Isolation and Characterization of Phage-Displayed Single Chain Antibodies Recognizing Nonreducing Terminal Mannose Residues. 1. A New Strategy for Generation of Anti-Carbohydrate Antibodies†

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ABSTRACT: Phage-display technology is probably the best available strategy to produce antibodies directed against various carbohydrate moieties since conventional hybridoma technologies have yielded mostly low-affinity antibodies against a limited number of carbohydrate antigens. Because of difficulties in immobilization of carbohydrate antigens onto plastic plates, however, the same procedures used for protein antigens cannot be readily applied. We adapted phage-display technology to generate human single chain antibodies (scFvs) using neoglycolipids as antigens. This study describes the isolation and characterization of phage-displayed antibodies (phage Abs) that recognized nonreducing terminal mannose residues. We first constructed a phage Ab library with a large repertoire using CDR shuffling and VL/VH shuffling methods with unique vector constructs. The library was subjected to four rounds of panning against neoglycolipids synthesized from mannotriose (Man3) and dipalmitoylphosphatidylethanolamine (DPPE) by reductive amination. Of 672 clones screened by enzyme-linked immunosorbent assay (ELISA) using Man3-DPPE as an antigen, 25 positive clones encoding scFvs with unique amino acid sequences were isolated as candidates for phage Abs against Man3 residues. TLC-overlay assays and surface plasmon resonance analyses revealed that selected phage Abs bound to neoglycolipids bearing mannose residues at nonreducing termini. In addition, binding of the phage Ab to RNase B carrying high mannose type oligosaccharides but not to fetuin carrying complex type and *O*-linked oligosaccharides was confirmed. Furthermore, first round characterization of scFvs expressed from respective phages indicated good affinity and specificity for nonreducing terminal mannose residues. These results demonstrated the usefulness of this strategy in constructing human scFv against various carbohydrate antigens. Further studies on the purification and characterization of these scFvs are presented in an accompanying paper in this issue.

As generally known, immunization with carbohydrates often leads to a primary IgM response and no response in some cases because many carbohydrates are self-antigens (*1*, *2*). Phage display technology, which allows one to generate antibodies against self-antigens, is thus considered the best available strategy to produce antibodies directed against carbohydrate moieties. The possibility of isolating antibody fragments with specificity and affinity for carbohydrates by this technique is appealing since conventional hybridoma technologies have proven to be ineffective in producing monoclonal antibodies (mAbs)[1] directed against a variety of carbohydrate moieties. This technology has been used mostly to generate antibodies against proteins, whereas its use for carbohydrate antigens has been limited. Because of difficulties in immobilization of carbohydrate antigens alone onto plastic plates, the same procedures generally used for protein antigens cannot be readily applied to carbohydrate

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[1] Abbreviations: Fv, antibody variable domain; scFv, single-chain Fv; Ab, antibody; mAb, monoclonal Ab; VH, immunoglobulin heavy chain; VL, immunoglobulin light chain; BSA, bovine serum albumin; IGF, insulin-like growth factor; DPPE, dipalmitoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; Man1, mannose; Man2, mannobiose (Manα1−6Man); Man3, mannotriose [Manα1−6(Manα1−3)Man]; Man5, mannopentaose [Manα1−6(Manα1−3)-Manα1−6(Manα1−3)Man]; GN2Man3, GlcNAcβ1−2Manα1−6(GlcNAcβ1−2Manα1−3)Man; GN2, di-*N*-acetylchitobiose (GlcNAcβ1−4GlcNAc); FG, Fucα1-2Gal and LNT, lacto-*N*-tetraose (Galβ1−3GlcNAcβ1−3Galβ1−4Glc); IGF, insulin-like growth factor; HRP, horseradish peroxidase; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; TOF-SIMS, time of flight secondary ion mass spectrometry; FWR, framework region; PCR, polymerase chain reaction; IPTG, isopropyl-thio β-D-galactopyranoside.

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antigens. Previous studies thus utilized glycoproteins, heteroglycans, and carbohydrate−BSA conjugates as antigens to produce anti-carbohydrate single chain antibodies (scFvs) by phage display methods (*1*, *3*−*7*). Since the majority of anti-carbohydrate antibodies generated thus far have relatively low affinity, they are not suitable for in vivo diagnostics or therapy. Low affinity to carbohydrate antigens as observed is most likely due to the intrinsic nature of carbohydrates, which may not be easily overcome. There have been, however, attempts to overcome the low-affinity nature of anti-carbohydrate antibodies by increasing their multivalency, since efficient binding of anti-carbohydrate antibodies depends on multivalency (*7*−*11*).

The current research adapted phage-display technology to generate human scFvs using neoglycolipids as antigens (*12*−*14*). The major reasons we decided on pursuing this research were not only to utilize our resources of more than 30 different neoglycolipids readily available at our institute but also to test whether neoglycolipids can be used as antigens to produce antibodies against desired carbohydrate moieties. Carbohydrate antigens, which are soluble, can be immobilized via lipid anchors on plastic titer-plates for panning and screening by enzyme-linked immunosorbent assay (ELISA) to isolate candidate phage antibodies against respective carbohydrate antigens. If successful, the generation of antibodies of interest would become possible as long as neoglycolipids with desired carbohydrates can be prepared.

To first establish methodologies to build up a set of scFvs directed against various carbohydrate moieties, phage-display libraries representing over 10^{11} independent human scFvs were prepared, by protocols similar to those in a previous report (*15*), and screened. Man3-DPPE was synthesized from mannotriose (Man3) and dipalmitoylphosphatidylethanolamine (DPPE) by reductive amination as described (*12*−*14*). Characterization of positive phage clones by TLC-overlay assays and surface plasmon resonance analyses revealed that the phage antibody clones bound to neoglycolipids bearing mannose residues at nonreducing termini. In addition, binding of the phage Ab to RNase B carrying high mannose type oligosaccharides but not to fetuin carrying complex type and *O*-linked oligosaccharides was observed. Furthermore, first round characterization of scFvs expressed from respective phages indicated good affinity and specificity for nonreducing terminal mannose residues. This study thus demonstrated the validity of a strategy employing phage display technology in constructing human scFv against various carbohydrate antigens. In an accompanying paper in this issue, further studies on isolated anti-Man3 scFvs described here are presented.

MATERIALS AND METHODS

Materials. Escherichia coli strains used were the suppressor strain TG1 and the nonsuppressor strain TOP10F' from Invitrogen (Carlsbad, CA). Helper phages M13KO7 were from Amersham Biotech (UK). Bovine serum albumin (BSA), fetuin, asialo-fetuin, RNase A, RNase B, DPPE, and dipalmitoylphosphatidylcholine (DPPC) were purchased from Sigma-Aldrich (St. Louis, MO). Mannose (Man1), mannobiose (Man2; Manα1−6Man), mannotriose (Man3) with the structure Manα1−6(Manα1−3)Man, mannopentaose (Man5) with the structure Manα1−6(Manα1−3)Manα1−6(Manα1−3)Man, di-*N*-acetylchitobiose (GN2; GlcNAcβ1−4GlcNAc), lacto-*N*-tetraose (LNT; Galβ1−3GlcNAcβ1−3Galβ1−4Glc), Fucα1−2Gal (FG), GlcNAcβ1−2Manα1−6(GlcNAcβ1−2Manα1−3)Man (GN2Man3), Man1−BSA, and Man3−BSA were obtained from Dextra Laboratories (Reading, UK). Glucose-sp-biotin and Man3-sp-biotin were from GlycoTech (Gaithersburg, MD). ABTS/H_2O_2 was from Roche Diagnostics (Mannheim, Germany). Oxalic acid was from Wako Pure Chemical (Osaka, Japan). Anti-M13 antibodies were purchased from GE Healthcare Bio-Sciences (Piscataway, NJ) and Exalpha Biologicals (Watertown, MA). Anti-insulin-like growth factor (IGF)-I receptor scFv-displaying phage antibody was constructed from 1H7 mAb-derived scFv (*16*) using the GE Healthcare Bio-Science Expression Module/Recombinant Phage Antibody system.

