of TF and its establishment as tumour specific antigen

Prior to the generation of mAbs toward TF, TF was detected with lectins, in particular peanut agglutinin, PNA (Arachis hypogaca agglutinin) (Lotan et al., 1975; Novogrodsky et al., 1975). However, the lectins are not strictly TF-specific, and PNA binds terminal β-galactose, and shows a rather broad reactivity toward normal tissue (Cao et al., 1996).

Historically, TF was found on the plasma membrane of a variety of carcinoma cells (Springer, 1984). Subsequently, TF expression was found on breast, colon, lung, kidney, ovary and rectum carcinomas using mAb RS1-114 (Stein et al., 1989), colon carcinoma using mAb AH9-16 (Itzkowitz et al., 1989), and bladder carcinoma using mAb HH8 (Langkilde et al., 1992). After these first reports, a comprehensive investigation of the expression of TF revealed that it is virtually absent from normal adult tissues (Cao et al., 1996). The few tissues where minor TF exposure was detected are inaccessible areas with respect to antibody based therapy.

The establishment of the outstanding tumour specificity of TF increased the focus on the expression frequency of TF in the various tumours. The major investigations are listed in **Table 1**

Table 1: Investigations of TF frequency in various tumours

Tumour type	Positive Patients	Antibody applied	Reference
Breast carcinoma	11 of 13 (85%)	mAb RS1-114	(Stein et al., 1989)
Colon carcinoma	7 of 12 (58%)	mAb RS1-114	(Stein et al., 1989)
Lung carcinoma	11 of 16 (69%)	mAb RS1-114	(Stein et al., 1989)
Colon cancer	21 of 29 (71%)	mAb AH9-16	(Itzkowitz et al., 1989)
Colorectal carcinoma	31 of 52 (60%)	mAb A78-G/A7	(Cao et al., 1995)
Colorectal adenomas	22 of 45 (49%)	mAb A78-G/A7	(Baldus et al., 1998)
Colorectal carcinoma	33 of 48 (69%)	mAb A78-G/A7	(Baldus et al., 1998)
Hepatocellular carcinoma	16 of 42 (38%)	mAb A78-G/A7	(Cao et al., 1999)
Pulononary adenocarcinoma	60 of 126 (48%)	MAb (DAKO)	(Takanami, 1999)
Renal cell carcinoma	7 of 53 (13%)	mAb A78-G/A7	(Cao et al., 2000)
Colorectal carcinoma	171 of 264 (64.8%)	mAb A78-G/A7	(Baldus et al., 2000)
Gastric adenocarcinomas	143 of 208 (68.8%)	mAb A78-G/A7	(Baldus et al., 2001)

Many of the TF localisation studies have been performed with the antibody A78-G/A7, which is considered to be the gold standard for TF detection with respect to specificity, and was consequently assigned to the recognition of CD176 (TF) in 2002 (Karsten, 2002). These studies have revealed that TF is a prognostic marker for colon and gastric carcinoma as well as a risk factor for the development of liver metastasis (Goletz et al., 2003).

1.2.3 Potential applications of TF in the clinic

The outstanding specificity of TF for various carcinomas makes it a suitable target for tumour diagnosis and/or therapy. Some of the obvious applications are:

- **TF antibodies in diagnostics**: TF-specific monoclonal antibodies have shown great potential as an indicator of prognosis in immunohistochemical examinations of gastrointestinal tumours.
- **TF as a therapeutic target**: As for virtually all tumour markers, the disadvantage of TF is the lack of homogeneity of the antigen in the tumour. The advantage is the exceptional tumour specificity, which can easily compensate for the low homogeneity if a suitable therapeutic strategy is chosen. The two most obvious strategies are radioimmunotherapy and therapy with prodrugs. Both strategies have strong bystander effects, which may help in case of tumours of lower homogeneity. Presently, the obstacle seems to be that all the established TF-specific antibodies are of rodent origin and nearly all of them are IgMs, which renders them not suitable for human therapy.

