Detection of anti-preS1 antibodies for recovery of hepatitis B patients by immunoassay

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AIM: To establish a convenient immunoassay method based on recombinant antigen preS1(21-119aa) to detect anti-preS1 antibodies and evaluate the clinical significance of antibodies in hepatitis B.

METHODS: The expression plasmid pET-28a-preS1 was constructed, and a large quantity of preS1(21-119aa) fragment of the large HBsAg protein was obtained. The preS1 fragment purified by Ni²⁺-IDA affinity chromatography was used as coated antigen to establish the indirect ELISA based on streptavidin-biotin system for detection of the anti-preS1 antibodies in sera from HBV-infected patients. For follow-up study, serial sera were collected during the clinical course of 21 HBV-infected patients and anti-preS1 antibodies, preS1 antigen, HBV-DNA and other serological HBV markers were analyzed.

RESULTS: preS1(21-119aa) fragment was highly expressed from the plasmid pET-28a-preS1 in a soluble form in E.Coli (30mg·L⁻¹), and easily purified to high purity over 90% by one step of Ni²⁺-IDA-sepharose 6B affinity chromatography. The purity and antigenicity of the purified preS1(21-119aa) protein was determined by 150gL⁻¹ SDS-PAGE, Western blot and a direct ELISA. Recombinant preS1(21-119aa) protein was successfully applied in the immunoassay which could sensitively detect the anti-preS1 antibodies in serum specimens of acute or chronic hepatitis B patients. Results showed that more than half of 19 acute hepatitis B patients produced anti-preS1 antibodies during recovery of the disease, however, the response was only found in a few of chronic patients. In the clinical follow-up study of 11 patients with anti-preS1 positive serological profile, HBsAg and HBV-DNA clearance occurred in 6 of 10 acute hepatitis B patients in 5-6 months, and seroconversion of HBsAg and disappearance of HBV-DNA occurred in 1 chronic patients treated with lamivudine, a antiviral agent.

CONCLUSION: The high-purity preS1(21-119aa) coated antigen was successfully prepared by gene expression and affinity chromatography. Using this antigen, a conveniently detective system of anti-preS1 antibodies in sera was established. Preliminary clinical trial the occurrence of anti-preS1 antibodies in acute hepatitis B patients suggests the clearance of HBV from serum in a short-term time, and anti-preS1 positive in chronic patients means health improvement or recovery from the disease.

INTRODUCTION

Human hepatitis B virus (HBV) is a small enveloped DNA virus which causes acute and chronic hepatitis in humans[1]. Worldwide, the number of infected persons is predicted to reach 400 million during 2000. Areas with high prevalence of HBV include China[2,3], Southeast Asia and Africa, where approximately 10% of the population are chronic carriers[4]. The envelope of HBV contains three related proteins, encoded by the S open reading frame (ORF) of the viral genome, composed of three regions: preS1, preS2 and S. The major protein, termed small HBs protein (SHBs), is encoded by the S gene, whereas the two minor envelop proteins, termed the middle protein (MHBs) and the large protein (LHBs), are encoded by the preS2 region and preS1+preS2+S region, respectively[5]. The preS1 region contains epitopes that elicit immune responses at the B-cell and T-cell level over a broader range of MHC haplotypes than those on the preS2 and S protein[6]. The preS1 domain also contains potential viral attachment sites to hepatocytes[7] and elicit antibodies capable of neutralizing HBV in the chimpanzee[8].

The preS1 epitope has been extensively analyzed using synthetic peptides, anti-peptide sera and anti-preS1 monoclonal antibodies. B cell epitopes have been mapped to residues 27-35aa, 72-78aa, 32-47aa, 41-53aa, 94-105aa and 106-117aa in the preS1 region[9-11], and T cell epitopes mainly located in residues 12-21aa, 21-30aa, 29-48aa and 94-117aa of the preS1 region[12-14]. These findings explain why the preS1 region has good immunogenicity and can easily elicit the anti-preS1 responses[15]. Additionally, nearly all preS1 epitopes concentrate on residues 21-119aa in the preS1 region (ad subtype; 8 residues on N-terminus of preS1 are absent in ay subtype). Because the preS1 region locates on the outside of the mature virion and has many overlapping epitopes, anti-preS1 immune response occurred early in the course of the disease and is involved in the clearance and neutralization of HBV. As anti-preS1 antibodies have been mainly detected early during acute hepatitis B[16,17], the anti-preS1 antibodies could represent an early serological marker for HBV clearance[18]. About 1/2 of subjects in the convalescence phase of acute hepatitis B were serological positive for anti-preS1 antibodies, whereas persistence of preS1 antigen and lack of corresponding antibodies have been the predict of the evolution of a chronic course of disease[19]. In this study, preS1(21-119aa) peptide was chosen and highly expressed in a soluble form. Using this protein as the antigen, an effective enzyme-linked immunosorobent assay was established. The sensitive test can provide a basis for monitoring anti-preS1 in sera of patients with hepatitis B and offers a prognostic implication for patients.
MATERIALS AND METHODS

