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Engineering Antibody Heavy Chain CDR3 to Create a Phage Display Fab Library Rich in Antibodies That Bind Charged Carbohydrates

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A number of small charged carbohydrate moieties have been associated with inflammation and cancer. However, the development of therapeutic Abs targeting these moieties has been hampered by their low immunogenicity and their structural relationship to self-Ag. We report the design of an Ab repertoire enriched in Abs binding to small charged carbohydrates and the construction of a human Fab phagemid library, "FAB-CCHO." This library combines L chain Ig sequences from human donors and H chain synthetic diversity constructed in key Ag contact sites in CDRs 1, 2, and 3 of the human framework V_H3–23. The H chain CDR3 has been engineered to enrich the library in Abs that bind charged carbohydrates by the introduction of basic residues at specific amino acid locations. These residues were selected on the basis of anti-carbohydrate Ab sequence alignment. The success of this design is demonstrated by the isolation of phage Abs against charged carbohydrate therapeutic target Ags such as sulfated sialyl-Lewis X glycan and heparan sulfate. *The Journal of Immunology,* **2008, 181: 6213–6221.**

Over the last 15 years, knowledge of the biological functions of protein-carbohydrate interactions has increased dramatically (1, 2). Glycoconjugates participate in biological processes as diverse as cell-matrix interactions, chemoattraction, inflammation, angiogenesis, tumor development, and viral attachment (3, 4). Carbohydrates involved in disease states of nonpathogenic origin are very often self-Ags. Among them are tumor-associated carbohydrate Ags (TACA) such as sialyl-Lewis-X, 2-deoxy-2-acetamido-α-D-galactopyranosyl-*O*-serine (TACA-Tn), sialyl-Tn, Thomsen-Freidenreich Ag, GM2 gangliosides, and glycosaminoglycans (GAGs)[3] like heparan sulfate (HS) (5–11). The relatively low affinities that are characteristic of carbohydrate binding proteins (e.g., Abs, selectins) constitute a major challenge for efforts to produce therapeutics that will intervene in carbohydrate recognition events. Given the low immunogenicity of carbohydrates (12, 13), immunization procedures often result in only a weak primary IgM response or no response at all. Conventional hybridoma techniques have generated anti-carbohydrate Abs of the IgM class, unsuitable for in vivo diagnostics or therapy (14–16).

Phage display is an alternative technique and probably the best available strategy for providing anti-carbohydrate Abs. However, there are only a few reports describing their isolation by this means. Most of these reports describe Abs recognizing long polysaccharides such as HS, heparin, and virus polysaccharide, whereas only a few reports on the use of phage display to isolate Abs against small charged carbohydrate moieties such as sialyl-Lewis-X, a sulfated glycan, are available. Described anti-carbohydrate Abs typically have affinities that are in the low micromolar range (2, 17), and the difficulty in isolating these Abs by phage display was initially assigned to problems of display valency. A multivalent display system that would confer an increased avidity of the anti-carbohydrate Abs toward their target has been proposed (15, 18–21), reasoning that monovalent binders with rapid dissociation kinetics are lost during the washing steps. To be useful therapeutics, Abs isolated through multivalent display systems are likely to need affinity maturation, a laborious and lengthy process.

One key to success in isolating anti-self Abs with carbohydrate binding activity might be the composition of the Ab gene diversity. There has been some success in identifying anti-carbohydrate Abs from immune phage display libraries (22), but the majority of successful reports describe the isolation of Abs against self-Ags from libraries with a semisynthetic or completely synthetic Ab gene source. These libraries, initially developed as an alternative to the use of animals for Ab production (23–25), consisted mainly of diversity introduced in vitro within the six CDR loops of the combining site. In a recent report, Persson et al. (26) created a hapten-focused library based on a FITC-specific Ab sequence with a built-in specificity for hapten-sized Ags. Because the H chain CDR3 has the greatest flexibility and conformational variability of the six CDRs, it could have the greatest influence on Ag binding (27). Semisynthetic combinatorial libraries with randomized H chain CDR3 domains have yielded Fabs that bind haptens (28) and form coordination complexes with metal ions (29). Libraries of single chain variable fragments (scFv) with a H chain CDR3 synthetic repertoire were used to select Abs that bind to a variety of haptens and self-Ags with affinities not better than 700 nM (24).

We have therefore investigated whether the engineering of a synthetic H chain CDR3 repertoire designed to specifically bind

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[3] Abbreviations used in this paper: GAG, glycosaminoglycan; HS, heparan sulfate.

