doi: 10.1111/j.1365-3083.2008.02164.x

Modifying Antibody Specificity by Chain Shuffling of V_H/V_L between Antibodies with Related Specificities

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Received 16 January 2008; Accepted 10 July 2008

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Abstract

Histo-blood group antigens are important markers of developmental stages and as such also often of tumours. Generation of antibodies towards these carbohydrate structures is still a challenging task as they may lack specificity, affinity or are only of the IgM class. We have examined four own antibodies to Lewis Y/H type 2 for their fine specificities using a large panel of mono- and oligosaccharides. Sequence alignment to other antibodies with similar specificity revealed an overall limited variation, and that our antibodies constitute a novel set. Based on produced and analysed chimeric mouse–human antibodies, extensive chain shuffling experiments were performed in order to analyse influences of the respective H and L chains on the specificity of the antibodies, and to generate modified antibodies with improved properties. One chIgG1 out of the shuffled antibodies revealed improved specificity and markedly enhanced functional affinity to Lewis Y compared to the parental chIgG1 antibodies. Therefore, the combinatorial approach of chain shuffling provides a platform to improve specificity and/or affinity of anti-carbohydrate antibodies.

Introduction

Carbohydrate epitopes such as the structural isomers Lewis Y [Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ-], Lewis b [Fucα1-4(Fucα1-2Galβ1-3)GlcNAcβ-] and Globo-H (Fucα1-2Galβ1-3GalNAcβ-) have been shown to be tumour-specific or -associated antigens. Epithelial cancers, such as breast, bladder, colon, stomach, pancreas, prostate, ovarian cancer and small-cell lung cancer, express Lewis Y and Globo-H more abundantly than any cell surface protein antigen [1–4]; reviewed in Refs [5, 6]. The expression of Lewis Y in cancers has been found related to poor prognosis [7–9]. Lewis b is abundant in cancers of distal colon, pancreas, endometrium, lung and urinary bladder [3]; reviewed in Ref. [5]. These carbohydrate antigens are therefore potential targets for tumour imaging, and active or passive immunotherapy [10–14].

Generating and using antibodies against these carbohydrate structures have several pitfalls: First, carbohydrate antigens often generate immune responses of the IgM type [15, 16], which is not considered to be a suitable antibody format for therapy. Using recombinant antibody technologies, class switching from IgM to IgG can be performed. However, considerably functional affinity is lost due to the low intrinsic affinity of carbohydrate-binding antibodies [17–19]. Second, unwanted side effects may occur due to the presence of identical or closely related carbohydrate structures found on normal tissues such as H type 2 expression on erythrocytes, or Lewis X on certain cell lineages in the spleen [3]. In addition, cross-reactivities to other glycotopes are a common problem. For instance, most of the existing antibodies recognizing Lewis Y show cross-reactivity towards other carbohydrate structures that will diminish their therapeutic potential [3, 20, 21][a]. Lastly, mouse antibodies are not suited for therapeutic purposes.

Cloning of the antibody variable domains from mouse hybridomas and the generation of mouse–human chimeric (ch) antibodies, humanized antibodies or single-chain variable fragments (scFv) does often lead to a considerable loss in affinity or stability. Methods including phage display, guided selection and light chain replacement have been used to circumvent these problems with the aid of large antibody libraries [22–27]. The method of variable domain shuffling can also be used to generate new antibody variants [28].

[a]Exclusive Lewis Y specificity of the genuine antibody A70-C/C8 mIgM from Glycotope was confirmed (doi: 10.1093/glycob/cwn049).

SCANDINAVIAN JOURNAL OF Immunology AN INTERNATIONAL MONTHLY JOURNAL

Here we describe specificity and sequences of four mouse monoclonal antibodies (MoAb) binding to the tumour-associated antigen Lewis Y. We generated ch mouse-human IgM and IgG1, which proved to be as specific as the parent antibodies. The new ch antibodies were used for variable domain shuffling to generate novel chIgs. chIgG1 A/A9-C/C8 was created via this method and was found to have two important features: (1) exclusive recognition of Lewis Y, and (2) enhanced reactivity to Lewis Y. This proves that the combinatorial approach of chain shuffling using antibodies with similar specificity can provide a platform for generating better carbohydrate-binding antibodies.

Materials and methods

Cell culture. Cell lines: The human cell lines are MCF-7 (ATCC No. HTB-22, breast adenocarcinoma) OVCAR-3 (ATCC No. HTB-161, adenocarcinoma of the ovary), HepG2 (ATCC No. HB-8065, hepatocellular carcinoma), HCT-15 (ATCC No. CCL-225, colorectal carcinoma), MDA-MB-435S (ATCC No. HTB-129, breast carcinoma), JEG-3 (ATCC No. HTB-36, choriocarcinoma), MT-3 (DSMZ No. ACC 403, breast carcinoma), SK-OV-3 (ATCC No. HTB-77, adenocarcinoma of the ovary), HT29 (ATCC No. HTB-38, adenocarcinoma of the colon), U266 (ATCC No. TIB-196, plasmacytoma). Human tumour cell lines were grown in RPMI-1640 or DMEM medium supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine at 37 °C in a humidified atmosphere with 8% CO_2. All media and supplements were purchased from Biochrom, Berlin, Germany.