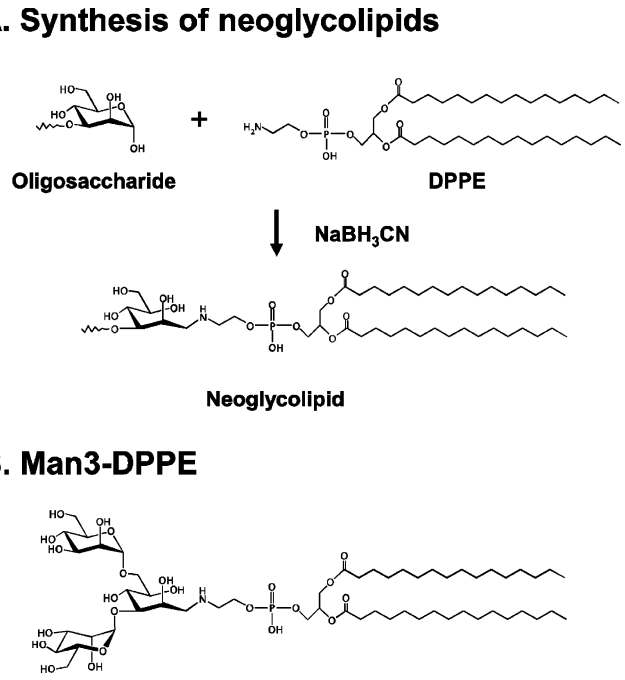

FIGURE 1: Schematic of synthesis of neoglycolipids (A) and Man3-DPPE (B): Neoglycolipids were synthesized from oligosaccharides, such as Man1, Man2, Man3, Man5, GN2, LNT, FG, and GN2Man3, whose structures are shown in Figure 2C, and dipalmitoylphosphatidylethanolamine (DPPE) by reductive amination as described in the methods.

Preparation of Neoglycolipids. Neoglycolipids were synthesized from carbohydrates (Man1, Man2, Man3, Man5, GN2, LNT, FG, or GN2Man3) and DPPE by reductive amination as described previously (*12*−*14*). A diagram of this reaction is shown in Figure 1A. Briefly, each carbohydrate (1−2 mg) in 60 μL of water was mixed with 9.4 mg of DPPE in 940 μL of chloroform/methanol (1:1, v/v) and 4 mg of sodium cyanoborohydride in 200 μL of methanol. The reaction was carried out at 80 °C for 5 h with occasional sonication. Neoglycolipids in the reaction mixtures were then purified by HPLC on a silica gel column (Shim-pack PREP-SIL, Shimadzu, Kyoto, Japan) and reversed-phase chromatography on a Bond Elut C18 cartridge column (Varian, Harbor City, CA) as described previously (*14*). The purity and structure of each neoglycolipid were confirmed with TLC and MALDI-TOF mass spectrometry.

Construction of Phage Libraries. cDNA, PCR Amplification, and Cloning of VH and VL Gene Repertoire To Construct Primary Phage Libraries. PCR templates used in

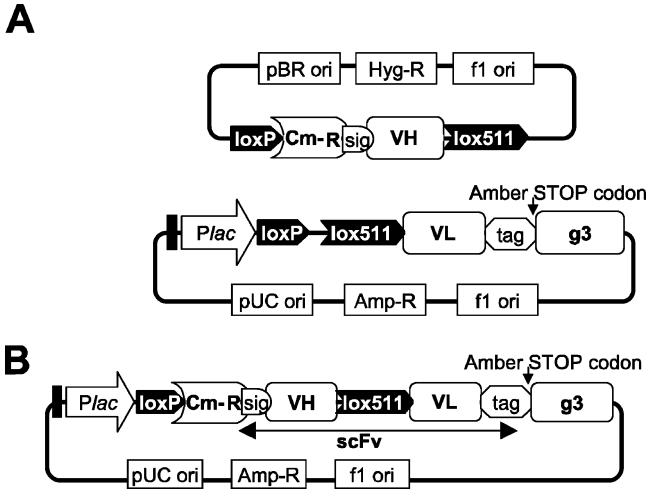

FIGURE 2: Construction of phage library displaying human scFvs. (A) Primary VH and VL phage libraries constructed using nonexpression vectors pVH-Hyg and pVL-Amp. The PCR products of the variable regions VH and VL were inserted into each of these two vectors. The pVH-Hyg and pVL-Amp vectors have neither promoters nor ribosomal binding sites. (B) ScFv expression vector. Recombination of the two identical lox sites between phagemids took place during culturing, resulting in the conversion of the pVL-Amp vector to an scFv expression vector. The symbols indicate sig, signal sequence; f1 ori, phage replication origin; plac, *lac* promoter; Hyg-R, hygromycin resistance; *Cm*-R, chloramphenicol resistance; tag, 6xHis- and E-tags; g3, M13 g3 protein; Amp-R, ampicillin resistance; pBR ori and pUC ori, plasmid replication origin derivative from pBR322 and pUC19, respectively.

this study were human spleen cDNA and human leukocyte cDNA, purchased from BioChain (Hayward, CA) and BD Biosciences (Palo Alto, CA), respectively. The framework region (FWR) 1 to FWR 3 and FWR3-CDR3-J segments were amplified with appropriate primer sets, designed according to previously published information (*17*), to cover the entire repertoire of immunoglobulin variable regions. Next, equal amounts of PCR products were mixed and ligated by PCR for assembly. This CDR shuffling was essential for diversity of the library. Briefly, reaction mixtures (50 μL) contained 0.5 μL of the cDNA solution, 25 pmol of back- and forward-primers, 200 μM dNTPs, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, pH 8.8, 2 mM $MgCl_2$, 100 μg/mL BSA and 1 unit of KOD plus DNA polymerase (Toyobo, Osaka, Japan). The first round PCR products purified by agarose gel electrophoresis were mixed in the same quantity as used for templates in the following PCR to amplify immunoglobulin variable regions. Resulting DNA fragments of the variable regions were purified and digested with appropriate restriction enzymes followed by insertion into pVH-Hyg or pVL-Amp vectors as illustrated in Figure 2A. After ligation, phagemid DNA was electroporated into *Escherichia coli* DH10B harboring F' plasmid derived from *E. coli* XL1-Blue and colonies were grown at 30 °C on agar plates containing 1% glucose and hygromycin (50 μg/mL) or ampicillin (50 μg/mL). The colonies were collected and converted to phages bearing VH or VL genes by superinfection with helper phage M13KO7 in 2× YT medium including 1% glucose at 30 °C overnight. Phages were obtained by centrifugation and precipitation with polyethylene glycol with a standard protocol.