  Another potential application of TF specific antibodies is the prevention of metastasis. Cao and co-workers reported a correlation between the expression of TF in primary colorectal tumours and an increased risk for development of liver metastases (Cao et al., 1995). The level of TF expressing metastases (91%) was shown to be significantly higher than the level in primary colorectal tumours (60%). Furthermore, it was found that patients with TF positive tumours have a higher risk (60%) for developing liver metastasis compared to patients with TF-negative tumours (15%). It seems plausible that elimination of TF-positive tumour cells in the circulation could minimise the risk of metastasis. This strategy is supported by a liver metastasis prevention study in mice, where TF-positive tumour cells in the circulation were targeted with A78-G/A7 (Shigeoka et al., 1999).
- **TF in vaccination strategies**: The potential of this strategy is very clearly illustrated by the results of G.F. Springer. The results of his non-randomised clinical trials with advanced breast cancer patients using enzymatically desialylated glycophorin from type O red blood cells were very promising (asialoglycophorin carries 15 TF groups, see chapter 4.1.1) (Springer,

1997). From 32 breast cancer patients (stages II – IV) all survived the first 5 years and 14 more than 10 years; in a second study of 20 patients 15 had no evidence of disease after 3-5 years and 5 showed distant metastases. The vaccination was shown to elicit both an anti-TF antibody response and a TF-cell-mediated autoimmune response (measured by *in vivo* delayed-type skin hypersensitivity).

- **Block of interactions**: Blocking the interactions of TF with either the asialoglycoprotein receptor or galectin-3 would inhibit the docking of the tumour cells in the liver or the vasculatory endothelium, and consequently the formation of metastasis (Beuth et al., 1988; Glinsky et al., 2001; Shigeoka et al., 1999; Stahn & Zeisig, 2000). For the TF masking strategy, it is important to establish, that there is no stimulation of proliferation of the tumour cells, as was observed for the TF-binding lectin PNA (Irazoqui et al., 2001; Yu et al., 2001) and the TF antibodies TF2, TF5, 5A8, 8D8 and BM22 (Yu et al., 1997). Fortunately, this effect was not observed with other antibodies investigated by Yu and co-workers, and was not observed with mAb A78-G/A7 (personal communication, J. Rhodes to U. Karsten), and the effect can therefore not be generalised.

1.3 Antibodies

The function of the vertebrate immune system is to protect against "invasion" of the organism. This defence is maintained through a complex interplay of cells and molecules, of which antibodies are one of the most important molecular components in the defence against pathogens. The efficiency of the antibody response is dependent on the ability to recognise a broad variety of antigens and is enabled by the enormous diversity of the antibody repertoire. The pioneering work on the generation of monoclonal antibodies (mAbs) by Köhler and Milstein (Kohler & Milstein, 1975) initiated the era of antibody-derived diagnostics and therapies. Whereas the diagnostic strategies were very successful, most of the mAbs-based therapeutic trials against cancer were initially disappointing, except for a few anecdotal successes. To improve the properties of antibodies for therapeutic trials, a plethora of recombinant antibodies have been and are being engineered and tested (suggested reviews (Hudson & Souriau, 2003; Milstein & Waldmann, 1999; van Spriel et al., 2000)).

1.3.1 Recombinant antibody fragments

The majority of the first mAbs generated were of rodent origin. These are associated with several disadvantages in respect to therapy. They have relative short half-lives in human serum, human effector functions are only activated by a few of the different antibody classes, and they often trigger a strong human-anti-mouse-antibody (HAMA) response. Consequently, efforts have been invested in recombinant antibodies, which could bypass some of these problems.

It was a major breakthrough to demonstrate that antibody derivatives such as the "Fragment Antigen Binding" (Fab), the "Fragment variable" (Fv) and the "single chain fragments variable" (scFv) could be expressed in bacteria and secreted into the oxidative environment of the bacterial periplasm, where they could be processed in a manner that resembles the process occurring in the endoplasmic reticulum (Better et al., 1988; Bird et al., 1988; Skerra & Pluckthun, 1988). **Figure 2** shows a schematic representation of an IgG and a number of antibody derivatives. The developments in recombinant antibody technology enabled fast, easy and efficient modification and optimisation of the antibodies. Still, correctly folded and active antibody derivatives are not always retrieved from the bacterial periplasm, presumably because the bacterial chaperone system is overwhelmed by the amount of over-expressed recombinant protein, or simply not capable of processing it. Some of the limiting parameters have been investigated in order to increase the yield of functional antibodies (Bothmann & Pluckthun, 1998; Ryabova et al., 1997; Wall & Pluckthun, 1995).

The smallest entity containing the antigen-binding site is the Fv fragment, which is composed of the variable domains of the heavy and light chain (V_H and V_L, respectively). Each variable domain is

composed of a conserved β-sheet framework. The binding site is made up of 6 loops termed the "complementarity determining regions" (CDRs). Three CDRs are mounted on each domain. The antigen-contacting residues of the CDRs and the antigen interactions have been investigated based on crystal structures (MacCallum et al., 1996) and the canonical structures of the variable domains have been established (Chothia & Lesk, 1987; Chothia et al., 1992; Chothia et al., 1989; Tomlinson et al., 1995).