Antibody production. A70-A/A9 (IgG1, κ) recognizing Lewis Y and partially Lewis b [29], A46-B/B10 (IgM, κ) recognizing Lewis Y and H type 2 [30, 31] and A70-C/C8 (IgM, κ) recognizing Lewis Y [31] have been published. A51-B/A6 (IgA, κ) recognizing Lewis Y, H disaccharide and H type 2 was generated using a sequence of different glycoconjugates and MCF-7 cells as immunogens. BALB/*c* mice were used as spleen donors, and X63-Ag8.653 cells as fusion partners. Common hybridoma techniques were employed. Screening assays included cellular (immunofluorescence) and ELISA tests. For the production of antibodies, hybridoma cells were transferred into serum-free RPMI-1640 medium for at least 1 week, leaving only small amounts of FCS in the supernatant before purification. All MoAb were produced by Glycotope.

COS-7 cells (ATCC No. CRL-1651) or CHO cells (ATCC No. CCL-61) were maintained in DMEM medium supplemented with 2 mM glutamine and 10% FCS. For transient and stable expression, cells were transfected with the plasmids encoding an antibody light chain (κ chain) and heavy chain (γ or μ chain) of interest by lipofectamine™ 2000 (Invitrogen, Karlsruhe, Germany) transfection according to the instructions of the manufacturer. After 48–72 h, the cell culture medium of transiently transfected COS-7 cells was collected. Stable transformants (CHO cells) were cultivated in the selection medium DMEM supplemented with 10% FCS, 2 mM glutamine, 5 μg/ml puromycin and 400 μg/ml neomycin (both antibiotics from Sigma-Aldrich, Taufkirchen, Germany), which was subsequently changed to the serum-free CHO-S-SFMII Medium (Invitrogen).

Other antibodies. Cy3-conjugated class-specific antisera (goat anti-mouse IgM or IgG), Cy2-conjugated antiserum (goat anti-mouse Ig) were from Sigma-Aldrich. Mouse anti-human κ chain was purchased from BD Bioscience, Heidelberg, Germany. Peroxidase-conjugated goat anti-human IgM, $Fc_{5\mu}$-specific and goat anti-human IgG, F(ab')2-specific antisera were from Jackson ImmunoResearch Labs., West Grove, PA, USA. Peroxidase-conjugated rabbit anti-mouse Ig antiserum (P260) was from Dako Cytomation, Hamburg, Germany.

Antibody purification. All supernatants were prior to chromatography centrifuged for 30 min at 4000× *g* and passed through a 0.22-μm filter to remove cells and cell debris. Purification of the mouse IgG1 A70-A/A9 and the chIgG1 C/C8 was performed on a HiTrap Protein A column (GE Healthcare, Freiburg, Germany) by supplementing the supernatant with 2 M NaCl. About 1 mg antibody per run was eluted with buffer at pH 4. The mouse IgM MoAb A46-B/B10 and A70-C/C8, and the IgA MoAb A51-B/A6 were purified using rabbit anti-mouse Ig (Z0259; Dako Cytomation, Hamburg, Germany) immobilized on a 5-ml HiTrap NHS-activated sepharose column (GE Healthcare). Coupling 10 mg antibody was performed according to the instructions of the manufacturer. The capacity of the column was about 200 μg antibody per run, and the column could be used for more than 50 runs. Elution was performed with 50 mM citric acid, 150 mM NaCl, pH 2.2. The purified antibodies were quantified by OD_{280} measurements ($\varepsilon_{IgG/A}$ 1.35/cm/mg; ε_{IgM} 1.2/cm/mg) and analysed by both reducing and non-reducing SDS-PAGE (Figure S1) and standard ELISA.

Cloning. To identify the variable antibody sequences of the four Lewis Y-binding antibodies (A70-C/C8, A51-B/A6, A70-A/A9 and A46-B/B10), the antibody producing hybridoma cell lines were cultured and cell pellets generated to isolate RNA and determine the V_H and V_L sequences. In brief, total RNA was extracted using the Qiagen RNeasy kit, the mRNA was isolated by using the Dynabead technology (Dynal Biotech, Hamburg, Germany), and the cDNA was synthesized by using the Omniscript reverse transcriptase (Qiagen, Hilden, Germany). Specific amplification of the cDNA encoding the variable regions of the specific antibodies was achieved by using a mixture of forward and reverse primers, respectively, covering 95–98% of known mouse germline sequences. Primer sequences were adapted

according to Ref. [32]. For cloning purposes, all primers were elongated with sequences containing recognition sites for restriction enzymes. Amplified products were digested with ApaLI/NotI (V_L) and NcoI/XhoI (V_H) and inserted into a prepared cloning vector. Sequencing was performed by Sequence Laboratories (Göttingen, Germany).

For the expression of chIgG1 or chIgM antibodies, variable sequences were cloned in eukaryotic expression vectors containing human genomic sequences of the μ, γ1 or κ chains respectively. Generation of expression vectors for the ch antibodies was performed according to Patent applications WO2004050707 and US2006251668. In brief, the chosen cDNAs encoding the light and heavy chain variable regions were subcloned into the expression vector system from Glycotope. In a first step, the cDNAs were transferred into the BS-leader vector in order to fuse a sequence coding for a secretory signal 5′ and a splice donor sequence 3′ to the cloned cDNA. These cDNA constructs were, in a second and final subcloning step, transferred into the expression vectors encoding the human constant κ and the human constant γ1 or the human μ chain respectively. Plasmids of independently obtained V_L and V_H expression clones were prepared by the Qiagen Midi-prep kit, and sequencing was performed by Sequence Laboratories.

