3 Discussion

3.1 Lewis Y binding antibodies and specificity

Several antibodies recognising Lewis Y have been generated and published (described in chapter 1.2.6). The key problem has been the specificity or cross-reactivity with other carbohydrate structures as only the antibody BR55-2 have by literature survey not been found reported to cross-react to other smaller carbohydrate structures. The specificities can be determined on synthetic carbohydrates by ELISA techniques or on purified antigens e.g. glycoprotein or glycolipids. Generally no or only small deviations have been observed between the methods and the deviations mainly concern the signal intensity (Benhar and Pastan 1994; Kitamura, Stockert et al. 1994). Results of the specificity determination of the carbohydrate binding antibodies can though also vary with the laboratory performing the experiment as BR96 has been reported specific for Lewis Y (Hellstrom, Garrigues et al. 1990), but also as cross-reacting to Lewis X (Zhang, Zhang et al. 1997).

Table 10: Binding specificity of the Nemod mouse monoclonal and chimeric antibodies. (++) denotes strong recognition, (+) denotes minor binding and (-) denotes lack of binding.

Trivial name	Carbohydrate structure	A51-B/A6	A46-B/B10	A70-C/C8	A70-A/A9	cIgG CC8	cIgG AA9/CC8
Lewis Y	Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ	++	++	++	++	++	++
Lewis b	Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ	-	-	-	+	-	-
Lewis X	Galβ1-4(Fucα1-3)GlcNAcβ	-	-	-	-	-	-
Lewis a	Galβ1-3(Fucα1-4)GlcNAcβ	-	-	-	-	-	-
Globo H	Fucα1-2Galβ1-3GalNAcβ	(+)	-	-	+	-	-
H type 3	Fucα1-2Galβ1-3GalNAcα	-	-	-	-	-	-
H type 2	Fucα1-2Galβ1-4GlcNAcβ	++	++	-	+	-	-
H type 1	Fucα1-2Galβ1-3GlcNAcβ	-	-	-	-	-	-
H disacch.	Fucα1-2Galβ	++	-	-	-	-	-

Here we report on the analysis of four Lewis Y recognising antibodies generated from 1983 to 1991 by U. Karsten, (MDC Berlin). The mouse monoclonal antibodies are A51-B/A6 (IgA), A46-B/B10 (IgM), A70-C/C8 (IgM), and A70-A/A9 (IgG1). We used 86 different synthetic polyacrylamide-coupled oligosaccharides for our specificity determinations. This panel of carbohydrates range from mono- to tetrasaccharides including sulphated or sialylated compounds. The specificity characterisation of the Nemod antibodies is summarised in Table 10. Also shown are the closely related non-interacting carbohydrate structures.

A51-B/A6 showed binding to four carbohydrates: Lewis Y, H type 2, and H type disaccharide, in which the H disaccharide (Fucα1-2Galβ1) is present (Figure 7 A chapter 2.1), but it did not recognise Lewis b, H type 1 or H type 3, which also contain the H disaccharide. A vital point in the recognition of the H disaccharide for A51-B/A6 is therefore the chain type. The recognition site for A51-B/A6 is located in the H disaccharide on a type 2 chain basis (Table 1 chapter 1.2.1). The trace binding to Globo H indicates that fitting of H disaccharide on the type 4 chain to the paratope is also possible, whereas the H type 3 is not able to fit to the paratope. The

only difference between the type chains 3 and 4 is the α and β forms of the GalNAc, which form the basis for the H disaccharide.

A46-B/B10 reacted only with the Lewis Y and H type 2 structures (Figure 7 B chapter 2.1). This suggests a binding epitope consisting of the (Fucα1-2Galβ1-4GlcNAc) turning away from the fucose attached 1-3 to the GlcNAc.

A70-C/C8 showed a clear binding specificity to Lewis Y with no cross-reactivity to other tested carbohydrates (Figure 8 A chapter 2.1). This specificity proposes the epitope to include the two fucose rings as major binding points, since no binding to either H type 2 or Lewis X was observed.

A70-A/A9 mainly recognises Lewis Y and Lewis b, with a small cross-reaction to Globo H and a trace binding of H type 2 (Figure 8 B chapter 2.1). This binding pattern also suggests the H disaccharide as the epitope though also in this case the lack of binding to several other compounds containing the H disaccharide is remarkable. On the other hand the binding might be more independent of the chain type since superposition of Lewis b and Lewis Y indicates, despite the chain type differences, that they share a common topography. This common topography excludes the base of the chain type, since the N-acetyl group is projected on opposite sites of the difucosylated structures according to modelling (Blaszczyk-Thurin, Murali et al. 1996; Luo, Agadjanyan et al. 1998).

The alignment of the obtained sequences (Figure 10 chapter 2.2) showed that the Lewis Y binding Nemod antibodies generally have a high sequence similarity. For the VH the BB10 sequence is quite different from the others and the AA9 CDR3 also contains some changes, which are bound to influence the binding site. The VH of CC8 and BA6(1) differ by only one residue in the framework. This amino acid is not involved in the antigen binding site or at a position believed to have impact on the canonical structures (Morea, Lesk et al. 2000), although an aspartic acid to tyrosine exchange is a big difference. That this change might influence the antigen binding e.g. through structural modifications is seen in the low production and low binding to Lewis Y of the chain shuffled variant BA6(1)μ/CC8κ (Figure 14 chapter 2.4). The VL sequences generally also show a high similarity with the CC8 being different from the others. This is though most prominent in the framework residues. The VL AA9 and CC8 differ in seven positions in the CDR 1-3 sequences. These seven differences could very well determine the difference in binding specificity between A70-A/A9 and cIgG AA9/CC8 from the chain shuffling experiment.

Figure 42 shows an alignment of Lewis Y binding antibody sequences available from the literature and the sequences obtained of the Nemod Lewis Y binding antibodies. The sequence for 3S193 is obtained from a humanised version of the antibody (Scott, Geleick et al. 2000). In the alignment here, the humanised amino acids are excluded. A general comparison of the sequences show that the already known antibodies have a high sequence similarity with each other except for the MSL5, whereas three of four Nemod antibodies differ much from the these already known sequences and seem to constitute a third set of sequences.

Most of the variation between the first two sets of sequences is located in the framework and is not expected to have a great influence on the specificity. From this it was found that the germline origins are not the same. The VH-VL interface is also influenced by the presence of different amino acids, and these interface residues determine the relative positions of the VH and VL and will therefore also influence the binding site.

Figure 42: Aligment of available sequences of Lewis Y binding antibodies. The sequences are adapted after (Blaszczyk-Thurin, Murali et al. 1996; Rheinnecker, Hardt et al. 1996; Scott, Geleick et al. 2000). The colour of the amino acid corresponds to its abundance at this position. Most abundant is indicated with the colour red followed by blue, green, cyan, gray, and black (which is used for single amino acids). The numbers above the alignment denotes an important position for the canonical structures of the CDRs. 1 for CDR1, 2 for CDR2, and 3 for CDR3, respectively. In the case of more implication on more than one CDR, the arrow indicates the position. The lines denote VH-VL interface positions. The canonical structure positions and VH-VL interface residues are defined as in (Morea, Tramontano et al. 1998; Morea, Lesk et al. 2000). The asterisk below the alignment denotes positions identified in BR96 as contact residues to Lewis Y (Jeffrey, Bajorath et al. 1995). Sequences are aligned with Clustal W (Thompson, Higgins et al. 1994). The sequence for 3S193 is obtianed from a humanised version. The human residues are omitted here. The CDR sequences of the Nemod antibodies are blackend due to patenting reasons

VH

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                 Framework 1           CDR1      Framework 2        CDR2                Framework 3                         CDR3        Framework 4
                                   1   11 1     1_____   _____     2 22                2                    ____1,3↓             3  ________
BR96     EVNLVESGGGLVQPGGSLKLSCATS GFTFSDY YMYWVRQTPEKRLEWVAYIS QGGD ITDYPDTVKGRFTISRDNAKNTLYLQMSRLKSEDTAMYY CARG--LDDGAWFAYWG QGTLVTVSA
B5       EVKLVESGGGLVQPGGSLKLSCATS GFTFSDY YMYWVRQTPEKRLEWVAYIS NGGG STYYPDTVKGRFTIDRDNAKNTLYLQMSRLKSEDTAMYY CARG--LSDGSWFAYWG QGTLVTVSS
H18A     EVKLVESGGGLVQPGGSLKLSCATS GFTFSDY YMYWVRQTPEKRPEWVAYIS NGGD IPYYVDTVKGRFTISRDNAKNTLYLQMSRLRSEDTAMYY CARG--KYDGAWFAYWG QGTLVTVSA
BR55-2   EVKLVESGGGLVQPGGSLKLSCATS GFTFSDY YMYWVRQTPEKRLEWVAYIS NGGG SSHYVDSVKGRFTISRDNAKNTLYLQMSRLRSEDTAMYH CARG--MDYGAWFAYWG QGTLVTVSA
B3       DVKLVESGGGLVQPGGSLKLSCATS GFTFSDY YMYWVRQTPEKRLEWVAYIS NDDS SAAYSDTVKGRFTISRDNARNTLYLQMSRLKSEDTAIYS CARG--LAWGAWFAYWG QGTLVTVSA
B1       EVQLVESGGGLVKPGGSLKLSCAAS GFIFSDN YMYWVRQTPEKRLEWVATIS DGGT YIDYSDSVKGRFTISRDNAKNNLYLQMSSLRSEDTGMYY CGRSPIYYDYAPFTYWG QGTLVTVSA
3S193                           TS GFTFSDY YMYWVRQAPGKGLEWVAYMS NVGA ITDYPDTVKGRFTISRDNSKNTL         EDTGVYF CARG--TRDGSWFAYWG QGT
CC8      EVKLVESGPELVKPGASVKISCKAS [redacted] NIDWVKQSHGKSLEWIGYIY [redacted] GTGYNQKFTNKATLTVDKSSSTAYMELHSLTSEYSAVYY [redacted] QGTLVTVSA
BA6(1)   EVKLVESGPELVKPGASVKISCKAS [redacted] NIDWVKQSHGKSLEWIGYIY [redacted] GTGYNQKFTNKATLTVDKSSSTAYMELHSLTSEDSAVYY [redacted] QGTLVTVSA
BA6(3)   DVKLVESGPDLVKPGASVKISCKAS [redacted] NMDWVKQTHAKSLEWIGYIY [redacted] YSDYNQKFTNKATLTVDKSSSTAYMELHSLTSEDSAVYY [redacted] QGTLVTVSA
AA9      KVKLQQSGPDLVKPGASVKISCKAS [redacted] NMDWVKQTHAKSLEWIGYIY [redacted] YSDYNQKFKSKATLTVDKSSSTAYMELHSLTSEDSAIYY [redacted] QGTLVTVSA
BB10     QVQLKESGPELKKPGETVKISCKAS GYTFTNY GMNWVKQAPGKGLKWMGWIN TYTG EPTYADDFKGRFAFSLETSASTAYLQINNLKNEDMATYF CAN---DYDGAWFAYWG QGTLVTVSS
MSL5     QVQLQESGPELKKPGETVKISCKAS GYTFTNY GMNWVKQAPGKGLKWMGWIN TYTG EPTYADDFKGRVALSLETSASTAYLQINNLKNEDTATYF CAN---DYDGAWFAYWG QGTLVTVSS
Contact residues                     ** * *               *   *                                              *  ** ***
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VL