The Fv is relatively unstable because the V_H and V_L domains are non-covalently associated. In the scFv, the V_H and V_L domains are connected covalently with a peptide linker, which provides stability to the molecule. The scFvs have some very attractive properties. They are small, 25-30 kDa, and relatively stable, which makes them well suited for the *in vitro* selection methods (Amstutz et al., 2001). These methods enable fast and efficient generation antibody fragments against almost any antigen.

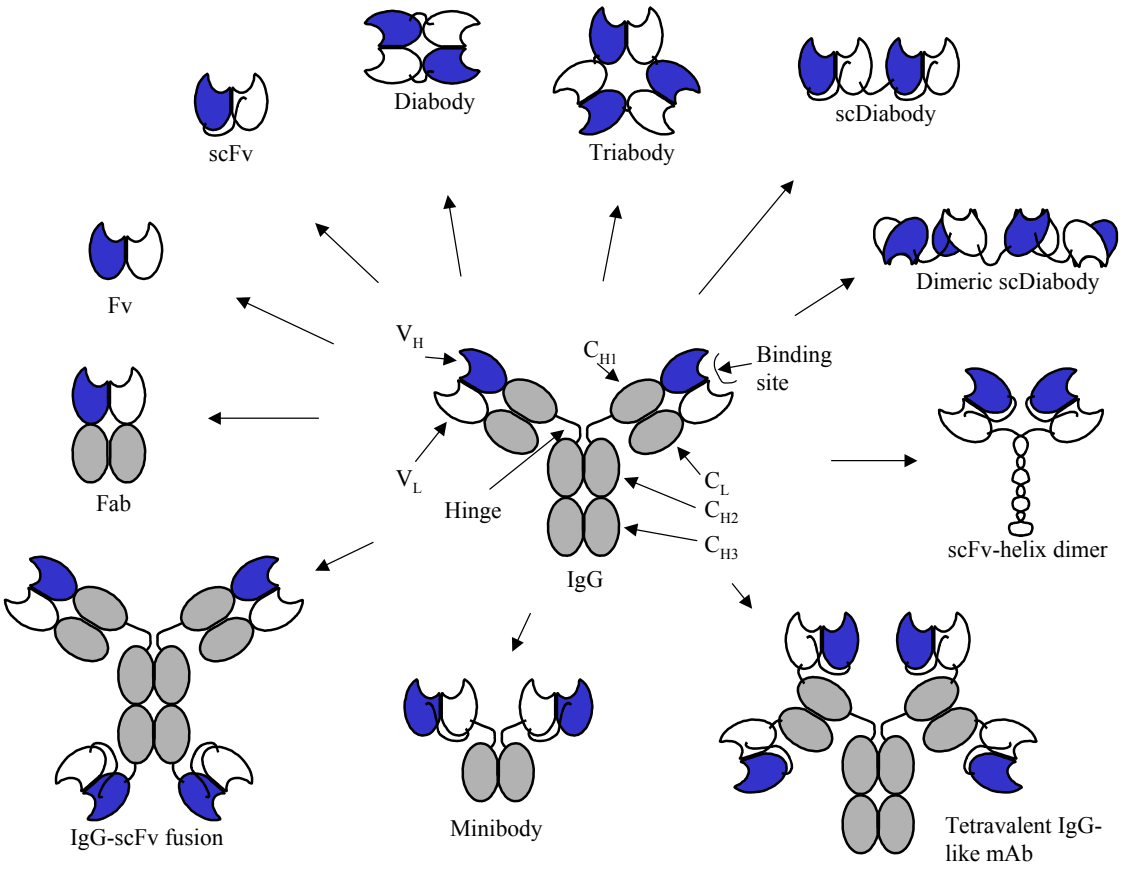

Figure 2 Different antibody formats
Schematic representation of various antibody formats. See main text for references.

1.3.2 Multimerisation

Multivalency seems to be one of the evolutionary advantages of antibodies, by which enormous gains in functional affinity is achieved (the result of the avidity effect of multivalency on the intrinsic affinity of the individual binding sites). This has drawn the focus onto the generation of multivalent complexes. Furthermore, the multivalent complexes often have improved pharmacokinetics and a more selective localisation to the tumours in comparison to monovalent antibody fragments. Consequently, several strategies have evolved to achieve multimerisation of recombinant antibodies.