ELISA assays. The carbohydrate specificity of the antibodies or antibody concentrations was determined in ELISA. Eighty-six different mono- and oligosaccharide-polyacrylamide (PAA) conjugates were purchased from Syntesome, Munich, Germany. Either 0.5 μg/well of carbohydrate conjugate or 0.2 μg/well of anti-human κ-chain antibody were coated in PBS at 4 °C overnight using MaxiSorp 96-well ELISA plates (Nunc, Wiesbaden, Germany). After washing with PBS and blocking with 3% BSA/PBS for 1 h, the wells were incubated with antibody supernatants or dilutions of purified antibodies for 1.5 h at RT. The wells were washed and incubated with peroxidase-conjugated rabbit anti-mouse Ig (Dako P260) diluted 1:2000 in 3% BSA/PBS for 1 h at RT. After washing, the wells' colour was developed with 3.3′,5.5′-tetramethylbenzidine (TMB) (Sigma-Aldrich) as substrate. The reaction was stopped with 2.5 N H_2SO_4, and staining was measured at 450 nm against 630 nm.

Surface plasmon resonance binding analysis. Biotinylated carbohydrate–PAA conjugates (Syntesome) were immobilized on streptavidin-coated (type SA) sensor chips (Biacore, Freiburg, Germany) according to the instructions of the manufacturer, resulting in carbohydrate densities in the range of 661–915 resonance units, which has been reported to be an appropriate range [33]. Subsequently, the sensor chips were washed with 10 μl of running buffer containing 2 M NaCl, followed by 10 μl running buffer containing 1 M NaCl. Binding analysis was conducted with increasing concentrations of the respective antibody (100, 150, 300, 500, 700 and 1000 nM) on a Biacore 2000 instrument. The buffer for the regeneration of the chip was citric acid (pH 2.2). Kinetic parameters were determined using the BIAevaluation program version 3.1 (Biacore). To obtain a functional affinity of the antibody, the sensorgrams were fitted to a Langmuir 1:1 model. The K_{obs} are means of two sets of data measured on different chips.

Results

Specificity analysis of the antibodies

Binding properties of the antibodies were analysed by screening on 86 different mono- and oligosaccharide structures conjugated to PAA using the ELISA technique. Fine specificities of the antibodies A70-C/C8 (IgM, κ) and A51-B/A6 (IgA, κ) are shown in Figs. 1A and B respectively. For A70-C/C8, exclusive binding to Lewis Y was found. This antibody did not bind to any of the other investigated carbohydrates. Figure 1B shows strong binding of A51-B/A6 to Lewis Y, H type 2 and the H disaccharide. In addition, a very small signal towards Globo-H was also found. These data reveal a specificity of A51-B/A6 for the H disaccharide, either alone or attached to a type-2 chain. The binding patterns of the two MoAb were verified even at high antibody concentrations (50 μg/ml) (data not shown). The fine specificities of the antibodies A70-A/A9 and A46-B/B10 were similarly analysed (published in Refs [29, 30]). Specificity data of all four antibodies are summarized in Table 1.

Sequence analysis of the variable domains of Lewis Y-binding antibodies

For characterizing the V_H and V_L chains of the four Lewis Y-binding antibodies (A70-C/C8, A51-B/A6, A70-A/A9 and A46-B/B10), the V_H and V_L chains were cloned in a multistep process, and the sequences were determined. In total nine products were successfully cloned. Each V_H and V_L cloning gave rise to one product except A51-B/A6, which gave rise to two different heavy chains, B/A6(1) and B/A6(3).

CLUSTAL W [34] was used to align and compare the obtained V_H and V_L sequences respectively, with known sequences of other Lewis Y-binding antibodies (Fig. 2). A general comparison of the sequences revealed that the already known antibodies had high sequence similarities with each other except for MoAb MSL5, whereas three of our four antibodies differed much from these already known sequences and seem to constitute a third set of sequences. The alignment showed that the heavy chains C/C8, B/A6(1), B/A6(3) and A/A9 have a high sequence similarity. V_H C/C8 and B/A6(1) differed only by one residue in the framework. This amino acid is