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                 Framework 1             CDR1       Framework 2    CDR2             Framework 3                 CDR3   Framework 4
          1                       1    1    1     1______  _____2              2      1            ______3    3  3_______
BR55-2   DVLMTQTPLSLPVSLGDQASISCRS SQSIVHSNGNTY LEWYLQKPGQSPKLLIS KVS NRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYYCFQ GSHVPF TFGSGTKLEIK
B5       DVLLTQTPLSLPVSLGDQASISCRS SQSIVHSNGNTY LEWYLQKPGQSPKLLIY KVS NRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYYCFQ GSHVPF TFGSGTKLEIK
H18A     DVLMTQTPLSLPVSLGDQASISCGS GQSIIHTNGNTY LEWYLQKPGQSPKLLIY KVS NRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYYCFQ GSHVPF TFGSGTKLEIK
BR96     DVLMTQIPVSLPVSLGDQASISCRS SQIIVHNNGNTY LEWYLQKPGQSPQLLIY KVS NRFSGVPDRFSGSGSGTDFTLKISRVEAEDLEVYYCFQ GSHVPF TFGSGTKLEIK
B3       DVLMTQSPLSLPVSLGDQASISCRS SQIIVHSNGNTY LEWYLQKPGQSPKLLIY KVS NRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYYCFQ GSHVPF TFGSGTKLEIK
3S193     V                    CRS SQRIVHSNGNTY LEWY       PKLLIY KVS NRFSG      G       F             YYCFQ GSHVPF TFGQGTK
B1       DVVMTQTPLSLPVSLGDQASISCRS SQNLVHSDGKTY LHWFLQKPGQSPTLLIY KVS NRFSGVPDRFSGSGSGTDFILKISRVEAEDLGVYFCSQ STHVPL TFGAGTKLELK
CC8      DIVLTQSPLFLHVSLGDQASISCTS [redacted] LDWHLQKSDQSLQLLIY [redacted] KRNSGVPDRFSGSGSGKDFTLKISRVEPEDLGIYYCFQ [redacted] TFGAGTKLEIK
BB10     DIVMTQTPLTLSVTIGQPASISCKS SQSLLDSDGKTY LNWLLQRPGQSPKRLIY LVS KLDSGVPDRFTGSGSGTDFTLKISRVEAEDLGVYYCWQ GTHFPQ TFGGGTKLEIK
BA6      DIVMTQTPLTLSVTIGQPASISCKS SQSLLDSDGKTY LNWLLQRPGQSPKRLIY LVS KLDSGVPDRFTGSGSGTDFTLKISRVEAEDLGVYYCWQ GTHFPQ TFGGGTKLEIK
MSL5     DIVMTQTPLTLSVTIGQPASISCKS SQSLLDSDGKTY LNWLLQRPGQSPKRLIY LVS KLDSGVPDRFTGSGSGTDFTLKISRVEAEDLGVYYCWQ GTHFPQ TFGGGTKLEIK
AA9      DIVMTQTPLTLSVTIGQPTSISCKS [redacted] LNWLLQRPGQSPKLLIY [redacted] NLESGVPDRFSGSGSGTDFTLKISRVEAEDLGVYYCLQ [redacted] TFGAGAKLELK
Contact residues                     *     *                                                                  *
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Even though there is some variation in the amino acids influencing the canonical structures of the CDRs, the variations in these residues in the VLs are not deviating from the allowed constrained variation for any of the CDRs. This means that the CDRs in all VLs in theory should belong to the same canonical structures class. For the VHs this is different. Regarding the CDR1 all antibodies belong to the same canonical structure class. Since variation and length of the amino acids sequence of the CDRs will influence the binding site and specificity, and since the major binding specificity is believed to be located in the VH, it is reasonable to believe that due to the major changes in the CDR2 and CDR3 in the VH, the binding of Lewis Y will be different from that described for BR96 (Jeffrey, Bajorath et al. 1995).

The antibodies MSL5 (Rheinnecker, Hardt et al. 1996) and A46-B/B10 are almost completely identical and constitute a third sequence set. These two antibodies vary from each other at only two positions outside the primer generated region. According to the canonical structure theory none of these positions are believed to influence the binding site (Morea, Lesk et al. 2000). The antibody MSL5 has though not been reported to recognise H type 2 as the A46-B/B10 does, on the other hand no test of this binding has been reported either.

Comparing the contact residues found in the crystal structure of BR96 (Jeffrey, Bajorath et al. 1995)(denoted with a asterisk in Figure 42) in the VLs it is seen that the antibodies BR96, B3, BR55-2, 3S193, B5, and H18A all have the same amino acids at these positions. B1, AA9, and CC8 deviate at one position, and the BA6, BB10, and MSL5 deviate at two positions. For the VH, only the contact residues in CDR1 can be compared to each other, the variation in the canonical structures of the CDR2 and CDR3 makes comparison for these CDRs impossible. In the CDR1 the contact residues of the Nemod antibodies do not deviate from the consensus found in the other antibodies but two contact residues located in the beginning of the framework 2, directly after the CDR1, do vary from the consensus in the other antibodies at both positions. At these two positions the consensus is two tyrosines and the Nemod antibodies have at these positions glutamic acid and aspartic acid, two changes to acidic residues. These changes also indicate that the binding of Lewis Y of the Nemod antibodies will deviate from the BR96 binding.

Obtaining the sequences and cloning of the different antibodies was in all cases but the A70-A/A9 VL relatively easy. For all VL clones a background consisting of one specific sequence was observed. The background sequence is clearly an antibody sequence, which contains one or more frame shifts and stop codons. It is possible that this sequence originates from the fusion cell line used to generate the hybridomas (X63-Ag8.653). In case of the A70-A/A9 VL a total of 500 sequences were screened before one not being the nonsense sequence was retrieved. Verifying that the obtained sequence corresponds to the antibody was done by testing recombinantly produced antibody in a specificity assay. This was successfully done for A70-C/C8 and A46-B/B10 (Figure 11 chapter 2.3). For A51-B/A6 only one of the obtained sequences was successfully expressed. Unfortunately, the expressed antibody did not show carbohydrate binding. For the BA6(3) and the AA9 sequences no successful expression was obtained. This does however not exclude the possibility of these sequences belong to the hybridoma antibodies.

Due to the high similarity between the variable domains and the high similarity to germline sequences a shuffling of the variable domains was performed. We shuffled all obtained light chains with all obtained heavy chains and we expected to find new or changed specificities against some of the already identified carbohydrate antigens.

The shuffling of the Nemod antibody variable domains and testing for expression of antibody showed that five new combinations gave rise to expressed antibody (Table 3 and Figure 14 chapter 2.4). Investigating the binding against Lewis Y and some related structures showed that the VHs BA6(1) and AA9 when combined with the VL sequence CC8 bound Lewis Y and not H type 2 nor Lewis b. The specificity test of the cIgM and cIgG AA9/CC8 (Figure 15 chapter 2.4) showed that these two antibodies exclusively bound to Lewis Y in the panel of 86 carbohydrate PAA conjugates tested. The exchange of the light chain did in this case narrow the specificity of the VH as the cross-reactivity of the A70-A/A9 to Lewis b and Globo H disappeared. For the antibody cIgM BA6(1)/CC8 it is not surprising that Lewis Y is recognised due to only one amino acid making up the difference between the VHs (discussed above). Unexpected it is though that two very different VH, CC8 and AA9, combined with the same VL, CC8, will be able to bind exclusively to the same antigen, Lewis Y.

