1 Introduction

1.1 Preface

Cancer is a growing concern in the western world, where it is the cause of death for many. Therefore knowledge on cancers, cancerantigens, and the possible methods of treatment is necessary. Lewis Y, a blood group related difucosylated oligosaccharide (Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ-) seems to be an attractive target for the development of new therapeutics. It is a tumour associated carbohydrate antigen, which is overexpressed in 60-90% of human carcinomas of epithelial origin such as breast, bladder, colon, stomach, pancreas, ovarian, and lung cancer. For example, Lewis Y is expressed in more than 70% of all breast cancers, whereas the Her-2/neu marker is only detectable in 25-30% of the patients (Hellstrom, Garrigues et al. 1990; Scott, Geleick et al. 2000). Overexpression, high density on tumour cell surfaces, and a relatively homogeneous expression in primary tumours are promising preconditions for the development of widely applicable therapeutic agents. Several reports have also found that patients having Lewis Y expressing tumours tend to have poorer prognosis than patients with cancers not expressing Lewis Y. In this context the development of immunotherapeutics is of special interst, because of the possibility of specific targeting.

Different strategies are possible for immunotherapeutic treatment of cancer patients:

(1) Passive immunotherapy: Using naked antibody for generation of antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity, or using the antibody coupled to a toxin, an enzyme, or an isotope for selective therapy or tumour imaging.

(2) Active immunotherapy by vaccination: Vaccination with the carbohydrate antigen itself or a compound mimicking the carbohydrate antigen, which makes idiotypic antibody and anti-idiotypic antibodies interesting.

A small number of references are available for these strategies for the Lewis Y antigen.

Idiotypic antibodies against Lewis Y are of upcoming interest in immunodiagnostics and immunotherapy as the expression patterns have been well investigated by in vitro histology (Hellstrom, Garrigues et al. 1990; Zhang, Zhang et al. 1997; Ravn and Dabelsteen 2000; Scott, Geleick et al. 2000). Most important is the availability of antibodies with unique specificity for a therapeutic application, because of a possibly cross-reactivity to blood group related carbohydrate structures especially Lewis X and H-type 2 and the resulting risk of erythrocyte agglutination. On the other hand Lewis Y is not shedded in large amounts into the serum of patients, which is a big advantage for an immunotherapeutic application. Numerous anti-Lewis Y antibodies are generated and published however most of them are cross-reactive to related structures. Examples are the B3 antibody which is cross-reactive to Lewis X, dimeric Lewis X, extended Lewis X or trifucosylated Lewis Y (Pastan, Lovelace et al. 1991), or the 3S193 which is Lewis Y specific but reacts with Lewis X at antibody concentrations which are necessary for a therapeutic administration (Kitamura, Stockert et al. 1994). This underlines the need of highly specific and well characterised antibodies for the development of therapeutics. Nevertheless promising results from clinical trials have been obtained with the B3 Lewis Y antibody as toxin

coupled and as isotope coupled for tumour imaging (Pai, Wittes et al. 1996; Pai-Scherf, Carrasquillo et al. 2000). For this and for other antibodies further clinical trials are planned, examples beeing the antibody IGN311 from Igeneon (www.igeneon.com/download/FactSheet_IGN311.pdf) a humanised version of the murine antibody BR55-2, and the hu3S193 (http://www.mskcc.org/mskcc/html/2270.cfm?IRBNO=03-069).

Only a few references are available for results obtained in clinical trials with carbohydrate vaccines. Several of these studies focus on the Lewis Y related carbohydrate Globo H (Fucα1-2Galβ1-3GalNAcβ-) (Wang, Williams et al. 2000; Gilewski, Ragupathi et al. 2001). These studies showed positive induction of anti-carbohydrate IgG antibodies, whereas only IgM antibodies was found in patients vaccinated with a Lewis Y-keyhole limpet hemocyanin conjugate (Sabbatini, Kudryashov et al. 2000). These groups does however report a new polyvalent vaccine under development (Musselli, Livingston et al. 2001; Ragupathi, Cappello et al. 2002). Reasons for the lack of proper IgG response could be a deficient immunogenicity and the lack of appropriate adjuvants for the Lewis Y. Anti-idiotypic antibodies could be a step forward in solving these problems.

The thesis presented here focus on basic research to enhance the knowledge on the characterisation of Lewis Y and Lewis Y binding antibodies for development of Lewis Y specific therapeutics. Both fields mentioned above are included:

(1) Idiotypic antibodies for development of passive immunotherapeutics: Most important is the investigation of the antibody specificity and the availability of assays for this. ELISA measurements on a panel of 86 carbohydrate conjugates, immunofluorescence and FACS-analysis were performed to determine the fine specificities of four different and beforehand less characterised Lewis Y recognising mouse monoclonal antibodies. To get an idea which reasons are responsible for a more or less specificity an analysis and comparison of antibody sequences was carried out. Next step was the generation of chimeric antibodies from the mouse monoclonals without loss of specificity, and the generation of other formats and the development of new antibodies from the parent antibodies. The results of this part are criteria to decide on a further development of one of the investigated or newly generated antibodies for a clinical application.

(2) Anti-idiotypic antibodies mimicking Lewis Y: Investigations of this part are performed to evaluate the possibility of inducing an immune response to a carbohydrate structure by a mimicking anti-idiotypic antibody. First of all a new method was developed for efficient generation of large repetoires of anti-idiotypic antibodies from synthetic phage display libraries. Secondly a proof of principle for carbohydrate mimicry of anti-idiotypic antibodies developed by this method was done for the first time. This was shown by the successful induction of a carbohydrate immunoresponse in mice by the mimicking anti-idiotypic antibody. Finally a new method for presentation of scFv to the immune system was developed. This was made by fusion of the scFv to domain I of the phage protein 3 and it proved to have beneficial effects, including adjuvant effect, when presented to the immune system.

1.2 Lewis Y

The difucosylated type 2 chain was first identified in 1968 by Lloyd and Kabat (Lloyd and Kabat 1968) and the name Lewis Y was later designated to this carbohydrate by Sen-itiroh Hakomori. The name is used for the terminal tetrasaccharide structure irrespectively of the carrier of the tetrasaccharide. It is structurally close related to the ABH histo-blood group antigens and consists of two α-L-fucoses (Fucα) a β-D-galactose (Galβ) and a N-acetyl-glucosamine (GlcNAcβ) which shortly written including the couplings is Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ- or just Lewis Y. Through the last decades several groups have been involved in the work of identifying the normal and tumourigenic expression of Lewis Y in different tissues, mainly by use of monoclonal antibodies towards this carbohydrate, reviewed in (Le Pendu, Marionneau et al. 2001).

1.2.1 Structure and pathway of the Lewis carbohydrates

Lewis Y is a terminal tetrasaccharide, which is structurally related to the ABH histo-blood group antigens. All of these terminal oligosaccharides are found as both O-glycosylations, N-glycosylations, and on glycolipids. The oligosaccharide chains are fashioned by a series of enzymes (glycosyltransferases and glycosidases), which acts in specific sequence in different sub cellular compartments, mainly in the ER-Golgi-plasmalemma pathway. The generation of N-glycosylations deviate from the generation of O-glycosylations and glycolipids by being initiated by *en bloc* addition of large multiantennary oligosaccharides to the Asn residues, before further trimming and fashioning. The multiantennary nature of the N-glycosylations is also a point of deviation from the O-glycosylation, which can be biantennary or unbranched, and the unbranced nature of glycolipids.

