CLONING, EXPRESSION AND IDENTIFICATION OF THE GENE OF HUMAN SINGLE-CHAIN VARIABLE FRAGMENT ANTIBODY AGAINST HEPATITIS B VIRUS SURFACE ANTIGEN

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Summary. – Expression of single-chain variable fragment (scFv) antibodies on the surface of bacteriophage is widely used to prepare antibodies with pre-defined specificities. A phage antibody library containing the gene for scFv antibody against Hepatitis B virus surface antigen (HBsAg) was panned with HBsAg immobilized on microtiter plate wells. After five rounds of panning 30 phage clones specific to HBsAg were obtained and one selected clone was sequenced. It was found to consist of 789 bp and its amino acid sequence and specifically detected the respective antigen in the patients but not in healthy persons.

Key words: Hepatitis B virus surface antigen; phage display; single-chain variable fragment antibody

Introduction

Hepatitis B virus (HBV) is one of the agents of human liver diseases including acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma (Birrer *et al*., 2003). The disease, with an estimate of 350 million chronically infected persons is worldwide, is a great public health problem (Davis, 2002). Effective HBV vaccines preventing HBV infection have been developed. Antibody responses to the common epitope of HBsAg are considered to confer protection against HBV infection regardless of viral subtype. HBV infection still occurs in spite of the presence of HBsAg antibodies (Lee *et al*., 2001; Kidd-Ljunggren *et al*., 2002; Nainan *et al*., 2002). In some cases HBV variants have appeared. A mutation destroying the antigenicity of HBsAg would have direct impact on the diagnostics and therapy of HBV infection (Fischer *et al*., 2001). The variability of the HBV S gene causes difficulties in serological detection of the virus and vaccine design. A MAb to HBsAg could passively prevent HBV infection. However, the preparation of MAbs by hybridoma technique is relatively time-consuming. In addition, clinical testing has shown that murine MAbs may evoke a human anti-mouse antibody response.

Phage display technology offers a means of cloning human anti-pathogen antibodies-coding genes of a defined specificity that may have potential therapeutic use (Canaan-Haden *et al*., 1995). In addition, a HBsAg-binding phage has been used for phage-linked immunosorbent assay. In detecting HBsAg, the assay was more sensitive than conventional ELISA (Lu *et al*., 2004). A semi-synthetic human scFv antibody library and solid-phase-bound HBsAg are currently used in our laboratory to screen out the phage display antibody that recognizes HBsAg (Cheng *et al*., 2000; Kim and Park, 2002).

Materials and Methods

Materials. A recombinant HBsAg from Virostat (USA) was employed. A human scFv antibody phage library in which the genes

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Abbreviations: HRP = horseradish peroxidase; HBsAg = Hepatitis B virus surface antigen; HBsAg scFv antibody = scFv antibody against HBsAg; HBV = Hepatitis B virus; MAb = monoclonal antibody; scFv = single-chain variable fragment

encoding VL and VH were amplified by PCR with degenerate primers and connected with a glycine linker [$(Gly_4Ser)_3$], was purchased from Novagen (USA). M13K07 phage was employed as helper (Pharmacia).

Phagemid rescue. To rescue phagemids from the library, 5 ml of 2×TY broth containing 100 mg/ml ampicillin and 1% glucose (2×TY-AMP-GLU) was inoculated with 10 µl of *Escherichia coli* TG1 taken from the library stock and grown for 3 hrs at 37°C. The bacteria were spun down, resuspended in 50 ml of 2×TY broth containing 100 mg/ml ampicillin (2×TY-AMP), and shaken until A_{600} reached 0.5. The bacteria were inoculated with M13K07 phage (1×10^{10} PFU) and incubated for 30 mins at 37°C without shaking. After pelleting the cells were resuspended in 200 ml of 2×TY broth containing 100 mg/ml ampicillin and 25 mg/ml kanamycin (2×TY-AMP-KAN), and shaken for 12 hrs at 37°C. Phage particles were purified and concentrated using polyethylene glycol and resuspended in 2 ml of distilled water.