Previous chain shuffling experiments have been made with the antibodies B3 and B5, both recognising Lewis Y (Benhar and Pastan 1995). This study reported a less active scFv when the B5 VH and the B3 VL was combined and a more stable and active scFv when the B3 VH and the B5 VL was combined, both compared to the parent antibody B3. In between the two VLs B3 and B5 there are though only three mutations, two in the framework 1 and one in the CDR1. No change in specificity was reported upon the chain shuffling. Furthermore, the individual chains of the antibody B3 showed the ability to bind to the surface of Lewis Y expressing cells (Brinkmann, Lee et al. 1993). It is reasonable to assume that this interaction is through Lewis Y, since the interaction could be inhibited with the full B3 antibody. This could also be an explanation for the results found here, that the chains involved in chainshuffling VL of CC8, VH of CC8 or AA9 alone could be able to bind to Lewis Y specifically.

The question concerning which of the two VH chains in the hybridoma A51-B/A6 produces the A51-B/A6 antibody can possibly be answered by the chain shuffling experiments. Two results point to the BA6(3) chain: (1) The antibody cIgM BA6(1)/BB10 was found to lack carbohydrate binding (Figure 11 chapter 2.3), (2) the antibody cIgM CC8/BB10 antibody lack carbohydrate binding (Figure 14 chapter 2.4). The latter supports the BA6(3) since only one mutation makes up the difference between the VH CC8 and BA6(1), which indicates that the combination of CC8 VH and BB10 VL should be able to produce a carbohydrate binding antibody if BA6(1) was the correct sequence.

To sum up these experiments it was shown that the mouse monoclonal antibodies A51-B/A6, A46-B/B10, and A70-A/A9 bound to Lewis Y and cross-reacted to substructures or related structures. The antibody A70-C/C8 was found to exclusively bind Lewis Y. A chimeric human-mouse IgG antibody was successfully generated from the A70-C/C8. Finally we were able to generate a new chimeric IgG antibody (cIgG AA9/CC8) showing high specificity for Lewis Y.

3.2 Expression and purification of the antibodies

The expression level of the murine antibodies by the hybridomas reached in our hands 20µg/ml for IgG, 15µg/ml for IgA, and 5µg/ml for IgM. Production of the murine IgM and IgA antibodies was done in media containing calf serum, which makes the supernatants contain an impurity of bovine antibodies. To remove

unwanted proteins and bovine antibodies, purification was performed by immunoaffinity chromatography on anti-mouse Ig sepharose. This single purification step enabled us to obtain pure and active murine antibodies from A51-B/A6, A46-B/B10, and A70-C/C8 (partly shown in Figure 16 A and C chapter 2.5). The murine IgG1 A70-A/A9 hybridoma was able to adapt to serum free media, which allows an effective purification of the IgG1 antibody by protein A chromatography. This single purification step was used for A70-A/A9 and produced pure and active antibody (Figure 16 B and C chapter 2.5).

We were able to establish stable CHO cell lines expressing the chimeric antibody cIgG CC8. The expression level of this antibody was estimated to be around 1µg/ml. The CHO cell line expressing cIgG CC8 was also able to adapt to serum free media, which again enabled the purification of cIgG CC8 by protein A sepharose chromatography. This procedure gave rise to pure cIgG CC8 (Figure 16 C chapter 2.5).

A cIgG Fab fragment of CC8 has also been cloned. Transient (COS-7) and stable (CHO) transfections of the CC8 Fab plasmid showed that we were able to express the chimeric Fab. The production of the Fab was analysed by sandwich ELISA with coated anti-human κ antibody as a catcher and anti-human IgG $F(ab)_2$ antibody as POD conjugated antibody for detection. However, Fab binding to Lewis Y-PAA has not been detected. The observed results might be due to weak antibody avidity or too low Fab concentrations in the supernatant to generate an ELISA signal.

Alternatively a protocol was established to isolate different formats by an immuno-affinity chromatography with Lewis Y conjugated to sepharose. Successful purification of the antibodies or antibody fragments on Lewis Y sepharose would facilitate purification of Lewis Y binding antibody fragments and ensure that the purified protein is active. However, we failed because the terminal fucose in the Lewis Y only is stable at pH above 7 and below 12, which limits the possible elution conditions (V. Atanov, Lectinity, personal communication).

A totally different purification method was established to isolate antibodies from media containing Prolifix, a serum substitute which contains low molecular weight compounds of plant origin only. Purification of A70-C/C8 and PankoMab, a MUC1 binding antibody, from culture supernatants with the serum subsitute Prolifix was successfully done by ultrafiltration. These antibody preparations contained though a considerable amount of DNA (Table 4 and Figure 17 chapter 2.5). The traditional method for removing DNA is by anion-exchange chromatography. We established a very simple new method by coprecipitation, which was only known in the context of transfecting cells (Graham and van der Eb 1973). The addition of a $MnCl_2$ solution to the phosphate-buffered antibody preparations and subsequent centrifugation resulted in a DNA-manganese-phosphate co-precipitation and efficient removal of DNA indicated by UV absorption and gel electrophoreses (Table 4 and Figure 17 chapter 2.5). We have estimated by absorbance measurements that the efficiency of DNA removal by the method described here is comparable to that of using ion exchange chromatography (data not shown). Similar results were obtained with $CaCl_2$, which is less preferable because of the danger of activation of possibly existing residual proteases.

The quantity of A70-C/C8 and PankoMab, purified by ultrafiltration, with respect to other proteins as well as its quantity before and after the purification steps, showed that no protein was lost in the precipitation procedure. An ELISA test for bioactivity proved that the binding activity also is retained after the precipitation (Chapter 2.5). The results show that the manganese precipitation step in combination with defined media and

ultrafiltration is a simple, fast, cheap and effective way for purifying antibodies of different isotypes. It is especially valuable for the purification of antibodies which are sensitive for the interaction with chromatographic matrices.

Furthermore, the results indicate the high potential of manganese precipitation for the effective removal of DNA from other protein samples to avoid loss of yield and activity, which might occur with other purification procedures. We also found that the successful removal of residual DNA from periplasmic preparations (TES fractions) of bacterially expressed single chain antibody fragments facilitated the column flow in further chromatographic purification.

In summary, we were able to isolate pure mouse monoclonal as well as recombinant antibodies by well known chromatographic methods and established a new simple ultrafiltration method including removal of DNA by coprecipitation.

3.3 H type 2 epitope

Using STD NMR spectroscopy it is possible to perform a detailed epitope mapping of small molecules bound to e.g. a receptor or antibody, and thereby obtain critical information about the size of the binding epitope in the ligand. Here we have used this system to analyse the epitopes of A51-B/A6 and A46-B/B10 in H type 2 trisaccharide (Fucα1-2Galβ1-4GlcNAc), which is a part of Lewis Y. The binding affinity of one binding site in the range of 1mM to 10nM for STD NMR experiments is a precondition to read successful analysis. A stronger binding is often a result of a lower off rate, which would prevent the measurement of the ligand in the unbound state. The functional affinities estimated by SPR analysis of the Lewis Y binding antibodies presented here (chapter 2.8 and 3.4) resulted in binding affinities below 10nM, which made STD NMR applicable.

We started with the H disaccharide (Fucα1-2Gal) to establish the analysis. The most intensive STD signal of H disaccharide with A51-B/A6 was found for Fuc H1 (Figure 19 chapter 2.6.1). Gal H4 also gave rise to a strong STD signal corresponding to a tight contact to the antibody. Two other proton signals (Gal H3 and Fuc H3) are not dispersed, but together they also show strong saturation. This experiment shows that at least three strong contacts are found in the interaction of A51-B/A6 to H disaccharide. Five protons (Gal H1, Gal H5, and the three Fuc H6 protons) gave very limited STD signals corresponding to the intensity of low spacer protons and no antibody contact.

The STD signals obtained with the more complex trisaccharide H type 2 and the A51-B/A6 antibody showed again that Fuc H1 and additionally the three GlcNAc H8 are in closest contact with the protein when considering the proton signals which are not overlayed (Figure 20 A chapter 2.6.2). The two strongest saturated signals do though originate from Gal H3, H4 (one signal) and Gal H2, GlcNAc H3 (one signal). Comparing the intensities of these STD signals to the H disaccharide signals, it was found that the GlcNAc H3 and Gal H3 are the major contributors to these two STD signals. The contribution from Gal H4 to the STD signal was lower compared to Gal H3 but is to some extend still be involved in the epitope. In this experiment limited or no STD signal of single integrated peaks was, in addition to the ones obtained with the disaccharide, obtained for GlcNAc H1, GlcNAc H5, GlcNAc H6, and Fuc H5. The very low signal to noise ratio obtained in this

experiment was the reason of inaccuracies in determining the individual STD signal intensities and mainly affected the small signals.

For A46-B/B10 a similar analysis was only performed for the H type 2 trisaccharide, because SPR results showed not detectable binding. The STD experiment with A46-B/B10 identified a similar binding epitope in H type 2 as found for A51-B/A6. Here the two strongest signal intensities for single proton signals were found for the three GlcNAc H8 and Fuc H1 (Figure 20 B chapter 2.6.2) and the two strongest signals arose by the protons Gal H3 and H4 (one signal) and Gal H2 and GlcNAc H3 (one signal). Figure 43 shows a graphical representation of the protons identified in both A51-B/A6 and A46-B/B10 recognition of H type 2.