1.3.2.1 Fusion-derived multimers

Fusion of scFvs to multimerisation domains has been a popular strategy to obtain multivalency. The simplest of these is probably the addition of a C-terminal cystine to the scFv, thereby enabling disulphide bridge formation between two scFvs (King et al., 1994). Alternatively, peptides directing the formation of multimers such as helix bundles (Pack & Pluckthun, 1992) or coiled-coils such as leucine zippers can be fused to the scFv to create dimers or/and tetramers (reviewed in (Pluckthun & Pack, 1997) (**Figure 2**)). Tetramers have also been reported to form when scFvs are fused to the oligomerisation domain of p53 (Rheinnecker et al., 1996) or when the scFv is biotinylated and subsequently allowed to bind to streptavidin (Cloutier et al., 2000).

1.3.2.2 Antibody-derived structures

A group of recombinant antibodies have been engineered exclusively of immunoglobulin folds in order to generate small multivalent molecules. A well established format is the minibody (scFv-hinge-CH_2(IgG)), which forms bivalent molecules of approximately 80 kDa (Borsi et al., 2002; Carter & Merchant, 1997; Hu et al., 1996; Roovers et al., 2001; Williams et al., 2001; Wu et al., 2000; Yazaki et al., 2001) (**Figure 2**). The enlarged molecular size combined with bivalency and higher stability (due to covalently linkage) makes minibodies superior in biodistribution schemes compared to the non-covalent formats. Alternative antibody formats have been engineered with focus on bi-specificity, either by fusing scFvs to the C-terminus of IgG heavy chains (Coloma & Morrison, 1997), or by fusing scFvs to the N-terminus of light and heavy chain constant domains (Muller et al., 1998; Zuo et al., 2000). Finally, a pentamer-forming construct has been reported applying the multimerisation sequence of an IgM in fusion with C_{H3} (Olafsen et al., 1998).

1.3.2.3 ScFv multimers

Initially, the flexible polypeptide linker in the scFv format was designed to restrain the V_H and V_L domains and increase the stability of the scFv. However, it was observed that shortening the linker could dictate the formation of multivalent complexes simply because the variable domains were sterically unable to associate with each other, and were therefore prone to associate with the variable domains of another scFv (Holliger et al., 1993; Holliger & Winter, 1993; Perisic et al., 1994) (**Figure 2**). The general focus was initially on the engineering of dimeric complexes (diabodies). Monospecific bivalent diabodies were expected to have improved pharmacokinetics due to lower dissociation rates and slower serum clearance compared to monomeric scFvs (Adams et al., 1993; King et al., 1994; Yokota et al., 1992). Bispecific diabodies were predicted to have great potential in therapeutic applications for recruitment of complement effector functions or for directing and activating a cellular immune response (Holliger et al., 1996; Holliger et al., 1997; Kontermann et al., 1997).

The group of Peter J. Hudson has done the majority of the pioneering characterisation of scFv multimerisation (Atwell et al., 1999; Atwell et al., 1996; Dolezal et al., 2000; Hudson, 1999; Hudson & Kortt, 1999; Hudson & Souriau, 2003; Iliades et al., 1997; Kortt et al., 2001; Kortt et al., 1997; Lawrence et al., 1998; Malby et al., 1998; Power et al., 2001; Power et al., 2003; Power & Hudson, 2000; Tahtis et al., 2001; Todorovska et al., 2001). It is generally believed that scFv formats with linkers longer than 15-18aa will predominantly form monomers at lower concentrations and dimers at higher concentrations. Dimer formation is usually observed for constructs with linkers between 4aa and 12aa, while linkers shorter than 4aa usually results in the formation of trimers (**Figure 2**). Direct fusion of the variable domains (0aa linker) may lead to the formation of tetramers or higher multimeric complexes. Another important finding was that the grade of multimerisation at a given linker length is dependent on the direction of the scFv (V_HV_L or V_LV_H) and on the antibody sequence (reviewed in (Todorovska et al., 2001)).

1.3.2.4 Single-chain diabodies

Diabodies generated by direct fusion of two scFvs have been engineered in many laboratories (Gruber et al., 1994; Kurucz et al., 1995) (**Figure 2**). The format is especially appealing for the construction of bispecific antibodies, because it minimises the often-problematic purification protocols. In therapeutic settings, the single-chain diabodies may have applicability as monomeric divalent antibodies (Power et al., 2001) or as dimeric tetravalent antibodies (Kipriyanov et al., 1999).