Recombination and Generation of Secondary Phage scFv Libraries. The F' plasmid derived from *E. coli* XL1-Blue was introduced into *E. coli* NS3529, which expresses Cre-recombinase constitutively, to obtain *E. coli* NS3529/F'. NS3529/F' was infected with VH and VL phages at MOI = 50 and left for 1 h at 37 °C without shaking, and then superinfected with M13KO7 helper phages at MOI = 20. After samples were incubated for 1 h at 37 °C, ampicillin and kanamycin were added at 50 and 25 μg/mL, respectively. During overnight culture at 37 °C, Cre/lox recombination resulted in formation of scFv genes because of insertion of a VH fragment into the pVL-Amp vector in the NS3529/F' cells. Figure 2B shows the resulting phagemid, which contains His- and E-tags at the carboxyl terminal of the scFv gene. Growing XL1-Blue was infected with the recombinant phages obtained from the culture at MOI < 0.1 and left at 37 °C for 1 h. ScFv displaying phages on surface g3 protein were obtained from culture in 2× YT medium (glucose free) with 20 μg/mL chloramphenicol, 50 μg/mL ampicillin, and 25 μg/mL kanamycin at 25 °C overnight. Phages concentrated by polyethylene glycol precipitation were suspended and stored in SM buffer including 1% gelatin and 6% DMSO at −80 °C.

Phage Selection Methods. Panning Procedures. The library was subjected to four rounds of panning. Fifty and 16 wells of a 96-well plate were coated with 2 μg of Man3-DPPE/well and used for first and second panning, respectively. Five and 2 wells coated with 2 μg of Man3-DPPE/well were used for third and fourth panning, respectively. Phage selection was basically carried out according to previously published procedures (*18*) with some modifications. Briefly, coating of wells with Man3-DPPE was achieved by applying 50 μL of Man3-DPPE (40 μg/mL methanol) and drying the solvent at 37 °C, followed by incubation with 150 μL of Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl (TBS) and 3% BSA (blocking buffer) at 4 °C overnight. Wells were rinsed twice with 50 μL of 0.2% Tween 20/TBS (TBS-T), and once with 200 μL of TBS. Fifty microliters of phage suspension in TBS containing 0.1% Tween 20 and 1.4% BSA were added to the wells, which were then incubated for 60 min at 37 °C under continuous rotation. After wells were washed 3 times with 200 μL of TBS-T and twice with 200 μL of TBS, bound phages were eluted by addition of 50 μL of 100 mM triethylamine and incubation at 25 °C for 10 min and then neutralized by mixture with 100 μL of neutralizing solution (1 M Tris-HCl, pH 7.4/3% BSA/TBS = 2:1, v/v) in wells of the control plate, which had been treated with the blocking buffer. Bound phages were further eluted by addition of 50 μL of 100 mM triethylamine and incubation at 25 °C for 20 min and were recovered in the neutralization solution as described above. Eluted phages were used to infect 100 μL of logarithmic growing *E. coli* TG1 at 37 °C for 1 h. Infected bacteria, grown on LB agar plates (ϕ = 10 cm) containing 1 mM NaOH, 0.1% glucose, and carbenicillin (50 μg/mL) at 25 °C for 16 h, were scraped from the plates using a spreader after addition of 2 mL of LB-10 mM Tris-HCl, pH 7.5 (SBS) per plate. One of the 38 mL of this suspension was inoculated into 40 mL of SBS containing carbenicillin and grown with shaking at 37 °C for 2 h. Phages were rescued after addition of 40 μL containing 3.5×10^9 pfu of helper phages and incubation at 37 °C for 1 h without shaking, followed by addition of kanamycin (25 μg/mL)/ chloramphenicol (10 μg/mL) and incubation with rotation at 25 °C for 40 h. Phage

particles were concentrated by PEG-precipitation and dissolved in 400 μL of TBS, 400 μL of 3% BSA/TBS, and 40 μL of 10% Tween 20/TBS by incubation at 37 °C for 1 h. After centrifugation at 18000*g* for 5 min at 4 °C, 800 μL of phage suspension were recovered and used for second panning. Second, third, and fourth panning were carried out similar to first panning except for washing conditions. Bacteria picked from single colonies after four rounds of panning were grown in 50 μL of SBS/carbenicillin in 96 well plates at 37 °C for 1 h with rotation, to which 50 μL of helper phages were added and incubated at 37 °C for 1 h with rotation. After SBS/kanamycin/chloramphenicol mixture (50 μL/well) was added, phage suspensions were obtained by incubation at 25 °C for 16 h with rotation and after centrifugation at 200 g for 15 min at 4 °C. Fifty microliters of the supernatants were added to wells containing 100 μL of 3% BSA/TBS, incubated at 37 °C for 1 h, and kept at 4 °C.

Screening of Phage Clones Expressing Antibodies Directed against Man3-DPPE by ELISA. Analysis of phages binding to Man3-DPPE by ELISA was performed on bacterial supernatants containing phages. Wells of a 96-well plate were coated with 1 μg of Man3-DPPE/well as described above and blocked by incubation with 150 μL of 3% BSA/TBS at 4 °C overnight. Control plates were prepared as above without the antigen. Seventy-five microliters of phage suspensions were added to the wells and incubated at 37 °C for 1 h. The wells were washed 5 times with 200 μL of TBS-T. Bound phage antibodies were detected by incubation with horseradish peroxidase (HRP)-conjugated anti-M13 antibody at 37 °C for 1 h, after which they were washed 10 times with 200 μL of TBS-T and once with 200 μL of TBS. Peroxidase activity was detected by reaction with ABTS/H_2O_2 for 30 min and termination with 1% oxalic acid, and absorbance was measured with a BIO-RAD plate-reader at 415 nm.

Characterization of Phage Antibodies. Colony PCR and Determination of DNA Sequences. scFv genes were amplified from respective *E. coli* TG1 colonies infected with phages by PCR with a primer set (forward primer *Cm*-f: 5′-TGTGATGGCTTCCATGTCGGCAGAATGCT-3′, reverse primer g3-r: 5′-GCTAAACAACTTTCAACAGTCTATGCGGCAC-3′). After preheating the sample at 94 °C for 2 min, PCR was carried out with 35 cycles under conditions of denaturing at 94 °C for 20 s, annealing at 53 °C for 20 s, and extension at 68 °C for 1 min. After purification and confirmation in 2% agarose gel electrophoresis, the resulting scFv genes were subjected to DNA sequencing. DNA sequences of scFvs were determined using a 3730 DNA analyzer (Applied Biosystem, Foster City, CA).

Binding Assays on TLC Plates. Four hundred picomoles of neoglycolipids dissolved in chloroform/methanol/water (50:55:18, v/v) were dot-blotted onto TLC plates. Alternatively, mixtures containing 1 μg each of neoglycolipids were spotted and developed on aluminum-backed silica gel 60 high-performance TLC plates (10 cm-length; Merck, Darmstadt, Germany) with a solvent system of chloroform/methanol/water (60:35:8, v/v). After drying, these plates were soaked for 30 s in *n*-hexane containing 0.1% (w/v) Plexigum P28 (Sigma-Aldrich) and then blocked with 3% BSA/TBS at room temperature for 1 h followed by washing with TBS. The plates were then overlaid with phage antibody (10^{13} cfu/mL) at 4 °C overnight followed by overlay with mouse anti-M13 phage coat protein (p8) IgG (5000-fold dilution) at room temperature for 1 h. Phage antibodies binding to neoglycolipids were detected by a combination of HRP-conjugated anti-mouse IgG (MBL, Nagoya, Japan) and a chemiluminescent reagent (ECL Western blotting detection reagents, GE Healthcare Bio-Sciences) according to the manufacturer's instructions.