Table 1: Precursor chains for the Lewis and ABH oligosaccharides, their structure and how they appear. **R** represents a core structure, a lipid, or an amino acid residue.

Precursor type chain name	Structure	Presence
Type 1	Galβ1-3GlcNAcβ1-**R**	O-, N-glycoproteins, and glycolipids (lactoseries)
Type 2	Galβ1-4GlcNAcβ1-**R**	O-, N-glycoproteins, and glycolipids (neolactoseries)
Type 3	Galβ1-3GalNAcα1-**R**	O-glycans, plus existence of a lipid associated derivate
Type 4	Galβ1-3GalNAcβ1-**R**	Glycolipids

The length of the carbohydrate antennas on all three kinds of glycosylations can vary due to various numbers of lactosamines (Galβ1-4GlcNAc) added, ranging from none to several. Furthermore, small variations are found in the terminal of these core structures, but the four main precursor types defined for the ABH and Lewis carbohydrates all terminates with a β-galactose (Table 1).

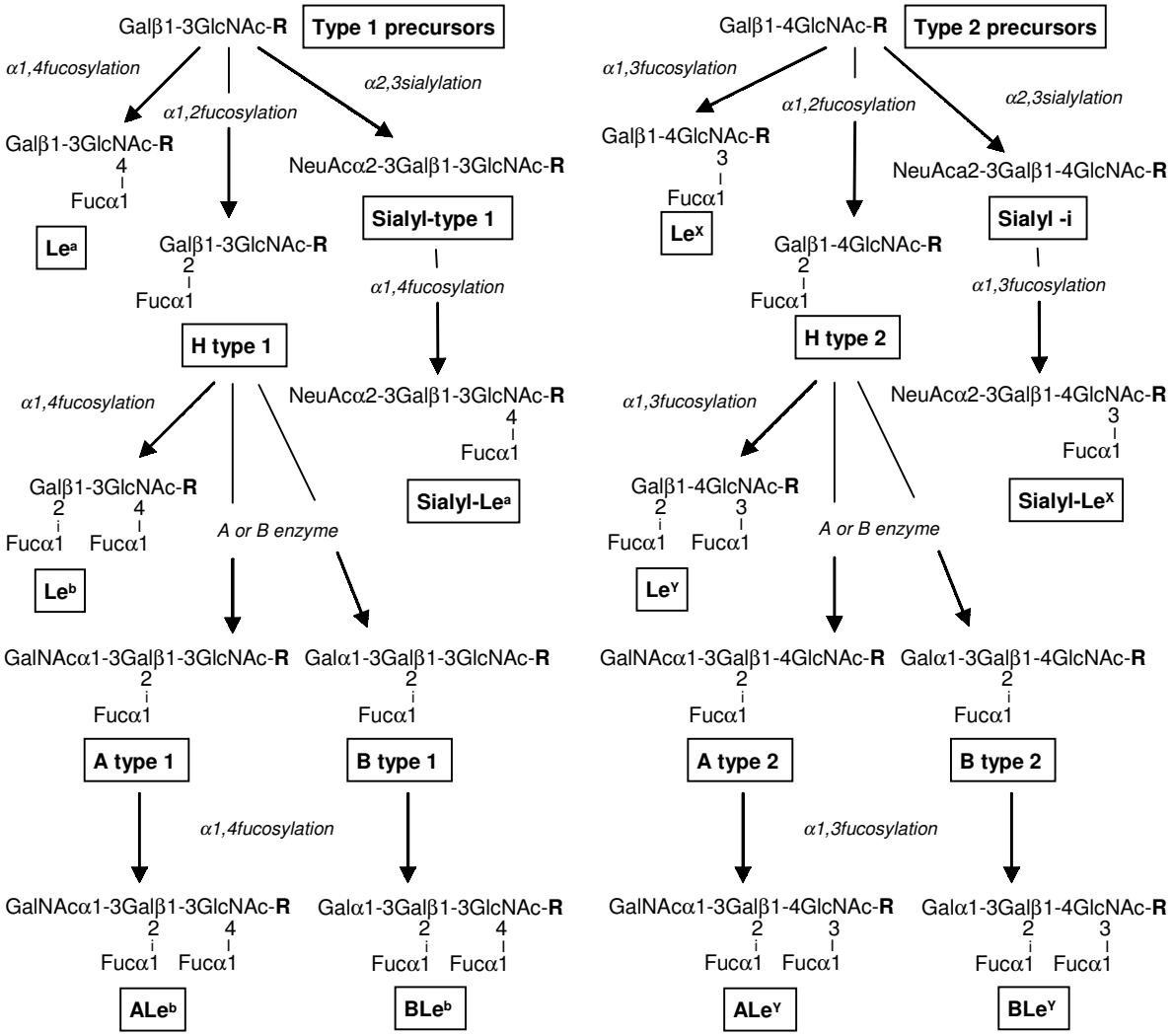

Figure 1: The major biosynthesis pathways for the ABH and Lewis antigens. On the left is the biosynthesis of Lewis b, where the precursor chain is type 1 and on the right is the biosynthesis of Lewis Y, where the precursor chain is type 2. Le is short for Lewis and the a, b, X, and Y is indicated with a suffix. **R** represents a core structure, an amino acid, or a lipid. Adapted after (Le Pendu, Marionneau et al. 2001)

The terms ABH and Lewis antigens refer to the terminal and outermost structures consisting of tri-, tetra-, or penta-saccharides. Their biosynthesis proceeds via sequential addition of monosaccharides to their precursors, which are the type chains. An overview of the major biosynthesis pathways for the generation of ABH and Lewis antigens from the type 1 and type 2 chains is shown in Figure 1. The terminal α1,2-fucosylations in the Lewis antigens are carried out by α2fucosyltransferases encoded by the Secretor or H genes. The α2fucosyltransferase encoded by the Secretor gene works preferentially on type 1 chains, whereas the α2fucosyltransferase encoded by the H gene preferentially works on precursor type 2 chains. However, both α2fucosyltransferases can work less efficiently on the other precursor chain. The α1,3-fucosylations can be carried out by one of several fucosyltransferases, whereas the α1,4-fucosylation only is carried out by the Lewis blood group fucosyltransferase. In A and B deficient individuals (carrying the blood group O) more H antigen may be available for the Lewis Y or Lewis b synthesis, which again leads to more Lewis Y and Lewis b

expression in some normal tissues (Oriol, Mollicone et al. 1992; Ravn and Dabelsteen 2000; Becker and Lowe 2003).

