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Selection, Expression and Purification of Single-Chain Fv Antibodies Against Non-structural Proteins of GB Virus C

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Abstract

The GB virus C (GBV-C) exists worldwide, and GBV-C infection is common among healthy blood donors as well as among immunocompromised individuals. It has been shown that GBV-C may have a beneficial impact on HIV disease progression. The results of recent studies also demonstrated that GBV-C may increase survival of Ebola virus patients. However, there is no commercially available GBV-C detection system which would identify past infection. The aim of this study was to select and express functional single-chain, variable fragment (scFv) antibodies against GBV-C non-structural proteins. In order to achieve this aim, we expressed GBV-C non-structural proteins in E. coli and constructed a naïve scFv library. This study is the first to identify recombinant antibodies against GBV-C by using phage display. Six scFv that showed the highest affinity score by ELISA were successfully expressed. These recombinant scFv antibodies may provide a valuable tool for further study of GBV-C and contribute to the improvement of a rapid and accurate test for identification of GBV-C infection.

Keywords: GBV-C; scFv expression; ELISA

Introduction

GB virus C (GBV-C, also known as hepatitis G) is a single-stranded, positive sense RNA virus recently classified as a member of *Pegivirus* within the family Flaviviridae [1]. GBV-C is a common virus and spread worldwide. Prevalence of GBV-C infection among blood donors varies from 0.9% to 7% in Europe and North America [2]. There are higher GBV-C infection rates among immunocompromised individuals. Depending on the population, GBV-C co-infection with HCV varies from 11% to 43.6% [3-7], from 14% to 36% with HIV-1 [8], and from 30 to 36% with HIV/HCV [9,10].

A number of studies have independently shown a beneficial effect of GBV-C infection on HIV disease progression and better medical treatment outcomes [11]. Despite these advantageous impacts of GBV-C, the results obtained from several studies have shown an association between GBV-C viremia and non-Hodgkin lymphoma (NHL) [12-14]. The subjects with detected GBV-C RNA had higher risk of NHL than those who tested negative for GBV-C. Based on transfusion frequency, at least 7,000 people worldwide each day receive blood products contaminated with GBV-C [15]. This may suggest that additional precautions and testing should be taken during blood transfusion [14].

At the moment, no commercial systems to detect GBV-C infection are available. The basic marker used to diagnose active GBV-C infection is viral RNA detection by RT-PCR [16]. There were several trials to develop an enzyme-linked immunosorbent assay (ELISA) by using antibodies against the E2 recombinant protein of GBV-C [17,18]. However, the assays gave many false positives, and using more than one epitope for GBV-C detection was suggested [19].

Among the conventional methods used to produce monoclonal antibodies, the antibody phage display technology has been widely used since its discovery [20,21]. Phage libraries displaying various types of antibody fragments have been applied broadly in diagnostics, research, and development of therapeutics [22-24]. Previous studies have reported that high-affinity neutralizing human anti-viral antibodies for human immunodeficiency virus type 1 (HIV-1), respiratory syncytial virus (RSV), and herpes simplex virus (HSV) successfully were selected from phage display [25].

In this study, we expressed GBV-C non-structural proteins and showed that the recombinant proteins NS3, NS4A/B, and NS5A contain unspecific thrombin cutting sites. Using phage display technology, we constructed a naïve antibody library against GBV-C recombinant proteins and selected specific scFv fragments that could potentially be used to diagnose GBV-C and to aid to study the functions of GBV-C non-structural proteins. The selection of phage antibodies was based on affinity chromatography by keeping target antigens in solution to preserve their native conformation.

Materials and Methods

Bacterial strains

Escherichia coli TG1 suppressor strain (K12, del(lac-pro), supE, thi, hsdD5/F'traD36, proA+B+, lacIq, lacZdelM15), *E. coli* XL1-Blue strain (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIqZΔM15 Tn10 (Tetr)]), *E. coli* BL21(DE3)pLysS (F-, ompT, hsdSB (rB-, mB-), dcm, gal, λ (DE3), pLysS, Cmr).

Construction of recombinant plasmids

The genes of GB virus C non-structural proteins were PCR amplified from the GBV-C cDNA of genotype 2a previously identified in our laboratory [7] with Phusion HF DNA polymerase (ThermoScientific, USA), using outer and inner primers: NS3_outer_Fw: 5'CAG TGC GTC ATG GGT TTA CCC GTG GT; NS3_outer_Rv 5'CCC CTT CAC ATC CCA GTC TGT;