Materials

Plasmids, E. coli strain The plasmid pADR-1 containing HBV genome (adr subtype) was constructed by Wu et al.[26]. Expression vector pET-28a (+) was purchased from Novagen. E. coli strain BL21 (DE3) ployS was used for the production of the preS1(21-119aa) peptide.

Enzymes and Reagents Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, Boehringer Mannheim and GIBCO BRL. DNA sequence kit was from USB. Agrose and LMP agarose were from GIBCO BRL. Acrlyamide, bisacrylamide, isopropylthio-β-D-galactoside (IPTG), imidazole and iminodiacetic acid (IDA)-Sepharose 6B, biotinaminidocaproatate-N-hydroxysuccinimide ester (BNSE) and 3,3’,5,5’-tetramethylbenzidine (TMB) were from Sigma. Protein A was from Pharmacia. Diaminobenzidine (DAB) and streptavidin-horseradish peroxidase (HRP) conjugate were from Sigma. Tetrabutyl ammoniumborohydride monoclonal antibody (mAb) 125E11 and 125E11 conjugated with (DAB) and streptavidin-horseradish peroxidase (HRP) conjugate were from Sigma. Protein A was from Pharmacia. Diaminobenzidine (DAB) and streptavidin-horseradish peroxidase (HRP) conjugate were from Sigma.

Serum specimens and kits for HBV markers Serum specimens were collected from patients admitted to the Rui-Jin Hospital with clinical and biochemical evidence of hepatitis B. As a control, sera of healthy persons with normal liver function and without any markers of HBV infection were used. HBsAg and anti-HBs, HBeAg and anti-HBe, anti-HBC were determined by commercially available ELISA kits from Abbott laboratories. PreS1 protein were detected by a double mAb sandwich ELISA[22]. HBV-DNA level in serum samples was assayed by HBV DNA sequence kit was from USB. Agrose and were purchased from New England Biolabs, Boehringer Mannheim.

Methods

Construction of expression plasmid containing preS1(21-119aa) DNA sequence.

Preparation of plasmid DNA, PCR, digestion of DNA with restriction enzymes, agarose gel electrophoresis, recovery and purification of DNA fragment, ligation of terminus created by restriction enzymes and transformation of competent E. coli were performed according to Sambrook[23].

Cell culturing and harvesting E. coli strain BL21(DE3) ployS cells harboring the expression plasmid pET-28a-preS1 were inoculated into LB medium containing kanamycin (50mg·L⁻¹). The seed culture was inoculated into 100-fold volume of fresh LB medium (kanamycin, 50mg·L⁻¹) and cultured at 37°C up to 0.4-0.6 of A600. To this culture IPTG was added to give a final concentration of 0.2mmol·L⁻¹, and incubation was continued at 37°C for 6h or 22°C for 24h. The induced cells, as well as the uninduced cells, were harvested by centrifugation, and cell lysates were analyzed by 150g·L⁻¹ SDS-PAGE.

Western analysis of the preS1(21-119aa) fusion protein After SDS-PAGE, proteins were transferred to nitrocellulose membrane (Schleicher & Schuell), and the membrane was incubated with a murine monoclonal antibody 125E11 (1:1000, volume ratio), which recognizes preS1(21-47aa) fragment within the preS1 region, followed by goat anti-mouse IgG peroxidase conjugate (1:1000, volume ratio). After washing, substrate solution containing DAB (0.5g·L⁻¹) and H₂O₂ (0.02mL·L⁻¹) was added to have 125E11 specific binding protein bands visualized.

Purification of the preS1(21-119aa) fusion protein In order to characterize whether the expressed fusion protein is soluble or insoluble, the cell pellet from 100ml culture was resuspended in 4ml buffer A (20mmol·L⁻¹ Tris-HCl pH7.9, 0.5mol·L⁻¹ NaCl, 100g·L⁻¹ glycerol, 1mmol·L⁻¹ PMSF, 100mmol·L⁻¹ imidazole), and sonicated in ice bath. Then the sonicate was centrifuged at 18000r.min for 15min, and the resulting supernatant was concentrated to 2mL, and the pellet was resuspended in 2mL buffer A as the insoluble protein sample. The soluble and insoluble samples were subjected to 150g·L⁻¹ SDS-PAGE and Western blot analysis.