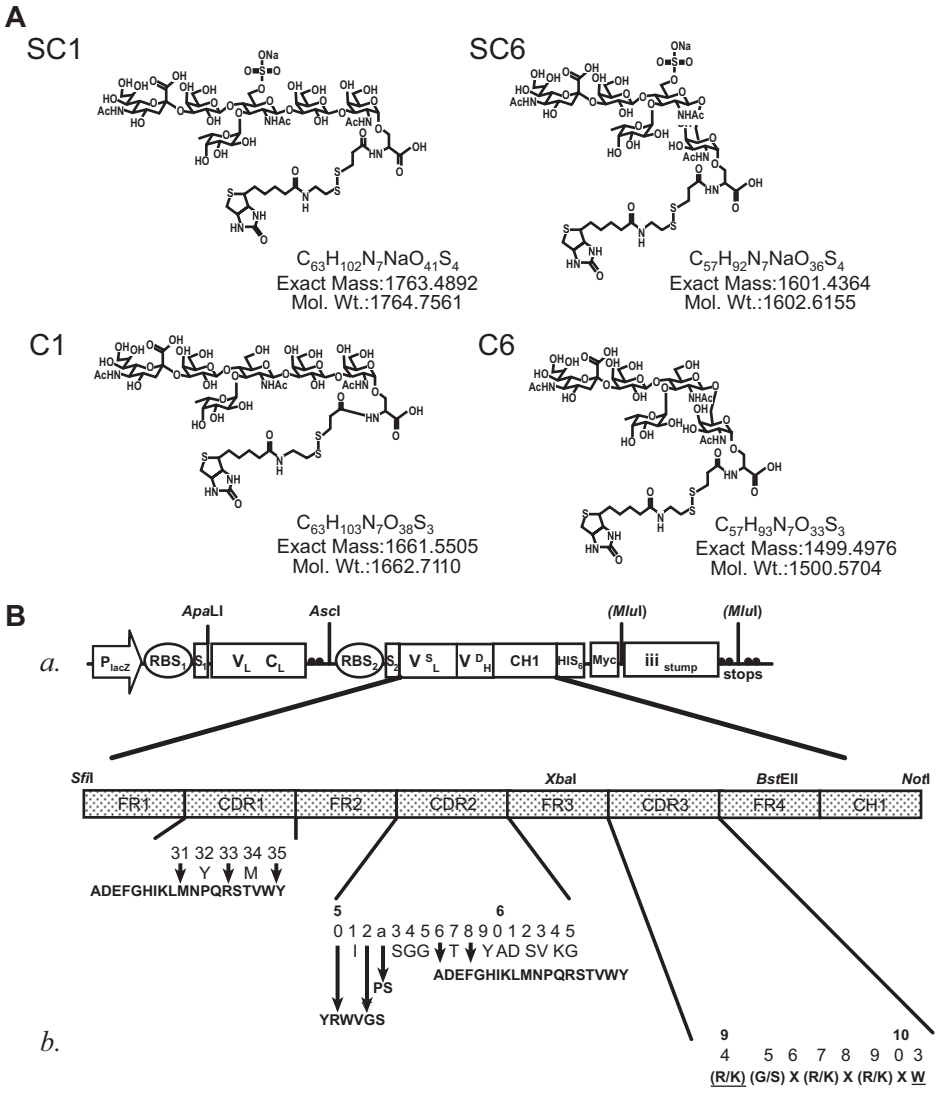

FIGURE 1. Targets and FAB-CCHO H chain CDR3 design. *A*, Structure of the 6-sulfo sialyl Lewis X core 1 (SC1) and core 6 (SC6) carbohydrate moieties and their non-sulfated counterpart's core 1 (C1) and core 6 (C6). Mol. Wt., molecular weight. *B*, FAB-310 library design as described in Hoet et al. *a*, Amino acid sequence of the H chain CDR1-CDR2 repertoire embedded in FAB-310 vector backbone *b*, Amino acid sequence of the H chain CDR3 repertoire embedded in FAB-310 vector backbone. *c*, Lower-strand nucleotide sequence of the oligonucleotide primer encoding the FAB-CCHO H chain CDR3 diversity. (Restriction endonuclease *Bst*EII site, boldface; M, equimolar A and C; N, equimolar A, C, G, and T.) Diversity is introduced through the ARG codon, which encodes R or K, the RGT codon encoding G or S, and the NNK codon, which encodes any of the 21 amino acids and only allows the amber termination signal (read through in *E. coli* TG1 strain).

charged carbohydrate moieties would help to select Abs with the desired carbohydrate specificity. Its rational design is based on sequence alignment of anti-carbohydrate Ab H chain CDR3s. We postulate that a H chain CDR3 repertoire mainly composed of residues with basic properties will prevail over large diversity. A library was created by introducing basic residues at specific amino acid locations of the H chain CDR3. We report the design and the construction of FAB-CCHO, a semi-synthetic human Ab Fab phage library. We have confirmed the utility of FAB-CCHO by selecting specific Abs against charged carbohydrate Ags.

Materials and Methods

Design of the H chain CDR3 consensus sequence

The H chain CDR3 region was designed with the following amino acid sequence: TAVYYCA(R/K)$_{94}$(G/S)X(R/K)X(R/K)X$_{100}$ W$_{103}$GQGTLVTVSS (underscored amino acids are in frameworks 3 and 4; X can be any amino acid; position numbers are in subscript to the right of the amino acid or mutation). The oligonucleotide (H chain CDR3 primer) encoding this sequence is shown in Fig. 1*B*.

Construction of the FAB-CCHO library

The diversity in Ab L chain, and H chain CDR1 and CDR2 was taken from a human semi-synthetic Ab phage display library (FAB-310) (30). Essentially, H chain CDR1 and CDR2 are variegated at specific positions and embedded in the V_H3–23 human framework. Ten nanograms of the FAB-310 Ab repertoire DNA were used as template for a PCR with a kappa constant upper-strand primer (5′-CCATCTGATGAGCAGTTGAAATCT-3′) as 5′-end primer and the H chain CDR3 primer as 3′-end primer (Fig. 1*B*) to amplify the full H chain repertoire. Amplification was performed for 35 cycles in 25 ml using Advantage 2 DNA polymerase (Clontech) (1 min at 95°C, 1 min at 50°C, and 2 min at 68°C). Ten micrograms of the 650-bp DNA product was digested with 40 U of restriction endonuclease *Bst*EII (New England Biolabs), followed by cleavage with 100 U of restriction endonuclease *Xba*I (New England Biolabs). The resulting 140-bp DNA fragments containing the H chain CDR3 repertoire were then purified on agarose gel and 1.5 μg was ligated using T_4 DNA ligase (New England Biolabs) with 6 μg of similarly cut phagemid vector pMID21 (GenBank AY754024) containing both the κ and λ L chain repertoires.

Next, 2.5 μg of a desalted ligation mixture was separately electroporated into the *Escherichia coli* strain TG1 using 100 ng of ligation mixture per electroporation event. The Fab library contains 2×10^9 representatives. Forty-eight isolates from the newly built library were picked at random, amplified, and sequenced to check that all conformed to the design.

Targets

Sulfated sialyl-Lewis X glycans and their non-sulfated counterparts were . provided by Thios Pharmaceuticals (Fig. 1*A*). The synthesis of 6-sulfo sialyl Lewis X oligosaccharides corresponding to the core 1 and core 6

branches of the L-selectin ligand are reported elsewhere (31). These synthetic targets possess a serine residue at the reducing terminus, providing a functional handle for further conjugation. Biotin was conjugated to the targets to allow a phage display selection procedure using streptavidin-magnetic beads and elution of target-bound phage particles with DTT.