NS3_inner_Fw 5'CGC GAA TTC CCA TGG CGC CTG TTG TCA TCC GAC GG; NS3_inner_Rv ATA TAA GCT TCA GCA GCG GAC GTA; NS4A/B_outer_Fw 5'GTG GCC GGC CAC CAC ATA GTG GA; NS4A/B_outer_Rv 5'ACC ACG CGC ACT TGG CGC AT; NS4A/B_inner_Fw 5'CGC GAA TTC CCA TGG ACG CTG GGC CGA TCT TGA TG; NS4A/B_inner_Rv 5' CTC GAG TCA CCC CAC CTG TAC CTC; NS5A_outer_Fw 5'GTG GTT GCC CTG GTC AAC AGG GAG C; NS5A_outer_Rv 5'TCC TCA TAA GTG TAA CCC ATG CT; NS5A_inner_Fw 5'CGC GAA TTC CCA TGT ATG TCT GGG ACC TGT GG; NS5A_inner_Rv 5' TAT TCT AGA TTA GGC CAA GGT TTC; NS5B_outer_Fw 5'AGA ACC ATA CAG CCT ATT GTG AC; NS5B_outer_Rv 5'CCT TGC CGC AAC AGA TGA ATT TAG TTC A NS5B_inner_Fw 5'CGC GAA TTC CCA TGT CCT TCT CTT ACA TTT GGT C; NSB5B_inner_Rv 5'TAT TCT AGA TTA CCC GAA GAG GGC TAC GA. The PCR products were cloned in pGEM-T vector (Promega, USA) to obtain pGEM-T-GBV-C-NS. The sequences of GBV-C non-structural proteins were confirmed by DNA sequencing and subcloned into pGEX-4T-2 vector between *EcoRI* and *SalI* (NS3, NS4A/B, NS5B) or *EcoRI* and *NotI* (NS5B) sites. The orientations of the inserts were confirmed by restriction enzyme digestion and sequencing.

Expression of GBV-C non-structural recombinant proteins in *E. coli*

The obtained pGEX-4T2-NS vectors were transformed into XL1-Blue cells. The single colonies of transformed *E. coli* XL1-Blue were inoculated into 5 ml LB media containing 100 μg/ml ampicillin and incubated overnight at 37°C with shaking at 160 rpm. The next day, 200 μl of the overnight cultures were transferred into 15 ml LB medium containing 100 μg/ml ampicillin and incubated at 37°C with shaking at 160 rpm until the OD600 reached 0.6. One milliliter of the culture was harvested by centrifugation at 4000 g for 15 min. The supernatant was removed, and 300 μl of 2x Laemmli sample buffer (10% SDS, 1M Tris-HCl (pH=6.8), 1% bromphenol blue, 1M DTT) and 100 μl of PBS (pH=7.2) were added onto the pellets and boiled at 98°C for 5 min. This fraction was considered the uninduced control. At this point, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to the rest of the flasks to a final concentration of 0.5 mM and incubated at different temperatures for various times with shaking at 160 rpm (18°C for overnight, 28°C for 6 h, and 37°C for 3 h). After incubation, the cultures were harvested by centrifugation, and the pellets were boiled as described above.

Purification of recombinant GBV-C proteins expressed in *E. coli*

E. coli XL1-Blue cells were transformed with pGEX-4T-2-GBV-C-NS, and a single colony was used to inoculate a 5 ml volume of LB medium, supplemented with 100 μg/ml ampicillin, and incubated overnight at 37°C with shaking at 160 rpm. The next day, the overnight cultures were transferred into 500 ml LB medium containing 100 μg/ml ampicillin. The cultures were incubated at 37°C until the OD600 reached 0.6, after which the temperature of the cultures was reduced to 18°C, and expression was induced by the addition of 0.5 mM IPTG overnight. The cell pellets were resuspended in lysis buffer (10 mM Tris-HCl (pH=8.0), 1 mM EDTA, 150 mM NaCl), and lysozyme was added to 1 mg/ml. The cell suspensions were incubated on ice for 15 min, after which 10 mM of DTT were added and the cell lysates were sonicated and centrifuged at 9000 g for 30 min at 4°C. The supernatants were transferred to 50 ml falcon tubes and 2% TritonX-100 and up to 20 ml lysis buffer were added, after which the supernatants were kept at room temperature for 30 min. One milliliter of glutathione-Sepharose 4B was added for each liter of protein expressing culture, and the suspensions were incubated at 4°C overnight with slight agitation. The glutathione–Sepharose 4B resin was collected by centrifugation at 500 g for 5 min at 4°C and washed three times with PBS (pH=7.0). The washed resin was resuspended in PBS supplemented with 1U thrombin at 4°C for 1 h, after which the eluents were collected.

Library construction

The library construction was performed as described previously with slight modifications [26]. Briefly, in the first step, the 75 independent PCR reactions were performed on human peripheral blood cDNA (AllCells, USA) in order to amplify V gene segments by using Phusion HF polymerase (Fermentas). The primers introduced either *SfiI* or *NotI* restriction sites, which were used for cloning into pMOD1 vector (provided by Montarop Yamabhai laboratory). The amplified V_H and V_L fragments were fused together through a (G4S) 3 linker. The constructed library (total 4.6 μg) was electroporated into *E. coli* TG1 cells. Examination of 23 randomly selected TG1 colonies revealed that all 23 clones contained the expected 800 bp insert.