2mL supernatant was applied to 2mL Ni²⁺-IDA sepharose 6B column (2mL) equilibrated with buffer A at 4°C. The column was washed with 30mL buffer B (20mmol·L⁻¹ Tris-HCl pH7.9, 0.5mol·L⁻¹ NaCl, 100g·L⁻¹ glycerol, 1mmol·L⁻¹ PMSF, 45mmol·L⁻¹ imidazole), and the bound protein was eluted with 4mL buffer C (20mmol·L⁻¹ Tris-HCl pH7.9, 0.5mol·L⁻¹ NaCl, 100g·L⁻¹ glycerol, 1mmol·L⁻¹ PMSF, 100mmol·L⁻¹ imidazole) and the elution samples were collected[24, 25]. The fractions enriched for preS1(21-119aa)-His, tag fusion protein was identified by 150g·L⁻¹ SDS-PAGE and Western blot analysis.

Biotin labeling of protein A The procedure is essentially the same as that described by Kittigul et al.[26]. Protein A (2.0g·L⁻¹) is dialyzed against 0.01mol·L⁻¹ NaHCO₃, at 4°C. After dialysis, 1mL of the protein A solution was mixed with 120µL of BNSE (1.0g·L⁻¹ in dimethyl sulfoxide). The mixture was incubated at 4°C overnight and dialyzed overnight against PBS at 4°C with several changes of PBS.

Antigenicity analysis of preS1(21-119aa) domain of the fusion protein The antigenicity of preS1(21-119aa) fusion protein was analyzed by a direct ELISA. The microtiter plate was coated with preS1(21-119aa) fusion protein from 10 to 2500ng·well, and mAb 125E11 labeled with HRP was added to each well and incubated at 37°C for 1h. The plate was washed, and substrate solution containing TMB (7.5g·L⁻¹) and H₂O₂ (0.3mL·L⁻¹) was added to each well to develop a color change, then the reaction was stopped with 1.0mol·L⁻¹ H₂SO₄ and A (absorbance) value was measured at 450nm in an ELISA reader (Labsystem Multiscan, Finland).

Establishment of indirect ELISA for detecting anti-preS1 antibodies in serum The optimal dilutions of the reagents are

Figure 1 Construction of expression plasmid pET-28a-preS1

Figure 1 depicts the steps leading to the final construction of the expression plasmid pET-28a-preS1 which expresses a fusion protein containing preS1(21-119aa) peptide with His6 tags. PCR primers were designed as follows:

Forward primer P₁: 5'-CATAGGATCCCTCTGGATACCTT-3' BamHⅠ
Reverse primer P₂: 5'-ATCGAGCTCTGATCTGACTTG-3' HindⅢ EcoRⅠ
determined by checkerboard titration. Mirotiter plates (Nunc, Denmark) were coated by incubation overnight at 4°C with 2mg·L⁻¹ preS1(21-119aa) protein in 100µL volume of per well in carbonate buffer (15mmol·L⁻¹ Na₂CO₃, 35mmol·L⁻¹ NaHCO₃, pH9.6). The microplates were then washed three times with a washing solution (20mmol·L⁻¹ Tris·HC1 pH7.4, 0.5mL·L⁻¹ Tween-20), and postcoated with 5g·L⁻¹ bovine serum albumin (BSA) in PB (2.7mmol·L⁻¹ KCl, 0.5mmol·L⁻¹ KH₂PO₄, 6.5mmol·L⁻¹ Na₂HPO₄, pH7.5) for 1h at 37°C. After washing, 100µL diluted serum samples [1:30 in PBFST (100mL·L⁻¹ fetal calf serum (FCS), 0.5mL·L⁻¹ NaCl and 0.5g·L⁻¹ Tween-20 in PB)] were added and incubated for 2h at 37°C. After washing, 100µL biotin labeled protein A solution (2mg·L⁻¹ in PBFST) diluted in PBFST was dispensed into wells, and the plates were incubated for 1h at 37°C. After washing 4 times, streptavidin-HRP conjugate diluted in PBFST was added into each well of the plates, and the plates were incubated for 1h at 37°C. Finally, after washing, 100µL of the substrate mixture (1.0mmol·L⁻¹ TMB, 0.2mmol·L⁻¹ TBABH, 0.2mol·L⁻¹ potassium citrate and 0.5mL·L⁻¹ H₂O₂, pH7.4) was added and incubated for 20min at 37°C. The reaction was stopped by the addition of 50µL of 1.0mol·L⁻¹ H₂SO₄. The optical density was measured at 450nm (A₄₅₀) with the ELISA reader. To determine cut-off value for the established ELISA, A₄₅₀ value of negative control for anti-preS1 antibodies was evaluated by testing a serum mixture from 100 normal individuals. The cut-off value was defined as 2.1 folds of mean of negative control. Specimens were considered to be positive when the absorbance value exceeded the cut-off value.