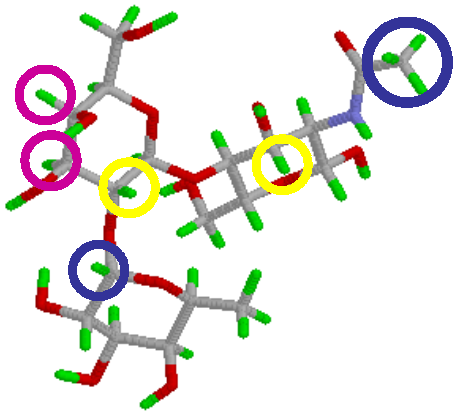

Figure 43: A figure of H type 2 showing the protons identified to be involved in the binding to the antibodies A51-B/A6 and A46-B/B10. A blue circle denotes protons involved in the binding. Yellow and pink circles denote pairs of protons which are overlayed in the NMR spectra. The carbohydrate is build with the program Sweet (Bohne, Lang et al. 1998) and the figure is prepared with RASMOL (Sayle and Milner-White 1995)

The dilution ELISA and functional affinity measurement for A51-B/A6 suggest that there is an additional binding determinant in the H type 2 compared to the disaccharide, which makes a stronger binding to the trisaccharide possible. A comparison of these results to the epitopes identified supports this, since the GlcNAc H3 proton and three GlcNAc H8 protons are in tight contact with the antibody. The difference in H disaccharide binding between A51-B/A6 and A46-B/B10 could be speculated to originate from deviating interactions to the GlcNAc carbohydrate ring e.g. the intermediate interaction to GlcNAc H1 identified in A46-B/B10 binding to H type 2 (Figure 20 chapter 2.6.2). If the results of the dilution ELISAs, functional affinity experiments, and epitope mapping are extrapolated to include Lewis Y, it could be speculated that this (Figure 43) is the common epitope of H type 2 (or H type 2 “in Lewis Y”) recognised by H type 2 and Lewis Y binding antibodies. This is plausible since neither A51-B/A6 nor A46-B/B10 shows lower functional affinity (in SPR) or reactivity (in ELISA) towards H type 2 than towards Lewis Y.

Comparing the binding epitope shown in Figure 43 to the crystal structures of BR96 and hu3S193 in complex with Lewis Y it appears that the interactions are not similar, which is supported by the sequence differences of the antibodies. The crystal structures of BR96 identified the interactions between the antibody and the carbohydrate mainly to involve three of the four carbohydrate residues GalNAc, Gal, and Fuc4 (Jeffrey, Bajorath et al. 1995). This is of cause not possible with our antibodies since the H type 2 structure does not

include the Fuc4, but we also speculate the Fuc4 not to be involved in the H type 2 “in Lewis Y” epitope, which is supported by the dilution ELISA and SPR analysis. This then shows the differences in the epitopes of antibodies recognising Lewis Y and cross-reacting to H type 2 (A51-B/A6 and A46-B/B10) and those cross-reacting to Lewis X (BR96).

Generally the spacer protons, which are not involved in the binding, are saturated to around 30% in the H disaccharide experiment and even lower in the H type 2 experiments. This low saturation correlates well to spin-diffusion effects in low-molecular mass molecules binding to large proteins. This spin-diffusion is mainly due to protein protons, and the saturation transferred to a ligand will experience only limited distribution within the ligand molecule once this has left the binding pocked (Vincent, Zwahlen et al. 1997). A problem is though the signal separation, which especially for experiments with these very similar carbohydrates makes the region around 3.7-3.85 very crowded. The overlayed signals for five and nine protons saturated to 50% and 42%, respectively, from the disaccharide and trisaccharide spectra, cannot be evaluated for their possible involvement in the binding. The approximately 45% saturation is an average of all protons integrated in the peak, which also means that most likely there are some protons hidden here with a much higher saturation degree.

The STD NMR spectroscopy has been used to determine other carbohydrate epitopes as well. As examples are the determination of the antibody mAb 2625 recognizing an N-methylated epitope in O-chain polysaccharide in Legionella pneumophila, where the epitope was not purified to homogeneity (Kooistra, Herfurth et al. 2002) and the epitope mapping of Chlamydia lipopolysaccharide with use of synthetic disaccharides (Maaheimo, Kosma et al. 2000). Antigens or ligands other than carbohydrates have also been investigated with STD NMR e.g. Gibberellin (Murata, Hemmi et al. 2003) and the MUC-1 glycotope (Moller, Serttas et al. 2002). Both studies found that the binding epitope determined by STD-NMR correlated to their respective X-ray structures, except for the artifacts arising from crystal packing.

To sum up the results the STD NMR results we have found similarities and differences in the binding epitope of the two antibodies A51-B/A6 and A46-B/B10. The results confirm the differences in specificity as indicated by ELISA. Furthermore, this epitope describes the recognition of H type 2 not restricted to the trisaccharide but also in Lewis Y.

3.4 Detection of Lewis Y on cancer cells

Over the years many groups have published results concerning Lewis Y expression in cancer by immunohistology and on cancer cell lines with immunocytochemistry. The histology data from literature points to a Lewis Y overexpression on various types of cancers, which means that Lewis Y is expressed in normal tissue but more extensively on cancers (Zhang, Zhang et al. 1997). The immunohistology published by Zhang et al. used the antibodies 3S193 and BR96 to elucidate the expression of Lewis Y (Zhang, Zhang et al. 1997). This revealed that the Lewis Y is strongly expressed on colon, breast, ovarian, prostate, lung, and pancreatic cancers. As for the Lewis b, a moderate expression was found on colon and pancreatic cancers. Here we used flow cytometric analysis and immunocytochemistry to compare the binding of the antibodies A51-B/A6, A46-B/B10,

A70-C/C8, A70-A/A9, cIgG CC8, and cIgG AA9/CC8 to cellular expressed Lewis Y and Lewis b (Table 5 chapter 2.7 and Table 6 chapter 2.8).

Several different cancer cell lines were included in the analysis: eight for flow cytometry and 12 for immunocytometry. The breast cancer cell line MCF-7 was investigated with both techniques and comparable results with this cell line show that both techniques are usable to detect different structures and differences in expression level. Moreover this cell line is well known for its expression of Lewis Y (among others shown in (Hellstrom, Garrigues et al. 1990; Pastan, Lovelace et al. 1991) with the antibodies BR96, B1, and B3).

Breast cancer cell lines included in these analyses were ZR-75-1, T47D, MCF-7, MDA-MB 435, and MT-3, and except for MDA-MB 435 they were all found to express Lewis Y due to recognition of all Lewis Y binding antibodies. ZR-75-1 has previously been published to bind 3S193 as well (Kitamura, Stockert et al. 1994). The MCF-7 cells were furthermore found to express Globo H due to the binding of A69-A/E8 and the lack of competition between A70-C/C8 and A70-A/A9. The MCF-7 cells were also previously found to express Globo H (Menard, Tagliabue et al. 1983). T47D cells do due to the reversal competition between A70-C/C8 and A70-A/A9 not express Lewis b or Globo H on the surface. MDA-MB 435 cells are from mammary gland duct metastatic site and they were here found negative for Lewis Y expression. Generally breast cancers should be positive for Lewis Y expression according to histology (Zhang, Zhang et al. 1997), but a down regulation in the expression of Lewis Y can be seen in metastatic cells (Le Pendu, Marionneau et al. 2001). The immunocytochemistry staining of the non-tumorigenic breast epithelial cell line H184A1 (Figure 25 A chapter 2.8) showed expression of Lewis Y on some, but not on all cells as demonstrated by counter staining of the nuclei.

Colon cancer cell lines found positive for Lewis Y expression were LS174T, SW480, and HCT15. HT29 and HCT116 did not bind any of the antibodies tested. Both cell lines were also negative with the Lewis Y recognising antibodies B1 and B3 (Pastan, Lovelace et al. 1991). In contradiction to this, BR55-2 has been reported to stain Lewis Y on the HT29 cells (Flieger, Hoff et al. 2001). HCT116 cells have previously been published as Lewis Y negative with the BR96 antibody (Hellstrom, Garrigues et al. 1990), and SW480 was found Lewis Y positive by staining with BR55-2 (Flieger, Hoff et al. 2001). The competition experiments showed that on SW480 cells A70-C/C8 and A70-A/A9 compete for binding which means that they do not express Lewis b or Globo H. On the LS174T cells A70-C/C8 and A70-A/A9 did not compete for binding which then means that they express either Lewis b, Globo H, or both.

Ovarian cancer has also been reported to express Lewis Y (Zhang, Zhang et al. 1997) and our testing of two ovary cancer cell lines gave the result that the OVCAR-3 cells were positive and the SK-OV-3 cells were negative. These results are similar to the results found by Yin et al. by use of the monoclonal antibody 3S193 (Yin, Finstad et al. 1996), whereas the B1 and B3 antibodies did not stain the OVCAR-3 cells, and no report was given on the SK-OV-3 cell line (Pastan, Lovelace et al. 1991). The differences seen between these two cell lines can possibly be explained, as above, by the origin of the SK-OV-3 cell line, which is an ascites adenocarcinoma from the metastatic site, where the Lewis Y expression might be downregulated.

Additionally the four cancer cell lines: HepG2 originating from liver, the HEK293 from kidney, U266 a B lymphocyte cancer and JEG-3 from placenta were tested for Lewis Y expression. These cell lines were all found negative for Lewis Y expression. No previous reports on Lewis Y expression have been reported on any of

these cell lines, whereas the results are in agreement with the histology data previously reported (Zhang, Zhang et al. 1997). The HEK293 was though found to express the H type 2 or the H disaccharide epitope as judged due to binding of A46-B/B10 and A51-B/A6. The expression of fucose was verified with binding of the lectin Ulex Europaeus A I to the cells.