Diversity analysis of antibody fragments

In order to analyze the diversity of antibody fragments and to evaluate the quality of the primary library, DNA segments encoding the scFv genes from 23 randomly picked clones were amplified and examined by digestion with *BstNI*. The fingerprint patterns were analyzed on agarose gel, compared with each other, and sequenced. The sequencing analysis was done by using CLC Main Workbench 6 (CLC, Inc., Aarhus, Denmark), IgBLAST [27], and V BASE [28] software. Library size was determined by counting transformants on ampicillin agar plates.

Phage Selection Experiments

TG1 cells carrying unique scFv phagemid DNA were grown to the mid-log phase, infected by the M13K07 helper virus (New England BioLabs) and incubated for 1 h at 37°C with gentle agitation. After incubation, the culture was centrifuged at 2000 g for 10 min at 4°C. The cell pellet was resuspended in 10 ml of 2x LB/amp/kan, and the culture was grown overnight at 28°C with shaking at 250 rpm. The bacterial cells were pelleted at 10000 g for 20 min at 4°C, and the supernatant, which contained the phage library, was transferred to a fresh tube. The secreted phage particles were purified from the supernatant by precipitation in 20% polyethylene glycol (PEG 8000) for 1 h on ice. The phage particles were centrifuged at 10000 g for 20 min, and the phage pellet was resuspended in 1 ml of PBS and used for further selection experiments.

The GBV-C nonstructural proteins were expressed in bacteria as the GST fusion proteins, immobilized on glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech, USA), and used as the basis for affinity selection of specific antibody fragments from a naïve scFv library, the products of which are displayed on the surface of filamentous bacteriophage. After 15 min preincubation at room temperature (RT) in PBS containing 3% bovine serum albumin (BSA), the naked Sepharose beads were incubated for 1 h on a rotating shaker at RT with the primary antibody library. Then the same library was incubated with GST protein immobilized on glutathione-Sepharose 4B

beads, which before were preincubated as naked beads. After 1 h at RT incubation, the library was transferred on the beads coated with the GST-target fusion protein. The Sepharose beads were collected by centrifugation and washed five times with 1 ml of ice-cold PBS-0.05% Tween 20 (PTW). Selected phages were recovered by adding to the Sepharose beads of 0.25 ml fresh *E. coli* TG1 bacteria (A600 nm 0.4) grown in 2x LB/amp and incubated at 37°C for 30 minutes. For the output titering, 10^2, 10^3, and 10^4 dilutions were made. The non-diluted culture and the three dilutions were separately spread on LB agar plates containing 100μg/ml ampicillin and incubated at 37°C overnight to generate individual colonies. The grown colonies were scraped from agar plates using 2.5 ml of 2x LB medium per plate, and 50 μl of the suspension were diluted into 500 ml of 2x LB medium supplemented with 4% glucose and 100 μg/ml ampicillin. The culture was grown to OD600=0.5 at 37°C with shaking at 160 rpm, infected with helper phage, and grown overnight. The phage was purified by PEG purification, as described above, and used for a second and third round of selection against the same target with slight changes. During the second selection round, the naked Sepharose beads and glutathione-Sepharose 4B beads with immobilized GST protein were incubated for 1 h 15 min, where the beads coated with the GST-target fusion protein were incubated for 45 min. The incubations for third-round selection lasted 1 h 30 min and 30 min, accordingly.

Preparation of candidate phage clones for ELISA

After the third selection round, the single colonies were picked and added to separate prelabeled 1.5 ml microcentrifuge tubes with 400 μl of 2x LB/amp/glucose. The tubes were incubated at 37°C overnight with shaking at 160 rpm. The next day, 50 μl of each overnight culture were transferred to the fresh tubes, and 400 μl of 2x LB/amp/glucose medium containing 10^8 pfu of $M_{13}KO_7$ helper phage were added to each tube. To the remaining 350 μl of overnight culture, sterile glycerol was added to a final 15% concentration and stored at -80°C. The tubes were incubated at 37°C with shaking at 160 rpm. After 2 h, the samples were centrifuged at 14000 rpm for 5 min at room temperature. The supernatant was discarded, and each cell pellet was resuspended in 400 μl of 2x LB/amp/kan. The tubes were incubated at 37°C overnight with shaking at 160 rpm. The phage clones were purified by PEG precipitation, as described previously.

