Genetically Engineered Single-Chain Fvs of Human Immunoglobulin against Hepatitis C Virus Nucleocapsid Protein Derived from Universal Phage Display Library

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Hepatitis C virus (HCV) is the major causative agent of post-transfusion non-A, non-B hepatitis. This virus is of clinical importance because more than half of HCV-infected patients subsequently develop chronic hepatitis which may progress to cirrhosis and liver cancer. HCV is transmitted mainly via blood transfusion and other parenteral contacts. The majority of patients with chronic hepatitis C infection, and can transmit the virus, are however asymptomatic. Therefore, the screening of blood and blood products for antibodies to HCV is currently the most effective method for prevention of HCV in the population. HCV can be found worldwide, with the prevalence in Thai blood donors of around 1-5%. The northeastern part of Thailand has a relatively high prevalence of this viral infection, and up to 8.3% of male blood donors aged between 31-40 years have antibodies to HCV.

HCV is a single stranded RNA virus and belongs to the family *Flaviviridae*. The viral genome encodes a single large polyprotein which is subsequently cleaved into a series of structural and nonstructural proteins. The analysis of nucleotide sequences and deduced amino acid sequences of HCV isolates from different parts of the world showed high degrees of heterogeneity of the viral genome. HCV can be classified into at least 6 major genotypes and a

**SUMMARY** Specific single-chain Fvs (scFvs) of human immunoglobulin that specifically recognized the recombinant hepatitis C virus (HCV) nucleocapsid protein were isolated from a large phage display antibody library. This universal library of genetically engineered filamentous phagemids displayed random pairings of the variable regions of both human heavy and light chain immunoglobulin in the scFv format. Specific clones were isolated by affinity selection with purified recombinant HCV protein fused to glutathione-S-transferase (GST). The GST-specific clones were excluded by blocking the phagemid library with GST prior to the selection. After 4 rounds of selection, the HCV-reactive clones were enriched by a factor of 100,000. About 4% and 9% of the clones from rounds 4 and 5, respectively, specifically reacted to the HCV portion of the fusion protein in an enzyme immunoassay. The specificity was confirmed by specific binding inhibition with plasma from an HCV-infected individual. Nucleotide sequence analysis of 3 HCV-specific clones indicated that all 3 clones contained an almost identical VH gene sequence which was derived from the VH3 germline gene family. These clones had different VL gene sequences of the lambda type. There were some differences between nucleotide and amino acid sequences of the HCV-specific scFv genes and those of the closest matched germline genes, indicating the presence of somatic mutation. This study illustrated the feasibility of using antibody engineering technology with the universal phage display library to isolate human antibodies with predefined specificity to important microbial pathogen which may be useful for future therapeutic purpose.
The desired specificity of antibodies to affected genotypes, and is therefore important in the treatment of this infection. The antibody to this protein can be found in most HCV patients regardless of the infected genotypes, and is therefore very important in the diagnosis of the virus. It may also be useful in studying the pathogenesis and in the treatment of this infection.

In recent years, there have been rapid developments in the area of antibody engineering by combining the knowledge on the functional activity and specificity of the antibody molecules and the ability to genetically modify the molecules to perform desired functions. Since it is fundamentally difficult to produce useful human monoclonal antibodies by hybridoma technique, the antibodies can be obtained by replacing the constant domain and most of the framework region of the variable domain of the mouse immunoglobulin genes with their human counterparts. Such technique is very powerful but it is time-consuming and requires expensive procedures. Recently, large repertoires of the variable region of human immunoglobulin genes can be cloned into bacteriophage and expressed on the surface of the phage as a fusion protein with phage coat proteins. This approach enables the isolation of human monoclonal antibodies with the desired specificity in vitro by selecting the "phage display" antibody libraries with the appropriate antigen. It overcomes the problems associated with the production of human monoclonal antibodies by hybridoma technique. The libraries used for selection have been constructed from immunized or naive individuals or can be synthesized in vitro. Powerful and rapid selection techniques have been developed. Since these antibodies are of human origin, they can be used for both diagnostic and therapeutic purposes. The quality of the libraries have been markedly improved and they can be used as a source for selecting antibodies to a vast number of antigens.