HS from bovine kidney, a chemically modified HS (CDSNS), and mouse anti-HS Ab (clone 10E4) were purchased from Seikagaku Kogyo.

Selection procedures

The phagemid particles were rescued with helper phage M13KO7 according to Marks et al. (32) on a 3-liter scale. The total number of phagemid particles was 1.2×10^{14} phage.

Selection on 6-sulfo sialyl Lewis X core 6. 10^{13} phage were used for selection with 500 nM soluble biotinylated 6-sulfo sialyl Lewis X core 6 Ags. The same Ag concentration was kept constant during all three rounds of selection.

Selection on HS. Phage (10^{13}) was used for selection with a mixture of HS and its chemically modified form CDSNS, immobilized at 100 μg/ml on immuno-tubes (Maxisorp tubes; Nunc) (32) in 90% saturated $(NH_4)_2SO_4$. The procedure was repeated three times with the same concentration of Ags. Eluted phage was used to infect *E. coli* TG1 cells.

Screening by ELISA using Fab on phage and sequencing procedures

Phage displaying Fab was produced from 384 individual clones as described (32). Culture supernatants or purified phage preparations were tested by ELISA using biotinylated Ag captured indirectly via immobilized BSA-streptavidin (Maxisorb; Nunc). To screen for 6-sulfo sialyl Lewis X core 6 binders, the ELISA plate was coated with 50 ng of biotinylated target 6-sulfo sialyl Lewis X core 6 Ag in PBS, 50 ng of nonsulfated core 6, or 500 ng of streptavidin per well as negative controls. To screen for HS-specific binders, plates were coated overnight at 4°C at 500 ng per well with a mix of HS/CDSNS (in 90% $(NH_4)_2SO_4$) or 100 ng BSA (as negative control). ELISA was performed as previously described (33). The Ab genes from clones giving a positive signal in ELISA (>2× background) were amplified using 5′ and 3′ vector backbone primers, and PCR products were sequenced for both L and H chains.

Conversion of Fab to IgG format

Conversion of Fab to IgG molecules was performed as described earlier (34). Briefly, the Fab cassettes were released from the phagemid vector by *Apa*L1 (New England Biolabs) and *Nhe*I (New England Biolabs) enzymatic treatment and cloned into a mammalian expression vector treated with the same endonucleases. The internal regulatory element (internal ribosome entry site (IRES) motif and a eukaryotic H chain leader) was introduced in the construct as an *Asc*I/*Mfe*I fragment.

Kinetic analysis of C3 purified IgG antibody

The specificity of the Ab was further characterized by surface plasmon resonance (BIAcore 3000; BIAcore Life Sciences, GE Healthcare). The IgG constructs were transiently expressed in HEK293T cells and purified using protein A-coupled beads (Amersham Biosciences, GE Healthcare) as described earlier (34). Biotinylated 6-sulfo sialyl Lewis X core 6, its non-sulfated counterpart core 6, biotinylated 6-sulfo sialyl Lewis X core 1, and its non-sulfated counterpart core 1 were immobilized on a streptavidin-coated CM5 chip (low density, ~7 resonance units; high density ~25 resonance units). Serial dilutions of the purified IgG in PBS were injected for 3 min at 30 μl/min in HBS-EP with 500 mM NaCl (pH 7.8), and dissociation of bound analyte was allowed to proceed for 5 min. The data obtained on the lower density giving an appropriate signal were analyzed with the help of the BIAevaluation software 3.2 (global fitting).

Three-dimensional modeling of C3 Fab and "docking" with models of 6-sulfo sialyl Lewis X core 1 and 6

The three-dimensional structures of 6-sulfo sialyl Lewis X glycans core 1 and core 6 were modeled using Insight II (Accelrys Software) and based on the sugar composition provided by Thios Pharmaceuticals (31). The potentials of different atom types were assigned in a consistent valence force field by the Builder module in Insight II. The 6-sulfo sialyl Lewis X core 1 and core 6 models were subjected to 500 steps of energy minimization using the conjugate gradient method.

The three-dimensional structure of C3 Fab was modeled using Modeler in Insight II. The crystal structure of the Fab that was selected to provide H chain CDR3 reference coordinates differed from the sequence of the C3 Ab but had the same number of residues. Affinity docking with 6-sulfo sialyl Lewis X core 6 yielded >500 structures, of which two were chosen based on low energy and orientation of the sulfate group. The final structure of the C3 Fab was optimized with 3000 steps of conjugate gradient. 6-Sulfo sialyl Lewis X core 1 and core 6 were docked to the C3 Fab independently with docking program Affinity (Insight II) using the consistent valence force fields. Both carbohydrates were initially set in the center of the Ag-binding cavity formed by the CDRs of the C3 Fab. The binding subset was defined as containing residues within 20 Å of the sulfate atom. The remaining part of the Fab was held rigid during the docking process. The ligands were moved randomly within 10 Å of the initial position, generating a new docking structure. After 500 steps of conjugate gradient minimization of the docked structure, the resulting structure, which had energies within 20 kilocalories of the lowest energy of the docked structures, was taken as the new docked structure. The docked structures were evaluated based on their energies and the orientation of the sulfate in the binding pocket.

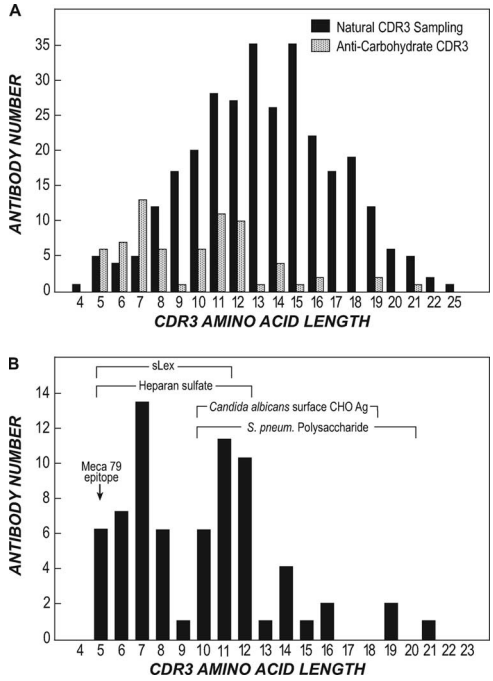

FIGURE 2. Length of H chain CDR3 in non-immune repertoire. *A*, Distribution of H chain CDR3 length within a sampling of 300 Abs from a nonimmune random repertoire and within a sampling of 71 anti-carbohydrate Abs. Mean length is ~13 and ~7 amino acids, respectively. *B*, Length of H chain CDR3 length for 71 Abs described in the literature related to the charge and size of the carbohydrate target.