The myelogenous leukaemia cell lines K562, NM-D4, and NM-F9 (NM-D4 and NM-F9 are both derived from K562 by selection for expression of the disaccharide Thomsen-Friedenreich (Schoeber, Schneider et al. submitted)), were all found to be negative for Lewis Y expression. NM-F9 cells were recognised by the A70-A/A9 and not by A69-A/E8, which means that Lewis b is expressed on these cells. In contrast to K562, NM-D4 and NM-F9 both bound UEA I which is in good agreement with the reported expression of Lewis X on these cell lines. K562 only showed a minor expression, whereas NM-F9 and NM-D4 show high expression (Schoeber, Schneider et al. submitted). The expression of a Fcα receptor on myeloid cells is very likely the reason for the nonsense results obtained by the use of IgA antibody A51-B/A6 (Monteiro, Cooper et al. 1992).

Generally it is seen that the antibodies A70-C/C8, cIgG CC8, and cIgG AA9/CC8 show identical recognition of cell lines, and that this recognition pattern is identical or very close to other Lewis Y specific antibodies. The expression pattern of Lewis Y on the different cell types is also in good agreement with the histology reports. It is however, also necessary to take into account the blood group of the individual from who the cells originate, as it is more likely that Lewis Y will be expressed on individuals having the blood group O than individuals having the blood groups A or B.

Summarising the results it can be stated that this antibody panel allows to distinguish between expression of the carbohydrate structures H disaccharide, H type 2, Lewis Y, and Lewis b on cell surfaces. A major result is that the recognition of Lewis Y on the surface of cell lines by the mouse monoclonal antibodies and the recombinant chimerics is fully comparable.

3.5 Affinities of the antibodies

Antibody-antigen affinities were estimated by SPR on a BIACORE using purified antibodies and carbohydrate-PAA-biotin was used to measure the functional affinities by SPR on a BIACORE (Table 7 chapter 2.9). These measurements showed that the mouse monoclonal antibodies A51-B/A6 (IgA), A46-B/B10 (IgM), and A70-C/C8 (IgM) all had K_{obs} around 5nM, whereas the A70-A/A9 (IgG1) had a higher K_{obs} of around 50nM all measured on Lewis Y. The K_{obs} indicate a strong binding of all the antibodies to Lewis Y, and the same is measured in ELISA, which is a highly sensitive method. Antibody concentrations less than 50ng/ml resulted in strong signals to Lewis Y. However, a comparison of the ELISA signals caused by different isotypes is not possible.

The binding to the H type 2 as measured by SPR was of the same order of magnitude like to Lewis Y for A51-B/A6 and A46-B/B10 and correlates again with the ELISA data. However, differences were observed against the H disaccharide, since the A46-B/B10 interaction to H disaccharide was detected in ELISA when high concentrations of antibody was used, but the interaction was too weak to be detected by SPR. The binding of

A51-B/A6 to the H disaccharide on the other hand could be measured with both techniques and was found to be approximately 10nM by SPR.

The A70-A/A9 functional affinity against Lewis b was determined by SPR to approximately 200nM. Generally the SPR experiments correlate very well with the dilution ELISA experiments (Figure 9 chapter 2.1), compared for each antibody individually. The dilution ELISA also indicated that the functional affinity against Lewis Y and H type 2 was of the same order of magnitude for both of the two antibodies A51-B/A6 and A46-B/B10. In a similar way it was also in both experiments found that A51-B/A6 bound the H disaccharide slightly weaker than H type 2.

Analysis of recombinant antibodies by SPR was only perfomed for cIgG CC8 because of the necessary amount of antibody available. The functional affinity of cIgG CC8 was measured to be about 1mM, which is much lower than the functional affinity 2nM of the IgM A70-C/C8. A difference between these two affinities was expected, since valency plays a great role in measuring the functional affinity (MacKenzie and To 1998).

The dissociation curves (Figure 27 chapter 2.9) show that the cIgG CC8 has a faster dissociation compared to the mouse monoclonal antibodies, and since the associations are approximately equal for all antibodies (data not shown), this fast dissociation causes the higher functional affinity. The dissociation curves of the A51-B/A6 and the A70-C/C8 are identical, which is surprising as the one is an IgA and the other is an IgM with four and 12 binding sites, respectively. This similarity could in part be explained by the assumption that only part of the binding sites of the IgM are able to bind to the carbohydrate at a time.

Comparing the functional affinities to Lewis Y for the cIgG CC8 and A70-A/A9 an approximately 20 fold higher functional affinity for cIgG CC8 was measured. Both antibodies have Lewis Y as their major antigen and they are both divalent. It appears that the natured maturation process from IgM to IgG which occurred with the A70-A/A9 had some influence on the binding affinity. Differences in the Fab sequence were of minor magnitude with respect to their influence on the functional affinities, since multivalency amplifies the loss or gain of intrinsic affinity (Yelton, Rosok et al. 1995). The role of the valency is also seen in comparing the interaction of the humanised scFv and IgG of 3S193 to Lewis Y. They show functional affinities of 7µM and 0.1µM with one and two binding sites, respectively (Power, Caine et al. 2001).

Because of to low amounts of the chain shuffling variant cIgG AA9/CC8 available, an estimation of the functional affinity was done by comparing its reactivity in ELISA. We compared the reactivity of the two cIgGs, CC8 and AA9/CC8, to Lewis Y in ELISA. This should be possible because the same isotype is recognised by the detection antibody. Our analysis showed a 10 fold increase in the reactivity to Lewis Y for the chain shuffled variant over the cIgG CC8 (Figure 28 chapter 2.9). Previous studies have found that the increase in reactivity measured by ELISA correlate approximately to the increase in functional affinity measured by SPR (Yelton, Rosok et al. 1995). We therefore postulate that the functional affinity of the cIgG AA9/CC8 should be in the range of 100nM. This functional affinity would be close to that of the IgG1 A70-A/A9. This fits with the theory of the VH harbouring the major binding site, since the two antibodies cIgG AA9/CC8 and IgG1 A70-A/A9 contain the same VH, and show the same functional affinity towards the antigen. A possible advantage of the newly generated antibody cIgG AA9/CC8 over the cIgG CC8 is the VH isotype origin. The A70-A/A9 is an IgG1, which means that the antibody has undergone a maturation process in the mouse. During the maturation process somatic mutations might take place, which will potentially increase the antibody affinity to the antigen.

The affinity of the antibodies can greatly affect their ability to localise to tumours, which has been shown with a panel of scFvs to the antigen HER-2/neu with affinities from 100nM-10pM. It was found that the specific tumour localisation reached a plateau at 1nM for scFvs (Adams, Schier et al. 2001). The Rituximab IgG antibody, which is one of the antibodies used for therapy, is reported to have an functional affinity to the antigen of 8nM (http://www.rituxan.com/rituxan/pi/). A similar order of magnitude functional affinity (13nM) is also reported for the hu3S193, which is one of the Lewis Y antibodies being prepared for clinical trials (Boghaert, Sridharan et al. 2004). Comparing these functional affinities to those obtained for the antibodies cIgG AA9/CC8 and mouse IgG1 A70-A/A9 it is found that the Nemod antibodies would need further affinity optimisation to reach the essential binding affinity needed for an immunotherapeutic.

3.6 Mimicry between histone H1 and a mixed carbohydrate epitope

The definition of mimicry between two molecules is that they resemble each other in either shape or function. A narrower description of immunological mimicry is that the two molecules cannot be distinguished by the immune system. An initial indication for immunological mimicry was found when tumour cells showed a diffuse staining of the cell nucleus with the antibody A70-A/A9. The staining could be a hint of an antibody cross-reactivity to a nucleus protein because freezing breaks the cell membrane and allows antibody diffusion inside the cell. Therefore a possible binding was investigated to histones, which are a major group of nucleus proteins. An initial ELISA showed that only the antibody A70-A/A9 but not A51-B/A6, A46-B/B10, A70-C/C8, or cIgG AA9/CC8 bound to the histone H1 (Figure 29 chapter 2.10 and data not shown). To exclude the possibility that a carbohydrate was responsible for this interaction a subsequent ELISA with and without periodate treatment of the antigen was made. This ELISA showed that the binding to the bovine histone H1 was independent of carbohydrate structures (Figure 29 chapter 2.10). Western blotting of a gel loaded with a natural mixture of the bovine histones H1, H2A, H2B, H3, H4, the human histone H1.2, and the bovine histone H1 showed that the antibody A70-A/A9 could bind the bovine histone H1 and the human histone H1.2, whereas the other classes of histones H2A, H2B, H3, and H4 were not recognised (Figure 30 chapter 2.10). These data indicate that the histone class H1 could contain a structural mimic of the Lewis b carbohydrate, since only the antibody A70-A/A9 and not A70-C/C8 or cIgG CC8 binds to these histones. Another possibility is that the histone contains a mimic of not one of the carbohydrates but instead the mixed epitope recognised by A70-A/A9.

A cell competition assays was used to estimate the relation in affinity, and this assay based on the binding of the A70-A/A9 antibody to Lewis b expressing NM-F9 cells resulted in IC50 values of 0.012mM for human histone H1.2 and 0.17mM for Lewis b (Figure 31 chapter 2.10), i.e. the binding to the human histone H1.2 is 15 times stronger than the binding to Lewis b.