Enzyme-linked immunosorbent assay (ELISA)

The binding of individual phage clones to the GBV-C nonstructural proteins was analyzed by ELISA. The glutathione-Sepharose 4B beads coated with the GST-target fusion protein were mixed with 50 μl of the prepared phage in PBS containing 0.1% BSA and incubated for 1 h at RT with occasional shaking. After the beads were washed three times with PTW, 100 μl of horseradish peroxidase (HRP)-conjugated anti-M13 phage antibody (Sino Biological), diluted 1/5000 in PBS containing 0.1% BSA was added, and incubated at RT for 1 h with occasional shaking. The beads were then washed three times with PTW, and 100 μl of a solution containing ABTS (2,2-azino-di-3-ethyl-benzthiazoine-6-sulfonate) peroxidase substrate (Sigma Aldrich, USA) was added. After incubation at RT for 20 min, the reaction was stopped with 50 μl of 1% SDS. A 100 μl, bead-free aliquot was transferred to a 96-well microplate, and the optical absorbance was measured at 405 nm. The coding regions of positive clones were sequenced.

scFv antibody expression and purification

The selected scFv fragments were PCR-amplified from the glycerol stock with Phusion HF DNA polymerase (ThermoFisher, USA). The PCR products were cloned in pET23a vector (Novagen, USA) between the *EcoRI* and *XhoI* sites. The sequences of scFv fragments were confirmed by DNA sequencing. The plasmid DNA was transformed into E. coli BL21 (DE3) pLysS competent cells. The next day, the single colonies were inoculated in 4 ml LB medium containing 100 μg/ml of ampicillin and incubated at 37°C overnight with shaking at 160 rpm. Then 4 ml of the overnight culture were diluted in 200 ml LB medium containing 100 μg/ml of ampicillin and incubated at 37°C with shaking at 160 rpm until OD at 600 nm reached about 0.5-0.7. The cultures were induced by adding IPTG to a final concentration of 0.5 mM and incubated at 37°C for 4 h with shaking at 160 rpm. Cells were harvested by centrifugation at 4000g for 15 min, resuspended in lysis buffer (PBS (pH=7.0), Protease Inhibitor Cocktail Tablets, cOmplete (Roche, USA)) and snap frozen in liquid nitrogen. To lyse the bacteria cells, frozen bacteria suspensions were thawed in a water bath at room temperature, gently mixed, and stored on ice. After the cells were completely disrupted by sonication, cell lysates were centrifuged at 15000 g for 30 min. The supernatants were separated from the pellets, which were then dissolved in a denaturing buffer (6 M guanidine-HCl, 50 mM Tris-HCl, 10 mM CaCl2, 50 mM KCl (pH=7.5)) and incubated at room temperature for 2 h with mild rotations. The lysed cell pellets were diluted in refolding buffer (50 mM Tris-HCl, 10 mM $CaCl_2$, 50 mM KCl, 0.1 mM PMSF (pH=7.5)) and incubated at 4°C overnight. The cellular debris was centrifuged at 20000 g for 30 min at 4°C, and the supernatants were transferred to the clean tubes. The six histidine-tagged scFv were purified by using Ni-NTA resin according to the manufacturer's instructions. The scFv fragments were eluted by 1 M imidazol.

Western blot

The purified scFv antibodies were separated by 12% separating and 4% stacking slab sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). After the proteins were transferred to a polyvinylidene fluoride membrane (PVDF) the membrane was blocked for 1 h at room temperature with 5% (w/v) skim milk in PBS containing 0.1% Tween-20 and then incubated overnight at 4°C with anti-His tag antibodies. The next day, the membrane was washed with PBS containing 0.1% Tween-20 and incubated for 1 h at room temperature with HRP-conjugated goat anti-mouse IgG antibody. Signals were visualized by enhanced chemiluminescence (ECL).

Protein dot blot assay

Protein dot blot assay was performed as described previously with minor modifications [29]. A 4 μl aliquot of a recombinant GBV-C protein was transferred onto a prepared PDVF membrane. The membrane was blocked with 5% Milk-PBST for two hours at room temperature. The membrane was then incubated overnight with the purified scFv at 4°C, followed by labeling with a 1:3000 dilution of the primary anti-His tag antibody (SigmaAldrich) for 2 hours. Immunoreactivity was detected following 1 h incubation with a 1:5000 dilution of a HRP conjugated goat anti-mouse IgG antibody. Signals were visualized by enhanced chemiluminescence (ECL).

Enzyme-linked immunosorbent assay (ELISA) with purified scFv

ELISA was performed as described above with some modifications. After the beads coated with the GST-target fusion protein were washed three times with PTW, 100 µl of the purified scFv in PBS containing 0.1% BSA was added, and incubated at RT for 1 h with occasional shaking. The beads were then washed three times with PTW, and 100 µl of anti-His antibody, diluted 1/3000 in PBS containing 0.1% BSA was added, and incubated at RT with occasional shaking. After 1 h incubation the beads were washed three times with PTW and 100 µl of HRP-conjugated goat anti-mouse IgG antibody diluted 1/5000 in PBS containing 0.1% BSA was added, and incubated at RT with occasional shaking. Measurements were done as described above.