Similar to the chains type 1 and 2 a fashioning of the type 3 and 4 chains is found. Type 3 and 4 chains consists of Galβ coupled 1-3 to N-acetyl-galactosamine α/β (GalNAc α/β). These precursor chains can also be α1,2fucosylated and give rise to the trisaccharides H type 3 and Globo H, respectively. Additional monosaccharides can be added by the A or B enzyme to give rise to A/B type 3/4 structures in a similar manner to Figure 1. Generally the antigenic determinants are genetically determined, but the final products of the glycosylations are dependent on a number of factors which includes: The initial protein message, the processing, tissue- or cell-type, and availability of enzymes and substrate. For recent reviews see (Hakomori 1999; Lloyd 2000; Le Pendu, Marionneau et al. 2001).

The glycosyltransferases, which carry out the sequential addition of the monosaccharides, add monosaccharides one at the time to specific positions on specific precursors. For each reaction there might though be more than one enzyme. The enzymes require conversion of monosaccharides to activated sugar nucleotides, which then can act as sugar donors in the glycosylation. The monosaccharides used in the biosynthesis can be retrieved from three different sources. They can be imported into the cell by different transporters (energy dependent or independent), or they can be salvaged from degraded glycoconjugates through an ordered and highly specific degradation process. A third option is that the sugars can be derived from other sugars within the cell via the epimerases (Allard, Giraud et al. 2001).

1.2.2 Expression of Lewis Y on normal cells

The ABH histo-blood group antigens were originally identified as surface antigens on erythrocytes. The expression of these antigens is however not constrained to these cells, since they also have been identified on most epithelial tissue and their secretions (Ravn and Dabelsteen 2000). Immunohistology stainings with the anti-Lewis Y antibodies BR96 and 3S193, showed an expression of Lewis Y on epithelial tissues such as lung, breast, prostate, colon, stomach, pancreas, uterus, ovary (Zhang, Zhang et al. 1997), though the authors noted that they observed a broader specificity for BR96. Reports have also been given on a weak expression of Lewis Y on resting granulocytes (Kitamura, Stockert et al. 1994; Dettke, Palfi et al. 2000). In humans the antigen have also been shown to be expressed at different stages in embryonic development of some tissues and organs (Miyake, Zenita et al. 1988), as free oligosaccharide in human seminal plasma (Chalabi, Easton et al. 2002), and on hematopoietic progenitor cells (Cao, Merling et al. 2001).

1.2.3 Function of Lewis Y

Lewis Y has been reported implicated in a diverse range of processes. Recognition and adhesion of cells is one, where especially the sialylated or sulphated derivatives are implicated (Kansas 1996). Upon stimulation of granulocytes their expression of Lewis Y have been shown to increase, which supports the involvements in cellular cytoadhesiveness, the precise mechanism is however still unknown (Dettke, Palfi et al. 2000). Cellular motility also involves Lewis Y or a partial epitope, since antibodies directed towards these epitopes were shown to inhibit cellular motility of Lewis Y bearing tumour cells, and that Lewis Y was found expressed on buccal epithelial cells in the course of wound healing (Miyake and Hakomori 1991; Garrigues, Garrigues et al. 1993; Garrigues, Anderson et al. 1994). Experiments with transfections of A or B gene results in a consistent reduction of haptotactic motility of H type expressing tumour cells (Ichikawa, Handa et al. 1997; Ichikawa, Handa et al. 1998), whereas tumour cell motility is enhanced upon transfection with a α1,2fucosyltransferase gene (Goupille, Hallouin et al. 1997) and the resistance to apoptosis is increased (Goupille, Marionneau et al. 2000). Two other groups have also indicated that Lewis Y expression is related to apoptosis, either as an increase in expression upon induction of apoptosis (Yamada, Ohwada et al. 1996) or as a co localisation with predictive markers of apoptosis in both tumours and normal tissue (Hiraishi, Suzuki et al. 1993), although none of the groups found the correlations to be more than ~50%. Lewis Y has also been reported as a stage-specific developmental antigen of the human lung in human embryos (Miyake, Zenita et al. 1988). Other inhibition studies with antibodies in the mouse model found that accessibility of Lewis Y was essential for the initial stage of embryo implantation (Zhu, Kojima et al. 1995; Wang, Zhu et al. 1998). Furthermore, one of these reports showed that Lewis Y can make carbohydrate-carbohydrate interaction with H type structures (Zhu, Kojima et al. 1995), an interaction that could be involved in adhesion (Dettke, Palfi et al. 2000). Another recent report indicates angiogenetic properties of shed Lewis Y and H type 2 (Halloran, Carley et al. 2000).

1.2.4 Lewis Y changes in cancer

Alteration of glycosylation seems to be a general feature of cancer cells. The altered structures are either truncations of normally expressed oligosaccharides (e.g. Tn) or it can be generation of novel oligosaccharide structures as a result of unusual termination of the oligosaccharide sequence (e.g. Lewis X/a) as a result of changed regulation of some glycosyltransferases (Kim and Varki 1997). Other aberrant glycosylation patterns occur due to loss, appearance, or over expression of known structures (e.g. Lewis Y/b). That the oligosaccharide changes, which occur in malignancy could be a very important feature was underlined as it was found that several monoclonal antibodies raised against various types of cancers cells frequently were found to recognise glycosylations. Though many of the generated antibodies did not recognise novel structures, recognition of normal tissue or cells was not possible due to the low density of the oligosaccharide (Feizi 1985; Hakomori 1986; Sell 1990), this lead to the expression tumor-associated-carbohydrate antigen (TACA).

Over the years several reports and reviews have been given on the changed expression of Lewis Y in cancers (Abe, McKibbin et al. 1983; Hellstrom, Garrigues et al. 1990; Yin, Finstad et al. 1996). Careful

evaluation of the results is necessary since (1) different methods have been used, (2) only one antibody against the antigen was tested, and (3) it has been found that several of the reported Lewis Y specific antibodies showed cross-reactivity to related antigens such as H type 2, Lewis X, Lewis a, and Lewis b (Furukawa, Welt et al. 1990; Kitamura, Stockert et al. 1994; Zhang, Zhang et al. 1997). One of the most consistent and recent studies found that several cancers of epithelial origin express the Lewis Y antigen more abundantly than normal tissue. This concerns cancers such as breast, bladder, colon, stomach, pancreas, ovarian, and lung cancer. Especially strong expression was found on colon, ovarian, lung, breast, and pancreatic cancers. In contrast hereto normal and malignant prostate cells expressed Lewis Y equally. Generally it was found that all normal tissues showed a complete restriction of the antigen expression to the secretory borders of the epithelial cells, which suggests this antigen normally may be relatively inaccessible to administered antibody (Zhang, Zhang et al. 1997). The expression of Lewis Y in some cancers have been, apart from glycolipids and lower molecular weight glycoproteins, found on two ovarian cancer antigens (CA125 and MUC-1) (Yin, Finstad et al. 1996), and LAMP-1 (Garrigues, Anderson et al. 1994).