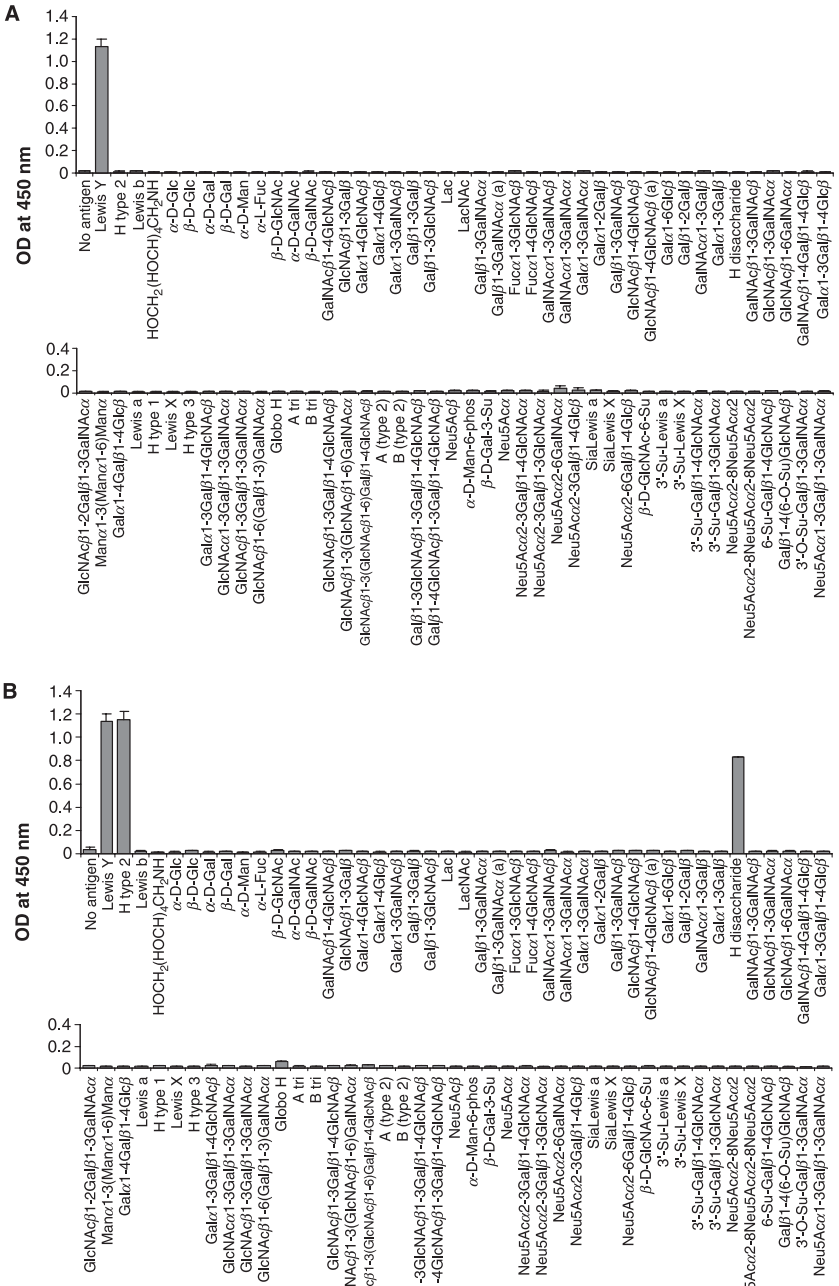

Figure 1 Specificity ELISA with (A) supernatant from the murine hybridoma A70-C/C8 and (B) from the murine hybridoma A51-B/A6 on 86 different immobilized carbohydrate structures. Supernatants were used undiluted and the signals shown are means of two measurements.

Table 1 Summary of binding specificities of murine antibodies studied.

Carbohydrate structure	A46-B/B10 mIgM	A51-B/A6 mIgA	A70-C/C8 mIgM	A70-A/A9 mIgG1
Lewis Y	+	+	+	+
H type 2	+	+	–	–
H disaccharide	–	+	–	–
Globo-H	–	–	–	(+)
Lewis X	–	–	–	–
Lewis b	–	–	–	+

neither involved in the antigen binding site nor at a position believed to have impact on the canonical structures [35]. V_H B/A6(3) and A/A9 were also very similar in their sequences. The A/A9 V_H CDR3 contains some changes that are assumed to influence the binding site. Finally, the B/B10 V_H sequence was identical to the sequence of the MSL5 antibody. The V_L sequences generally also showed high similarity, with C/C8 being different from the others. The V_L sequences obtained from A51-B/A6 and A46-B/B10 are identical to MSL5.

V_H

	Framework 1	CDR1	Framework 2	CDR2	Framework 3	CDR3	Framework 4
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
C/C8	EVKLVESGPELVKPGASVKISCKAS	GYTFTDY	NIDWVKQSHGKSLEWIGYIY	PNNG	GTGYNQKFTNKATLTVDKSSSTAYMELHSLTSEYSAVYY	CAR---ELG--RLPYWG	QGTLVTVSA
B/A6(1)	EVKLVESGPELVKPGASVKISCKAS	GYTFTDY	NIDWVKQSHGKSLEWIGYIY	PNNG	GTGYNQKFTNKATLTVDKSSSTAYMELHSLTSEDSAVYY	CAR---ELG--RLPYWG	QGTLVTVSA
B/A6(3)	DVKLVESGPDLVKPGASVKISCKAS	GYTFTDY	NMDWVKQTHAKSLEWIGYIY	PYNG	YSDYNQKFTNKATLTVDKSSSTAYMELHSLTSEDSAVYY	CAR---ELG--RLPYWG	QGTLVTVSA
A/A9	KVKLQQSGPDLVKPGASVKISCKAS	GYTFTDY	NMDWVKQTHAKSLEWIGYIY	PYNG	YSDYNQKFKSKATLTVDKSSSTAYMELHSLTSEDSAIYY	CAR---QLG--PGTFWG	QGTLVTVSA
B/B10	QVQLKESGPELKKPGETVKISCKAS	GYTFTNY	GMNWVKQAPGKGLKWMGWIN	TYTG	EPTYADDFKGRFAFSLETSASTAYLQINNLKNEDMATYF	CAN---DYDGAWFAYWG	QGTLVTVSS
MSL5	QVQLQESGPELKKPGETVKISCKAS	GYTFTNY	GMNWVKQAPGKGLKWMGWIN	TYTG	EPTYADDFKGRVALSLETSASTAYLQINNLKNEDTATYF	CAN---DYDGAWFAYWG	QGTLVTVSS