Results

Design of the library

We used the semi-synthetic phage display library, described by Hoet et al. (30), to introduce the specific H chain CDR3 repertoire. This phagemid display system, which requires the use of helper phage to make phage particles, displays Fab Ab fragments fused to the domain 3 of M13 protein III (pIII). The pIII coat protein from the helper phage competes with pIII domain fusion protein for incorporation into the phage particle. As a result, the phage population consists of phage bearing between zero and four copies of Fab per phage, with most displaying phage particles having one fusion protein (35). In addition, this library offers the advantage of containing a nonimmune L chain repertoire combined with synthetic H chain CDR1 and CDR2 regions. The synthetic diversity mimics the pattern of mutations that give rise to high affinity Igs after immunization and clonal selection.

The design of the synthetic H chain CDR3 repertoire is based on two observations. Although the mean length of human H chain

Table I. *Carbohydrate-binding antibodies*

Carbohydrate Target	V_H Germline	CDR3 Size (Amino Acids)	H Chain CDR3[a]	Source
HS	DP-38 (V_H3)	5	GM**R**P**R**	53
HS	DP-40 (V_H3)	5	LGF**H**S	54
HS	DP-65 (V_H4)	5	WVTEP	53
Lex-BSA and sLex-BSA		5	QLGEN	11
sLex and Lex S7	DP-47 (V_H3)	5	SVVGY	56
Meca 79 epitope	DP-71	5	GPLDF	16
Haemophilus influenzae b polysacch.	V_H3	6	GYGFDY	57
HS	DP-42 (V_H3)	6	GA**R**L**KR**	54
HS	DP-58 (V_H3)	6	GM**R**P**R**L	53
HS	DP-38 (V_H3)	6	G**RR**L**K**D	42
HS	DP-42 (V_H3)	6	GY**R**P**R**F	53
Human skeletal muscle GAG RB4CD12	DP-38 (V_H3)	6	GM**R**P**R**L	55
Lex-BSA and sLex-BSA		6	**R**GTTAA	11
Gal (Galα1–3Galβ1–4GlcNAc-R)	V22.1 (mouse)	7	PGYAMDY	58
HS	DP-38 (V_H3)	7	G**K**M**K**LN**R**	53
HS	DP-32 (V_H3)	7	**RR**YALDY	53
HS	DP-8 (V_H1)	7	YY**H**Y**K**VN	42
Heparin	V_H3	7	E**R**NTI**RR**	59
Heparin	V_H3	7	G**K**V**K**LPN	59
Heparin	V_H3	7	G**R**TVG**R**N	59
Heparin	V_H3	7	GT**KK**LG**K**	59
Homogalacturonan		7	F**H**P**R**VYD	60
Homogalacturonan		7	**K**W**R**LALF	60
Human skeletal muscle GAG RB4EA12	DP-32 (V_H3)	7	**RR**YALDY	55
Lex-BSA and sLex-BSA		7	WLL**R**QS**H**	11
Mouse skeletal muscle GAG AO4B05	DP-53 (V_H3)	7	L**K**QQGIS	55
Heparin	V_H3	8	GGTT**RI**K**R**	59
Heparin	V_H3	8	G**R**L**H**LP**RK**	59
Heparin	V_H3	8	G**RRHK**LI**R**	59
Heparin	V_H1	8	GT**K**L**K**MT**K**	59
sLex and Lex S10	DP-75 (V_H1)	8	V**K**SGAFDI	56
sLex and Lex S8	DP-75 (V_H1)	8	AG**R**FGELY	56
Heparin	V_H3	9	L**R**GT**K**MF**RH**	59
Blood group B Ag	V_H4(?)	10	EGGPY**R**PADY	61
Candida albicans surface CHO Ag	V_H1	10	D**R**SGSYAFDI	62
Candida albicans surface CHO Ag	V_H4	10	D**R**SGTYAFDI	62
Glycosphingolipid GM3 on BSA	V_H4	10	WNGQNNAFDT	63
Heparin	V_H1	10	SSS**RHHR**L**HR**	59
Pneumococcal polysaccharide vaccine	V_H3 DP47	10	GGNWNWNFGL	64
HS	DP-38 (V_H3)	11	**H**APL**R**NT**R**TNT	53
HS	DP-42 (V_H3)	11	SL**R**MNGCGA**H**Q	42
HS	DP-47 (V_H3)	11	SL**R**MNGW**RA**H**Q**	53
HS	DP-3 (V_H1)	11	S**RK**T**RK**PFM**RK**	53
Heparin	V_H3	11	PVS**HRK**W**R**VTV	59
Heparin	V_H1	11	S**RK**TP**K**PFM**RK**	59
Human skeletal muscle GAG RB4CB9	DP-38 (V_H3)	11	**H**APL**R**NT**R**TNT	55
LPS	(mouse)	11	D**H**DGYYE**R**FSY	65
Mouse skeletal muscle GAG AOB08	DP-47 (V_H3)	11	SL**R**MNGW**RA**H**Q**	55
Pneumococcal polysaccharide vaccine	V_H6 DP74	11	DG**R**SGSYSLDY	64
sLex and Lex S6	DP-75 (V_H1)	11	GGYGP**R**GYFDI	56
Glycosphingolipid GM3 on BSA	V_H1	12	DLMNWG**R**FPLDY	63
Heparan sulfate	DP-47 (V_H3)	12	SISMNGVGV**R**IQ	53
Heparin	V_H3	12	A**R**MTG**H**V**R**NVMI	59
Heparin	V_H3	12	D**RR**NTQ**K**T**R**Y**R**T	59
Heparin	V_H7	12	Q**R**W**K**PAVTP**K**LV	59
Heparin	V_H3	12	SG**R**QA**R**QG**R**FP**K**	59
Human skeletal muscle GAG RB4EG12	DP-4 (V_H1)	12	SG**RK**YF**RA**RDMN	55
Mouse skeletal muscle GAG AOF12	DP-7 (V_H1)	12	AMTQ**KK**P**RK**LSL	55
Pneumococcal polysaccharide vaccine	V_H3 DP38	12	DLTYAPVNGFDY	64
Pneumococcal polysaccharide vaccine	V_H3 DP38	12	GYSS**R**GF**H**GMDV	64
Pneumococcal polysaccharide vaccine	V_H3–8	13	DNTP**KR**GPYYFDY	64
Biotinylated α-Gal BSA G9V_H	V3–43	14	EGWYVPAAAAGMDV	66
Biotinylated α-Gal BSA P19V_H	V3–15	14	ED**H**MSGWWG**K**GMDV	66
glycosphingolipid GM3 on BSA	V_H3	14	DSSYVDWPPYGL**H**V	63
Glycosphingolipid GM3 on BSA	V_H4	14	TGTSSDIG**R**YNYVS	63
Pneumococcal polysaccharide vaccine	V_H3 30.3	15	DPYCSSTSC**HH**GMDV	64
Glycosphingolipid GM3 on BSA	V_H1	16	A**R**YSGSY**R**PLDYYMDV	63
Pneumococcal polysaccharide vaccine	V_H3 DP50	16	D**R**ISGDYDIYYYGMDV	64
Candida albicans surface CHO Ag	V_H1	19	APDCGGGSCYSF**H**YYGMDF	62
Carbohydrate moiety of midgut mosquito	DP-48 (V_H3)	19	TPTPYDFWSGYDQNWFFDL	22
Pneumococcal polysaccharide vaccine	V_H1 DP8	21	G**R**ATL**R**FLEWVNWEEGCLFDY	64