The objective of the present study is to isolate the phagemid clones expressing human antibodies specific to hepatitis C virus nucleocapsid protein by using a universal phage display library. The usage of germline gene segments in the phagemids producing scFvs to HCV nucleocapsid protein is also illustrated.

**MATERIALS AND METHODS**

**Purification of recombinant HCV nucleocapsid and GST proteins**

Purified HCV nucleocapsid protein was obtained by expressing a recombinant *Escherichia coli* transfected with plasmid pCGBBS51-g which contained the HCV nucleocapsid gene. The protocols for protein expression and affinity purification have been described. In brief, the gene encoding the first 123 amino acids of HCV nucleocapsid protein of HCV isolated from a Thai patient was cloned into the 3' end of a gene encoding glutathione-S-transferase (GST). Protein expression was induced by the addition of isopropyl b-D-thiogalactoside (IPTG). The protein was expressed as a fusion protein containing GST at the amino terminal and HCV nucleocapsid protein at the carboxyl terminal. This fusion protein was purified by affinity chromatography using glutathione-Sepharose 4B beads (Pharmacia Biotech, U.S.A.). The purified protein was dialyzed extensively with phosphate-buffered saline (PBS) in a microdialyzer (Pierce, U.S.A.). Protein concentration was quantified by a Bradford Protein Assay Kit (Bio-Rad, U.S.A.). The protein was stored at -70°C. Purified GST protein was obtained by expressing plasmid pGEX-3X (Pharmacia Biotech, U.S.A.), containing the gene for GST protein. The protein was expressed, affinity purified and dialyzed as described above.

**Plasma samples**

An anti-HCV antibody-positive plasma sample was obtained from a Thai blood donor who was infected with HCV. The viral genome from this sample (BB51) was used for constructing the recombinant plasmid containing the viral recombinant nucleocapsid protein as described above. In addition, an anti-HCV antibody-negative sample was obtained from a healthy blood donor. Antibody to HCV was detected by enzyme immunoassay and confirmed by an immunoblot assay (HCV ELISA and HCV Blot 3.0, Diagnostic Biotechnology, Singapore). The plasma samples were thawed once, aliquoted and immediately stored at -70°C.

**Phage display antibody library of scFv**

The phage display library used in this study contained the variable regions of the human heavy and light chain immunoglobulin genes (VH and VL, respectively), arranged in the single...
chain Fv format (scFv). The VH and VL genes were randomly cloned into a phagemid (phage plus plasmid), allowing the expression of scFv as a fusion protein with the minor coat protein (pII) of the filamentous phage. The construct contained an amber codon for selective expression as a bacteriophage pII surface protein or as a soluble protein. The library size was estimated to be around 5 x 10^8-5 x 10^9 diverse clones. This phage display antibody library was a gift from Dr. G. Winter, MRC Centre for Protein Engineering, Cambridge, U.K.