Screening of HBsAg scFv antibody clones. Delta surface plates (Nalge Nunclon International, Denmark) were coated with 1 ml of HBsAg (80 µg/ml) per plate in a coating buffer (50 mmol/l carbonate/bicarbonate pH 9.6) overnight at 4°C. After washing with Tris-borate-saline (TBS), the plates were incubated in 2% BSA in PBS for 2 hrs at 37°C (blocking). The washing was repeated and 1 ml of purified phage in 2% BSA per plate was added. The plates were rotated gently for 10 mins, left undisturbed for 90 mins at 37°C, and washed 20 times with PBS with 0.1% Tween 20 and 20 times with PBS. The bound phage particles were eluted from the plates with 1 ml of 100 mmol/l triethylamine per plate. The phage-containing elutate was immediately neutralized with 0.5 ml of 1.0 mol/l Tris-HCl pH 7.4 and stored at 4°C. The phage was used to infect 10 ml of log-phase *E. coli* TG1 plated on TYE-AMP-GLU plates and grown overnight at 37°C. The colonies were picked up into 2 ml of TYE-AMP-GLU per colony with 30% (v/v) glycerol and stored at -20°C. The panning (amplification-adsorption-elution) was repeated 5 times, and 30 phage clones were picked up randomly from well-isolated colonies on the top-agar plates. Each clone was grown in 400 µl of 2×TY-AMP-GLU at 37°C overnight. Aliquots (20 µl) of each clone were transferred to 400 µl of 2×TY-AMP and further cultured until A_{600} reached 0.5. After adding a helper phage the cultures were incubated at 30°C overnight. To collect samples for ELISA the supernatants after centrifugation at 12,000 r.p.m. for 1 min at 4°C were saved.

Identification of phage clones. Wells of 96-well-plates were coated overnight with 8 µg HBsAg in 100 µl of coating buffer per well. After blocking for 2 hrs at 37°C 50 µl aliquots of culture supernatants and 50 µl of 2% BSA were added per well for 1 hr at 37°C. After washing with 0.05% Tween 20 in PBS a 1/5000 dilution of horseradish peroxidase (HRP)-labeled anti-M13 secondary antibody was added for 1 hr at 37°C. The plates were washed with 0.05% Tween 20-PBS and a tetramethylbenzidine solution was added for 10 mins at 37°C. The reaction was stopped with H_2SO_4 and A_{450} was read.

ELISA. Wells of 96-well-plates were coated overnight with 8 µg HBsAg per well in the coating buffer. After blocking 100 µl aliquots of diluted (1:50) culture supernatants were added per well for 1 hr at 37°C. The plates were washed and loaded with the secondary antibody as described above. M13K07 phage served as negative control.

DNA sequencing. Plasmid DNA was prepared from the culture of a selected positive clone using Wizard Plus Minipreps DNA Purification System (Promega) and sequenced in an ABI3700 automated DNA sequencer (Perkin Elmer).

Expression of soluble HBsAg scFv antibody in E. coli. To express the scFv antibody in soluble E-tagged form the selected clone was subcloned into the pCANTAB5E expression vector. Restriction digestion and subsequent 1% agarose gel electrophoresis confirmed the identity of the recombinant pCANTAB5E-scFv vector. Competent *E. coli* XL1-Blue was transformed with pCANTAB5E-scFv and induced with IPTG for 20 hrs. The culture was centrifuged at 10,000 r.p.m. and the supernatant was subjected to SDS-PAGE and Western blot analysis.

Western blot analysis. The culture supernatant was diluted 1:1 with 2×SDS loading buffer, heated at 100°C for 10 mins, briefly centrifuged again, and 20 µl of the supernatant was used for SDS-PAGE. After the run the gel was blotted onto a PVDF membrane (Millipore). The blot was blocked with 5% non-fat dry milk for 2 hrs, incubated with an anti-E-tag MAb for 1.5 hr and with a secondary HRP-goat anti-mouse IgG antibody for another 1 hr, and stained with DAB and hydrogen peroxide.