[a] Boldfaced amino acids highlight the basic residue content of the CDR3.

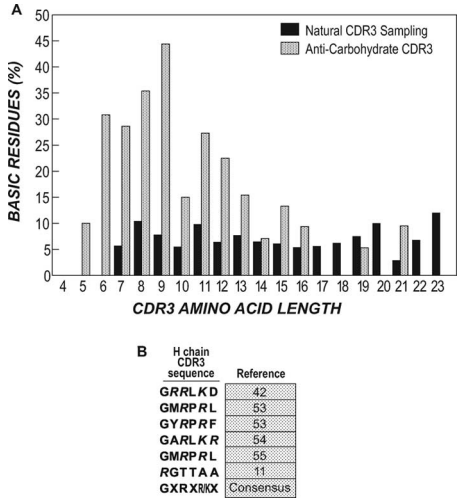

FIGURE 3. H chain CDR3 amino acid content. *A*, Percentage of basic residues per H chain CDR3 in FAB-CCHO compared with FAB-310. *B*, Derivation of H chain CDR3 consensus sequence focused on binding to carbohydrate. Alignment of 6-aa residue H chain CDR3s described in literature.

CDR3 is ~13 amino acids (36, 37), we observed that the mean length of H chain CDR3s from Abs to charged carbohydrate is 6–8 aa (Fig. 2*A* and Table I). Secondly, from an analysis of the literature, we observed a trend to short H chain CDR3 sequences when binding self-charged carbohydrates (Fig. 2*B*). Hence, we limited the H chain CDR3 size to 6-aa residues.

We also evaluated and compared the average percentage of basic residues found in the H chain CDR3 sequences from known anti-carbohydrate Abs with those captured from a nonimmune repertoire. The V_H sequence of a random sample of 300 clones was analyzed. Basic residues were assessed in H chain CDR3s of the same length. The average percentage of basic residues per H chain CDR3 is reported for each length category in Fig. 3*A*. Of the amino acid residues in naturally occurring H chain CDR3s, 5–10% are basic for each H chain CDR3 length. For anti-carbohydrate Abs, 30–45% of the residues are basic in H chain CDR3s of 6–9 amino acids. This percentage decreases strongly for longer H chain CDR3s. In addition, from H chain CDR3 sequence alignment, we determined the positions where basic residues were most frequently found. We also determined amino acid positions where we could find either a limited or an extended diversity (Fig. 3*B*). Based on this reasoning, we decided to introduce basic residues (R or K) at positions 94, 97, and 99, whereas positions 96, 98, and 100 can be any of the 20 aa. Position 94 is nominally the end of framework region 3 (FR3), and a very large fraction of Abs have either R or K in this position; hence, we allowed R or K. The codon variegation strategy used is detailed in Fig. 1*B*. Because we very frequently observed G (20/71) or S (10/71) as the first residue in the Abs described in the literature, we allowed the first residue to be either G or S, consistent with the small size and flexibility of these two amino acids allowing a small binding pocket. With (R/K)(G/S)X(R/K)X(R/K)X as consensus sequence, the resulting H chain CDR3 repertoire will contain at least 33% basic residues. The theoretical diversity that we generated is 1.28×10^5 (Fig. 3*B*).

Validation of the library

The constructed library contains 2×10^9 transformants. The entire H chain CDR3 diversity allowed by the synthetic oligonucleotides has been captured, each H chain CDR3 occurring in $\sim 1.6 \times 10^4$ contexts. We evaluated the quality of the unselected library by sequencing 48 random clones. Each of the 48 H chain CDR3 sequences is distinct, indicating that there is no large bias in the gene diversity introduced. Additionally, analysis of the complete V_H and V_L sequences showed that the H chain CDR1, CDR2, and L chain diversities in the newly built library were unaltered from those in the original library (data not shown). Although the first H chain CDR3 residue was always S or G, S was present in 70% of the clones. Also, K predominates over R at position 97 (85% of the clones). Similarly, 83% of the clones also contain K at residue 99. This bias has been introduced by the oligonucleotide primers.