V_L

	Framework 1	CDR1	Framework 2	CDR2	Framework 3	CDR3	Framework 4
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
C/C8	DIVLTQSPLFLHVSLGDQASISCTS	SQSLVHSNGNSY	LDWHLQKSDQSLQLLIY	EVS	KRNSGVPDRFSGSGSGKDFTLKISRVEPEDLGIYYCFQ	RTHLPL	TFGAGTKLEIK
B/B10	DIVMTQTPLTLSVTIGQPASISCKS	SQSLLDSDGKTY	LNWLLQRPGQSPKRLIY	LVS	KLDSGVPDRFTGSGSGTDFTLKISRVEAEDLGVYYCWQ	GTHFPQ	TFGGGTKLEIK
B/A6	DIVMTQTPLTLSVTIGQPASISCKS	SQSLLDSDGKTY	LNWLLQRPGQSPKRLIY	LVS	KLDSGVPDRFTGSGSGTDFTLKISRVEAEDLGVYYCWQ	GTHFPQ	TFGGGTKLEIK
MSL5	DIVMTQTPLTLSVTIGQPASISCKS	SQSLLDSDGKTY	LNWLLQRPGQSPKRLIY	LVS	KLDSGVPDRFTGSGSGTDFTLKISRVEAEDLGVYYCWQ	GTHFPQ	TFGGGTKLEIK
A/A9	DIVMTQTPLTLSVTIGQPTSISCKS	SQSLLHGNGKTY	LNWLLQRPGQSPKLLIY	LVS	NLESGVPDRFSGSGSGTDFTLKISRVEAEDLGVYYCLQ	ATHFPL	TFGAGAKLELK

Figure 2 Alignment of available sequences of Lewis Y-binding antibodies. The sequences are adapted from Refs [2, 19, 51]. The colour of the amino acid corresponds to its abundance at this position. Most abundant presence is indicated with the colour red followed by blue, green, cyan, grey and black. Sequences were aligned with CLUSTAL W [34]. The sequence for 3S193 is obtained from a humanized version. The human residues are omitted here. The CDR are defined after Chothia and Lesk [52].

Expression and analysis of chIgMs

In order to verify that the obtained sequences corresponded to the antibodies with the observed binding specificities, the V_H sequences were subcloned into a human μ-chain expression vector. A μ-chain vector was chosen for these experiments for the potential of multivalent display of the binding sites and therefore higher binding sensitivity. Multimerization is though not guaranteed as transient expression in COS-7 cells not necessarily renders IgM integration. The V_L sequences were subcloned into a human κ-expression vector. Two vectors, one μ and one κ, corresponding to a chIgM antibody, were transiently transfected into COS-7 cells. Antibody expression was examined in a sandwich ELISA specific for human IgM, and binding to Lewis Y and the related carbohydrate structures H type 2, H disaccharide and Lewis b was analysed as aforementioned. The chIgM C/C8 was produced and was found to bind to the Lewis Y but not to H type 2 (Fig. 3) or to Lewis b (not shown). Thus, the observed binding pattern corresponded to that of A70-C/C8. The chIgM B/B10 clone recognized Lewis Y and H type 2. Therefore, the specificity of chIgM B/B10 was identical to that of the murine A46-B/B10 antibody. These specificity data indicated that the correct sequences of the variable light and heavy chains were obtained. In contrast to these antibodies, the cells transfected with B/A6(1) μ and B/A6 κ produced an antibody, but the chIgM was not found to bind to any of the carbohydrates coated in the ELISAs. Expression of the chIgM B/A6(3) and chIgM A/A9 was not achieved.

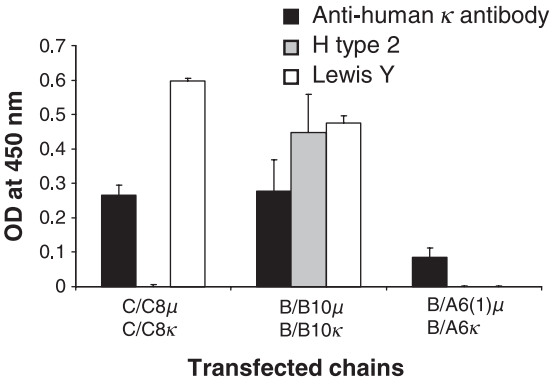

Figure 3 Sandwich and specificity ELISA of supernatants from COS-7 cells transfected with the respective μ and κ chains. Legends above indicate the immobilized compound.

Expression and analysis of chIgG CC8

A70-C/C8, the most promising antibody with respect to specificity, is a murine IgM and thus not suitable for clinical application. Therefore, the C/C8 V_H chain was cloned into a γ1 chain expression vector to express a ch antibody in the IgG1 format. The chIgG1 C/C8 supernatant was tested on the panel of 86 carbohydrate conjugates. The specificity of the chIgG1 antibody was identical to the mouse antibody in Fig. 1A (data not shown).