Specificity of established ELISA for detection of anti-preS1 antibodies Twenty-five µL anti-preS1 antibodies positive serum with the same volume of 0.15g·L⁻¹ preS1(21-119aa) protein or PBS was incubated at 37°C, and mixture was stepwise diluted and added to wells coated by preS1(21-119aa), and then indirect ELISA was performed. A parallel test with normal human serum was also performed in the same experiment.

Follow-up study The serum specimens from 11 anti-preS1 positive patients and 10 anti-preS1 negative patients with hepatitis B were collected at monthly intervals and their HBV markers including HBV-DNA, HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HBc, preS1 and anti-preS1 were analyzed.

RESULTS
Construction of expression plasmid pET-28a-preS1 PreS1(21-119aa) gene fragment was synthesized by PCR using the primers P₁, P₂ and the plasmid pADR-1 containing HBV genome (adr subtype) as the template. The PCR product was digested with BamHI and HindIII and then subcloned into the BamHI-HindIII sites of pET-28at+ to yield expression plasmid pET-28a-preS1 (Figure 1). Two His tag fragments which coded a stretch of six histamine residues located in both terminus of inserted fragment, respectively. This made it easy to purify preS1(21-119aa) fusion protein by Ni²⁺-IDA-sepharose 6B affinity chromatography. DNA sequence analysis confirmed that the expression plasmid coded the correct nucleotide sequence. The nucleotide sequence and deduced amino acid sequence of the preS1(21-119aa) region gene fragment inserted in the pET-28a-preS1 is shown in Figure 2.

Expression of preS1(21-119aa) fusion protein In order to express the preS1(21-119aa) fusion protein, the expression plasmid pET-28a-preS1 was transformed into E.Coli BL21(DE3) plysS, and the cells were cultured and induced by IPTG. The expressive levels were determined by SDS-PAGE and Western blot analysis of the cell lysates. As show in lane 2 of Figure 3, more than 30% of total stainable proteins was about 17000 fusion protein. The analysis of solubility showed that about 80% of the fusion protein was soluble (lane 3 of Figure 3). Thus, the soluble form was convenient for the followed purification and clinical application.

Purification and antigenicity analysis of the preS1(21-119aa) fusion protein The fusion protein was purified by affinity chromatography on Ni²⁺-IDA sepharose 6B column as described in Materials and Methods. The purity of purified fusion proteins was evaluated to be over 90% (Lane 5, Figure 3). In order to analyze antigenicity of the fusion proteins, microtiter plates were coated with stepwise diluted solutions (from 2500ng to 10ng per well) of purified preS1(21-119aa) protein, GST-preS1(21-47aa) or artificially synthesized preS1(21-47aa) peptide. Direct ELISA showed that they all reacted well with the preS1-specific mAb 125E11 in a dose-dependent manner, meanwhile the antigenicity of preS1(21-119aa) protein was better than GST-preS1(21-47aa) and much better than synthesized preS1(21-47aa) peptide (Figure 4).

Detection of anti-preS1(21-119aa) antibodies in sera from hepatitis B patients In order to observe specificity of established indirect ELISA, anti-preS1 antibodies positive serum (confirmed by Western Blotting) was used to test. The result showed in a drop of the extinction to a value close to the negative control, thus demonstrating specificity of the method (Figure 5).
Immunoreactivity of preS1(21-119aa), GST-preS1(21-47aa) peptide. Microtiter plate was coated with different amount (10-2500ng) of the purified preS1 proteins or peptide. Then direct ELISA was performed.