Cell staining of periodate treated T47D cells showed no nuclear staining with A70-A/A9, whereas a control anti-human H1.2 antibody did show a spotted staining within the nucleus. This could not be due to too weak binding affinity to the histone H1 according to the inhibition experiment, but one possibility is that the binding site is not available when the histone is in its natural environment. In mammals several different histone H1 subclasses have been found. They all have a three domain structure with a very conserved central DNA

binding domain (Doenecke, Albig et al. 1997). The binding of the antibody A70-A/A9 to two different subclasses from two different mammals and no recognition of the histones in cells where the DNA binding domain is bound to DNA, leads to the hypothesis that the binding site of A70-A/A9 is located in the DNA binding region of the histone H1 subclass.

The presence of auto-antibodies to histones have been found related to different pathogens such as hepatitis C virus polyprotein, HIV, and different Mycobacteria strains (Eriksen, Kumar et al. 1995; Williams, Whalley et al. 1996; Cohavy, Harth et al. 1999; Gregorio, Choudhuri et al. 2003). Autoimmune diseases such as systemic lupus erythematosus (SLE) also give rise to self-recognising antibodies e.g. anti-dsDNA and anti-histone antibodies. A possible explanation for this is dysregulated apoptosis, which leads to the formation of immune complexes and their deposition in tissues, which again leads to organ damage (Lorenz, Herrmann et al. 2000). Very interesting are though the results of Costa et al. who found anti-histone H1 antibodies in 61% of SLE patients and around 6% of cancer patients (majority of patients with breast cancers) (Costa, Tchouatcha-Tchouassom et al. 1986). Other investigators have then examined the cross-reactivity of murine monoclonal antibodies to the surface of cancer cells (Raz, Ben-Bassat et al. 1993). This revealed that an anti-DNA antibody but not an anti-histone antibody bound to the surface of cancer cells. This study did though only include an anti-histone H2B, and no anti-histone H1 antibody. Surprisingly, cancer patients can on rare occasions develop lupus-like symptoms, and immunological cross-reactivity has been suggested to be the explanation for this occurrence (Chtourou, Aubin et al. 1998). Several autoimmune diseases are associated with the presence of molecular mimicry presented by the infectious agent (Rose and Mackay 2000; Christen and von Herrath 2004).

The existence of cross-reactive epitopes between histone H1 and the TACA Lewis b (Lewis b and Lewis Y), recognised by the antibody A70-A/A9, opens up for the hypothesis that this immunological mimicry could be the explanation of lupus-like symptoms in cancer patients, since cancer patients develop antibodies against TACAs. These antibodies will then in some occasions cross-react with histone H1 (as A70-A/A9). This mixed epitope could also be the explanation for the finding of anti-histone H1 antibodies in some cancer patients, and it could shed light on the controversies in the discussion of a possible protection of SLE patients from cancer (Hughes 2001; Abu-Shakra, Ehrenfeld et al. 2002). SLE can lead to the expression of anti-H1 antibodies (Costa, Tchouatcha-Tchouassom et al. 1986), which we found might exhibit cross-reactivity to Lewis Y (and Lewis b), as does the A70-A/A9. This interpretation is a hypothesis which needs further investigation but it surely opens new perspectives.

Mimicry between biological and chemical different compounds has been identified in a diverse range of fields. Epitopes, which can not be distinguished by the immune system or antibodies, are shared epitopes. Most shared epitopes identified in e.g. autoimmune diseases are based on sequence homology or the existence of the same compound in the pathogen and the host. The mimicry as defined by immunological similarity among dissimilar molecules, and identified here between the carbohydrate epitope Lewis b (or Lewis Y/Lewis b) and histone H1 is to the best of our knowledge the first immunological cross-reactivity described between a naturally occurring protein and naturally occurring carbohydrate structures.

3.7 Selection method of anti-idiotypic antibodies

Anti-idiotypic antibodies (Ab2s) are functional mimics of natural antigens. These functional mimics could be used in immunology to overcome some of the obstacles, which are associated with the nature of carbohydrate antigens. So far the generation of Ab2s using the hybridoma technique has been a tedious operation often resulting in one or very few Ab2s (Rosok, Eghtedarzadeh-Kondri et al. 1998; Sen, Chakraborty et al. 1998). In addition, phage display was used for generation of Ab2s from immunised mice (Magliani, Polonelli et al. 1998) or in selection from naïve phage libraries (Lamarre and Talbot 1997). Both resulted in only few Ab2s, however, only the Ab2s from the immunised mice was able to induce Ab1' in immunisations. Despite the great potential inherent in Ab2s, their application is still limited. This is in part caused by the difficulties inherent in their generation, especially in producing an array of different Ab2s, from which to choose the most suited ones. Here we establish a powerful and reliable technique by use of a specific elution and a proteolytic cleavable helper phage for generating large sets of Ab2s in form of scFv from phagemid libraries using phage display. The technique was evaluated on four different antigens, Lewis Y, H type 2, Thomsen-Friedenreich disaccharide, and the MUC1. We were able to generate 28 to 88 anti-idiotypic scFvs from 96 tested clones after two to three rounds of selection (Table 8 chapter 2.11.1). Out of these Ab2s, 55 to 90% revealed different amino acid sequences (Table 9 chapter 2.11.1). The possibility to generate such large diversities of Ab2 was somewhat unexpected. The reliability and broad applicability of the technique was shown with the successful generation of large anti-idiotypic scFv repertoires for two carbohydrate antigens of different size (di- versus tetrasaccharide), as well as for a conformational peptide epitope.

Key elements of this success were specific elution with the antigen followed by trypsin treatment in combination with a protease-sensitive helperphage (Kristensen and Winter 1998). In phagemid selection systems the scFv-p3 fusion protein competes with the p3 from the helperphage for incorporation into phages, therefore only about 1% of all phage particles display scFv (Bass, Greene et al. 1990). Due to the "sticky" properties of phages, non-specific elution with glycine buffer pH 2.2 or triethylamine leads to a large excess of empty phage in the eluate. This requires in conventional selections up to five rounds of selections resulting in an accumulation of only one or very few binders. In most cases scFv-phage with growth advantage and/or the highest affinity to any part of the antigen molecule, which often is not the desired one, are selected in this situation (Deng, MacKenzie et al. 1994). This is especially critical for the generation of Ab2 fragments since the antigen binding region is only a small part of the whole Ab1, and only rarely the immunodominant one. Therefore the specific elution with the antigen of Ab1 is of great advantage for this purpose.

The second critical component of our method is the use of a protease-sensitive helperphage. The modified p3 from KM13 is sensitive to tryptic digestion leaving phage lacking scFv non-infective, while those with scFv are infective via the wild type p3 of the scFv-p3(wt) fusion protein, which cannot be cleaved by trypsin (Figure C chapter 1.4.2) (Kristensen and Winter 1998). The specific elution alone elutes still a rather high amount of empty phage. The proteolytic elution alone, also called proteolytic selection, is a powerful technique for the isolation of antigen-specific scFvs (Ravn, Kjaer et al. 2000). This is reflected here by the accumulation of scFvs against immunoglobulin epitopes (Table 8 chapter 2.11.1). Apparently, those shared antibody epitopes dominate, causing a drastic decrease of phage displaying anti-idiotypic scFv from the first to the second round of selection. Nevertheless, the proteolytic elution can be used to generate at least a few anti-idiotypic scFv after a

single round of selection. The main advantage of the proteolytic selection as such is the limited number of eluted phage after the first round of selection which can be analyzed in total thereby retaining diversity. The data show that, in theory, maximum diversity of anti-idiotypic scFv could be obtained by the analysis of all eluted phage from the first round of the selection using the specific elution or the proteolytic elution alone. However, since the number of phage isolated with these two techniques is still in the range of one to several thousands, the use of the combined specific elution with subsequent trypsin treatment is much more practical and efficient. Two rounds of this selection type are sufficient to allow the fast generation of large anti-idiotypic repertoires. Somewhat surprising was the fact that a third round of selection apparently did not reduce the diversity (Table 9 chapter 2.11.1). As an exception to the generation of large diversities is the selection on A46-B/B10, which only gave rise to five different clones with a total diversity of 16% (Table 9 chapter 2.11.1). This may be caused by the limited amounts of total rescued clones (80) after the first round of selection, since the diversity is expected to decrease on subsequent selection rounds and the best binders will be enriched.

Selections with anti-IgG or -IgM magnetic beads allow the usage of low amounts of even non-purified idiotypic monoclonal antibody as antigen in comparison to larger amounts of purified idiotypes required for immunotube selections. Qualitatively there were no detectable differences between selections with magnetic beads and immunotubes. In addition magnetic beads allow the use of polyclonal antibody sera for the isolation of anti-idiotypic antibodies against a panel of antigen-specific antibodies.

The generation of these large diversities of anti-idiotypic scFv by the described technique is to our knowledge not matched by any other selection method to any antigen presented so far. We suppose that this technique will also enable the generation of large diversities of surrogate molecules like receptor ligands, agonists, or antagonists by using for example the ligands or parts of ligands for elution (Martin, Toniatti et al. 1996). Such surrogate molecules could be antibody fragments or other molecular formats displayed on phage. In addition, this technique should also be suitable for the selection of large diversities of antibodies against certain antigens, antigen parts and epitopes as long as specific elution is possible, for example with parts of the antigen or antibodies recognizing certain epitopes on the antigen (Meulemans, Slobbe et al. 1994). Due to the limited rounds of selections needed it is also possible to isolate antibodies with lower affinities. By carefully choosing the conditions for specific elution, for example by small amounts of soluble biotinylated antigen (Hawkins, Russell et al. 1992), the selections can be biased towards high affinity scFv using only few rounds of selection and thereby reducing the role of growth advantages. A multivalent phage vector system (Becerril, Poul et al. 1999) in combination with specific elution alone might also be a valuable method for generating varieties of Ab2s. However, the generation of large antibody libraries with the phage vector system is more difficult and time consuming. In addition, alternative methods and systems, which reduce or abolish the background of phage without scFv might also be well suited for generation of anti-idiotypic phage by specific elution as e.g. the hyperphage which lack a functional p3 gene (Rondot, Koch et al. 2001).