Results

Expression and purification of GBV-C nonstructural proteins

GBV-C non-structural proteins NS3, NS4A/B, NS5A and NS5B were expressed as GST-tagged fusion proteins from pGEX-4T-2. All fusion proteins were present in the insoluble fractions. However, induction of the non-structural genes at 18°C with reduced IPTG concentrations overnight brought these fusion proteins into the soluble fraction. These recombinant proteins were then immobilized on glutathione-Sepharose 4B beads. Thrombin protease was used to remove GST affinity tags from the purified GBV-C recombinant proteins in order to obtain pure samples for antibody fragment selection. The expected sizes of the cleaved proteins were NS3-GST 95 kDa (69 kDa from NS3 plus 26 kDa from GST tag), NS4A/B-GST 63 kDa (37 kDa from NS4A/B plus 26 kDa from GST tag), NS5A-GST 73 kDa (47 kDa from NS5A plus 26 kDa from GST tag), and NS5B-GST 90 kDa (64 kDa from NS5B plus 26 kDa from GST tag). However, the analysis of proteins by SDS-PAGE electrophoresis showed that thrombin appears to make cleavages at nonspecific sites in the GBV-C recombinant proteins. The bands corresponding to the NS3-GST, NS4A/B-GST, NS5A-GST, and NS5B-GST were observed to be completely missing on the SDS gel, suggesting that the cleavage was complete (Figure 1). Thrombin digestion of the recombinant protein NS3-GST revealed four bands on the SDS gel corresponding to NS3 (~55 kDa and ~18 kDa), GST (~26 kDa), and thrombin (~37 kDa). The digestion of NS4A/B-GST gave three bands corresponding to NS4A/B (~31 kDa), GST (~26 kDa), and thrombin (~37 kDa), and in the case of NS5A-GST, there were four bands corresponding to NS5A (~30 kDa and ~17 kDa), GST (~26 kDa), and thrombin (~37 kDa) (Figure 1). Only the digestion of NS5B-GST revealed three bands corresponding to NS5B (~64 kDa), GST (~26 kDa), and thrombin (~37 kDa), as was expected. After obtaining the results that showed the highly non-specific cleavage of the GST tag using thrombin, further experiments were done on the GBV-C recombinant proteins immobilized on glutathione-Sepharose 4B beads.

Library construction

Antibody genes were obtained from whole-blood PBMC cDNA (AllCells, USA). V_H and V_L chains were amplified by PCR primers described previously [26]. The correct size PCR products were cloned into phagemid vector pMOD1, which was electroporated in TG1 competent cells. After optimizing the steps of library construction, the final scFv library of about 1.6×10^6 different molecules with 0.03% background was obtained.