Figure 4

Specificity test of indirect ELISA for detection of anti-preS1 antibodies

In order to inspect established indirect ELISA, 192 serum specimens were collected from patients admitted to the Rui-Jin Hospital, Shanghai Second Medical University. Among them, there were 92 serum specimens with clinical and biochemical evidence of hepatitis B; and 100 sera of individuals without any markers of HBV infection and with normal liver function were assigned as negative controls. Additionally, 3 sera known to contain antibodies against preS1(21-119aa) with different level tested by Western analysis were used as positive controls. Through the indirect ELISA based on recombinant preS1(21-119aa) fusion protein, anti-preS1 antibodies were detected in none of 100 HBV negative controls. In HBsAg positive individuals (Table 1), anti-preS1 antibodies were noted in more than half of patients with acute hepatitis before or after recovery, but only found in a few of patients with chronic hepatitis, and the level of anti-preS1 antibodies had significant difference between acute hepatitis patients and chronic hepatitis patients (t test, \(P<0.01\)).

Table 1

| Subjects                      | n  | anti-preS1 Ab(+) positive/% |
|------------------------------|----|-----------------------------|
| Acute hepatitis              |    |                             |
| Before recovery (HBsAg+)     | 16 | 10                          | 62.5 |
| After recovery (HBsAg-)      | 17 | 9                           | 52.9 |
| Chronic carriers             |    |                             |
| Healthy chronic carriers     | 30 | 1                           | 3.3  |
| Chronic hepatitis            | 29 | 2                           | 6.9  |
| Total                        | 92 | 22                          | 23.9 |
| Control (negative for all HBV markers) | 100 | 0 | 0.0 |

Follow-up study

Diagnosed by clinical symptoms and serological profile, the inpatients for follow-up study were assigned to 10 acute hepatitis patients and 11 chronic hepatitis patients. Sera from 21 hepatitis B inpatients were collected at monthly intervals in half a year and anti-preS1 antibodies were detected by indirect ELISA. In Figure 6A, a typical profile of an acute episode of hepatitis B followed by seroconversion to anti-preS1 are presented. HBV-DNA and preS1 antigen were detectable simultaneously in the acute phase of the disease. Anti-preS1 developed early in infection, still in the presence of low level of preS1 antigen, and climbed the highest with disappearance of HBV-DNA and preS1 antigen. The results of detection of the other serological markers showed that anti-HBe and antiBs in sera of patients was followed by appearance of anti-preS1 antibodies.

Figure 6

The profiles of serologic markers during acute chronic HBV infection with immuno-diagnosis for preS1 antigen and anti-preS1 (21-119aa) antibodies. A: Typical serologic profile of HBV markers during acute infection, with disappearance of preS1 antigen and seroconversion to anti-preS1(21-119aa) antibodies; elimination of HBV-DNA in 2-4 months from the appearance of antibodies. B: Chronic patients with HBeAg+ serologic profile; high level of preS1 antigen and HBV-DNA but absence of anti-preS1(21-119aa) antibodies. C: Chronic patients with anti-HBe+ serologic profile; low level of preS1 domain and HBV-DNA and absence of anti-preS1(21-119aa) antibodies. D: One patient persisting of HBsAg, HBeAg and preS1 for almost 3 years until treated with Lamivudine. Appearance of anti-preS1(21-119aa) antibodies with simultaneous health improvement; disappearance of HBV-DNA and declining level of preS1 antigen in serum. OD\(_{400}\) value of Y axis (‘•’ preS1 antigen and ‘·’ anti-preS1 antibody) was mean value of inpatients in every group; different shade (■) in the rectangle (A) represented different level of HBV-DNA in patients.
Ten chronic hepatitis B inpatients were divided into two groups: one group was healthy chronic carriers (Figure 6B) who were seropositive HBsAg and high level of HBV-DNA and preS1 antigen; the other group was chronic hepatitis B patients (Figure 6C) who had seropositive anti-HBe and low level of HBV-DNA and preS1 antigen during the course of the disease. During follow-up period, anti-preS1 antibodies were not found and there were no apparent improvement in both groups. Interestingly, a patient (Figure 6D) treated with lamivudine, a anti-HBV-DNA replication agent, was different from the other chronic patients. Seroconversion of preS1 antigen to anti-preS1 antibodies was observed after lamivudine treatment. Although no elimination of HBsAg was observed in this patient, the development of anti-preS1 response correlated well with improvement in health.

**DISCUSSION**

The PreS1 domain is found exclusively in LHBs, which is a major component of the envelope of mature virions, but it is significantly less represented in subviral particles of HBV[5]. These findings suggest that PreS1 domain is located on the outer surface of the virion and thought to be involved in virus-host cell interaction[33-35]. Moreover, a that PreS1 domain is located on the out surface of the virion and application for detection of anti-preS1 antibodies.

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