1.2.5 Lewis Y expression and prognosis

Generally changes of glycosylation on tumour cells are associated with the stage and progression of the tumour and especially in the early stages the patient survival rate is correlated to particular types of aberrant glycosylation (Hakomori 1996). Several reports present correlations between patient prognosis and A, B and Lewis Y antigen expression. It has been found that the loss of the A or B antigens is associated with bad prognosis, invasive and metastatic properties of tumours. The expression of Lewis Y in cancers of breast, tongue, non-small cell lunge cancer, and oesophagus also indicates poor prognosis compared to patients with no Lewis Y expression in some reports (Narita, Funahashi et al. 1993; Ogawa, Sano et al. 1995; Xie, Boysen et al. 1999), whereas others have not been able to confirm this for the non-small cell lung cancer (Mehdi, Tatum et al. 1998). Some of the factors that can influence these controversies are: (1) the use of antibodies with different specificity (e.g. cross-reactivity) and (2) different definitions of Lewis Y positive tumours, as reviewed in (Hakomori 1996; Hakomori 1999; Lloyd 2000; Le Pendu, Marionneau et al. 2001). An explanation for the increased expression of Lewis Y in cancer cells is that the loss of A and B antigen expression leads to an increase in the precursor H type 2 (Figure 1). The H type 2 antigen is subsequently masked by addition of fucose, which then results in Lewis Y. In the later metastatic stages of cancer the fucosylated antigens such as Lewis Y are almost completely lost to the benefit of sialylated antigens (Le Pendu, Marionneau et al. 2001).

1.2.6 Anti-Lewis Y therapy

Due to the overexpression of Lewis Y in some cancers it is an obvious target for active or passive therapy or imaging of tumours. Engineered antibodies, carbohydrate vaccines or mimicry vaccines are some of the presently used methods employed against Lewis Y carrying tumours. The availability of monoclonal mouse antibodies specific for Lewis Y and the field of antibody technology (further described in chapter 1.3.1) have already given rise to several clinical trials. Vaccination with the carbohydrate compound itself is also promising although this mainly gives responses of the T cell-independent type (Mond, Lees et al. 1995). Vaccination by mimicry compounds is at present also under development.

Antibody therapy can be managed in many ways depending on the target. Several factors come in play concerning the patient, the antibody, and coupling of e.g. toxin or radioactivity. Is the therapy for catching metastasis, against a solid tumour, or is the antibody used for imaging? Can the antibody be of mouse origin or does it need to be chimeric or humanised to prolong the lifetime in serum and avoid a human anti-mouse antibody (HAMA) response? Should a whole antibody be used or only fragments thereof? Several preclinical trials and clinical trials have been made with different anti-Lewis Y antibodies in different formats and for different use. The main results will be summarised here.

Over the last decades several antibodies specific for Lewis Y have been generated, and from these especially the antibodies originating from the hybridomas AH-6 (Abe, McKibbin et al. 1983), B3 (Pastan, Lovelace et al. 1991), BR96 (Hellstrom, Garrigues et al. 1990), 3S193 (Kitamura, Stockert et al. 1994), and BR55-2 (hybridoma ABL364) (Blaszczyk-Thurin, Thurin et al. 1987) have special interest and some of them have been taken into clinical trials in different formats. Some of the most recent reports on these antibodies concern murine, chimerics, humanised, $F(ab)_2$, and single chain fragment variable (scFv) formats used either alone or conjugated to a toxin, as combination therapy or radiolabeled for e.g. imaging (Stahel, Lacroix et al. 1992; Schlimok, Pantel et al. 1995; Co, Baker et al. 1996; Tolcher, Sugarman et al. 1999; Pai-Scherf, Carrasquillo et al. 2000; Saleh, Sugarman et al. 2000; Wahl, Donaldson et al. 2001; Posey, Khazaeli et al. 2002).

One of the first studies with BR55-2 showed that it was well tolerated in small-cell lung cancer patients to an antibody serum level high enough to mediate in vitro lysis of target cells. No clinical response was found in the patients possibly due to a still too low in vivo serum antibody level (Stahel, Lacroix et al. 1992). In colorectal or breast cancer patients this antibody was able to reduce or eliminate micrometastases in the bone marrow (Schlimok, Pantel et al. 1995).

The chimeric antibody BR96 conjugated to doxorubicin was tested as treatment for metastatic breast cancer, but failed to produce a clinically significant number of responses though preclinical studies had shown that targeting and delivering of toxin to cancer cells was possible. The toxicities observed were mainly gastrointestinal, which made the authors suggest that expression of Lewis Y in the gastrointestinal tract acts as an antigen sink (Tolcher, Sugarman et al. 1999). Similar or modest results was found by others in treatment of Lewis Y expressing epithelial tumours (Saleh, Sugarman et al. 2000; Posey, Khazaeli et al. 2002). Partly positive or positive results were obtained with B3 as used for therapy and tumour imaging (Pai, Wittes et al. 1996; Pai-Scherf, Carrasquillo et al. 2000).

Vaccination with carbohydrates may not be capable of eliciting an effective immune reaction and specific TACAs often produce poor immunological reactions. Studies have shown that several problems arise by

use of synthetic carbohydrates (Globo H or Lewis Y) coupled to keyhole limpet hemocyanin (KLH) as vaccines (with or without adjuvant QS-21). Even though the vaccines are well tolerated with no autoimmunity in humans, only a modest response is found, the sera did in some cases only react with carbohydrates on glycolipids and not with carbohydrates on mucins, and only low amount of IgG was obtained (Sabbatini, Kudryashov et al. 2000; Gilewski, Ragupathi et al. 2001; Ragupathi, Coltart et al. 2002). Several other carbohydrate TACAs are also very weak immunogens and may also fail to produce a proper response, examples being Thomsen-Friedenreich (TF), Tn-antigen (Springer 1997) and (sialylated)-Lewis Y/X/a (Le Pendu, Marionneau et al. 2001). The generation of carbohydrate vaccines have been dominated by two problems: (1) the carrier and (2) the adjuvant. The use of KLH as carrier in combination with the adjuvant QS-21 has however brought the vaccination strategies a long way (Ragupathi 1996; Ragupathi, Coltart et al. 2002). We will try to circumvent the problems by using anti-idiotypic mimicry in combination with the domain I from the phage protein 3 as a carrier and adjuvant (Jensen, Larsen et al. 2002).

1.2.7 Crystal structures of antibodies with Lewis Y

A general detailed analysis of antibody-carbohydrate combining sites has yet not been made. Presently only a limited number of carbohydrate binding antibodies in complex with the antigen have been crystallised (Cygler, Rose et al. 1991; Zdanov, Li et al. 1994; Jeffrey, Bajorath et al. 1995; Villeneuve, Souchon et al. 2000; Vyas, Vyas et al. 2002; Nguyen, Seto et al. 2003; Ramsland, Farrugia et al. 2004; van Roon, Pannu et al. 2004). Two of these crystal structures are Lewis Y in complex with the BR96 Fab (Figure 2) (Jeffrey, Bajorath et al. 1995) and hu3S193 Fab (Ramsland, Farrugia et al. 2004), respectively. Both structures are similar in that all four sugar rings are involved in the binding and that the carbohydrate Fuc 4 is completely buried in the antigen combining site. The two antibodies bind the Lewis X carbohydrate in a similar manner, as all except two interactions are performed by the same residues in the two antibodies. Additional interactions to the Fuc 1 sugar ring were found for the hu3S193. Two solvent molecules and one residue in the heavy chain make up this interaction and it is involved in the specificity determination of hu3S193 (Ramsland, Farrugia et al. 2004). The Fuc 1 sugar ring is on the other hand only interacting with one residue located in the light chain of BR96. In a total the light chain only contributes to the interaction via three residues, whereas 19 are found in the heavy chain in the BR96 Fab (Figure 2) (Jeffrey, Bajorath et al. 1995).