Determination of Carbohydrate Specificity of Phage Antibodies through Examination of their Reactivity to Natural Glycoproteins. Fetuin, asialo-fetuin, RNase A and B (5 μg/lane) were separated by SDS−polyacrylamide gel electrophoresis (10% gel) and blotted onto a PVDF membrane. The SDS−PAGE gel was stained with Coomassie Brilliant Blue, whereas reactivity of phage antibody to natural glycoproteins was examined by Western blotting with 5A3 phage antibody followed by HRP-conjugated anti-M13 phage antibody as described above.

Expression and Preparation of scFv Proteins. Each isolated phage clone was subjected to infection to *E. coli* TOP10F' containing chaperon/repressor vector. The infected TOP10F' cells with 1A4-2, 1A4-12, 1G4, 5A3, or a control anti-FLAG phage were cultured in 200 mL of 2× YT medium containing 50 μg/mL of ampicillin, 50 μg/mL of spectinomycin, and 1 mM isopropyl-thio β-D-galactopyranoside (IPTG) at 30 °C for 3 h. Supernatants, periplasmic fractions, or whole cell extracts of the TOP10F' cells were collected by differential centrifugation. Whole cell extracts, derived from the cells in 40 mL culture, were solubilized in 1 mL of 50 mM Tris-HCl, pH 8, containing 6 M guanidine-HCl, 5% glycerol, 0.5 mM PMSF, and 0.1 mM DTT and kept on ice for 1 h. After centrifugation at 18000*g* for 30 min, supernatants were dialyzed against a decreasing concentration of denaturant and finally against 10 mM HEPES, pH 7.4, containing 150 mM NaCl. Aggregates were removed by centrifugation and then the supernatants were subjected to SDS−PAGE/Western blotting analysis. A total protein amount of 18 μg/lane each, except for 1A4-12, which contained 33 μg/lane, was separated by SDS−PAGE (4−20% acrylamide gel) (Daiichi Pure Chemicals, Tokyo, Japan) under reducing conditions and then transferred to a PVDF membrane. After blocking with 3% BSA/PBS, scFv proteins expressed were detected with an HRP-conjugated anti-E-Tag antibody (GE Healthcare Bio-Sciences). The scFv protein preparations were used for SPR analysis.

Characterization of Phage Antibodies and scFv Proteins by Surface Plasmon Resonance (SPR). SPR analyses were done at 25 °C. All solutions were freshly prepared, degassed, and filtered through 0.22-μm pores. Binding properties of phage antibodies were determined by SPR using Biacore X (Pharmacia Biosensor). Used in this analysis were HPA sensor chips, composed of aliphatic chains covalently bound to a gold surface, and 10 mM HEPES, pH 7.4, containing 150 mM NaCl (HBS) as a running buffer. Immobilization of neoglycolipids on the HPA sensor chip surface was carried out as described before (*19*). Briefly, a lipid monolayer with carbohydrates on the surface was formed on the HPA sensor chip by adding neoglycolipids bearing liposomes. Before immobilization, the surface was cleaned by injection of nonionic detergent, 40 mM MEGA9 (Dojindo, Kumamoto, Japan), and 50% ethanol at a flow rate of 5 μL/min. DPPC or Man3-DPPE/DPPC (1:10, mol/mol) was then applied to

Table 1: Deduced Amino Acid Sequence Alignment of VH Chains of 15 Selected Clones

clones	FWR1	CDR1	FWR2	CDR2
1A4-2[a]	QVQLQQSGPPTGESPRRPSHSPVPSPGTVS	LTRVLLG	TGSGSPHLRGLEWLG	RTYYR-SKWYNDYAVSVKS
1A4-12[a]	QVQLQQSGPRLVKPSQTLSLTCAISGDSVS	NKSAAWN	WIRQSPS-RGLEWLG	RTYYR-SKWYNDYAVSVKS
1A7	QVTLKESGPALVKPTQTLTLTCTFSDFSLT	TSGMRVS	WIRQPPG-KALEWLA	RIDW---DDDKFYSTSLKT
1G4[a]	QVQLVQSGGGLVKPGGSLRLSCAASGFPFS	N---AWMS	WVRQAPG-KGLEWVG	RITSKTDGGTADYAAPVKD
4C8	QVQLQQWGAGILKPSETLSLTCAIYG-TYI	GG-YHWT	WIRRPPG-GGLEWIG	EIEN---TGNIKYNPSLRN
4D8	QVTLKESGPTLVKPTQTLTLTCTFSGFSLS	TSGMGVG	WIRQPPG-KALEWLA	VIYW---DDDKRYSPSLKT
4H5	QVQLQQWGAGLLKPSETLSLTCAVSGYGSL	SG-YYWS	WIRQPPG-KGLEWIG	EINR---GGSTKYNPSLKS
5A3[a]	QVQLQQSGPGLVKASETLSLTCAVSGYSIS	SG-YYWG	WIRQPPG-KGLEWIG	NIYH---SGNTYYNPSLKS
5C10[a]	QVQLVESGSEVKKPGASVKVSCKASGYTFT	D---YYIH	WVRQAPG-QGLEWMG	WLNP---NGGSTNYPQKFQG
6H10	QVQLQESGGGLVQPGGSLRLSCTASGFTFS	N---HWMH	WVRQTPG-KGLVWVS	RIRG---DAGDRNYADSVKG
7A8	QVQLVESGGGVVQPGRSLSLSCTTSGFTFS	D---YYMH	WVRQAPG-KGLEWVA	FISS---DGGNKYYLDSVKG
7B2	QVTLKESGPALVKSTQTLTLTCNLSGFSVN	TRGVGVG	WLRQPPG-KALEWLA	MIYW---DDDRRYSPYVKD
7C1	QVQLVETGGGLVQPGGSLRLSCAASGLSFS	N---YAMS	WVRQAPG-KGLEWVS	TISN---SGDNTLYPDSVKG
7C7	QVQLVQSGGGLVKPGGSLRLSCAATGFSFR	H---YGMN	WVRQAPG-KGLEWVS	YISD---SSSHIYYADSVRG
7G4	QVQLQQWGAGLVKPSETLSLTCAIYG-GSF	SS−YYWN	WIRQPPG-KGLEWIG	EINH---SGGTNYNPSLKS