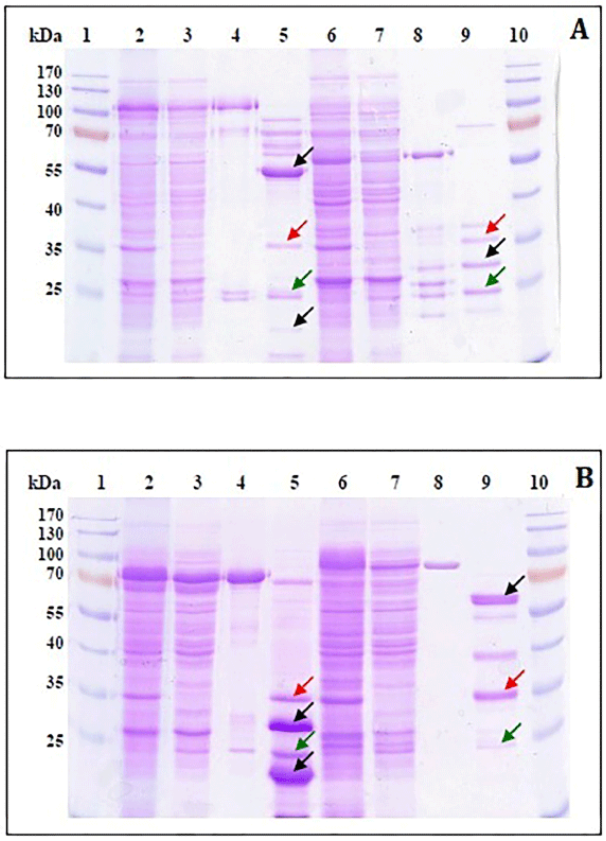

Figure 1: SDS-PAGE analysis of GBV-C non-structural proteins. **A:** 1, 10-Ladder (ThermoScientific, USA); 2-bacteria lysate (pGEX-4T2-GBV-C-NS3); 3-supernatant of bacteria lysate (pGEX-4T2-GBV-C-NS3); 4-protein bound to glutathione beads (pGEX-4T2-GBV-C-NS3); 5-glutathione beads cleaved with thrombin (pGEX-4T2-GBV-C-NS3); 6-bacteria lysate (pGEX-4T2-GBV-C-NS4A/B); 7-supernatant of bacteria lysate (pGEX-4T2-GBV-C-NS4A/B); 8-protein bound to glutathione beads (pGEX-4T2-GBV-C-NS4A/B); 9-glutathione beads cleaved with thrombin (pGEX-4T2-GBV-C-NS4A/B). **B:** 1, 10-Ladder; 2-bacteria lysate (pGEX-4T2-GBV-C-NS5A); 3-supernatant of bacteria lysate (pGEX-4T2-GBV-C-NS5A); 4-protein bound to glutathione beads (pGEX-4T2-GBV-C-NS5A); 5-glutathione beads cleaved with thrombin (pGEX-4T2-GBV-C-NS5A); 6-bacteria lysate (pGEX-4T2-GBV-C-NS5B); 7-supernatant of bacteria lysate (pGEX-4T2-GBV-C-NS5B); 8-protein bound to glutathione beads (pGEX-4T2-GBV-C-NS5B); 9-glutathione beads cleaved with thrombin (pGEX-4T2-GBV-C-NS5B). Black arrows indicate recombinant proteins; red arrows indicate thrombin; green arrows indicate GST protein.

In order to characterize the diversity of the scFv antibody fragments, 23 randomly picked clones were examined by the digest with *BstNI* and analyzed on a 1% agarose gel (Figure 2) and by sequencing.

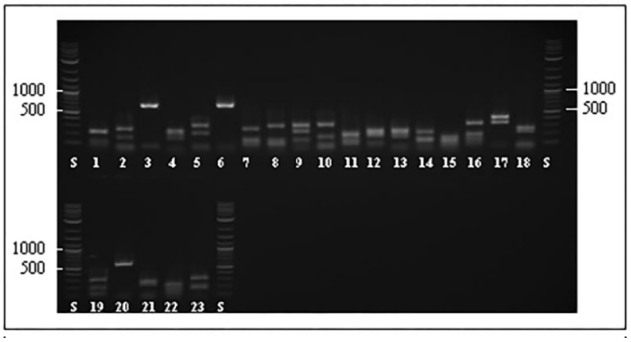

Figure 2: DNA fingerprinting of randomly picked clones from naïve library. Twenty-three clones were amplified by PCR, digested with *BstNI* and analyzed on 1% agarose gel.

Affinity selection

The naive human scFv display library was used for the selection against recombinant GBV-C non-structural proteins. The phages bound to recombinant proteins were amplified by adding log-phase TG1 cells onto the glutathione-Sepharose 4B beads coupled by recombinant proteins. Biopanning was repeated three times. Phage titers of input and output were determined by colony forming unit (CFU). The number of input phages was higher than the number of output phages in all four cases, which means that only antigen-bound phages were selected after each round of biopanning.

After three rounds of biopanning, 200 individual phage clones in total from each round were tested for specific binding by ELISA (Figure 3). The phages were counted as positive by ELISA if the absorbance values were two times greater than those for binding to GST protein. For all antigens, except for NS5A, at least one specific phage clone was obtained.

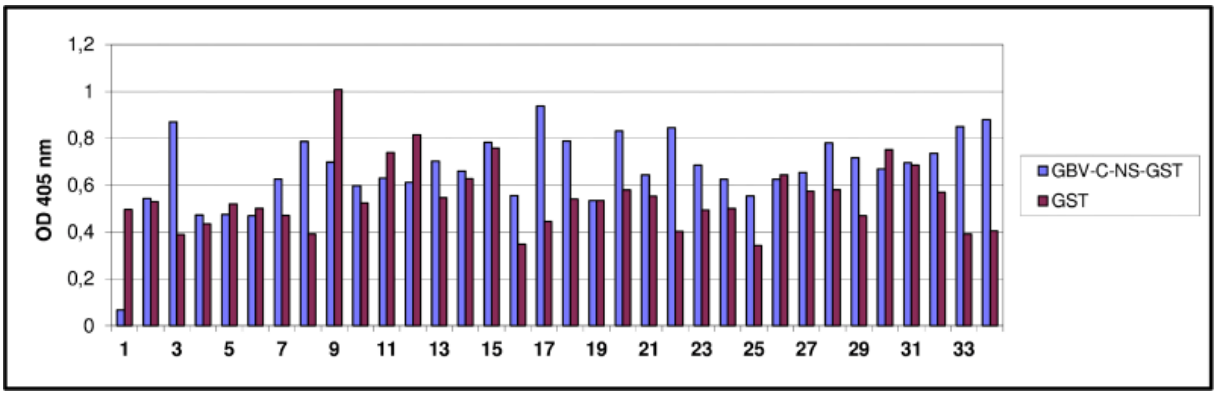

Figure 3: Specificity of the isolated binders. 1-3-target GBV-C-NS3 protein; 4-9-target GBV-C-NS4A/B protein; 10-16-target GBV-C-NS5A protein; 17-34-target GBV-C-NS5B protein.