Lewis X was originally not reported to be recognised by BR96 (Hellstrom, Garrigues et al. 1990), whereas a later study found this interaction (Zhang, Zhang et al. 1997). One of the other crystal structures solved recently is a Fab complex with Lewis X. And even though the binding pockets in the antibodies are of the same size the binding mode of the sugars to the antibodies is quite different (van Roon, Pannu et al. 2004).

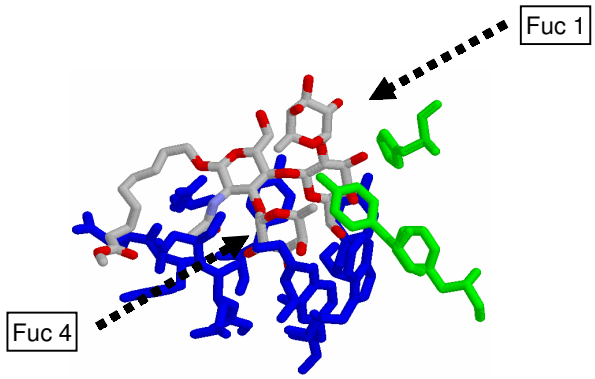

Figure 2: The crystal structure of BR96 antibody in complex with the Lewis Y antigen. Only the residues from the light chain (coloured green) and the heavy chain (coloured blue) identified to be involved in the binding to the antigen (coloured cpk) are shown (Jeffrey, Bajorath et al. 1995). Protein data bank PDB entry 1CLY. Figure prepared with RasMol (Sayle and Milner-White 1995).

Due to high sequence similarity molecular modelling of other Lewis Y binding antibodies has been possible. These modellings indicate that BR55-2 and B3 interact with Lewis Y in a similar fashion as does BR96 (Blaszczyk-Thurin, Murali et al. 1996; Murali and Kieber-Emmons 1997), and hu3S193 as found in the recent crystal structure (Ramsland, Farrugia et al. 2004). All four antibodies have by other investigators also been shown to have a minor affinity for Lewis X (Kitamura, Stockert et al. 1994; Zhang, Zhang et al. 1997), which seems possible as the Fuc 1 is almost pointing away from the antigen combining site and only makes one or a few interaction to the complementarity-determining regions (CDRs). A crystal structure of the unligated disulfidebridge-stabilised B1 antibody recognising Lewis Y and H type 2 found significant differences in the size of the antigen combining site and the surface residues. Modelled complexes of B1 with the two carbohydrates found that the Lewis Y could fit in the binding pocket as in the crystal of BR96, whereas the H type 2 could “turn around” and place the Fuc 1 buried in the binding pocket (Almog, Benhar et al. 1998). These findings lead to the conclusion that existing Lewis Y binding antibodies generally recognise the antigen in a similar manner, with only small deviations affecting the specificity. The general difficulties in obtaining crystal complexes make the use of other methods for studying the antibody-carbohydrate interactions necessary.

1.3 Technologies

1.3.1 Antibodies and antibody fragments

Antibodies are a very important part of the human defence against pathogens, and the immune system gives rise to great variability. Structurally antibodies consist of at least one of the heavy chains (μ, δ, γ, ε, or α) and one of the light chains (λ or κ), each made up of a variable portion (V) and a constant portion (C). The heavy chain constant portion determines the isotype of antibody (IgM, IgD, IgG, IgE, and IgA) and thereby the number of binding sites and the properties of the antibody. The isotype of an antibody is generated by class switch recombination and depends on many factors such as stimulation of helper T- and B lymphocytes. Some of the isotypes have several subclasses, which may show slightly different functions. In Figure 3 the structure of antibody is exemplified by an IgG, which contain two binding sites compared to 10-12 in IgM and four in IgA. The antigen binding site of an antibody resides in two variable domains, one from the light chain (VL) and one

from the heavy chain (VH) (Figure 3). The variability in the κ or λ VL originates from the recombination of a Vκ or Vλ germline gene segment and a Jκ or Jλ germline gene segment, respectively. In a similar way the VH variability originates from recombination of three germline gene segments the V, D, and J. The second wave of diversification arises from somatic mutation in the variable domains. Although these mutations only give a minor contribution to the diversity, by far most domains have undergone somatic mutation, reviewed by (Papavasiliou and Schatz 2002; Li, Woo et al. 2004). VH and VL each contain three hypervariable loops, which are surrounded by relatively invariant framework residues. These loops are known as complementarity-determining regions (CDRs) and they constitute the interaction site with the antigen (Wu and Kabat 1970; Kabat, Wu et al. 1977). The main chain conformations of five of the six CDR loops appear to be limited and the conformation has been found to be correlated with the length of the CDR and the amino acid type of some residues in the hypervariable regions plus a few framework residues. The sixth CDR loop, the CDR3 from VH, seems not to fall in specific classes, but some of the residues outside the loop might also have a limited structure, onto which the loop is extended. These commonly occurring main chain CDR conformations are referred to as canonical structures (Chothia and Lesk 1987). This gives the opportunity to evaluate if two antibodies theoretically have the same structure, based on sequence alignments and the constraints of residues allowed at a certain position (Morea, Lesk et al. 2000). The theory of the canonical classes is though still not complete since not all antibodies fall into the until now identified catagories as e.g. the 1F9 scFv recognising lysozyme (Ay, Keitel et al. 2000).