Immunohistochemistry. Paraffin-embedded liver tissue slices from patients with positive HBsAg and HBV-DNA were examined. After deactivating endogenous hyperoxidase the slices were submersed in 0.5% hydrogen peroxide in methanol at room temperature for 50 mins, washed with PBS 3 times, and kept in 5% BSA overnight at 4°C. Then the slices were incubated with the scFv antibody diluted 1:100 for 1 hr at 37°C and overnight at 4°C. A sheep HRP-anti-M13 antibody diluted 1:200 was dropped on tissue preparations and left to react for 40 mins at 37°C. The preparations were washed 3 times with PBS and a few drops of a DAB solution (9 mg DAB, 13.5 ml 0.01 mol/l Tris-HCl pH 7.6, 1.5 ml 0.3% $CaCl_2$, and 15 ml 30% hydrogen peroxide) were added. After 10 mins at room temperature the preparations were washed with PBS 3 times and 1% heamatin was used to stain the cell nucleus. After a standard dehydratation procedure the preparations were observed under microscope. Negative controls consisted of PBS instead of the scFv antibody and liver tissue preparations from healthy persons.

Results

Identification of HBsAg scFv-positive clones

After five rounds of panning (amplification-absorption-elution) 30 clones were picked up and tested for HBsAg scFv antibody by ELISA. Twenty of these were found positive. Five of these showed a low cross-reaction with BSA (Fig. 1). One positive clone with the highest reaction with HBsAg and the lowest reaction one with BSA was chosen for a confirmatory test with restriction digestion. The digestion with *Sfi*I and *Not*I and subsequent gel electrophoresis proved the presence of a 789 bp insert corresponding to the scFv antibody gene (Fig. 2).

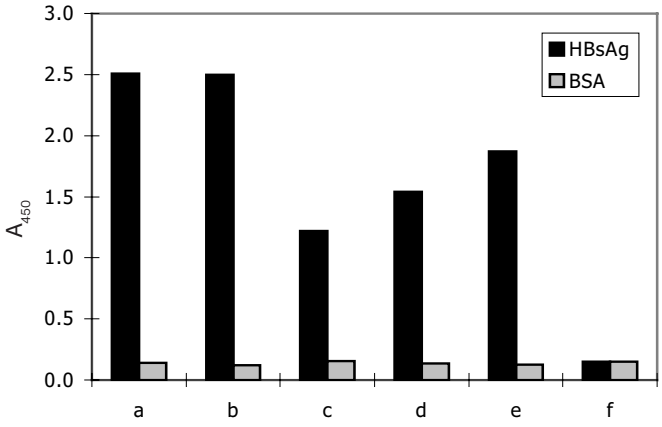


Fig. 1

Identification of phage clones positive for HBsAg scFv antibody

ELISA of the binding of five clones (lanes a–e) to HBsAg. Helper M13K07 instead of tested clones used as negative control (lane f).

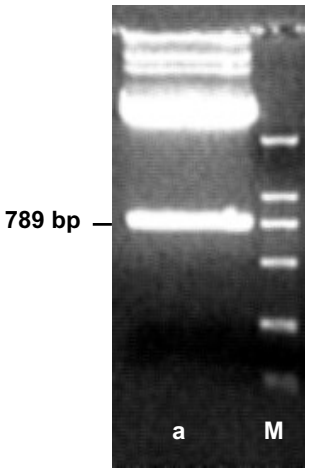


Fig. 2

Digestion of the recombinant pCANTAB5E-scFv vector with restriction endonucleases *Sfi*I and *Not*I

Agarose gel electrophoresis. pCANTAB5E-scFv (lane a) and DNA size marker (lane M).

Sequencing of HBsAg scFv gene

The nucleotide sequence of the selected clone was determined and submitted to GenBank (Acc. No. AF236816). The corresponding amino acid sequence was deduced and complementarity-determining regions and framework regions were determined (Table 1).

Expression of HBsAg scFv in E. coli

The HBsAg scFv antibody was expressed in *E. coli* and confirmed by Western blot analysis (Fig. 3). A negative control consisting of *E. coli* infected with empty vector did not show any specific protein. These results indicated that the soluble form of human HBsAg scFv antibody was successfully expressed in this system.