1.3.2.5 Recombinant antibodies in clinical trials

Recombinant antibodies in the IgG format such as human chimeric or fully humanised antibodies have been taken to clinical trials. Until 2 years ago, it was believed that only human or humanised antibodies would get an FDA approval, and thus the list of humanised or chimeric antibodies is long.
However, more and more scFv-derived antibodies are also entering the pre-clinical trials. Most of them are initially tested as simple monovalent scFvs, but it is becoming increasingly clear that the scFv format has pharmacokinetic disadvantages. Many of the recent studies with radiolabelled recombinant antibodies have been carried out with two or more formats of different size and valency, and thus helps generating valuable information. It should be obvious that the data reflect the individual parameters of the antibodies such as affinity, serum stability, immunogenicity etc. This will be further discussed in Chapter 3.

1.3.3 Generation of scFv antibodies

Established hybridoma clones are a rich source for generating scFvs. The variable domains of the antibodies expressed by the hybridoma cells can be derived relatively easily, and cloned into the scFv format. However, a major disadvantage is that not all scFv derived from existing antibodies are active. Another disadvantage is the non-human origin of most hybridoma clones, which may render the derived scFvs immunogenic.
Alternatively, scFvs can be selected from large naïve scFv repertoires or smaller "immunised" repertoires. The techniques and methods for such selections include phage display (Winter et al., 1994), ribosome display (Hanes et al., 1998; Hanes & Pluckthun, 1997; Hanes et al., 2000), bacterial display (Daugherty et al., 1998; Georgiou et al., 1997), yeast display (Boder et al., 2000; Boder & Wittrup, 1997), periplasmic expression cytometric sorting (PECS) (Chen et al., 2001), and potentially some two-hybrid systems to screen for intracellular antibodies. Phage display in a monovalent format is by far the most frequently applied choice, and the system has been extensively reviewed (Hoogenboom, 1997; Hoogenboom & Chames, 2000; Hoogenboom et al., 1998; Kristensen et al., 2000; Winter et al., 1994).

1.3.3.1 Morphology and life cycle of the filamentous bacteriophage Ff

The filamentous bacteriophage is a rod-shaped particle (**Figure 3**) measuring approx. 900 nm in length and 6.5 nm in diameter. The filamentous bacteriophage Ff propagates by infection of *Escherichia coli (E. coli)*, which contains the F conjugative plasmid. The genome of the Ff filamentous bacteriophage is a covalently closed single-stranded DNA molecule (ssDNA) of about 6400 nucleotides. It encodes

eleven proteins that can be grouped according to their functions in the phage morphology or to their roles in the phage lifecycle (reviewed in (Webster, 1996)).

The phage capsid consists of five different proteins of which pVIII (50aa) is the most abundant. pIII, pVI, pVII and pIX (406, 112, 33 and 32aa, respectively) are all present in a few copies at the ends. The actual wall of the rod-like phage particle is composed of pVIII (2700-2800 copies). All the capsid proteins have hydrophobic membrane spanning domains and reside in the inner membrane until they are assembled into the mature phage particle (**Figure 3**).