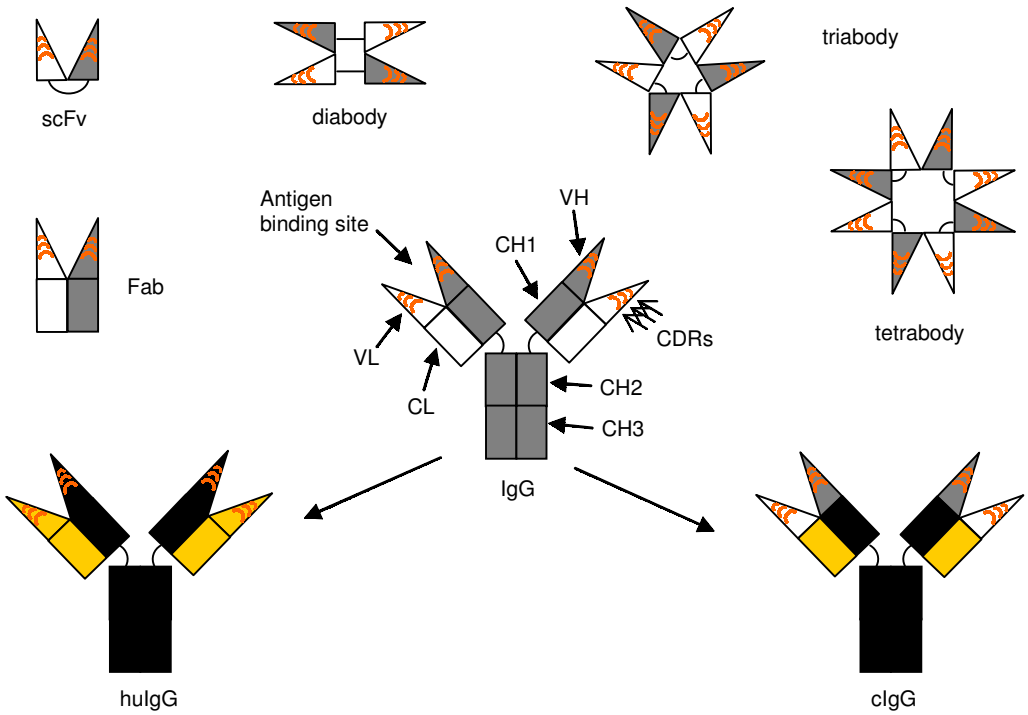

Figure 3: Different antibody formats. In the middle the structural elements of a mouse IgG antibody are shown. It consists of four heavy chain elements (three constant and one variable) and two light chain elements (one constant and one variable). The variable domains contain each three CDRs (shown in red colour). Mouse light chains are coloured white, mouse heavy chains: grey, human light chains: orange and human heavy chains: black. huIgG: humanised IgG and cIgG: chimeric IgG. For further information see text.

The biggest milestone in antibody technology was establishment of the hybridoma technique by Kohler and Milstein (Kohler and Milstein 1975). This technique made the generation of monoclonal antibodies possible, which also has led to the use of intact mouse monoclonal antibody as the first antibody to be used in therapy. The advantage by using whole antibodies is the presence of the constant regions, which harbour the abilities to recruit cytotoxic effector functions (reviewed in (Glennie and Johnson 2000)). On the other hand the use of mouse antibodies often cannot be repeated due to a human immune response against the mouse antibody (HAMA response). To surmount this problem several strategies have been applied, e.g. fusion between mouse variable domains and human constant domains (chimerisation, cIgG), and grafting of murine CDR regions onto human framework (humanisation, huIgG) (Morea, Lesk et al. 2000). A schematic representation of these fusions is shown in Figure 3.

Antibody fragments have also found a range of applications since smaller fragments exhibit better tissue penetration. The antigen binding fragment (Fab) was originally produced by proteolytic cleavage of a whole antibody and has later been expressed in bacteria, or it could be a bacterial expressed single chain fragment variable (scFv), which is constituted by a VL and a VH connected through a linker (Figure 3). However, the loss of multivalency is a disadvantage for both of these formats due to faster off rate and thereby poor retention time on the target. For recent reviews see (Carter 2001; Hudson and Souriau 2003).

Multivalency can, by careful design, be rebuilt into the Fvs. The length of the linker between the VH and VL in a monomeric scFv is longer than 12 amino acids and consists mainly of glycines and serines to ensure flexibility and protease resistance. Shortening the linker creates new forms of the Fv due to steric constrains. A length between 3 and 12 inhibits the folding into monomer, and two scFv associates to form a dimer also called a diabody. Reducing the linker length even further to below three can force the scFv to form trimers or tetramers (triabody or tetrabody) (Figure 3). In this way bacterial expressed multivalent antibody fragments can easily be obtained, which regain some of the lost properties of the original multivalent antibody. Furthermore, as well as the linker length, the orientation of the VL and VH domains may also influence the multimerisation (Todorovska, Roovers et al. 2001).

1.3.2 Phage display

The filamentous bacteriophage Ff is a rod-shaped particle, which propagates by infection of *Escherichia coli*. The genome is a single stranded DNA molecule and consists of about 6400 nucleotides encoding 11 proteins. The capsid consists of five different proteins (p3, p6, p7, p8, and p9). p8 is the most abundant, with 2700-2800 copies forming the wall of the particle. The other proteins are present in a few copies at the particle ends. Infection of *Escherichia coli* is initiated though interaction of p3 with the F-pilus on the bacteria. p3 is a three domain protein of which the two external domains are involved in the infection (Webster 2001).

The display of a recombinant protein on the phage was first published in 1985 (Smith 1985). A fragment of the restriction enzyme EcoRI was displayed in the central region of p3 and phage displaying recombinant protein could be enriched by selection on an antibody. Soon after, the first scFv was displayed on a phage (McCafferty, Griffiths et al. 1990) and libraries of scFv cloned (Marks, Hoogenboom et al. 1991). Most

phage coat proteins have been tested for display of antibody fragments but the insertion of the scFv genes upstream of gene 3 is the dominating choice, and results in a fusion protein upon expression (McCafferty, Griffiths et al. 1990; Gram, Marconi et al. 1992; Gao, Mao et al. 1999).

Various types of phage display libraries displaying scFv have been constructed. The libraries have been generated from many different species, both as naïve and immunised libraries. Immunised libraries originate, as the name indicates, from immunised donors, whereas the naïve library is from none-immunised donors. Furthermore, the libraries can be semi-synthetic or fully synthetic. These libraries consist of e.g. a human framework and synthetic CDRs (reviewed in (Kristensen, Ravn et al. 2000; Hust and Dubel 2004)).

Two different formats have been used for scFv display: The phage vector generates phage particles with scFv on virtually all p3s by insertion of the gene into the genome, whereas the phagemid only contains a p3 fusion gene and a phage packaging signal, resulting in higher transformation efficiencies which is needed for generation of large repetoires (reviewed in (Hoogenboom, de Bruine et al. 1998)). The phage from a phagemid library rely on a helper phage to provide the remaining phage proteins. Several different helper phage have been developed relying on the system of choice (Hoogenboom, Griffiths et al. 1991; Duenas and Borrebaeck 1995; Krebber, Spada et al. 1997; Kristensen and Winter 1998; Rondot, Koch et al. 2001). Each of these phage have their own strength and weaknesses. The general disadvantage of the phagemid system is that on average only one percentage of the phage carry a scFv and the majority do not display any scFv (Bass, Greene et al. 1990).

The phage display technology is a highly suited method for the rapid generation of specific recombinant human antibody fragments. In order to generate Fab fragments or scFv with the desired specificities, antibody libraries are selected against the corresponding antigens. However, these antigens are not always available in a pure form but may be part of an antigen mixture or a molecule amongst others on a cell surface. Even pure antigens consist of different epitopes. Furthermore, the selection strategy is important for the target epitope. Multiple rounds of selection are usually necessary in order to enrich and isolate specific antibody fragments. At the end of these rounds, antibody fragments will be obtained, which bind with the highest affinity, or phage, which have a growth advantage during the amplification between the rounds of selection. Therefore, antibody fragments against weak epitopes are not or only rarely selected. This leads to the loss of diversity of potential binders during sequential rounds of selection, and hampers the selection of antibodies of lower affinity or against minor components of antigen mixtures and cell surfaces (Winter, Griffiths et al. 1994).

Kristensen and Winter introduced the KM13 helper phage for the selection for protein fold of enzymes (Kristensen and Winter 1998). The principle behind the KM13 is a scFv-p3 (wild type) fusion phagemid and a modified helper phage, which is made proteolytically cleavable by the introduction of a trypsin cleavage site into p3 (Figure 4).