Analysis of specific scFv

The selected specific clones were sequenced and analyzed by use of the VBASE immunoglobulin V gene database and the IgBLAST program. The CDR3 sequences of the scFv that potentially bound to GBV-C are shown in Table 1.

Antigens	CDR1	CDR2	CDR3	Germline	Family
NS3-3	QSVSTN	GAS	QQYGSSPHT	IGKV3-20*01	VK3
	GFTLSRHE	ITDNGRSI	TIDWQMIFTNYYPMNV	IGHV3-48*03	VH3
NS4A/B-9	QDISNY	DAS	QQYDNLPPT	IGKV1D-33*01	VK1
	GYSFNTYW	IYPDDSDT	ARLRLPGADRTGLYPIYYYYIDV	IGHV5-51*03	VH5
NS5B-17	SLRSYY	GKN	NSRDSSGNHLGWV	IGLV3-19*01	VL3
	GGSISSYY	IYYSGST	ARGGGDIAAAGSDY	IGHV4-59*01	VH4
NS5B-22	QSVSSSY	GAS	QQYGSR	IGKV3-20*01	VK3
	GYSFTSYW	IYPGDSDT	ARHVRGITLYYYYMDV	IGHV5-51*01	VH5
NS5B-33	QSVSSSY	GAS	QQYGSSGYT	IGKV3-20*01	VK3
	GFSLSNARMG	IFSNDEK	ARIPGHYYYYMDV	IGHV2-26*01	VH2

NS5B-34	KLGDKY	QDS	QAWDSSTYV	IGLV3-1*01	VL3
	GFIFADFA	IRSEVGGGTI	ARAAGQQVVPPSYYYMDF	IGHV3-49*04	VH3

Table 1: Sequences of the selected scFv.

Purification of scFv antibodies

The constructed plasmid pET23a-scFv was transformed into BL21 (DE3) pLysS, an *E. coli* expression host. All the clones selected by ELISA were expressed, and the obtained bands were 32 kDa, matching the predicted size of the scFv (Figure 4). After cell disruption, all target proteins were found in insoluble protein fraction; therefore, treating the pellets with 6 M guanidine-HCl and refolding afterwards were needed. We expressed and purified one scFv against the GBV-C NS3 protein, one against the GBV-C NS4A/B protein, and four against the GBV-C NS5B protein.

Antigen binding activity of purified scFv

To determine the specificity of scFv a Dot Blot assay and ELISA (Figure 5) were carried out. The results demonstrated that the svFv were able to bind specifically to GBV-C non-structural proteins.

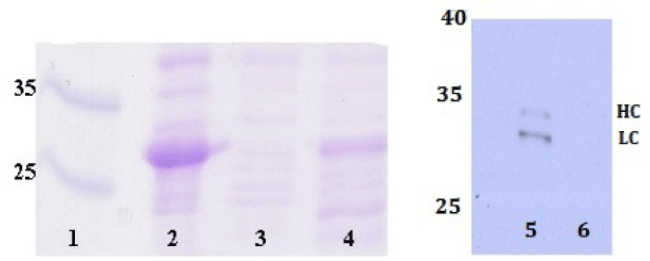

Figure 4: SDS-PAGE (1-4) and Western blot (5-6) analysis of expressed scFv against GBV-C-NS5B. 1-ladder, 2-bacteria lysate; 3-supernatant of bacteria lysate; 4-protein; 5-Western blot of bacteria lysate, 6-negative control. HC-heavy chain, LC-light chain.

Discussion

GBV-C is a non-pathogenic virus that has spread worldwide. GBV-C shares transmission mechanisms with HCV and HIV, resulting in coinfection with HIV (14%-36%), HCV (10%-25%), and HIV/HCV (30%-36%) [30,31]. Some studies have shown that co-infection with GBV-C in HIV-infected patients causes a reduction in mortality rates and better clinical outcomes [32]. On the other hand, there have been reports that GBV-C could possibly increase the risk of NHL. At the moment, there is no commercially available detection system that can rapidly and accurately identify GBV-C infection [33].