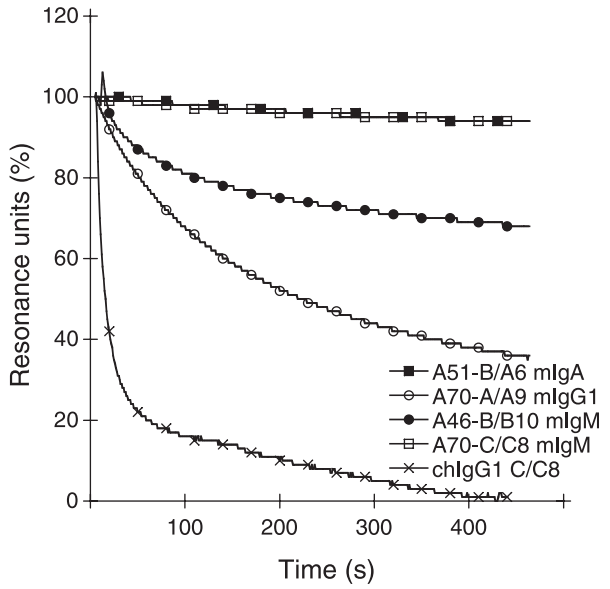

Figure 4 Overlay plot of individual dissociation curves on Lewis Y for A70-C/C8 (2211), A51-B/A6 (1103), A46-B/B10 (1450), A70-A/A9 (2300) and chIgG1 C/C8 (219), all at an antibody concentration of 500 nM. All curves are relative to the individual signals at the end of the association curve (resonance units at the end of association curves are given in brackets). A51-B/A6 and A70-C/C8 are almost identical.

Comparison of antibody off rates on the Lewis Y epitope

Surface plasmon resonance (SPR) technology on a Biacore 2000 instrument with a Streptavidin Sensor Chip SA was used to compare antigen binding. The binding pattern of the various antibodies was verified on a small set of related carbohydrates. Comparing the dissociation curves on Lewis Y, striking differences were seen (Fig. 4). A70-C/C8 and A51-B/A6 showed virtually no dissociation, whereas intermediate dissociation was seen for A70-A/A9 and A46-B/B10, and almost instant dissociation was observed for chIgG1 C/C8. Size exclusion chromatography of A51-B/A6 unexpected showed the antibody to be a mixture of monomer, dimer and multimer (Figure S2). The dissociation curve should therefore not be evaluated as belonging to a divalent molecule but include the possibility of significant rebinding comparable to an IgM molecule.

Chain shuffling – looking for new antibodies

Due to the high similarities of the four antibodies found in both their sequences and their recognition patterns of antigens, we speculated whether we could generate new antibodies with improved specificity and affinity by recombining the obtained V_H and V_L chains. This recombination was achieved by transfecting COS-7 cells with all possible combinations of the obtained μ and κ chains for transient expression of the antibodies. Supernatants from the transfected COS-7 cells were tested 2–3 days later in a sandwich ELISA to verify antibody expression and binding to Lewis Y, H type 2, H disaccharide and Lewis b. The results are summarized in Table 2.

By combining the A/A9 μ chain with the C/C8 κ chain, we obtained an antibody (chIgM A/A9-C/C8) which, when tested on the small panel of carbohydrate conjugates, showed the same specificity as the mouse MoAb y A70-C/C8. In a similar way did the chIgM B/A6(1)-C/C8 recognize Lewis Y, though generally a weaker signal was found both for expression and for binding to Lewis Y (data not shown). The chain-shuffled chIgM antibodies C/C8-B/B10, B/B10-C/C8 and A/A9-B/B10 could all be expressed but no binding of the antibody to any of the tested antigens was found. None of the transfection variants containing A/A9 κ or B/A6(3) μ were found to express an antibody when tested in sandwich ELISA.

Additionally, the A/A9 V_H sequence was cloned into a γ1 expression vector, and the A/A9 γ1 chain was also combined with the C/C8 κ chain, and this antibody (chIgG1 A/A9-C/C8) was transiently expressed in COS-7 cells. The supernatant was tested in ELISA on 86

Table 2 Summary of the results obtained from chain shuffling experiments with respect to expression and specificity of the antibody [B/B10 V_L is identical to B/A6 V_L (Fig. 2)].

				Specificity			
Antibody name	V_H μ chains	V_L κ chains	Expression	Lewis Y	H type 2	H disaccharide	Lewis b
A/A9-C/C8	A/A9	C/C8	+	+	–	–	–
A/A9-B/B10	A/A9	B/B10	+	–	–	–	–
B/B10-C/C8	B/B10	C/C8	+	–	–	–	–
B/B10-A/A9	B/B10	A/A9	–	n.d.	n.d.	n.d.	n.d.
B/A6(1)-C/C8	B/A6(1)	C/C8	+	+	–	–	–
B/A6(1)-A/A9	B/A6(1)	A/A9	–	n.d.	n.d.	n.d.	n.d.
B/A6(3)-C/C8	B/A6(3)	C/C8	–	n.d.	n.d.	n.d.	n.d.
B/A6(3)-A/A9	B/A6(3)	A/A9	–	n.d.	n.d.	n.d.	n.d.
C/C8-B/B10	C/C8	B/B10	+	–	–	–	–
C/C8-A/A9	C/C8	A/A9	–	n.d.	n.d.	n.d.	n.d.

+, positive result; –, negative result; n.d., not done.