clones	FWR3	CDR3	FWR4
1A4−2[a]	RITINPDTSKNQFSLQLKFVTPEDTALYYCSR	ERGTSSLYN---------WFDP	WGQGTLVTVSS
1A4−12[a]	RITINPDTSKNQFSLQLKFVTPEDTALYYCSR	ERGTSSLYN---------WFDP	WGQGTLVTVSS
1A7	RLTISKDTSKNQVVLTLTNMDPVDTATYYCAR	-----DIS---------KAHDY	WGQGTLVTVSS
1G4[a]	RFAFSRDDSKTTLYLQMSSLKTEDTAVYYCTT	-DLGYYGLG--------SLPDY	WGQGTLVTVSS
4C8	RVTISSDTSKRQVFLSLTSLTTADTAVYYCAK	GGGKEVTVLGLPVGWDYYGMDI	WGPGTTVTVSS
4D8	RLTITKDTSKNQVVLTMTNMDPVDTATYYCAQ	-----QRW---------SNFDY	WGQGILVTVSS
4H5	RITISVDTSKNQFSLNLNSVTAADTAVYYCAR	SGLYGTTPS---------TFDH	WGQGTLVTVSS
5A3[a]	RVTISRDTSKNHFSLKLKSVTAADTAVYYCAR	ADPGYMIFN---------WFDP	WGQGTLVTVSS
5C10[a]	CVTMTRDTATSTAFMELSGLKSDDTALYYCAR	AIKADSGFS--------YLLDV	WGQGTTVTVSS
6H10	RFTISRDNSQNTLFLQMNSLTAEDTAVYYCAR	--DVVLGSG--------ST-DQ	WGQGALVTVSS
7A8	RFTVSRDNSKNTLSLQMNSLKTEDTALYYCAG	-VPAVTSAW--------YF-DL	WGRGTLVTVSS
7B2	RISLTRDISKTQVVLTMTNMEPADTGTYYCAR	-----DYNP--------RSFDH	WGQGTLVTVSS
7C1	RFTISRDNSRDTLYLHLSGLGAEDTAIYYCA-	--NAPPYTH--------YF-DQ	WGQGTLVTVSS
7C7	RFSISRDNAKDSLFLEVNSLRPGDTAAYYCAR	-DTGTYARM--------SGMDV	WGQGTTVTVSS
7G4	RVTISLDMSKNHFSLNLNSVTAADTAVYYCAR	DAGNRGSTD---------WFDP	WGQGTLVTVSS

[a] Clones characterized in this study.

the sensor chip surface at a low flow rate of 2 μL/min. To remove multilamellar structures from the lipid surface, 5 μL of 10 mM NaOH was injected at a flow rate of 5 μL/min. Phage antibody samples that had been dialyzed against HBS were allowed to flow for 10 min over the surface of the chips at a flow rate of 5 μL/min. To examine the effect of competitive inhibitors on SPR, phage antibody samples were mixed with 10 or 100 mM D-mannose or α-methyl-D-mannopyranoside (Nacalai Tesque, Kyoto, Japan), and then incubated at 4 °C for 1 h.

SPR analyses of scFv protein samples were performed at a flow rate of 5 μL/min with BIAcore 3000 using the Sensor chip HPA or the Sensor chip SA (Streptavidin). Immobilization of DPPC, Man3-DPPE/DPPC (1:10, mol/mol), Man2-DPPE/DPPC (1:10, mol/mol), Man5-DPPE/DPPC (1:10, mol/mol) to HPA sensor chips was performed as described above. The amount of immobilized ligands were calculated from the molar ratio of the DPPE conjugates included in the lipid layer, which indicated that DPPC, Man2-, Man3-, and Man5-DPPE were 0.43, 0.31, 0.48, and 0.30 pmol, respectively. HBS containing 0.005% Tween 20 (HBS-T) was used as a running buffer when SA sensor chips were used. SA sensor chip surface was activated with three consecutive 1-min injections of activating buffer (1 M NaCl containing 50 mM NaOH) prior to immobilization of biotinylated ligands. Glucose-sp-biotin and Man3-sp-biotin (0.1 mM) were diluted with HBS-T to 0.05 μM, 10 μL of which were injected manually at a flow rate of 2 μL/min until RU of 100−150 is achieved. Unlike neoglycolipids used above, these ligands contained a spacer (sp) consisting of $-O(CH_2)_3NHCO(CH_2)_5NH-$. The amounts of immobilized biotinylated carbohydrates were estimated to be 0.25 and 0.23 pmol, respectively. The scFv samples that had been dialyzed against HBS-T were injected at a flow rate of 5 μL/min for 4 min over the surface of the chips. After one sample was assayed, the sensor chip surfaces were regenerated by treating with 10 mM glycine-HCl, pH 1.5, for 1 min, followed by washing with a continuous flow of HBS-T before the next sample was injected.

RESULTS

Screening of scFv-Displaying Phages Directed against Man3-DPPE. The phage displayed human scFv library was subjected to four rounds of panning against Man3-DPPE, the structure of which is schematically presented in Figure 1B. Two μg of Man3-DPPE/well were used for panning as described in the Methods. Of 672 clones screened by ELISA using Man3-DPPE (1 μg/well) as an antigen, over 40 clones showed ELISA positivity with S/N of >2. Of those, 25 positive clones encoding scFvs were selected as candidates for phage antibodies directed against the Man3 moiety. DNA sequencing of scFv regions of those phage clones revealed that all the clones thus far analyzed are different. Amino acid sequence alignments of VH and VL of 15 selected clones are presented in Tables 1 and 2, respectively. Of those, 1A4-2, 1A4-12, 1G4, 5A3, and 5C10 clones were used for further characterization in this study. It should be noted, however, that 1A4-2 and 1A4-12 are subclones derived from the same original clone, 1A4.

Carbohydrate Specificity of Phage Clones. To quickly identify which phage antibodies have specificity and high

Table 2: Deduced Amino Acid Sequence Alignment of VL Chains of 15 Selected Clones

clones	FWR1	CDR1	FWR2	CDR2
1A4-2[a]	EIVLTQSPDSLAVSLGERASINC	KSSQSVSHSFDIANYLA	WYQQRPGQPPKLLIY	WASTRES
1A4-12[a]	EIVLTQSPDSLAVSLGERASINC	KSSQSVSHSFDIANYLA	WYQQRPGQPPKLLIY	WASTRES
1A7	ETTLTQSPATLSVSLGDRASLSC	RASQSVGTK------LA	WYQHKPGQAPRLLFY	GASTRAP
1G4[a]	DIRVTQSPSSLSASVGDRVTITC	RASQGINSA------VA	WYQQKLGKGPTLLIY	DASTLES
4C8	DIQMTQSPSSLSASVGDRVTITC	RASQTLTTY------LN	WYQQRPGKAPKLLIY	LASTLDS
4D8	EIVLTQSPATLSLSPGERATLSC	RASQSVSGN------LA	WYQQKPGQAPRLLIF	ATSTRAT
4H5	QSVVTQPP-SVSGTPGSWVTISC	TGTRSNIGAPKG---VH	WYQHVPGTAPKLLIY	DSNSRSS
5A3[a]	NFMLTQPP-SASGTPGQRVTISC	SGSSSNIGNND----VY	WYQQFPGTAPKLLIY	RNNQRPS
5C10[a]	QPVLTQPP-SASGTPGQRVTISC	SGSSSNIGSNY----VY	WYQQLPGTAPKLLIY	RINQRPS
6H10	DIVMTQTPDSLAVSLGERATINC	KSSQSVLYSSNSKNYLA	WYQQKPGQPPKLLIY	WASTRES
7A8	DIQLTQSPDSLAVSLGERATINC	KSSQSVLYSSNNKNYLA	WYQQKPGQPPKLLIY	WASTRES
7B2	NFMLTQPP-SASGTPGQTVSISC	SGSDSNIGTYT----VN	WYQQLPGTAPKLLIY	SNDQRPS
7C1	DIRLTQSPDSLAVSLGERATINC	KSSQSILYRYNSNNYLA	WYQQKPGQPPKLLIY	WASTRES
7C7	DIVLTQTPSSLSASVGDRVTITC	RASQSISDW------LA	WYQQKPGKAPKLLIY	KASSLES
7G4	DIRMTQSPSSLSASVGDRVTITC	RASQGIRND------LG	WYQQRPGKAPNLLIY	AASTLQS