Fig. 3

Western b lot analysis of the supernatants from induced and non-induced *E. coli* transformed with pCANTAB5E-HBsAg-scFv

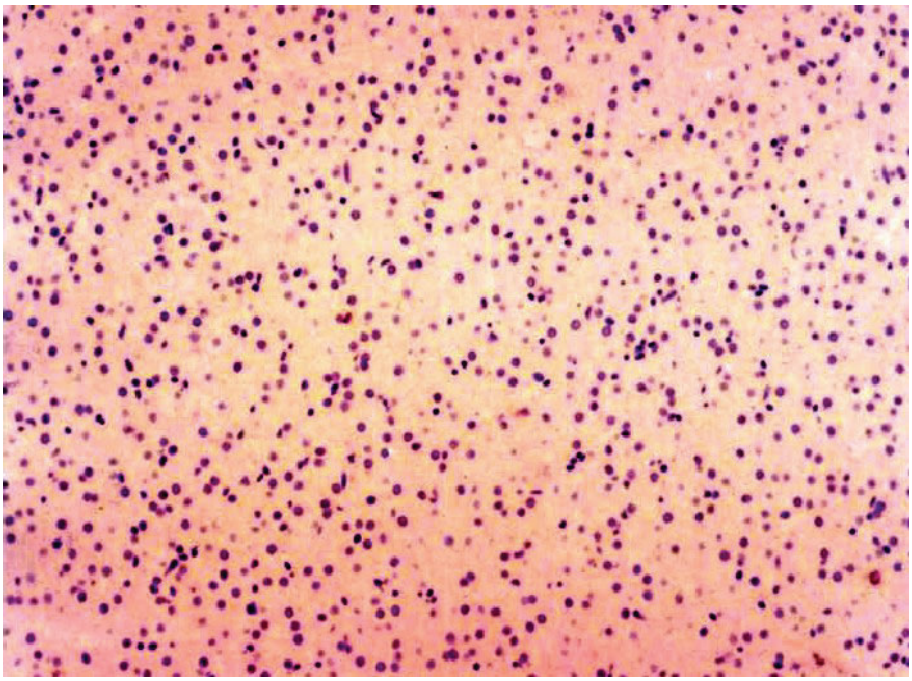
Non-induced (lane a) and induced (lane b) cultures.

Immunostaining of HBsAg of liver tissue sections

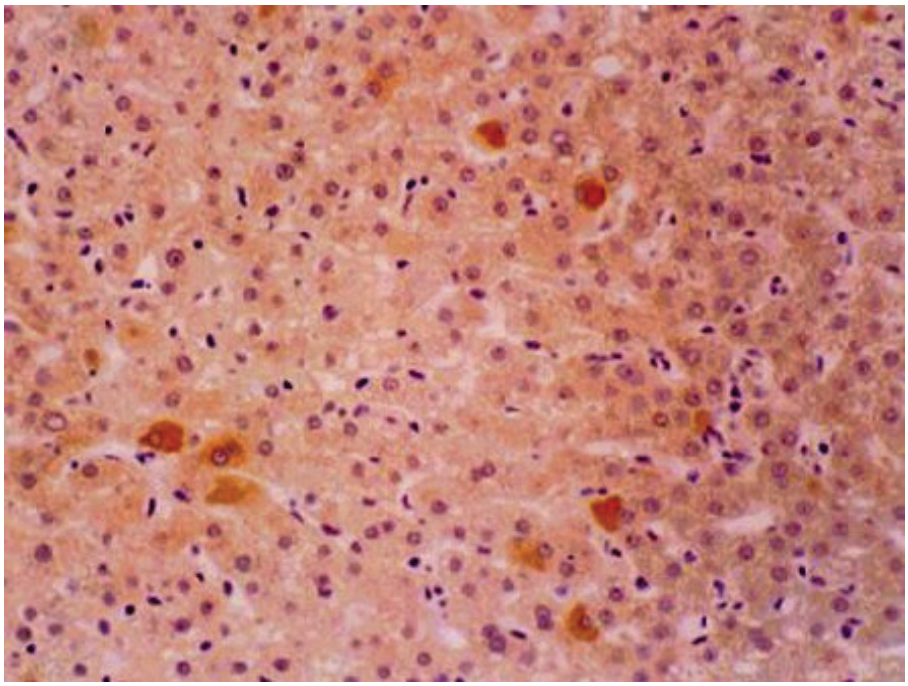
Immunostaining of sections of liver tissues from and patients with chronic hepatitis B and healthy persons gave positive results in the former but not latter group (Fig. 4A). HBsAg was mainly located in the plasma membrane of the hepatocytes.