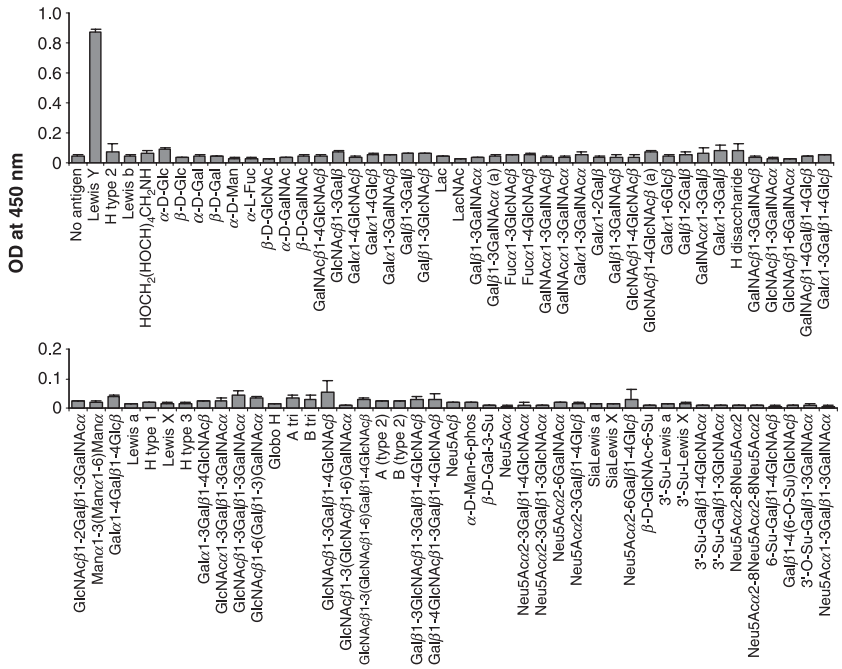

Figure 5 Specificity ELISA with supernatants from transiently transfected COS-7 cells producing the antibody chIgG1 A/A9-C/C8 on 86 different immobilized carbohydrate structures. Supernatants were used undiluted and the signals shown are means of two measurements.

carbohydrate–PAA conjugates. Figure 5 shows the specificity profile obtained for the chIgG1 A/A9-C/C8. The antibody was found to bind specifically to Lewis Y and showed no cross-reactivity to any of the other immobilized carbohydrates similar to the mouse antibody A70-C/C8.

For a comparison of the new chIgG1 A/A9-C/C8 with the chIgG1 C/C8 antibody, binding of equal concentrations of the antibodies were evaluated in ELISA. To this end, the concentrations of the antibodies in the supernatants containing chIgG1 C/C8 and chIgG1 A/A9-C/C8 were determined by a sandwich ELISA using purified human IgG as standard. According to these results, both supernatants were diluted to equal antibody concentrations and analysed in ELISA with Lewis Y as antigen (Fig. 6). It was found that the signal intensities of chIgG1 A/A9-C/C8 were approximately 10 times higher than that of the chIgG1 C/C8 at any given antibody concentration (Fig. 6). As comparable signals indicate comparable amount of antibody bound, the 10-fold different chIgG1 concentrations reflect true differences in affinity [36].

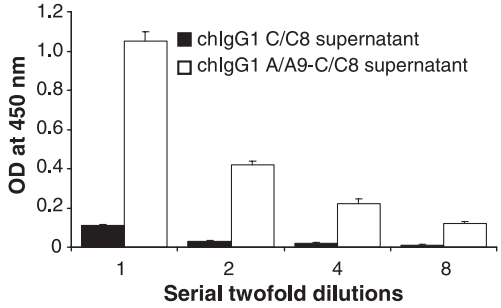

Figure 6 A comparative ELISA experiment of the activities of chIgG1 C/C8 and chIgG1 A/A9-C/C8 with equal relative concentrations of the antibodies. Solid-phase antigen was Lewis Y–PAA conjugate, and bound antibody was detected with an anti-human γ chain-specific antibody coupled to peroxidase.

Discussion

Among the histo-blood group carbohydrate antigens, many are developmentally regulated. They are characteristic surface markers of cells at certain stages of differentiation. Therefore, it is not surprising that some of them are also tumour markers, or more precisely oncofetal antigens [37]. The Lewis antigens, and in particular Lewis Y, Lewis X and some H-related structures belong to this category [30, 31, 38]. Among these antigens, Lewis Y is of special interest as a tumour-associated antigen with potential applications in diagnosis and therapy. However, as already outlined in the Introduction, the number of available antibodies with satisfying specificity and functional affinity is still limited [21]. The key problem has been their lack of specificity, as also the most specific antibody published BR55 [39] can reveal cross-reactivity by using other methods [21].

Here, we compared specificity and sequences of four mouse MoAb (A51-B/A6, A70-A/A9, A70-C/C8 and A46-B/B10) generated by us, which recognize Lewis Y. Cloning, chimerization, expression and verification of binding patterns were successfully accomplished, and subsequently a chIgG1 was generated from the Lewis Y-specific antibody A70-C/C8. The chIgG1 C/C8 proved

to be as specific towards Lewis Y as the parent antibody. Using the chain shuffling technique, we successfully generated two novel antibodies (chIgG1 A/A9-C/C8 and chIgM B/A6(1)-C/C8) to Lewis Y. The antibody chIgG1 A/A9-C/C8 was especially interesting as it recognized exclusively Lewis Y, and bound approximately 10 times stronger to the antigen than the corresponding parental chIgG1 C/C8. Binding of the antibodies A70-C/C8, chIgG1 C/C8 and chIgG1 A/A9-C/C8 to a panel of Lewis Y-positive and -negative cell lines showed recognition patterns identical or very close to other Lewis Y-binding antibodies [39–42], and again chIgG1 A/A9-C/C8 bound significantly stronger than chIgG1 C/C8 (Table S2). At first sight, also the chain shuffled antibody chIgM C/C8-B/B10 could have a potential for strong and specific binding to Lewis Y as the light chain from mIgA A51-B/A6 (identical to B/B10) potentially could contribute strongly to the affinity (evaluated from Fig. 4). Size exclusion chromatography of mIgA A51-B/A6 (Figure S2) unexpectedly identified this antibody as a mixture of monomers, dimers and something larger (possibly tetramers), with the dimer as the main population and a substantial amount of multimer.