Panning of the FAB-CCHO library on two human charged carbohydrate targets, HS and the newly described target 6-sulfo sialyl Lewis X core 6 (31), was followed by the ELISA analysis of 384 isolates for the 6-sulfo sialyl Lewis X core 6 selection and 96 isolates for the HS selection. The V_H and V_L sequences of the binders were determined (Table II), and target specificity of the unique clones was further confirmed using purified phage preparations. The specific binding of the three unique HS Fabs on phage to HS was confirmed, because no binding is observed in ELISA with the control Ags tested (Fig. 4*A*). In the case of the anti-6-sulfo sialyl Lewis X core 6 Abs, we selected one specific lead candidate (C3 clone) for which no ELISA signal is observed for the non-sulfated version of the carbohydrate (Fig. 4*B*). This observation indicates that the charged moiety is important for the binding of this Ab.

Alignments of the H chain CDR3s from the selected anti-carbohydrate Abs were performed. Interestingly, all selected H chain CDR3s contain G at the first amino acid position (residue 95) and R at the third (residue 97, R or K allowed) despite the initial ratios observed at these positions (70% S:30% G and 85% K:15% R), suggesting that the selection on carbohydrates preferred these residues. In addition, although all amino acid types were allowed at positions 96, 98, and 100, most of the H chain CDR3s have selected additional basic residues at one or two of these positions (R, K, and H). None of the selected Abs contained aspartic or glutamic acid residues in the H chain CDR3. Of the four Abs, three have three basic residues in H chain CDR3 and one has four. This

Table II. *CDR amino acid sequences of the anti-heparan sulfate and anti-6-sulfo sialyl Lewis X core 6 Abs*[a]

	L Chain CDR1	L Chain CDR2	L Chain CDR3	Family	H Chain CDR1	H Chain CDR2	H Chain CDR3
HS binders							
A2	KSSQSVLYSSNNKNYLA	WASTRES	QQYYSTPPT	VK1	NYTMA	SISSSGGHTPYADSVKG	GKRNRN
H1	ARASQSVSSSYLA	GASSRAT	QQYGSSPRT	VK1	GYRMN	SIGSSGGHTSYADSVKG	GKRNRT
A4	RSSQSLVHSDGNTYLN	KVSNRDS	MQGTHWPYT	VK2	EYIMS	YISPSGGTTKYADSVKG	GRRTKH
6-Sulfo sialyl Lewis X core 6 binder							
Clone C3	QGDSLRSYYAS	GKNNRPS	NSRDSSGNIWKV	VL3	VYPMH	YIGSSGGETMYADSVKG	GKRNKR

[a] Family affiliation is shown for the L chain.

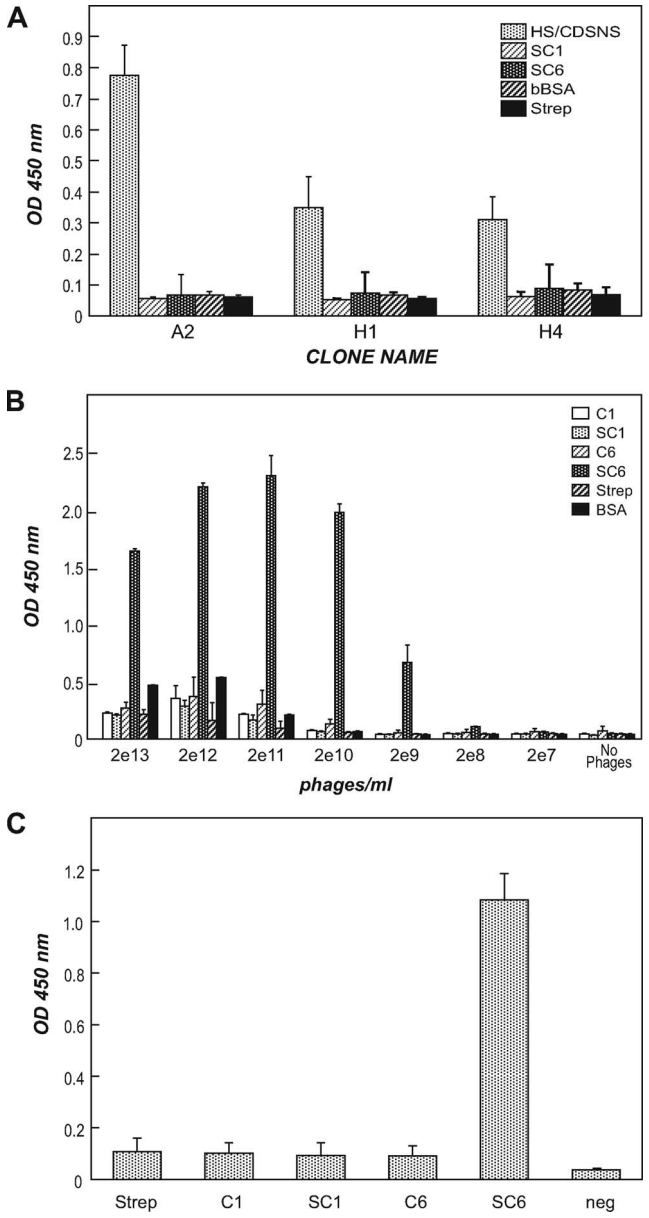

FIGURE 4. *A* and *B*, Specificity of selected anti-HS (*A*) and anti-6-sulfo sialyl Lewis X core 6 (*B*) Ab fragments determined by ELISA. The assay was performed by immobilizing HS on a polystyrene plate or by indirect coating of biotinylated 6-sulfo sialyl Lewis X core 6 on BSA-streptavidin-coated plates. Titrated phage-displayed Ab preparations (10^9-10^{10}) reactive with the coated Ag were detected with peroxidase-conjugated anti-M13 Ab (Amersham Biosciences, GE Healthcare). The results of the assay are shown as light absorbance at a 450-nm wavelength. SC6, 6-Sulfo sialyl Lewis X core 6; C6, sialyl Lewis X core 6; SC1, 6-sulfo sialyl Lewis X core 1; C1, sialyl Lewis X core 1; Strep, streptavidin; bBSA, biotinylated BSA. *C*, Specificity ELISA of 6-sulfo sialyl Lewis X core 6-specific C3 Ab was performed with purified IgG preparation on 6-sulfo sialyl Lewis X core 6, 6-sulfo sialyl Lewis X core 1, and their non-sulfated counterpart sialyl Lewis X core 6 and sialyl Lewis X core 1 (i.e., the target Ag and three other carbohydrate moieties of the L-selectin ligand) using streptavidin and BSA as negative controls (neg). Detection was performed using a peroxidase-conjugated rabbit anti-human IgG (1/6000; DakoCytomation).

observation confirms the necessity of basic properties for the binding of H chain CDR3 to the targeted negatively charged carbohydrates.