clones	FWR3	CDR3	FWR4
1A4-2[a]	GVPDRFSGSGSGTDFTLTISSLQAEDVAVYYC	QQYYSNPF---T	FGGGTKVEIK
1A4-12[a]	GVPDRFSGSGSGTDFTLTISSLQAEDVAVYYC	QQYYSNPL---T	FGGGTKVEIK
1A7	SVPARFSGSGSGTEFTLTISSLQSEDFAVYYC	QQYINWPR---T	FGQGTKVEIK
1G4[a]	GVSSRFSGSGSGTDFTLTISSLQPEDFATYYC	QHFNSHPI---T	FGQGTRLEIK
4C8	GVPSRFSGSASGTTFTLTITSLQPDDVATYYC	QQSYNIPR---T	FGQGTKVEIK
4D8	GIPVRFSGRGSGTDFTLTISRLEPEDFAVYYC	QQYGGSPRL---T	FGGGTKVEIK
4H5	GVPARFSGSKSGTSGSLAIAGLQPEDEADYYC	QSLDTNLN-AWV	FGGGTKLTVL
5A3[a]	GVPDRFSGSKSGTSASLAISGLRSEDEADYYC	AAWDDSL----I	FGGGTKLTVL
5C10[a]	GVRDRFSGSKSGTSASLAISGLRSEDEADYYC	SAWDDSLS-GWV	FGGGTKLTVL
6H10	GVPDRFSGSGSGTDFTLTISSLQAEDVAAYYC	QQYSTTPF---T	FGGGTKLEIK
7A8	GVPDRFSGSGSGTDFTLTISSLQVEDVAVYYC	QQYYTYSL---T	FGGGTKLEIK
7B2	GVPDRFSGSKSGTSASLAISGLQSEDEADYHC	AAWDANLYGHVV	FGGGTKVEIK
7C1	GVPDRFSGSGSGTDFTLTISSLQAEDVAVYYC	QQYYSTPQ---T	FGQGTKVEIK
7C7	GVPSRFSGSGSGTEFTLTISSLQPDDFATYYC	QQYNSY-F---T	FGPGTKVDIK
7G4	GVPSRFSGSGSGTDFTLTISSLQPEDSATYYC	LQDYAYPR---T	FGQGTKVEIK

[a] Clones characterized in this study.

affinity for the antigen used, it was necessary to first establish methods suitable for characterizing scFv-presenting phages as phage antibodies. Although expression, isolation, and characterization of scFvs proteins are ultimately required, expression and purification of scFvs proteins are labor-intensive and time-consuming, and yet primary information on phage clones is essential to selecting candidate clones for further characterization (see an accompanying paper). Two methods have been established for this purpose. In brief, a set of neoglycolipids was either chromatographed (Figure 3A) or individually spotted (Figure 3B) on TLC plates, and then phage antibodies were overlaid on the plates to allow their binding to the neoglycolipids as described in the methods. In Figure 3A, Man1-, Man2-, Man3-, and Man5-DPPE bands separated by chromatography were stained with primulin (P) and orcinol (O) reagents to identify the constituents of corresponding neoglycolipids, that is, lipids and carbohydrates, respectively. These experiments not only revealed that phage antibodies show specificity to Man3 as well as to Man2 and Man5 to a lesser extent (Figure 3A,B) but also demonstrated that the phage antibodies examined bound to mannose residues at nonreducing termini but not to the lipid portion of neoglycolipids since binding to GN2Man3 and DPPE or DPPC was not observed. The structures of neoglycolipids used in these experiments are illustrated in Figure 3C.

Binding of Phage Antibodies to Natural Glycoproteins. Since phage antibodies isolated by using Man3-DPPE as a target antigen were shown to have specificity to mannose residues at nonreducing termini of neoglycolipids, this research next examined whether they can bind to the mannose residues at nonreducing termini of glycoproteins. Figure 4 clearly shows that 5A3 phage antibody bound to RNase B carrying high mannose type oligosaccharides but not to non-glycosylated RNase A or to fetuin carrying complex type and *O*-linked oligosaccharides.

SPR Analyses of Man3-Specific scFv-Presenting Phage Antibodies. SPR was qualitatively used to observe the binding kinetics of phage antibodies. As shown in Figure 5, sensorgrams showing the binding of the 5A3, 1A4-2, and 1G4 phages to Man3-DPPE (the sensorgrams of the phage antibody binding to Man3-DPPE were minus binding to DPPC alone) revealed clear binding kinetics of all three phage antibodies examined (sensorgrams A−C), which confirmed the Man3-specificity of those phage antibodies. This conclusion was further supported by the fact that (i) the binding of phage antibody was competitively inhibited by mannose (sensorgram E) or α-methyl-D-mannoside (data not shown) and (ii) a control phage antibody displays human IGF-I receptor specific scFv did not bind to Man3-DPPE (sensorgram D).

Expression and Characterization of Man3-Specific scFvs as Soluble Proteins. scFv proteins were expressed as soluble proteins for further analysis with regard to their carbohydrate specificity. When the presence of scFv proteins was compared among supernatants, periplasmic fractions, and whole cell extracts by SDS−PAGE and subsequent immunoblotting with anti-E-tag antibody, whole cell extract fractions contained the most scFv proteins expressed (data not shown). The whole cell fractions as shown in Figure 6 were thus used for SPR analyses. Although the fractions used were