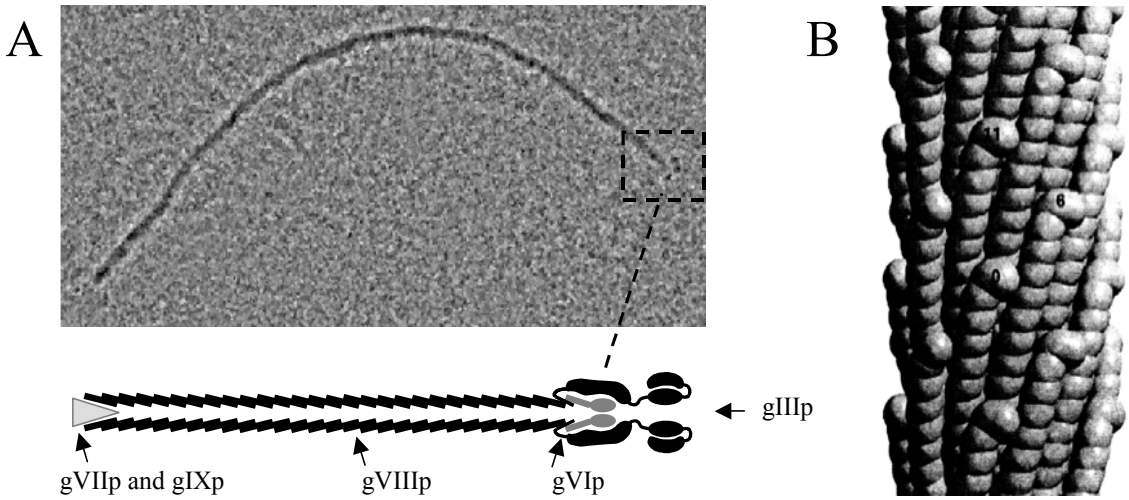

Figure 3 Phage particle and function
A) Electron micrograph of a phage particle and below a schematic representation of the capsid proteins. B) A model of the phage particle showing the organisation of the gVIIIps
The electron micrograph was obtained from (http://www.une/depts/bkay/phagedisplay.html) and the model was adopted from (Marvin, 1998).

The infection process is initiated when the phage particle binds to the first of the two bacterial receptors needed for infection, the F-pilus, via an interaction with domain D2 of the pIII (Deng et al., 1999) (**Figure 4A**). Subsequently, domain D1 of pIII can interact with the second bacterial receptor, TolA, and complete the infection (Click & Webster, 1997; Click & Webster, 1998; Deng & Perham, 2002; Holliger et al., 1999; Lubkowski et al., 1998; Lubkowski et al., 1999; Riechmann & Holliger, 1997).

Three proteins encoded by the phage genome are involved in the replication of phage DNA. pII (410aa) binds to the double-stranded replicative form of the phage genome and introduces a specific cleavage on the (+) strand at the origin of replication. Upon completion of the rolling circle replication, the ssDNA is circularised by pII, and a stabilising complex is formed with pV (87aa). pX (111aa) is the product of an internal initiation of gene II at methionine 300, and is probably controlling the DNA replication (Webster, 1996).

The last three proteins encoded by the genome are required for the assembly of the phage coat. The pI(348aa), pXI(108aa) and pIV(405(+21)aa) proteins form a channel in the bacterial membrane, and are required for the phage assembly and release (Brissette & Russel, 1990; Guy-Caffey et al., 1992; Rapoza & Webster, 1993; Rapoza & Webster, 1995) (**Figure 4B**). It has been speculated that this channel may set the restrictions on maximum size of proteins that can be displayed on phage.