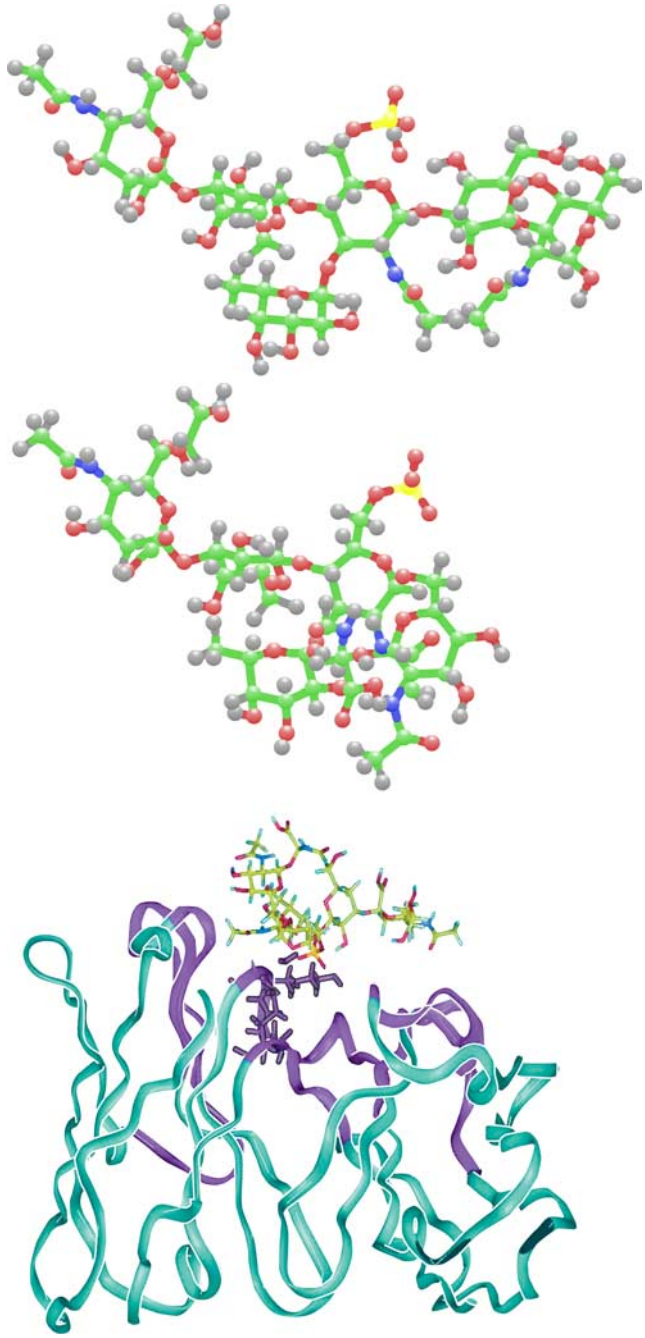

FIGURE 5. *Top and middle diagrams*, Three-dimensional structures. Structural models of 6-sulfo sialyl Lewis X core 1 (*top*) and 6-sulfo sialyl Lewis X core 6 (*middle*). The atoms are colored using the standard coloring code, with carbon atoms represented in green, oxygen in red, sulfur in yellow, and nitrogen in blue. *Bottom diagram*, Diagram of C3 V region binding to 6-sulfo sialyl Lewis X core 6. The ribbon diagram of the model structure of the variable region of C3 is shown in cyan. The CDR residues for both L and H chains are colored purple. The side chain residues of H chain CDR3 that are potentially involved in contact with the Ag are shown in purple.

Characterization of the C3 lead candidate

We further characterized the C3 lead candidate because of its potential as an anti-inflammatory drug candidate. C3 was converted to an IgG1 molecule. As a bivalent molecule, its K_D value was determined by BIAcore to be 54 ± 4 nM, which is a relatively high affinity when compared with other described anti-carbohydrate Abs (2, 17). In addition, because this carbohydrate Ag is present in

multiple copies in vivo, avidity effects will likely increase its binding potency. Interestingly the C3 Ab did not cross-react with 6-sulfo sialyl Lewis X core 1. This 6-sulfo sialyl Lewis X core 1 differs from 6-sulfo sialyl Lewis X core 6 only by the position of the linkage from the sulfate-bearing *N*-acetyl glucosamine to the next sugar (stereoisomer; Ref. 31) (Fig. 4*C*), indicating that the Ab recognizes a particular conformation of the 6-sulfated sialyl Lewis X. Computational studies of glycan-Fab interaction were performed in this study. The Ag modeling reveals a flat structure for the 6-sulfo sialyl Lewis X core 1 glycan whereas the 6-sulfo sialyl Lewis X core 6 appears sharply bent (Fig. 5*A*), suggesting that the Ab binding pockets required for these Ags are very likely to differ from each other. Affinity docking analysis performed between the Fab Ab (C3) and the 6-sulfo sialyl Lewis X core 6 Ag confirmed our hypothesis that the sulfated group, protruding from the bent core 6, is accommodated in the groove formed by the CDRs of both L and H chains. The orientation of the sulfate group is a critical factor, because C3 was shown experimentally to be specific to 6-sulfo sialyl Lewis X core 6 and not to its non-sulfated counterpart. This analysis also showed that the H chain CDR3 basic residue K99 is most likely important in binding the sugar because it is in close contact with the sulfate group (Fig. 5*B*). This critical interaction is not possible between C3 and 6-sulfo sialyl Lewis X core 1, given the flatter structure of this Ag. The second K96 makes hydrogen bonds to an oxygen atom, potentially conserved in core 6, in its sulfated counterpart and in core 1 and, hence, K96 is not critical in differentiating binding.