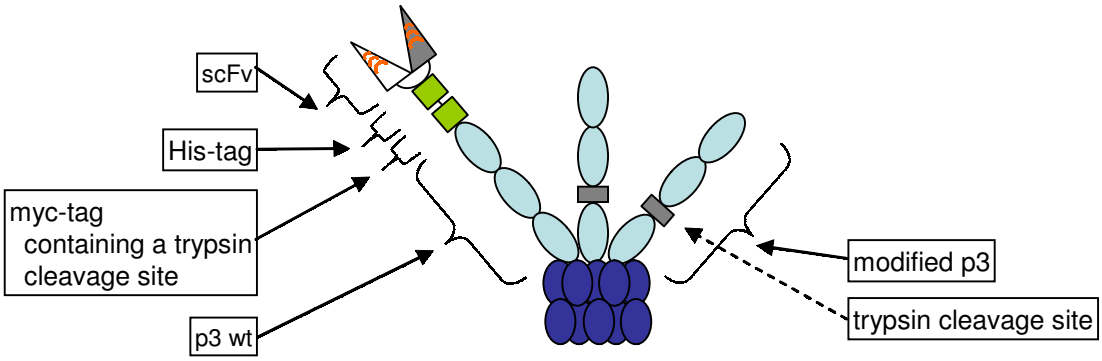

Figure 4: The head of the phage, showing the fusion protein of p3, tags, and scFv and the modified p3.

By combination of proteolytic and antigen-specific selection, we have obtained a high yield of specific clones with high diversity after only few rounds of selection (see chapter 2.11.1).

1.3.3 Anti-idiotypic antibodies and molecular mimicry

In nature mimicry exists in many different forms, for example as macromolecular mimicry between chemically very different compounds such as nucleic acid and protein (Nissen, Kjeldgaard et al. 2000) or as pathogenic use of molecular mimicry of the infected host e.g. to overcome immune surveillance. The latter can greatly influence the health of the host since the display of the same or similar carbohydrate structures on the surface of a pathogen as in the host might lead to autoreactive antibodies (Moran, Prendergast et al. 1996; Allos 1997; Hughes, Hadden et al. 1999; Willison and O'Hanlon 1999; Binder, Horkko et al. 2003). In a similar way several Lewis antigens (a, b, Y, X, and sialyl-Lewis X) have been found on lipopolysaccharides of some *Helicobactor pylori* strains (Aspinall, Monteiro et al. 1996; Monteiro, Chan et al. 1998). Infection with *H. pylori* can among other things lead to chronic gastritis, gastric atrophy, and gastric adenocarcinoma (Dunn, Cohen et al. 1997), reviewed in (Moran and Prendergast 2001). Other kinds of chemical different mimicry has also been found or selected as e.g. decoy RNA molecules as mimicry for human insulin receptor (Doudna, Cech et al. 1995) or peptides as mimicry of dsDNA (Putterman and Diamond 1998).

Immunological mimicry can be defined as a chemically different molecule binding specifically to an antibody like the authentic antigen (immunogen). The use of a mimicking substance as a vaccine is of considerable practical interest in the case of TACAs or tumour specific antigens, such as the Lewis Y and the disaccharide Thomsen-Friedenreich glycotopes, since carbohydrate structures may be less immunogenic, are difficult to purify, expensive to synthesize, and cannot be produced by recombinant technologies. Furthermore, carbohydrate structures induce mostly T cell-independent humoral responses of the IgM isotype (Mond, Lees et al. 1995).

The immunological network hypothesis of Niels Jerne (Jerne 1974) implies that anti-idiotypic antibodies (Ab2) are potentially useful as substitutes of antigens. If they bind to the antigen-combining site, they are displaying an “internal image” of the idiotypic antibody (Ab1). Such type β Ab2 can activate both the

humoral and the cellular branch of the immune system, giving rise to anti-anti-idiotypic antibodies (Ab3), of which some (Ab1') recognise the original antigen (Herlyn and Birebent 1999; Bhattacharya-Chatterjee, Chatterjee et al. 2000). The network hypothesis is schematically shown in Figure 5.

There are several ways in which the mimic can exert its mimicry of the antigen. Anti-idiotypic antibodies have in some cases been found to show sequence homology with the original antigen (Herlyn, Somasundaram et al. 1997; Tripathi, Qin et al. 1998; Luo, Qi et al. 2000; Vogel, Miescher et al. 2000). Since carbohydrates cannot be mimicked by sequence homology they are speculated to provide a functional mimic or to provide similar interactions to the idiotype (Westerink, Campagnari et al. 1988; Magliani, Polonelli et al. 1998). Comparison of crystal structures of Fab with bound carbohydrate and bound mimicry peptide showed that the mimicry is mainly functional and that structural mimicry is only a minor component (Vyas, Vyas et al. 2003).

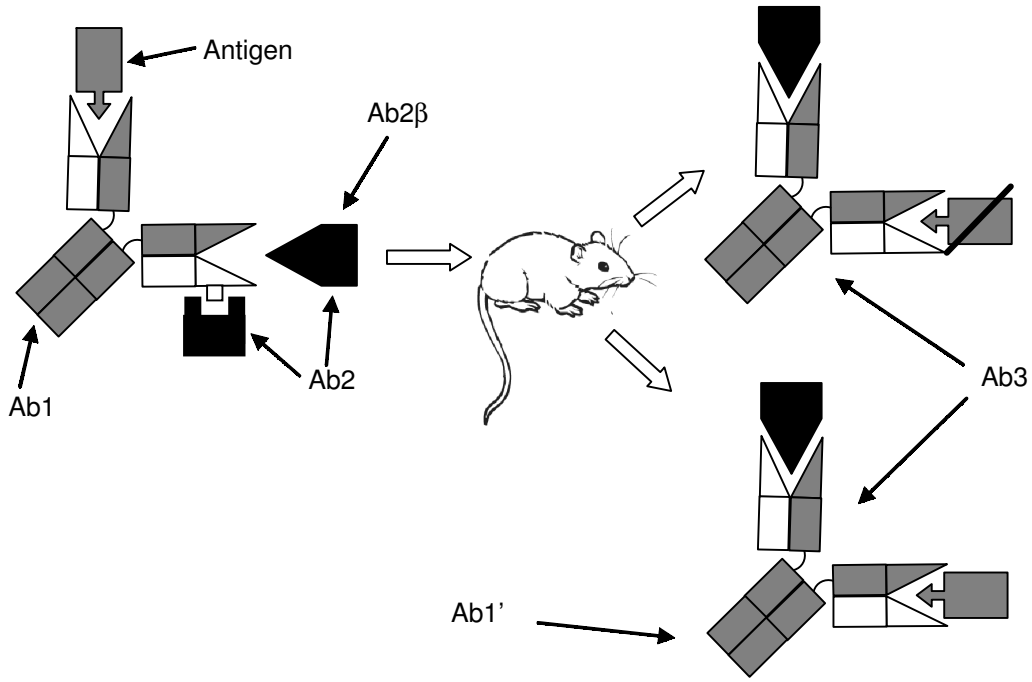

Figure 5: Generation of anti-antiidiotypic response in mouse. Ab1 recognising the original antigen is shown on the left. Two different kinds of Ab2s are shown. One (the Ab2β) is binding in the antigen binding site and displaying an "internal image". The other is recognising an epitope outside the binding site. Immunising a mouse with an Ab2β give rise to several Ab3s. Some of them (the Ab1') are expected to be able to bind the original antigen.