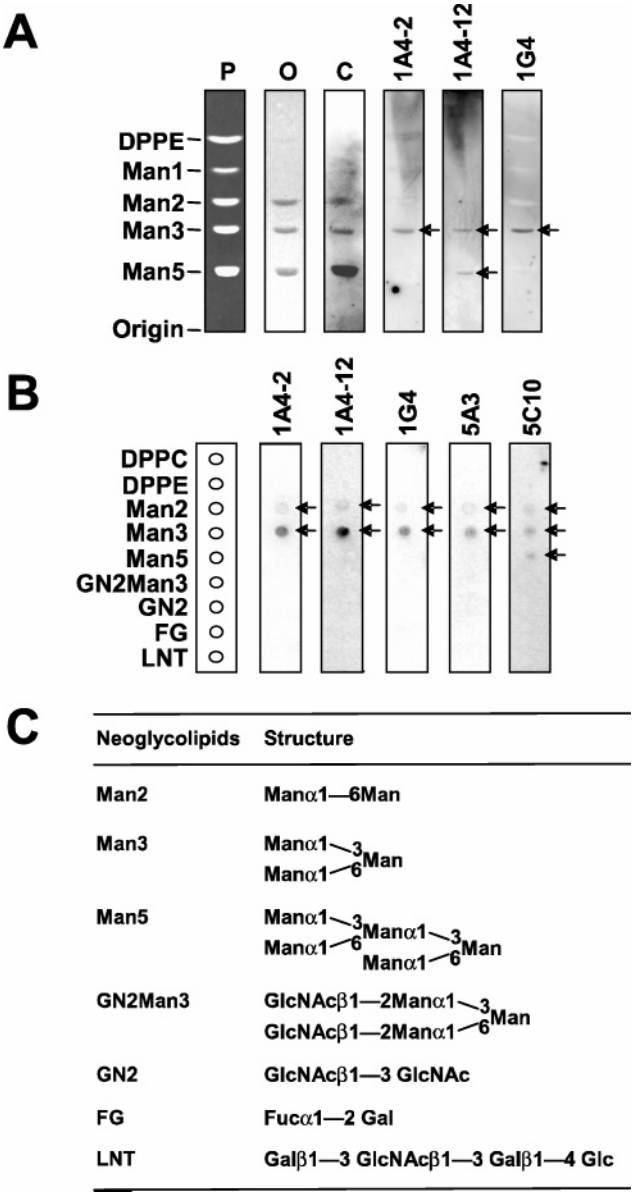

FIGURE 3: Carbohydrate specificity of phage antibodies screened by panning and ELISA with Man3-DPPE. (A) After mixtures of neoglycolipids were chromatographed on TLC plates, each plate was overlaid with anti-Man3 phage antibodies, 1A4-2, 1A4-12, or 1G4, followed by detection of bound phages as described in the methods. In lanes P and O, lipid and oligosaccharide moieties of neoglycolipids were detected with primulin reagent (lane P) and orcinol reagent (lane O), respectively. In lane C, the TLC plate was overlaid with HRP-conjugated Concanavalin A as a positive control. Arrows indicate where phages were bound. (B) After various neoglycolipids were dot-blotted on TLC plates, each plate was overlaid with anti-Man3 phage antibodies, 1A4-2, 1A4-12, 1G4, 5A3, or 5C10, followed by detection of phages as described in the methods. (C) The neoglycolipids used in this study are summarized as follows. Man1, mannose; Man2, mannobiose (Manα1−6Man); Man3, mannotriose [Manα1−6(Manα1−3)Man]; Man5, mannopentaose [Manα1−6(Manα1−3)Manα1−6(Manα1−3)Man]; GN2Man3, GlcNAcβ1−2Manα1−6(GlcNAcβ1-2Manα1−3)Man; GN2, di-*N*-acetylchitobiose (GlcNAcβ1−4GlcNAc); FG, Fucα1−2Gal and LNT, lacto-*N*-tetraose (Galβ1−3GlcNAcβ1−3Galβ1−4Glc).

impure (Figure 6A), they contained E-tag reactive scFv proteins (Figure 6B).

SPR Analysis of Man3-Specific scFvs as Soluble Proteins. scFv protein preparations as shown in Figure 6 were used to analyze the binding kinetics of Man3-specific scFv proteins by SPR using immobilized neoglycolipids or biotinylated carbohydrates. Sensorgrams shown in Figure 7A are binding kinetics of scFv proteins derived from 1A4-12, 1G4, and 5A3 scFvs to Man3-DPPE (the sensorgram of each scFv

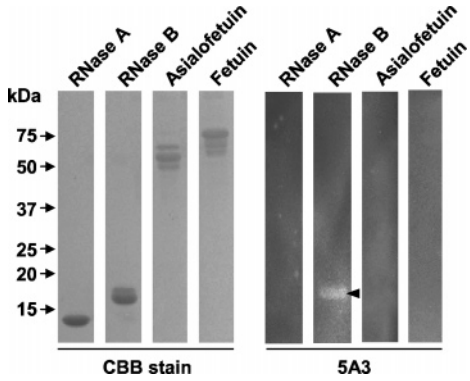

FIGURE 4: Binding of phage antibodies to natural glycoproteins. Reactivity of 5A3 phage antibody to natural glycoproteins was examined by Western blotting of fetuin, asialofetuin, RNase A, and RNase B as described in the methods. The protein bands were stained with Coomassie Brilliant Blue. Western blotting with 5A3 phage antibody followed by anti-M13 phage antibody-HRP was carried out to identify to which band the phage antibody bound.

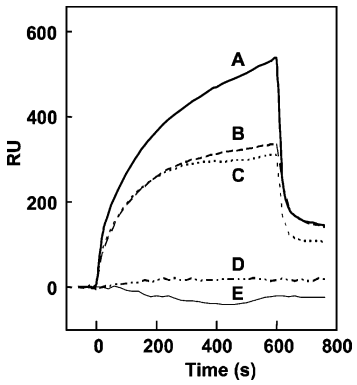

FIGURE 5: SPR analysis of Man3-specific scFv-presenting phage antibodies. Phage antibodies, 5A3 (A), 1A4−2 (B), and 1G4 (C), were subjected to SPR analysis with Man3-DPPE. Anti-IGF-I receptor scFv-phage was used as a negative control (D). A competitive inhibition test was performed with 5A3 phage antibody in the presence of 100 mM mannose (E). The sensorgrams shown are specific binding to Man3, which were obtained by subtracting the sensorgrams on DPPC from those obtained on specific Man3-DPPE.

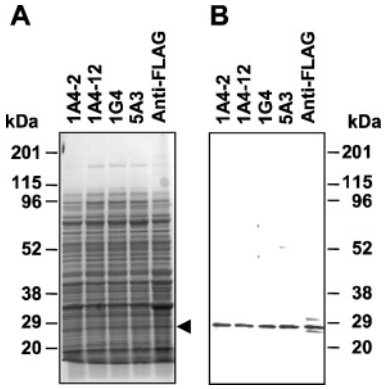

FIGURE 6: Expression of Man3-specific scFvs as soluble proteins. (A) SDS−PAGE of scFvs proteins (1A4-2, 1A4-12, 1G4, and 5A3) as well as anti-FLAG scFv protein, as a control, expressed in *E. coli* TOP10F' was carried out as described in the methods. The gel was stained with Coomassie Brilliant Blue. (B) Western blotting of scFv proteins with an anti-E-tag antibody was performed as described in the methods.

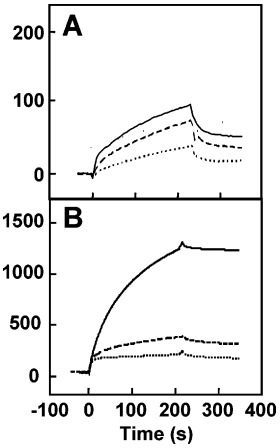

FIGURE 7: SPR analysis of Man3-specific scFvs proteins on Man3-bearing antigen immobilized chip. Experiments were carried out as described in the methods. (A) Binding of scFv proteins to Man3-DPPE is displayed by subtracting the sensorgrams with respect to DPPC. Solid line, 1A4-12; dashed line, 5A3; dotted line, 1G4. (B) Binding of scFv proteins to Man3-sp-biotin is displayed by subtracting the sensorgrams with respect to Glc-sp-biotin. Solid line, 1A4-12; dashed line, 5A3; dotted line, FLAG.

binding to Man3-DPPE was subtracted by that of DPPC alone). These scFv proteins showed similar relative kinetics of binding to Man3- and Man5-DPPE (data not shown). Figure 7B shows binding kinetics of 1A4-2, 5A3, and FLAG scFvs to Man3-biotin (the sensorgram of each scFv binding to Man3-sp-biotin was subtracted by that of glucose-sp-biotin alone, to which none of the three scFvs appeared to bind; data not shown). The relative binding affinity of those scFv proteins observed here, however, needs to be determined by SPR analysis after complete purification. Further studies on expression, purification, and binding kinetics of these scFv proteins are described in an accompanying paper in this issue.