The remarkable diversity of the anti-idiotypic scFvs which we were able to generate with the combined specific elution and trypsin treatment provides for the first time a toolbox large enough for gaining major insight into the molecular mimicry of carbohydrate and protein conformational epitopes by anti-idiotypic antibodies. Large varieties of Ab2s including those with lower affinities are essential because the molecular mimicry is independent of the affinity of Ab2β to Ab1 (Monafo, Greenspan et al. 1987; Raychaudhuri, Kang et al. 1990).

Very large sets of Ab2s should be suited to shed more light on Jerne's immunological network theory. For these purposes, immunisation and structural studies have to be performed. Such studies will also reveal which anti-idiotypic scFv truly mimic the antigen as an internal image of the idiotype (Ab2β), thereby differentiating from those which just sterically hinder the binding of the idiotype to its antigen. This cannot be distinguished with available biochemical assays but could be revealed by the induction of Ab1 from Ab2.

3.8 Anti-idiotypic mimicry

The generated anti-idiotypic antibodies selected against A70-C/C8, named PACB, were all but two found to consist of a short peptide and the VL of the scFvs. The binding reactivity against the idiotype of these RSD-phage was also shown to be reduced. The reduced binding was not found to be due to a lower expression of the RSD-phage (Figure 35 A chapter 2.11.2), but could probably be explained by the lack of a VH which normally harbour the main binding site. The advantage in expression of the single domain over the scFv on the phage (Figure 35 A chapter 2.11.2) might explain the overwhelming representation of the single domain phage. It does however not explain why these RSD-phage have an affinity towards the antibody A70-C/C8 at all.

In the search for possible explanations for the selection of the RSD-phage we reasoned that the N-terminal peptide attached to the VL (EVFDYWGQGTLVTVSS) could be included in the binding through the aromatic residues. This peptide is also present in the full-length scFvs but it might have a different conformation. Several studies have previously shown that peptides with a high prevalence of tyrosine and tryptophan are mimics of different carbohydrates (Oldenburg, Loganathan et al. 1992; Scott, Loganathan et al. 1992; Hoess, Brinkmann et al. 1993; Mirkov, Evans et al. 1995; Westerink, Giardina et al. 1995; Luo, Agadjanyan et al. 1998). Similarly, mimicry peptides previously identified against other compounds have been found to bind to the idiotype without being able to inhibit the idiotype binding to the antigen (Dr. Steffen Goletz, personal communication), as seen with the RSD-phage here. The testing of a dimér peptide consisting of the aromatic residues of the N-terminal part of the RSD-phage (EVFDYWGQGEVFDYWGQG) was negative. No binding of A70-C/C8 was found to this peptide, when coated directly in ELISA, or to the biotinylated peptide immobilised on a streptavidin plate, nor was the peptide able to inhibit A70-C/C8 binding to Lewis Y in ELISA (data not shown). Previous studies have shown that many mimicry peptides are able to inhibit the binding of the idiotype to the antigen (Luo, Agadjanyan et al. 1998). The lack of inhibition does though not exclude the possibility of peptide mimicry as the reason for RSD-phage selection, since only one peptide was tested but no other obvious mimicry peptide could be identified in the single domain. Also other studies have identified motifs with fewer aromatic residues (Luo, Canziani et al. 2000), which makes a theoretical identification of the exact location more difficult.

Another explanation for the rise of the single domain anti-idiotypic antibodies could be found in the crystal structure of an anti-carbohydrate antibody which generally contains a narrow and deep antigen binding cleft, making it impossible for the anti-idiotypic antibody to reach the pocket (Evans, Rose et al. 1994). Similarly Rosok et al. found by mutational analysis of BR96, a Lewis Y specific antibody, that residues important for the binding of an anti-idiotypic antibody are more exposed and accessible than the residues important for idiotypic

binding to the antigen Lewis Y (Rosok, Eghtedarzadeh-Kondri et al. 1998). Comparing this to the results obtained with camel single domain VH antibodies, which are able to reach narrow active site clefts in enzymes (Lauwereys, Arbabi Ghahroudi et al. 1998; Transue, De Genst et al. 1998) and the successful selection of anti-idiotypic single domain VH antibodies from llama mimicking carbohydrate structures (Tanha, Dubuc et al. 2002), it suggests that the RSD-phage are selected on the basis of a weak interaction of the VL to the idiotype. Non-specific interactions through the existence of an unpaired VH-VL interface of the VL can be excluded on the background of the specific binding to A70-C/C8 and lack of binding to the isotype control (Davies and Riechmann 1995). On other occasions the VL alone has proven able to retain the ability to bind the antigen, e.g. B3(VL)-PE38KDEL binding to Lewis Y expressing cells (Brinkmann, Lee et al. 1993), and VL from antibody F11 binding to ferritin (Dubnovitsky, Kravchuk et al. 2000). For both antibodies the VH was also able to bind to the antigen alone. A final support of the selection of single domain VL antibodies from libraries comes from the study of van den Beucken et al. This study also reports the selection of single VL domain binders against CD80 and CD86 from a library consisting of VL single domains (van den Beucken, van Neer et al. 2001).

The generation of anti-idiotypic antibodies against A70-B/B10 did not lead to the isolation of single domain antibodies. The PACA clones were all full length clones, and the inhibition potential and idiotypic reactivity (Figure 34 and 35 chapter 2.11.2) was considerably better than what was observed for the RSD-phage. Especially the textbook like inhibition exhibited by PACA17 was promising and the cross-reactivity to the antibody A63-D/B12 (Figure 37 chapter 2.11.2), which show the same reactivity to carbohydrates as A46-B/B10, suggested that PACA17 scFv is a true mimic of H type 2. This is the first report on an anti-idiotypic antibody, generated from an semi-synthetic antibody phage library, which mimics a carbohydrate structure. Previous studies have found anti-idiotypic against both H type 2 (Rochu, Crespeau et al. 1990) and Lewis Y (Rosok, Eghtedarzadeh-Kondri et al. 1998) but these have been generated via the classical pathway of immunising mice with the idiotype.

Clone PACA17 was selected to induce am immuno-response in mice, because it was the best clone caracterised in vitro as a carbohydrate mimicking anti-idiotypic antibody. Sera generated against PACA17 and derivatives generally gave rise to a response directed against relevant carbohydrate epitopes. In total five of the IgM sera from the 12 mice gave rise to a response only or mainly against H type 2. The majority of the IgM sera revealed a strong or stronger binding against the two smaller carbohydrates H disaccharide and fucose than to H type 2. Moreover, five mice responded with IgG antibodies against the investigated carbohydrate structures, though none of these sera recognised H type 2. On the other hand all but one mouse generated a response against H type 2 (Figure 39 chapter 2.11.3). By use of the anti-idiotypic antibody PACA17 we were able to generate a carbohydrate directed response, and all but one mouse showed an IgM response against the H type 2 in vivo. Measurable responses are also directed against parts of the H type 2 structure. Several immunisations with scFv mimicking other carbohydrate structures have been made by Nemod. The responses obtained were here also recognising smaller carbohydrate structures (Dr. Anja Löffler, personal communication). Subsequent generation of hybridomas from spleens of these mice showed that even though the generated response towards the more complex carbohydrates is low, it was possible to find a limited number of hybridomas which showed expression of antibodies with the desired specificity (Dr.s Uwe Karsten and Steffen Goletz). This confirms the findings here and suggests that by further optimisation of immunisation conditions it might be possible to direct the response

even further towards a specific complex carbohydrate. Remarkably, PACA17 was also able to generate a class switch from the IgM isotype to the IgG isotype, which is also an important feature of the anti-idiotypic antibodies.

Several studies have shown that vaccination of mice with anti-idiotypic antibodies can give rise to a specific response against carbohydrates from e.g. bacteria (Westerink, Campagnari et al. 1988; Magliani, Polonelli et al. 1998; Beninati, Oggioni et al. 2001) and also against several sialo-gangliosides (Chapman and Houghton 1991; Sen, Chakraborty et al. 1998; Grant, Kris et al. 1999). Generally these studies developed sera containing antibodies with a narrower specificity, and also the titers of IgG antibodies to the original antigen were much higher than what we obtained from our immunisations. One of the possible reasons why the specificity of these sera are better than ours is the nature of the original antigen. The bacterial antigens differ from the human and the sialo-gangliosides contain two or more charged sialo groups. One of the anti-idiotypic antibodies mimicking disialo-ganglioside has also been shown to prolong the survival of patients with small cell lung cancer (Grant, Kris et al. 1999). No reports have been given reporting the use of anti-idiotypic antibodies mimicking Lewis Y, but studies have shown that mimicking peptides are able to generate a response in mice and thereby prolong the survival time of mice with a Lewis Y expressing tumour (Luo, Agadjanyan et al. 1998; Kieber-Emmons, Luo et al. 1999).