The alignment of the sequences of our antibodies with the sequences known from the literature revealed three groups of variable heavy chains among Lewis Y-binding antibodies. Within each group, the sequences were very similar. The variations between the sequences of our antibodies and those of the previously reported antibodies were found at both domains, the framework V_H–V_L interface and CDR regions. The V_L sequences generally also showed high similarity with the exception of the V_L of C/C8, which was different from the others mostly in the framework residues. Although there were some variations in the amino acids influencing the canonical structures of the CDRs, they were not exceeding the allowed constrained variation for any of the CDRs, leaving the binding specificity only affected by the side chain type [35]. The antibodies MSL5 [19] and A46-B/B10 [30] are almost completely identical and constitute a third sequence set.

Analysing the V germline origins by IgBLAST search [43] (Table S1) showed that the three V_H sequences B/A6(1), C/C8 and A/A9 originated from the same germline V sequence (VH108A); however, all sequences revealed a varying number of deviations from the germline at different positions. The C/C8 V_L turned out to be mutated at 20 positions, being by far the V sequence with the highest deviation frequency from the germline. Additionally, the antibody A46-B/B10 (and MSL5) were found identical with a germline V sequence. Mouse germline antibodies hereby also establish their importance for human blood group antigen recognition as they already did for bacterial polysaccharides [44, 45].

The availability of a panel of slightly different V_H and V_L chains prompted us to perform chain shuffling experiments in order to examine potential changes in fine specificity, and to search for antibodies with improved properties. These extensive experiments revealed that the V_H, B/A6(1) and A/A9, when combined with the V_L sequence C/C8, bound Lewis Y but not H type 2 or Lewis b. It might not be surprising that chIgM B/A6(1)-C/C8 recognizes Lewis Y, as only a single amino acid exchange (D to Y) in the framework makes up the difference between the V_H sequences of B/A6(1) and C/C8. The second novel antibody chIgG1 A/A9-C/C8 generated by chain shuffling bound exclusively to Lewis Y among the panel of 86 carbohydrate–PAA conjugates tested. The exchange of the light chain in this case influenced the specificity of the antibody in that the cross-reactivity of the A70-A/A9 to Lewis b (and possibly Globo-H) disappeared. Unexpectedly, two very different V_H chains, C/C8 and A/A9, when combined with the same V_L, C/C8, are able to bind exclusively to the same antigen, Lewis Y. This suggests that by replacing the light chain of an antibody with broader specificity with another light chain from an antibody with a similar but narrower specificity, we were able to change the binding pattern. Obviously, the light chain plays a major part in the determination of the specificity, whereas it seems as though the affinity is mainly harboured in the heavy chain.

Previous chain shuffling experiments have been made with the antibodies B3 and B5, both recognizing Lewis Y [27]. This study reported a less active scFv when the B5 V_H and the B3 V_L were combined, and a more stable and active scFv when the B3 V_H and the B5 V_L were combined both compared to the parental antibody B3. The V_L chains B3 and B5 differed by only three mutations, two in the framework 1 and one in the CDR1. No change in specificity was reported upon chain shuffling. Furthermore, the individual chains of the antibody B3 showed the ability to bind to the surface of Lewis Y-expressing cells [46].

The functional affinity of the recombinant antibody chIgG1 C/C8 was measured by SPR on a Biacore instrument to be about 1 mM (data not shown). The dissociation curves showed that the chIgG1 C/C8 has a faster dissociation compared to the mouse MoAb, which causes the much weaker binding. The affinity of the antibodies can greatly affect their ability to localize to tumours with a localization plateau at 1 nM for scFv [47]. The functional affinities reported for the antibodies hu3S193 and BR96, which are two of the Lewis Y antibodies already in clinical trials, are 13–100 nM [2, 48, 49]. An estimation of the functional affinity of the chIgG1 A/A9-C/C8 was made by comparing its reactivity in ELISA as previously shown [50]. The analysis of binding of the two chIgG1s, C/C8 and A/A9-C/C8, showed a 10-fold increase in the reactivity to Lewis Y for the chain-shuffled variant over the chIgG1 C/C8. This functional affinity is of the same order of magnitude as that of the mIgG1 A70-A/A9, which contains the same V_H

sequence. Of note, the functional affinity is also of the same order of magnitude as that of the Lewis Y antibodies already used in clinical trials.

It was quite surprising that two so different V_H chains such as C/C8 and A/A9 can, together with the C/C8 V_L, bind exclusively to Lewis Y, especially considering the general difficulties in raising specific anti-carbohydrate antibodies. Comparing the reactivities of chIgG1 C/C8 and chIgG1 A/A9-C/C8, the latter proved to be able to bind the antigen approximately 10 times stronger than the former, which is an invaluable gain if the antibody is intended to be developed further for diagnostic or therapeutical applications. The method of shuffling of chains from antibodies binding similar antigens appears to be a valuable technique for introducing minor but potentially essential changes in specificity such as, e.g. removing cross-reactivities to unwanted antigens. This is especially interesting for anti-carbohydrate antibodies as their sequences are similar, close to germline sequences, and often allow cross-reactivities among related carbohydrate structures. Our data have also implications for the generation of potent carbohydrate-binding antibody libraries.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 SDS-PAGE of purified antibodies.

Figure S2 SEC of mIgA A51-B/A6.

Table S1 Germline origin by IgBLAST.

Table S2 Summary of cell staining.

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