Discussion

In this study, we have developed an Ab repertoire for binding negatively charged carbohydrate targets by varying the composition of a small, fixed-length, H chain CDR3. Because the H chain CDR3 is thought to have the greatest influence on Ag binding, we focused on this CDR to gain binding properties to negatively charged carbohydrates. In naturally occurring human Abs, H chain CDR3 size varies from four to >35 residues (36). Previously, we compared this distribution with the length distribution of the H chain CDR3 repertoire cloned in the phage library described above (30) and used as backbone in this study. The H chain CDR3 length distribution (Fig. 2*A*) is similar to that found in the literature. We also observed from published sequences that the mean length of H chain CDR3s from Abs to charged carbohydrate is 6- to 8-aa residues. Because of their small size, short H chain CDR3s are likely to offer a binding pocket for self-charged carbohydrates. Repeated selections with several Ab libraries having nonimmune H chain CDR3s with median lengths of 13–15 amino acids and no bias toward basic residues showed that Abs against small charged carbohydrates are very infrequent in phage libraries containing only nonimmune V gene repertoires. Interestingly, there are publications indicating that part of the V gene repertoire is depleted during the development of B cells. In particular, H chain CDR3 repertoires that are hydrophobic, charged, or having a length of eight or fewer residues tend to be excluded in splenic follicular and bone marrow while being present in more immature repertoires (38), reinforcing the rationale for a synthetic repertoire to obtain specific anti-carbohydrate Abs.

Consensus H chain CDR3 amino acid sequences for glycosaminoglycan GAG recognition were determined in earlier studies to be XBBXBX and XBBBXXBX, where B is usually a basic residue and X is any amino acid residue (39). These consensus sequence elements show a high proportion of basic amino acids and may form key features for the recognition of anionic carbohydrates bound to proteins. Because not all GAG ligands fit this paradigm, we were interested in the proportion of basic residues found in H chain CDR3 sequences from known anti-carbohydrate Abs. The percentage of basic residues is 30–45% in H chain CDR3s of 6–9 aa. This percentage decreases strongly for longer H chain CDR3s. In addition, from alignment of H chain CDR3 sequences of 6–9 amino acids we determined positions where basic residues were most frequently found, allowing us to design the consensus sequence of the FAB-CCHO H chain CDR3 repertoire.

Some carbohydrates are negatively charged, bearing sulfated or phosphorylated groups (40). This electrostatic charge appears to modulate their function and interaction with other molecules such as protein ligands (41). We aimed to find Abs to those negatively charged carbohydrates because they contribute to various pathologic states. We first used HS to validate the library, because this target was successfully used by others with phage display (42); next, we were particularly interested in obtaining Abs against the newly described target 6-sulfo sialyl Lewis X core 6, part of the L-selectin ligand (31), because it is thought to be involved in early inflammation events such as lymphocyte rolling and tethering (43, 44). Abs that would block lymphocyte tethering might be an interesting therapeutic approach for the treatment of inflammatory diseases. Selections on both Ags with various nonimmune libraries (33) were not successful using selection and screening procedures identical with the ones described in this article's *Materials and Methods* (our unpublished results), emphasizing the challenging nature of these carbohydrate targets. The isolation of a specific Fab on phage binders from this biased library against two carbohydrate targets validates the library concept. As expected, the H chain CDR3 sequences were found to contain a high proportion of basic residues. In addition to the introduced basic residues, most of the H chain CDR3s have selected additional basic residues at one or two positions (R, K, and H), although all amino acid types were allowed by design. This observation confirms the necessity of basic properties for the binding of H chain CDR3 to the targeted negatively charged carbohydrates. H chain CDR3s similar to those selected from FAB-CCHO would be exceedingly rare in large diversity libraries such as FAB-310 (30).

Earlier studies using site-directed mutagenesis (45) or structural characterization of protein-heparin complexes by x-ray crystallography (46), nuclear magnetic resonance (47), or molecular modeling (48) clearly indicated that heparan binding sites are not composed exclusively of linear sequences. They can also include conformational epitopes comprising distant amino acids organized in a precise spatial orientation through the folding of the protein (49). In addition, differences in the number and location of amino acid residues play an important role in recognition of Ags of different sizes (50, 51). Small Ags are recognized by residues located centrally in the Ag binding site, whereas larger Ags can have additional contact residues in more peripheral regions. These observations would suggest that small carbohydrate-antibody interactions would require formation of a charged pocket as opposed to the flatter surface typical of protein-antibody interactions. Modeling of small carbohydrate moieties in complex with proteins was recently reported, and the role of positively charged residues was shown to be crucial in stabilizing the negative charge of the bound sugar molecule (52). Such charged pockets may be quite rare in Ab libraries having H chain CDR3s with naturally occurring length and sequence diversity, explaining earlier failure when selecting from a naive phage library. Because the pocket should not be too large and should accommodate only one charged sugar ring, biasing the L chain may not be needed or even desirable. In reported sequences, L chains do not show any recognizable motifs; they are diverse and belong to both κ and λ families, suggesting that the L chain does not play a crucial role in the selection of anti-carbohydrate Abs. The computational studies performed in this

study for the 6-sulfo sialyl Lewis X glycans (core 1 and core 6) confirmed the earlier findings that the Ab binding pockets required for these Ags are very likely to differ from each other because they are organized in different conformations. The sulfated group, protruding from the bent core 6, is accommodated in the groove formed by the CDRs of both L and H chains of the Fab Ab (C3), confirming our hypothesis of a small binding pocket. These modeling data bring additional understanding of the binding properties of Abs to small charged carbohydrates and reinforce our initial hypothesis regarding the nature of Ab repertoire.

In summary, we successfully selected specific Abs to both carbohydrate targets. We also have demonstrated that it is possible to engineer the H chain CDR3 of an Ab by biasing its amino acid content with basic residues, facilitating binding to small molecules such as charged carbohydrates. This study supports the general concept that it is possible to create synthetic Ab repertoires that are biased for the recognition of Ags of predefined size or nature.

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Disclosures

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