The in vitro as well as in vivo mimicry of a complex carbohydrate structure, which is principally shown here, is the first anti-idiotypic mimicry by an semi-synthetic antibody. It is therefore hypothesised that a clinical application of anti-idiotypic antibodies selected from semi-synthetic phage display libraries could work in the future. The application and generation of anti-idiotypic antibodies or scFv as vaccines against carbohydrate antigens is still a field which deserves some attention. It has however one great disadvantage as the evaluation of an anti-idiotypic antibody as a good immunological mimic is empirical and thereby cost-intensive. Apart from the application as vaccines other uses have been suggested, e.g. as surrogate molecules in ELISA, and measuring of tumour penetration of idiotype (Liu, Panousis et al. 2003).

3.9 Adjuvant effect of DI of phage p3

The experiments shown in Figure 40 and 41 (chapter 2.12) document the adjuvant property of the N-terminal fragment of p3 of the filamentous bacteriophage, since immunisations with otherwise non-immunogenic scFvs fused to the N-terminal domain of p3 induce a thymus-dependent humoral immune response towards the scFv. We also determined the Th1 and Th2 type T cell profile via comparison of the antibody isotype responses IgG2a/IgG1 in mice, which showed that immunisations using IFA or DI fusion as adjuvant both give mixed IgG1 and IgG2a antibody responses with tendency towards Th2. The Th2 induction of IgG1 points to stronger induction of antibodies than what is induced through Th1 (IgG2a). Interestingly, the cytokine expression patterns of the two types of T helper cells has been proposed to be mutually antagonistic although this is still a controversial subject (Singh and O'Hagan 1999; O'Hagan, MacKichan et al. 2001; Singh and O'Hagan 2002), since also mixed Th1/Th2 profiles are seen (Albu, Jones-Trower et al. 2003), as in our results. Also the administration route and vaccination format can influence the Th1/Th2 profile as also shown in earlier studies

(Serezani, Franco et al. 2002; Herrick, Xu et al. 2003). To get a significant result a higher number of mice per group must be investigated, along with the administration rute and concentration of the recombinant proteins.

The filamentous bacteriophage particles has previous been used as adjuvant with the antigen displayed on either p3 (de la Cruz, Lal et al. 1988; Yip, Smith et al. 2001) or p8 (Greenwood, Willis et al. 1991; Yip, Smith et al. 2001; Frenkel, Dewachter et al. 2003), but no reports have appeared on the application of fusion proteins with N-terminal fragments of p3 as adjuvant. Although previous studies also assessed that p3 is indeed immunogenic (Minenkova, Ilyichev et al. 1993); we demonstrate that DI of p3 is sufficient to confer immunogenicity.

Recent results have shown that the fusion of a scFv to various fragments of p3 (especially DI) might have several beneficial effects, which include greater expression yields and higher activity than an expressed scFv alone. Furthermore, the formation of multimers increases the activity of the scFv fusions through avidity (Jensen, Larsen et al. 2002). The generation of higher order multimers during cellular expression of p3 DI fusion scFvs can either be due to high intracellular protein concentrations or due to structural regions of DI (and DII), which has been revealed by structural studies to interact (Holliger, Riechmann et al. 1999). Not only the higher activity but also the formation of multimers could be very beneficial in immunisations, since multimerisation leads to longer *in vivo* half-life due to the reciprocal proportional relation between the size of the molecule and its half life in blood (Willuda, Honegger et al. 1999; Powers, Amersdorfer et al. 2001). Theoretically this should result in prolonged contact between the antigen containing multimeric compound and the immune system. Also aggregation of soluble antigenic determinants by aluminum hydroxide precipitation likely helped to increase its antigenicity due to the prolonged half-life (Bhattacharya-Chatterjee, Chatterjee et al. 2000). This fact is used by a series of other vaccine studies primarily focusing on multivalent display of antigens on cell surfaces (Rode, Moebius et al. 1999) and virus particles (Jiang, Abu-Shilbayeh et al. 1997; McInerney, Brennan et al. 1999).

The few fusion protein systems which have been investigated, such as the pentameric cholera toxin B subunit and related structures (Liljeqvist, Stahl et al. 1997; Albu, Jones-Trower et al. 2003) show great potential, whereas others need Freund's adjuvant to generate a sufficient response (Schodel, Peterson et al. 1996). Based on our studies in mice, we suggest that the scFv-DI fusion system also shows great promise as an adjuvant for vaccine purposes, especially for anti-idiotype vaccines due to the induced active folding of the expressed scFv. We speculate that the potent effect of the scFv-DI fusion expression system is due to the multimerisation and increased active folding of the antibody fragments. The scFv-DI fusion system is especially valuable for scFvs, which are difficult to obtain in an active form (Jensen, Larsen et al. 2002). Therefore the scFv-DI fusion system is likely to be not only suitable for antibody fragments but also for other antigenic structures to be expressed in *E.coli*. This way of presenting a vaccine to the immune system is unique, both due to the induced active folding and for patent reasons, since the DI of p3 as an adjuvant has not been published. Further experiments are though necessary for a final conclusion if this is a preferable adjuvant for scFv immunisations.

3.10 Achievements and outlook

The first major achievement of this study is the specificity determination and cloning of the four Nemod Lewis Y binding antibodies. A successful expression and verification of binding patterns was obtained for two of the antibodies, and subsequently a chimeric IgG was generated from the Lewis Y specific antibody A70-C/C8. The chimeric antibody cIgG CC8 proved to be as specific towards Lewis Y as the parent antibody. The sequences of the Nemod Lewis Y binding antibodies proved to be quite different from the already existing Lewis Y binding antibodies. However, they were found to be highly similar when aligned to each other.

The second major achievement is the generation of the antibody cIgG AA9/CC8 by shuffling the chains of the antibodies. Surprisingly, this antibody showed a binding as specific as A70-C/C8 and the cIgG CC8 to Lewis Y. It is quite surprising that the two so different VH chains as CC8 and AA9 can, together with the CC8 VL, bind specifically to Lewis Y, especially when looking on the general difficulties in raising carbohydrate specific antibodies. Comparing the reactivities of the cIgG CC8 and cIgG AA9/CC8 the latter proved to be able to bind the antigen approximately 10 times stronger, which is an invaluable gain of affinity if the antibody is intended to be developed further eventually for diagnostic or therapy. The method of shuffling of the chains from antibodies binding similar antigens proved to be a valuable technique in making minor changes in specificities as, e.g., removing the cross-reactivity to the unwanted closely related antigens. Especially for carbohydrate antigens it is interesting as the sequences show some similarity, are close to germline sequences and often show cross-reactivity to related carbohydrates.

Even though a great enhancement of the functional affinity of the cIgG AA9/CC8 over the cIgG CC8 was achieved the binding is still not sufficient when compared to the threshold value of tumour localisation or an antibody successfully in use as therapeutica (Adams, Schier et al. 2001). Therefore it is suggested that a further affinity maturation of the antibody should be tried as has been done for other Lewis Y binding antibodies (Yelton, Rosok et al. 1995). It is also possible that a positive effect on affinity can found by optimising the VH-VL region in cIgG AA9/CC8 as there are several differences between the A70-C/C8 and A70-A/A9 in this region. The most important step in the further development of these Lewis Y binding antibodies, however, are cell assays, as it is very important to know if the antibodies are able to mediate complement-dependent cytotoxicity or antibody-dependent cellular cytotoxicity, and do not lyse erythrocytes.

The third major achievement is the detailed determination of the epitope in the H type 2 carbohydrate bound by the two antibodies A51-B/A6 and A46-B/B10. These studies using the saturation transfer difference NMR method resolved the epitope in the H type 2 carbohydrate, which also can be recognised in the Lewis Y structure by these two antibodies. The interesting aspect of this is the ability of similar antibodies like the Nemod Lewis Y antibodies to destinguish closely related carbohydrates like Lewis Y, X, a, b, and H type 2. To reach this goal more experiments will have to be performed including the tetrasaccharides and the remaining antibodies.

The fourth major achievement is the identification of a naturally occurring immunological mimicry between two carbohydrate structures and the histone H1. This is the first identification of an immunological mimicry between carbohydrate structures and a natural occurring protein. The interesting point on this mimicry is the occurrence of anti-histone antibodies in systemic lupus erythematosus (SLE) and the controversies about this auto-immune disease concerning frequency of cancer and the binding of SLE auto-antibodies to the surface

of cancer cells. The mimicry identified here proposes a possible explanation for these cross-reactions. It is interesting though to see whether this mimicry could provide an explanation for the exsistence of anti-cancer antibodies in SLE patients. To get an answer to this question a close investigation of antibodies in SLE patients was to be done.

The fifth major achievement is an improved method for selecting anti-idiotypic antibodies. Generally anti-idiotypic antibodies have been produced by immunising mice with the idiotype and generating hybridomas from the mice. We found that a phage display selection strategy including specific elution with the antigen and a proteolytic helper phage could generate many and diverse anti-idiotypic scFv from a phage display. The advantages by using this technique are that after only a few rounds of selection scFv of the wanted specificity are generated, whereby the maximum diversity is retained in the selected clones. Especially in the case of mimicry few selection rounds are very important since the best mimicry is not necessarily related to affinity. Subsequent rounds of selection will enrich for other features such as binding activity. This method is a promising method for selecting mimicry or surrogate molecules of large diversities.

The sixth major achievement is the detection of a novel adjuvant system generated by fusion between a scFv and the domain I of phage protein 3. The advantage is that no additional adjuvant is necessary due to the multimerisation and immunological properties of DI from the phage protein p3. Especially the expression and immunisation with anti-idiotypic antibodies will benefit from this, since the induced correct folding will ensure that the correct mimic is exposed to the immune system.