DISCUSSION

This paper described isolation and characterization of phage-displayed scFvs recognizing nonreducing mannose residues by phage display technology in combination with the use of a neoglycolipid as an antigen. The isolated scFvs, as both phage antibodies and soluble proteins, were shown to bind to neoglycolipids bearing mannose residues at nonreducing termini as well as mannose residues conjugated to proteins, which demonstrated the validity of this strategy for the generation of carbohydrate-specific human scFvs. To the extent known, this is the first report of isolation of phage displayed scFvs directed against carbohydrates immobilized via lipid anchors on plastic titer-plates. In essence, generation of anti-carbohydrate antibodies of interest is now possible as long as neoglycolipids with desired carbohydrates can be prepared.

Although a set of Man3-specific scFvs was obtained, optimizing conditions for successful panning and screening with glycolipids was extremely difficult. Why this phage display technology/neoglycolipid strategy has not been previously reported may be due to the following reasons. First, carbohydrates immobilized via lipid anchors can be easily washed away during extensive washing to reduce nonspecific phage backgrounds if one uses washing procedures that are normally designed for protein-based antigens. Thus, determining the washing conditions that would minimize nonspecific binding of phages without washing away the antigens is obviously the most critical step in establishing a phage display/neoglycolipid strategy. To circumvent this problem, other investigators previously used glycoproteins, heteroglycans, and carbohydrate−BSA conjugates as antigens to screen anti-carbohydrate antibodies (scFvs) (*1*, *3*−*7*). The major reasons that we chose to pursue this research were as mentioned in the introduction to utilize our resources, that is, more than 30 different neoglycolipids and phage-display libraries representing a large repertoire of human scFvs, which were readily available at our Glycotechnology Institute and Keio University, respectively, to isolate candidate phage antibodies against respective carbohydrate antigens.

Second, excessive coating of Man3-DPPE in wells for panning and screening may have helped isolate anti-carbohydrate phage antibodies. Man3-DPPE was immobilized on plastic wells at an amount of ≥ 1 μg ($\sim$770 pmol)/well. Since glycolipids consist of carbohydrate and lipid portions, binding of phage antibodies to the lipid portion is inevitable. The major task associated with the phage display/neoglycolipid strategy is thus how to eliminate nonspecific binding of phage antibodies to lipids. The excess amount used apparently resulted in nonuniform coating of Man3-DPPE on the plastic surface. Antigen epitopes to which phage antibodies bound were assumed to be aggregates of the glycolipids. This supposition was supported by the following observations. TOF-SIMS spectra of Man3-DPPE coated wells indicated that when wells were coated at an amount of 1 μg/well, Man3-DPPE densely localized within a $\sim$500 μm diameter from the center of the wells, whereas when one-tenth of that amount was used, the same trend was observed although it was less significant (unpublished observation). This is consistent with the current observation that when one-tenth of the antigen (0.1 μg/well) was coated for ELISA, Man3-specific scFvs or phage antibodies that had been isolated using Man3-DPPE at the level of 1 μg/well did not seem to bind to the antigen (unpublished observation). In addition, dilution of antigens with lipids to sparsely coat wells with neoglycolipids apparently resulted in far greater recovery of lipid-specific scFvs as compared to high amounts of neoglycolipids used as described in the original protocol (unpublished data).

It is common knowledge that monoclonal antibodies directed against carbohydrates produced in vivo mostly belong to the IgM class and therefore exhibit low affinity. Since this may be due to the intrinsic problems associated with carbohydrate antigens, which are self-antigens, in vitro production of scFvs or Fabs by phage display technology is an attractive alternative that can overcome this problem. To date, however, previously reported carbohydrate-specific scFvs showed their affinity (K_A) in a range of 10^{-7}−10^{-5} (*2*, *5*, *6*), which is better than those of anti-carbohydrate mAbs, but is still too low to be useful. The repertoire of phage libraries used previously was in a range of 10^6−10^9 (*5*, *6*), which may not have been large enough to represent carbohydrate specific antibodies with high affinity.

This study was conducted to test whether improving the diversity of scFv sequences would help obtain a variety of anti-carbohydrate scFvs with high affinity. During the past decade, more efficient techniques have been developed to build larger libraries of antibody fragments using sophisti-

cated in vivo recombination methods (*20*) or laborious cloning procedures (*21*, *22*). Such large libraries have yielded a greater number of human antibodies with higher affinity. To generate a scFv library with a large repertoire ($>10^{11}$ clones), a combination of PCR-mediated "CDR shuffling" and Cre-recombinase-mediated "VH/VL shuffling" was introduced (unpublished experiments). This strategy utilizes two independent primary phage libraries for CDR-shuffled VH or VL genes that are unable to express VH or VL themselves because the pVH-Hyg vector lacks a promoter (promoter-less) and the pVL-Amp vector lacks a ribosomal binding site (SD-less). Expression of scFv-displaying phages can be achieved only after both genes are put together through recombination (Figure 2). In addition, various conditions have been introduced to optimize amplification of scFv-displaying phages without sacrificing substantial diversity. The use of a scFv library with a large repertoire ($>10^{11}$ clones) may have been a critical aspect of the successful isolation of Man3-specific scFvs through use of the phage display/neoglycolipid strategy.

Two major aspects described above, the presentation of antigens and the size of the phage library, must have contributed to successful isolation of Man3-specific scFvs, as evidenced by TLC-overlay assays and SPR analyses, which revealed that selected phage Abs bound to neoglycolipids bearing mannose residues at nonreducing termini. In addition, results confirmed that the 5A3 phage Ab bound to RNase B carrying high mannose type oligosaccharides but not to fetuin carrying complex type and *O*-linked oligosaccharides and that the first round characterization of those scFvs expressed from respective phages exhibited good affinity and specificity for nonreducing mannose residues.

As far as affinity evaluation by SPR is concerned, the results shown in Figures 5 and 7 are considered to be the only preliminary data to primarily observe the binding kinetics of either Man3-specific scFv-presenting phage antibodies (Figure 5) or scFv proteins to immobilized Man3-antigens (Figure 7). It is interesting to note that the shape of the subtracted sensorgrams in Figure 5 show heterogeneous binding nature, indicating a possibility that scFv aggregates displayed on phages may bind with higher affinity than monomeric scFvs. Thus, although characterization of scFv-displaying phage antibodies as well as scFv protein preparations by various methods indicated good affinity and specificity for nonreducing terminal mannose residues, purification of expressed scFv proteins is required for more accurate evaluation of their specificity and further determination of kinetics. Expression and purification of scFv proteins as well as scFv-Fc as previously reported (*16*) are now completed in the laboratory (see accompanying manuscript in this issue).

In summary, the phage display/neoglycolipid strategy provides a simple introductory procedure for isolating scFvs directed against carbohydrates of interest. This technology should possibly lead to developments of future therapeutics since an improvement of affinity, if necessary, can be achieved by affinity maturation procedures and/or since constructions of human IgG proteins can readily be prepared.

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