**Selection of scFvs reactive to recombinant HCV nucleocapsid protein**

The scFvs were selected from the library using affinity selection technique with the recombinant antigen on solid surface. To avoid selecting the scFvs reactive to the GST component of the recombinant fusion protein, purified GST was added as a competing antigen during the selection process. This markedly reduced the frequency of GST-reactive clones. The selection process consisted of 4-5 rounds of repeated selection. In brief, a Maxisorp immunotube (Nunc, U.S.A.) was coated overnight with 10 μg/ml of the recombinant HCV-GST fusion protein at room temperature. The tube was washed three times with PBS and then blocked with 3% (w/v) skimmed milk powder in PBS (3% MPBS), followed by another 3 rounds of washing. In the first round of selection, the original universal phage display scFv antibody library was added to the fusion protein-coated immunotube at the titre of 2 x 10^13 colony forming units. The tube was incubated at 37°C for 120 minutes, with gentle rotation in the first 30 minutes followed by 90 minutes of static incubation. The unbound phagemids were removed by extensive washing for 20 times with PBS containing 0.1% Tween 20, and then 20 times in PBS without Tween 20. The bound phagemids were eluted from the immunotube using freshly made 100 mM Triethylamine (BDH Chemicals, U.S.A.). The reaction was neutralized by adding 1/2 volume of 1 M Tris-Cl pH 7.4. The eluted phagemids were used for infecting the mid-log phase *E. coli* strain TG1. The infected TG1 were then plated onto a large culture disk containing TYE agar with 100 μg/ml ampicillin and 1% glucose. The plate was incubated overnight at 37°C. After the incubation, bacterial colonies containing phagemids including those reactive to recombinant HCV-GST fusion protein were grown on the plate. The recombinant bacteria were then collected from the plate and grown in 2xTY media containing 100 μg/ml ampicillin and 2% glucose, to log phase. The phagemids were rescued by adding 10-fold excess of VCS-M13 helper phage (Stratagene, U.S.A.). The tube was incubated at 37°C without shaking for 20 minutes and then with shaking for 40 minutes. The culture was centrifuged and the cell pellet was resuspended in 2xTY media containing 100 μg/ml ampicillin and 50 μg/ml kanamycin, followed by incubation at 30°C with shaking. The phagemids were recovered from the culture supernatant by precipitation with 20% polyethylene glycol 6000 in high salt solution, and finally resuspended in PBS. The phagemids recovered after the first round of selection were then offered for selection in the second round. The selection process was repeated at least 4 times. After the first round, the selection was carried out in the presence of GST as competitor. The phagemids were preblocked in a buffer containing 50 μg/ml of purified GST, before incubating with the immunotubes coated with purified recombinant HCV-GST fusion protein.

**Quantitation of phagemids**

The phagemids offered and recovered from each round of selection were quantitated by serial dilutions. One microlitre of the phagemid suspension was used for infecting 1 ml of mid log-phase *E. coli* TG1. The resulting infected bacteria were diluted serially from 10^1 to 10^7 and plated onto TYE plates containing 100 μg/ml ampicillin and 1% glucose. The plates were incubated overnight at 37°C. The concentration of phagemids was calculated from the number of colonies grown on each plate.

**Induction of expression of soluble scFv by the phagemids**

A small aliquot of eluted phagemid after 3 to 5 rounds of selection was used for infecting *E. coli* strain HB2151 which allowed the expression of soluble scFv. The infected bacteria were randomly selected and then grew overnight by incubating at 37°C with shaking, in 100 μl of 2xTY media containing 100 μg/ml ampicillin and 1% glucose, in a 96-well culture plate (Corning, U.S.A.). Small innoculum of each clone was transferred to another 96-well plate and regrown to log phase and then induced with IPTG.
to the final concentration of 1 mM. Soluble scFvs were recovered from the supernatant and used in the ELISA.

**Detection of antibody activity to recombinant HCV nucleocapsid protein by ELISA**

Microtitre assay plates (Falcon, U.S.A.) were coated overnight at room temperature with 100 μl per well of 10 μg/ml of either the purified recombinant HCV-GST fusion protein or purified GST. The wells were blocked with 200 μl per well of 2% MPBS, for 2 hours at 37°C. Soluble scFvs from the phagemid culture were added to each well in a buffer containing 2% MPBS. The plate was incubated at room temperature for 1 hour and then washed 3 times with PBS containing 0.05% Tween 20, and 3 times with PBS without Tween 20. The bound scFv was detected using a mouse monoclonal antibody specific to c-myc peptide which was genetically engineered as tagged peptide at the carboxyl terminal of the scFv phage display library (antibody 9E10, a gift from Dr. G. Winter, Cambridge). The signal was amplified by using peroxidase-conjugated anti-mouse IgG, Fc-specific (Sigma, U.S.A.), and detected with TMB (3,3',5,5'-tetramethylbenzidine) as substrate. The optical density was read at 450 nm, using 650 nm as standard. For the inhibition ELISA, the soluble scFv was incubated with the recombinant HCV-GST fusion protein-coated plate in the presence of serial twofold dilution of anti-HCV-positive and anti-HCV-negative human plasma, started from 1:2 up to 1:64. The subsequent steps were similar to those described above.