In this study, we fused the open reading frames of GBV-C non-structural proteins with the open reading frame of GST, expressed GST-GBV-C-NS proteins in bacteria, and optimized and purified them by using thrombin from bovine plasma, which mostly specifically recognizes the amino acid sequence Leu-Val-Pro-Arg-Gly-Ser and digests between Arg and Gly [34]. After an analysis of the purified non-structural proteins of GBV-C, we found that the NS3, NS4A/B, and NS5A proteins have the nonspecific thrombin recognition sites and are digested non-specifically. Due to its relatively low cost and cleavage activity ratio, thrombin is one of the most popular restriction proteases used for removal of affinity tags from recombinant fusion proteins [35,36]. However, non-specific activity of thrombin is also common, especially in overexpressed recombinant proteins, and there are no procedures which would help to avoid the undesirable nonspecific cleavage [34].

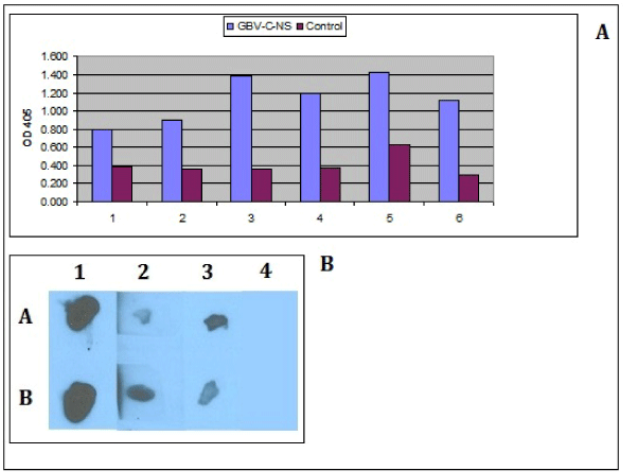

Figure 5: A: ELISA with purified scFv. Antigen: 1-GBV-C-NS3, control-GBV-C-NS5B, 2-antigen: GBV-C-NS4A/B, control-GBV-C-NS5B, 3-6-antigen: GBV-C-NS5B, control GBV-C-NS3. Recombinant antibodies: 1-scFv-9, 2-scFv-9, 3-scFv-17, 4-scFv-22, 5-scFv-33, 6-scFv-34. **B:** Dot Blot assay. A1-antigen: GBV-C-NS5B, antibodies: anti-GST; A2-antigen: GBV-C-NS5B, antibodies: scFv-33; A3-antigen: scFv-33, antibodies: anti-His; A4-antigen: GST, antibodies: scFv-33; B1-antigen: GBV-C-NS5B, antibodies: anti-GST; B2-antigen: GBV-C-NS5B, antibodies: scFv-34; B3-antigen: scFv-34, antibodies: anti-His, B4-antigen: GST, antibodies: scFv-34.

There are two mainly used strategies to produce humanized antibodies: transgenic mice or phage display methods [37]. In this study, we constructed a diverse scFv library of about 1.6×10^6 clones by electroporating only 4.6 μg DNA. Analysis of our primary library showed that variable regions were derived from six V_H (V_{H1}, V_{H2}, V_{H3}, V_{H4}, V_{H5}, and V_{H6}) and six V_L ($V_{κ1}$, $V_{κ3}$, $V_{λ1}$, $V_{λ2}$, $V_{λ3}$, and $V_{λ6}$) gene families. These gene families had been frequently found in other large phage display libraries [38,39]. These results demonstrate that our library is compact and highly diverse, and sufficient for antibodies selection against any antigen.

In order to select antibodies specific to any possible epitopes of the recombinant proteins of GBV-C, we used fusion proteins of GST and non-structural recombinant proteins immobilized on glutathione-

Sepharose 4B beads [40,41]. First, we incubated phages with naked glutathione-Sepharose 4B beads, then with GST-coated beads, and finally with antigen-coated beads. In the first two steps, by incubating phages with naked or GST-coated beads we eliminated antibody fragments binding to the beads and GST. The eluted phages showed high specificity to the target proteins. This was also demonstrated by ELISA.

From a naïve library, selected scFv were expressed in *E. coli* with a hexa-histidine tag at the C-terminus. The antibody fragments banded at ~32 kDa, with much greater intensity in the inclusion bodies fraction versus the soluble proteins fraction. However, the proteins were successfully purified without losing their dimeric forms. The purified scFv antibodies were tested by ELISA and Protein Dot Blot Assay which confirmed that our expressed and purifies proteins are specific to the recombinant GBV-C proteins.

In this study, we showed that even proteins with nonspecific cleavage sites can be used for further experiments such as scFv antibody selection without losing any possible epitopes. There is a lack of antibodies that would detect not only past GBV-C infection but also active viremia. This study is the first attempt to produce antibodies against GBV-C by using recombinant DNA technology. The antibodies selected, expressed, and purified in this study could be used for further experiments.

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