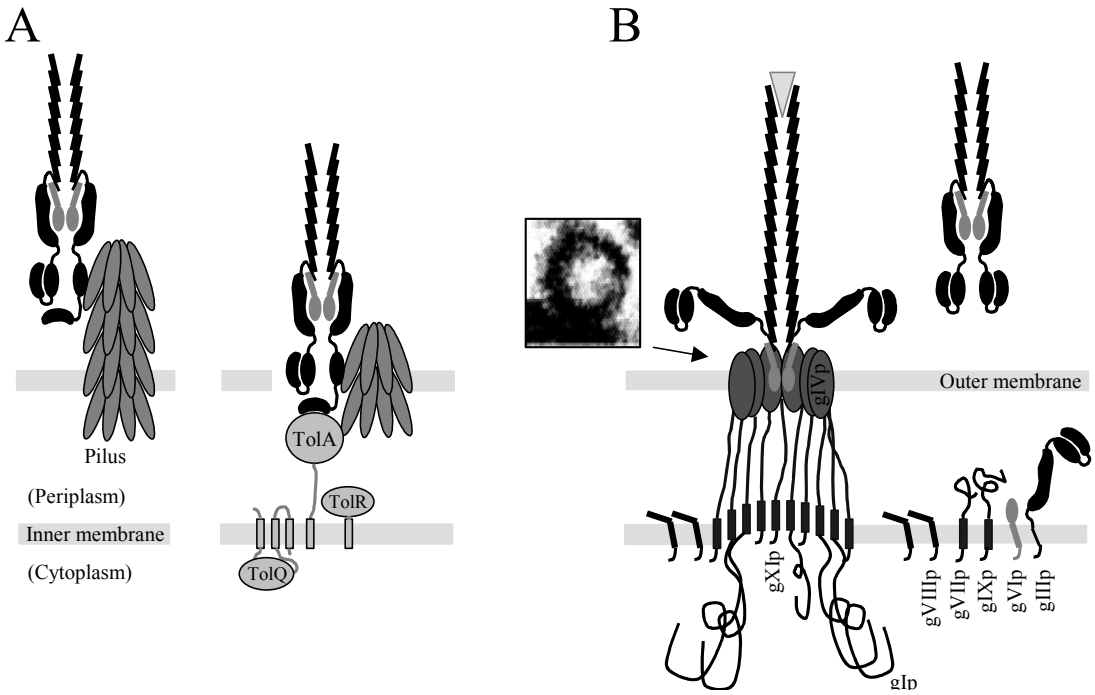

Figure 4 Infection and propagation of phage
A) Schematic illustration of the infection process via the two *E. coli* receptors needed: Pilus and TolA. Pilus is usually involved in the conjugal transfer of DNA between *E. coli*. It consists of a protein tube assembled by polymerisation of pilin subunits from the bacterial inner membrane. The pilin subunits are encoded by the *tra* operon on the F conjugate plasmid (Webster, 1996). Upon interaction with D2 of pIII, pilus retracts and D1 of pIII can interact with the TolA leading to completion of infection.
B) Schematic presentation of phage propagation. Following DNA replication, the phage genome is directed to the bacterial inner membrane, where pV is stripped of the genome while passing through the assembly channel and propagating into phage particles. The assembly channel consists of an oligomer of approximately 14 gIVp monomers embedded in the outer membrane, and a number of interacting gIp and gXIp situated in the inner membrane (Linderoth et al., 1997; Russel, 1993; Russel et al., 1997). The filamentous bacteriophage is non-lytic, and one bacterium can produce 100-200 phage particles per doubling time (Webster, 1996).

1.3.3.2 Display on phage particles

The first paper describing phage display was published in 1985 (Smith, 1985). Smith displayed a fragment of the EcoRI enzyme in the central region of pIII, and showed that the recombinant phage could be enriched in a phage pool by panning the pool on antibodies recognising EcoRI. The display of an anti-lysozyme scFv on phage in 1990 paved the way for the generation of antibody repertoires on phage (McCafferty et al., 1990).

Over the years, most of the phage coat proteins have been tested for display of antibody fragments, with pIII as the dominating choice (Gao et al., 1999; Gram et al., 1992; McCafferty et al., 1990). The scFv genes are inserted directly upstream of the gene for pIII, which results in fusion proteins upon expression. The phage particles produced from a phage vector are homogeneous in their content of pIII, whereas the phage particles produced from a phagemid vector in combination with a helper phage have pIII from both the phagemid (fusion proteins) and from the helper phage (non-fusion). The phage vectors offer the advantage that all phage particles display scFvs and therefore enable a more efficient selection. In contrast, the phage particles produced from phagemid systems are "monovalent". On average the phage particles displaying a fusion protein, display only one, whereas the vast majority do not display any (Bass et al., 1990). This is a clear advantage when high affinity is desired. The other advantage of phagemid vectors is the relative high transformation efficiency allowing the generation of libraries with higher diversity. A variety of antibody repertoires displayed on phage in different formats is listed in **Table 2**

Antibody repertories can be divided into categories based on the source used for their generation. Natural antibody repertoires can be created from B-lymphocyte mRNA obtained from either immunised or non-immunised donors. The "immune" repertoires will be biased by the immune response of the donor and will therefore be enriched for antibodies directed against the antigen used for immunisation. In addition, several of the antibody genes may contain somatic hypermutations, and therefore encode high affinity antibodies. However, there are several drawbacks: Immunisation may not result in an immune response, toxic antigens may kill the donor, tolerance will limit or prevent an immune response toward self-antigens, and finally, ethical concerns may hinder active immunisation of humans. Finally, the applicability range of a repertoire is limited to selections against the immunising antigen. In contrast, naïve repertoires can be applied in the selections against a wide range of targets, including those that cannot be used in immunisations. The caveats are the diversity requirement and the potential bias by the donors' immunoglobulin repertoire.