**DNA amplification and nucleotide sequencing**

The VH and VL genes from HCV-reactive phagemid clones were amplified by polymerase chain reaction. The PCR product was purified using Wizard PCR Prep system (Promega, U.S.A.). Nucleotide sequence of the PCR product was obtained using PRISM dye terminator FS sequencing system (Perkin-Elmer, U.S.A.). The sequence obtained was analyzed using Sequence Navigator and MacVector programs (Applied Biosystems, Inc., U.S.A.). The usage of germline V and J gene segments were analyzed by comparing with those catalogued in the V BASE GOLD directory of human V gene segments and alleles using DNAPLOT package.

**RESULTS**

**Isolation of HCV-reactive scFvs**

A total of 5 rounds of selection was performed for isolating scFvs reactive to the recombinant HCV-GST fusion protein from the universal phage display antibody library. The results are summarized in Table 1. After 4 and 5 rounds of selection, the frequency of the phagemids that bound to the recombinant HCV-GST fusion protein-coated immunotube was enriched by more than 10^5 fold from the original phage display antibody library.

**scFvs reactive to HCV nucleocapsid protein and to GST**

A total of 96 colonies each from rounds 3, 4 and 5 of the selection were randomly selected. The phagemids were then recloned from E. coli strain TG1 to strain HB2151, to allow the expression of

<table>
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<tr>
<th>Selection rounds</th>
<th>Numbers of phagemid</th>
<th>Frequency of reactive /total phagemids</th>
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<tr>
<td></td>
<td>Offered</td>
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<tr>
<td>Round 1</td>
<td>2x10^13</td>
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<td>Round 5</td>
<td>2x10^12</td>
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Table 2  The frequency of the phagemids from each round of selection that were reactive to recombinant HCV-GST fusion protein and to purified GST in ELISA test.

<table>
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<th>Numbers of phagemid reactive to HCV-GST fusion protein</th>
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<td>24/96</td>
<td>15/96</td>
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nd, not determined.

soluble scFvs because of the presence of an amber stop codon in the construct. This construct also contained the gene encoding parts of c-myc peptide, enabling the detection of scFv by murine monoclonal antibody (9E10) to the tagged c-myc peptide. The results are summarized in Table 2. After round 3, only 1 in 96 clones was reactive to the recombinant HCV-GST fusion protein. This clone was reactive to GST portion, but not the HCV portion of the fusion protein. After round 4, all 4 positive clones obtained (4%) were reactive to the fusion protein but not to GST, indicating the reactivity to the HCV

Fig. 1  Enzyme immunoassay for scFv antibody activity to recombinant HCV-GST fusion protein and to purified GST protein of the phagemids reactive to HCV (R4H2, R4F3, R4A3 and R4D6), and to GST (R5D4). Phagemid R4A1 reactive to neither of the two proteins were used as a negative control.
protein. The percentage of reactive clones increased to 24% after round 5, 9 clones (37.5%) of which were reactive to the HCV portion while the other 15 clones (62.5%) were reactive to GST.

Five HCV-reactive or GST-reactive phagemids were selected and recloned twice, then assayed by ELISA. Four clones (R4H2, R4F3, R4A3 and R4D6) were specific to HCV portion, and one (R5D4) was specific to the GST portion of the fusion protein (Fig. 1). Clone R4H2 was further used in the inhibition ELISA. The binding of R4H2 scFv were inhibited, in a dose-dependent manner, by plasma containing human antibody to HCV confirming the specificity of this scFv (Fig. 2).