Several studies have already shown that in case of tumour antigens, immunisation with Ab2β can generate a response recognising the antigen and prolong the lifetime of cancer patients (Grant, Kris et al. 1999; Bhattacharya-Chatterjee, Chatterjee et al. 2000; Durrant, Maxwell-Armstrong et al. 2000). In these studies, additional advantages in using Ab2 were observed, e.g. the ability to break host tolerance to the antigen or breakage of immunosuppression (Bhattacharya-Chatterjee, Chatterjee et al. 2000). Ab2β has also proven a valid technology for mimicry of carbohydrate structures (see e.g. (Westerink, Campagnari et al. 1988; Magliani, Polonelli et al. 1998; Grant, Kris et al. 1999)).

Peptides are another classical way of presenting mimicry of carbohydrate structures. Generation of mimicry peptides is often done by phage display (Hoess, Brinkmann et al. 1993). Some mimics does though have a more exotic origin e.g. anti-idiotypic antibody (Westerink, Giardina et al. 1995), or has been found by modelling and docking in silico (Luo, Canziani et al. 2000). The peptides that are mimics of carbohydrate structures have been found to have a high prevalence of tryptophan and tyrosine residues independent of the original epitope (Oldenburg, Loganathan et al. 1992; Scott, Loganathan et al. 1992; Hoess, Brinkmann et al. 1993; Mirkov, Evans et al. 1995; Luo, Agadjanyan et al. 1998). One problem with several of the sera generated with different Lewis Y mimicking peptides is that though the sera show recognition of Lewis Y, they also cross-react with related carbohydrate structures such as H type 2 (Agadjanyan, Luo et al. 1997; Luo, Agadjanyan et al. 1998; Kieber-Emmons, Luo et al. 1999).

1.3.4 Saturation transfer difference nuclear magnetic resonance

Only a limited number of carbohydrate-antibody complexes have been crystallized, possibly due to the general limited affinity found for antibody-carbohydrate interactions. This suggests that other methods than crystallography must be used to shed light on the molecular interactions making a specific recognition of carbohydrate antigens possible. Nuclear magnetic resonance (NMR) is an excellent method to study weak interactions and it has been used by several groups to reveal the structures of antibody-bound conformations of carbohydrates. In these experiments transferred Nuclear Overhauser Effects (trNOEs) are obtained from hydrogens in different carbohydrate residues and thereby reveal the distance between two hydrogen atoms in two different carbohydrate residues, which again gives the conformation of the carbohydrate in the bound state. See f.x. (Bundle, Baumann et al. 1994; Haselhorst, Espinosa et al. 1999). However, they fail to provide information on the actual binding epitope. A recent development in the field of NMR spectroscopy, the saturation transfer difference (STD) NMR spectroscopy, is based on a transfer of saturation from a large ligand to a small epitope. The antibody-carbohydrate interaction is therefore an excellent system for use of this method. In these cases the saturation transfer comes from the antibody to the bound carbohydrate for those carbohydrate protons that interact with antibody protons through an intermolecular NOE (Klein, Meinecke et al. 1999; Mayer and Meyer 2001). In this kind of experiment the dissociation constant for the complex must be low enough to get sufficient transfer of saturation to the carbohydrate and high enough for the carbohydrate to leave the binding site before all magnetisation has been equally distributed between all the protons of the ligand (Figure 6). Detection of saturation is made on free carbohydrates which also require some exchange with carbohydrates in solution. All unwanted signals are removed by generating a difference spectrum between an on-resonance spectrum, where the antibody is selectively saturated and an off-resonance spectrum with an irradiation frequency far from any signal. Resonances originating from large molecule, such as the antibody, are removed by recording the spectra with a $T_{1\rho}$ filter consisting of a 30-ms spin-lock pulse prior to acquisition. In this way signals from the antibody disappears due to shorter relaxation times. This leaves only transferred saturation signals from the ligand in the remaining spectrum. Preferred is a high ligand excess and long saturation time since this gives a larger build up effect in the protons in close contact than the ones with no contact (Mayer and Meyer 2001). Oligosaccharies are

especially suitable for this kind of epitope mapping because the glycosidic linkages provide bottlenecks for any spin-diffusion process (Maaheimo, Kosma et al. 2000)

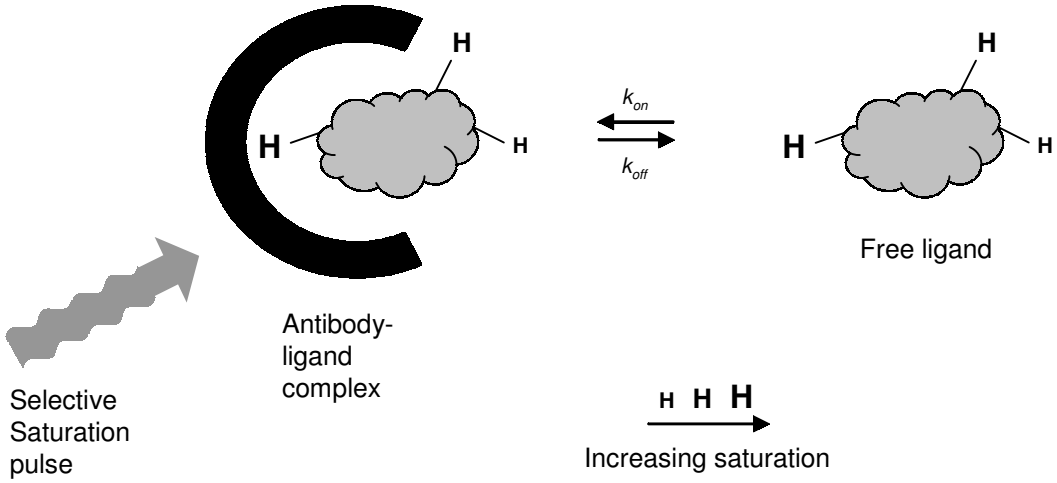

Figure 6: Illustration of the STD-NMR experiment. The antibody is irradiated with a saturation pulse far from any ligand resonances. This leads to selective saturation of the antibody by spin diffusion. Saturation is then transferred to the bound ligand in relation to the degree of contact with the antibody. Small protons to large protons = no contact to much contact. Adapted after (Mayer and Meyer 2001)

To circumvent the problems of severe overlap of carbohydrate proton resonances two dimensional NMR analysis applying the saturation transfer has also been used and was found applicable to improve epitope resolution of disaccharides (Maaheimo, Kosma et al. 2000). This technique could even map an epitope on the tetrasaccharide sialyl Lewis X recognised by E-selectin (Rinnbauer, Ernst et al. 2003).

We used the STD-NMR method to study the interactions between antibodies and the recognised related carbohydrates.