Table 1. The complementarity-determining regions (CDRs) and framework regions (FRs) of deduced amino acid sequence of HBsAg scFv antibody

	CDR1	CDR2	CDR3	FR1	FR2	FR3	FR4
H chain	GFTFSDY-YMS	YISSSGSTI-YYADSVKG	KLRNGRW-PLVY	EVQLVESGGGLVK-PGGSLRLSCADS	WIRQAPGKG-LEWVS	RFTISRDNAKNSLYLQ-MNSLRAEDTAVYYCAR	WGQGTLVTVSR
L chain	CQGDSLR-SYYAS	GKNNRPS	NSRDSSG-NHVV	SELTQDPAVS-VALGQTVRIT	WYQQKPGQA-PVLVIY	GIPDRFSGSSSGNTAS-LTITGAQAEDEADYYC	FGGGTKLTVLG



A



B

Fig. 4

Immunostaining of sections of liver tissues from healthy persons (A) and patients with chronic hepatitis B (B) for HBsAg using HBsAg scFv antibody

Discussion

The preferential attachment and entry of HBV into hepatocytes is an initial step in HBV infection. The HBV particle surface proteins have been considered crucial molecules in recognizing the receptors on the plasma membrane of human hepatocytes (Meyer *et al.*, 1997). HBsAg antibodies can block the molecular mechanism of attachment and penetration of HBV into human hepatocytes and indicate recovery from the infection.

The *a*-determinant of HBsAg, a predicted double-loop structure projecting from the surface of the HBV particle (Stirk *et al*., 1992), is the major neutralizing epitope. Antibodies to the *a*-determinant confer protection of adults from all common HBV subtypes. Within the predicted loop regions are also located subtype determinants *d* or *y* and *w* or *r*. A total of 8 major serological subtypes of HBV have been so far recognized. More recently, the serotypes were replaced with genotypes, represented by 8 different viruses, from HBV-A to HBV-E (Norder *et al*., 1992, 1993; Fauquet *et al*., 2005). These viruses differ in S gene sequences.

Phage libraries are a powerful tool for the selection of antibodies of important and useful specificities, particularly for humanized scFv antibodies (Marks *et al*., 1991; Hoogenboom *et al*., 1998; Lamarre and Talbot, 1997; Rondon and Marasco, 1997). It has many advantages. First, it is the only method to get specific antibody by passing the immunization step. It can mimic the maturation process of human antibody *in vivo*, so that it is possible to obtain a high affinity antibody from this selection. Second, a scFv antibody consisting of antigen-binding domains of heavy and light chain regions of immunoglobin connected by a flexible peptide linker is a small-size molecule compared with the full-length antibody. If an antibody library of human origin is used, the selected antibody is most suitable to human administration and is potentially applicable to clinical diagnosis and treatment of both infectious disease and cancer. Finally, as it contains no Fc fragment, its background in immunohistological study is very low. In contrast, a MAb against HBsAg prepared from hybridomas is of murine origin and hence immunogenic if used systemically in humans (Hoogenboom *et al*., 1998). In order to overcome the disadvantages of an intact MAb applied *in vivo* and to offer an antibody with a stable genetic source, soluble scFv antibodies are currently generated by advanced recombinant phage antibody technique, which may provide novel targeting vehicle for diagnosis and treatment of diseases.

In this study, we succeeded in cloning the HBsAg scFv gene by means of the phage display library technique. The cloned gene was sequenced and expressed in *E. coli.* These results illustrate the feasibility of using the antibody-engineering technology that may prove useful in the future for diagnostics and therapy of hepatitis B infection.

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