**Nucleotide sequence analysis of HCV-specific phagemids**

The VH and VL genes from 3 HCV-specific phagemids (R4H2, R4F3, and R4A3) were sequenced (Fig. 3 and Fig. 4). The VH gene segment of all 3 phagemids were almost identical to each other and were derived from the same germline gene segment in the VH3 gene family, with the closest similarity being to the DP-31 and DP-32 germline genes. The J regions were derived from JH4 germline segment. However, there were some differences from the closest matched germline genes, indicating the presence of somatic mutation. The putative amino acid sequences of VH were identical to each other (Fig. 4).
Fig. 3 Alignment of nucleotide sequences of (a) VH and (b) VL genes of 3 HCV-specific scFv phagemids.
(b) VL gene

R4H2-VL
R4F3-VL
R4A3-VL

CDR1
40

TCCTGACTGAAACTGGTACGTTGGTGGTTATGACTATGTCTCCTGGTACCACAG

CDR2
120

CACCCAGCCAAGCCCAACTCTCTCATCTATGTAACAGCAATCGGCCCTCAGGGTC

CDR3
180

CACGGCTAGGGATGAGGCTGATTATTTCTGCAGCACATATGCACCCCCC

240

CCTGATCGCTTCTCTGCCTCCAAGTCCGGCAATTCGGCCTCCCTGACCATCTCTGGACTC

320

AGGCTGAGGATGAGGCTGATTATTTCTGCAGCACATATGCACCCCCC

290

R4H2-VL
R4F3-VL
R4A3-VL

330

TTCGGCGGAGGGACCAAGCTGGAGATCAGA

R4H2-VL
R4F3-VL
R4A3-VL

TTCGGCGGAGGGACCAAGCTGGAGATCAGA
The V\(L\) gene segment of all phagemids were of the lambda type. Each of the 3 phagemids had different V\(L\) sequences including those in the CDR regions, although their V\(L\) segments were derived from the VL2 germline gene with closest similarity to the 2a2.272A12 gene. The J genes of R4H2 and R4F3 were derived from the JL2/JL3a and JL3b segments, respectively. The germline origin of the R4A3 J segment could not be identified. The V\(L\) genes of these 3 phagemids were also different from the closest matched germline genes, indicating the presence of somatic mutation.

**DISCUSSION**

This study demonstrated the isolation of phagemids producing single-chain F\(v\s\) specific to recombinant HCV nucleocapsid protein. The competing strategy was successfully employed to minimize the selection of scF\(v\) specific to GST, by blocking the scF\(v\) phage display antibody library with GST prior to offering to the recombinant HCV-GST fusion protein-coated immunotube. After 4 rounds of selection, the frequency of bound phagemids was increased by 100,000 fold, from \(3 \times 10^8\) to

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### Feb. 7, 2023

(a) VH putative amino acid sequence

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<tr>
<td>(\text{R4F3-VH})</td>
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CDR2 (cont)

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(b) VL putative amino acid sequence

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<td><em><strong><strong><strong>a_________ka________i________f________i</strong></strong></strong></em></td>
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<td>(\text{R4A3-VL})</td>
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CDR3

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Fig. 4 Alignment of putative amino acid sequences of (a) VH and (b) VL genes of 3 HCV-specific scF\(v\) phagemids.
3.6 × 10^3. This demonstrated the efficiency of multiple rounds of selection. More than 10^6 phagemids were obtained after eluting the bound phagemids from the antigen-coated immunotubes, about 5% and 9% of which were reactive to the HCV portion of the recombinant HCV-GST fusion protein, after round 4 and round 5, respectively.