To extent the repertoire diversity, "synthetic" repertoires have been generated in which parts of the natural antibodies are randomised. The classic approach is to exchange the CDR3 regions, which are normally created by the recombination of the V(D)J gene segments, with synthetic oligonucleotides. This strategy can be further extended to cover the other CDRs. The synthetic repertoires are not biased, and can generally be applied in selections against any antigen, including self-antigens and toxic antigens. The synthetical repertoires may contain high fractions of non-active or unfolded antibodies, which was addressed in the construction of the TomlinsonI and TomlinsonJ libraries that were pre-selected for folding by enrichment on protein A and protein L (Tomlinson, personal communication)

Table 2 Antibody phage display libraries

Donor	Source	Format (vector type)	Diversity	Reference
Immune				
Mouse	Spleen	scFv (3)	2×10^5	(Clackson et al., 1991)
-	Spleen	scFv (3+3)	6.5×10^6	(Kettleborough et al., 1994)
-	Spleen	scFv (3+3)	2.4×10^6	(Lorimer et al., 1996)
Rabbit	Spleen, bone marrow	Fab (3+3)	2×10^7	(Lang et al., 1996)
Chicken	Spleen	scFv (3+3)	1.4×10^7	(Yamanaka et al., 1996)
Camel	PBL	V_H (3+3)	5×10^5	(Arbabi Ghahroudi et al., 1997)
Human	Bone marrow	Fab (3+3)	10^7	(Barbas et al., 1993)
-	PBL	scFv (3)	5×10^8	(Cai & Garen, 1995)
-	Spleen (IgG-positive lymphocytes	scFv	10^6	(de Wildt et al., 1996)
-	Thymic lymphocytes	Fab (3+3)	4.8×10^6	(Graus et al., 1997)
-	PBL	scFv (3+3)	2×10^8	(Mao et al., 1999)
Mouse	Pre-selected repertoires	Bispecific diabody	7×10^4	(McGuinness et al., 1996)
Naïve				
Mouse	Bone marrow	Fab (8+8)	5×10^6	(Gram et al., 1992)
Chicken	bursal lymphocytes	scFv (3)	2.7×10^7	(Davies et al., 1995)
Human	PBL (IgM)	scFv (3+3)	2.9×10^7	(Marks et al., 1991)
-	PBL (IgG)	scFv (3+3)	1.6×10^8	(Marks et al., 1991)
-	PBL, tonsil, bone marrow	scFv (3+3)	1.4×10^{10}	(Vaughan et al., 1996)
Synthetic				
Mouse	V_H: HCDR3: $9^{(a)}$	V_H (3+3)	4×10^8	(Reiter et al., 1999)
Human	HCDR3: $16^{(a)}$	Fab (3+3)	5.0×10^7	(Barbas et al., 1992)
-	LCDR1: 4, LCDR3: 6, HCDR2: 2, HCDR3: $5^{(a)}$	Fab (3+3)	3×10^8	(Garrard & Henner, 1993)
-	HCDR3: 4, LCDR3: $4^{(a)}$	scFv (3+3)	3×10^8	(Pini et al., 1998)
-	HCDR3: $8^{(a)}$	scFv (3+3)	8×10^8	(Braunagel & Little, 1997)
-	49 V_H, HCDR3: $5^{(a)}$	scFv (3+3)	10^7	(Hoogenboom & Winter, 1992)
-	50 V_H, HCDR3: 4-$12^{(a)}$	scFv (3+3)	10^8	(Nissim et al., 1994)
-	49 V_H, HCDR3: 4-12, 26 V_κ, LCDR3: 0-2, 21 V_λ, LCDR3: 0-$3^{(a)}$	scFv (3+3)	6.5×10^{10}	(Griffiths et al., 1994)
-	49 V_H, HCDR3: (6-15) $^{(a)}$	scFv (3+3)	3.6×10^8	(de Kruif et al., 1995)
-	7 V_H and 7 V_L	scFv (3+3)	2×10^9	(Knappik et al., 2000)
-	VH: HCDR3: 5-$12^{(a)}$	V_H (3+3)	2×10^8	(Davies & Riechmann, 1995)

Examples of antibody repertoires displayed on phage. $^{(a)}$ The number of randomised residues in the CDR regions, and the number of applied V gene segments (only one if nothing is stated).
PBL: Peripheal Blood Lymphocytes

1.3.3 Humanised Antibodies

For human therapy, rodent antibodies have several disadvantages. They have a short half-life in serum, human effector functions are only activated by a few of the different antibody classes, and to a lower extent, and they often trigger a human anti-mouse antibody (HAMA) response. To solve these

problems, variable domains of rodent mAbs have been engineered onto constant domains of human antibodies (chimeric antibodies). Lately, humanised antibodies have been created by grafting the CDRs of rodent mAbs onto the frameworks of human antibodies. New framework-loop interactions in the grafted antibodies may result in decreased affinity, and it may be necessary to investigate and substitute some of the framework residues to restore binding activity. For a review on humanised antibodies see (Winter & Harris, 1993).