The specificity of the scFvs to HCV nucleocapsid protein was illustrated by their specific interaction with the recombinant HCV-GST fusion protein but not with purified GST in ELISA test and, using R4H2 scFv as a model, by the ability of antibody in human plasma from HCV-infected blood donor to block the binding of scFv to recombinant HCV-GST fusion protein, in a dose-dependent manner. Normal human plasma did not block this binding. This also demonstrated that this specific scFv recognized the same epitope as that which occurred during natural infection in humans.

Phage display antibody library selection is a very powerful technique for producing human antibody against several antigens. Instead of having to construct the library from immunized or infected individuals, the recent availability of universal libraries containing a large repertoire of human immunoglobulin genes has enabled us to isolate HCV-specific scFv from this unimmunized library. Although the selection method is still complicated and requires experienced investigators, scFv with good specificity can be isolated by trained scientists within weeks as compared to months by using hybridoma technique. Our group has recently reported the production of murine hybridoma clones reactive to the recombinant HCV-GST fusion protein. Both the scFvs and murine monoclonal antibodies were specific to the same antigen. The hybridoma-derived antibodies were of murine origin. Murine monoclonal antibodies are complete antibody molecules with intact effector function, and are good reagents for diagnostic purposes. It is expected that the phage display antibody technology will not replace hybridoma technology for producing specific diagnostic reagents. However, murine monoclonal antibodies have limited value for in vitro treatment. On the other hand, the variable regions of both heavy and light chain immunoglobulin of the scFvs are of human origin which enabled them to be used in humans. However, scFvs are small molecule without the effector functions of intact antibody molecule, and further cloning is needed to add effector function domains to this molecule. Complete antibody molecules can be constructed from the scFvs and the "cassette-type" cloning system is now available. In its present format, scFv can be used as a diagnostic reagent and the specific binding between scFv and antigen can be detected by utilizing the epitope tagged to scFv, such as c-myc peptide, as illustrated in this study. It should be noted that the scFvs isolated from the universal unimmunized phage display antibody do not have natural somatic mutations which occur after natural infection in vivo and therefore lack affinity maturation process which may be important if high-affinity antibodies are required. The changes in the nucleotide sequences of the scFv clones from the closest germline V gene sequences, as seen in this study, indicated mutations that occurred prior to the construction of the library and prior to the encounter with the antigens. However, affinity maturation, if required, can be genetically mimicked in vitro by several molecular biological techniques such as chain shuffling, point mutagenesis and affinity-driven selection.

There were two previous reports on the isolation of phage antibodies to HCV proteins, one in the Fab format and the other in the scFv format. In contrast to this study, in which the universal library was used, in those two reports the phage display libraries were constructed from HCV-infected individuals. The obtained phage antibodies, two scFv fragments and one Fab format, were reactive to the HCV synthetic nucleocapsid peptides. Interestingly, the VH of all phage antibodies specific to HCV nucleocapsid protein were derived from the VH3 segment of the germline immunoglobulin gene, similar to that demonstrated in this study. The analysis of V segment usage of a hybridoma-derived monoclonal antibody to HCV nucleocapsid protein also showed that VH3 gene segment was used. However, the nucleotide and deduced amino acid sequences of the phage antibodies isolated by ours and other groups were different from one another. All three scFvs sequenced in this study had almost identical VH sequences with only one change in the CDR3 segment of one scFv, which did not affect the amino acid sequence. It is possible that if more specific scFv clones are sequenced, those with different nucleotide and deduced amino acid sequences in the VH gene might be obtained. The VL of all three scFv were of lambda type but had different sequences. This indicated that the VH component may contribute more than VL in the
specificity to HCV nucleocapsid protein.

In summary, this study demonstrated the feasibility of isolating genetically engineered scFv antibodies to hepatitis C virus nucleocapsid protein by using universal phage display antibody library. The scFvs have been isolated and confirmed to be specific to the viral antigen. The analysis of nucleotide sequences showed that these scFvs contained identical VH amino acid sequence, but utilized different VL sequences.

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REFERENCES