

# Establishment and preliminary use of hepatitis B virus preS1/2 antigen assay

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## INTRODUCTION

Hepatitis B virus (HBV) is a DNA virus with an envelope, its membrane protein gene preS/S have three parts-preS1, preS2 and S, there is a translation initiation codon ATG in each of them. By different ATG, the membrane protein gene can be translated into three kinds of products, i.e. the large protein (preS1 + preS 2 + S), medium (preS2 + S) and small (S) (also called primary protein). In patients with acute hepatitis B, PreS antigen occurred together with other HBV markers and parallels the level of HBV DNA. Disappearance of PreS antigen predicts a benign outcome<sup>[1]</sup>. Its presence in chronic hepatitis B is somewhat correlated with HBsAg, the ratio of PreS1/HBsAg antigen titers can reflect the level of DNA replication<sup>[2]</sup>. Meanwhile, the PreS domain in the membrane protein include the binding sites for hepatocyte receptor and a number of epitopes of B cells and T cells. The PreS domain possesses very strong immunogenicity in mouse and man. In mice with consanguinity, the PreS domain can induce broad-spectrum protective antibodies and overcome their nonresponsiveness to protein S. In human body, it has been proved the PreS domain is an effective immunogen at both B and T cell levels. The antibodies directing against synthetic peptides (PreS: 21 - 47) mimicking receptor-binding sites can protect chimpanzee from HBV infection<sup>[3]</sup>. During the disease course appearance of antibody against PreS1 is considered an early marker for elimination of HBV infection<sup>[4,5]</sup>. Therefore PreS antigen/ antibody is a pair of important indexes for diagnosis of hepatitis B. As one part of a research project granted by the EC Foundation, we have

prepared a recombinant PreS protein with high purity, a polyclonal anti -PreS antibody and established the ELISA method for detection of PreS antigen, preliminary clinical studies had been carried out.

## MATERIALS AND METHODS

### *Immunologic reagents*

HRP-conjugated murine anti-human IgG was provided by Department of Molecular Immunology, Institute of Basic Medical Sciences, Academy of Military Medical Sciences; HRP-conjugated murine anti-human HBs were provided by the Department of Immunology, No.302 Hospital of PLA; and HRP-conjugated sheep anti-rabbit IgG was provided by the Department of Molecular Biology, No.307 Hospital of PLA.

### *Blood specimens*

They were provided by the Central Blood Bank of PLA units under Beijing Command and No.301, 302 Hospitals of PLA.

### *Preparation of PreS antigen*

The engineered *E. coli* for hepatitis B viral PreS/2 recombinant antigen was provided by Department of Virology, Max-Planck Institute of Biochemistry, Germany. The PreS1/2 recombinant antigen containing PreS1:10-108PreS2: 1-55/S1-22 (ayw subtype) was expressed by using pQe8 (Quiagen) expression vector and six histidin residuals were fused to the N-terminal of the product, which was purified by affinity chromatography using Ni-NTA agarose column (Quiagen) to a purity above 95%. The purified protein was stored at -20°C for use.

### *Preparation of recombinant antigen-directed immune sera*

New Zealand large-ear white rabbits were immunized by four injections of renatured PreS recombinant antigen, once each on the wk 0, wk 2, wk 7 and wk 11. One hundred micrograms of the antigen were injected subcutaneously on multiple sites at one time. In the first injection, Freund's complete adjuvant was used, while Freund's incomplete adjuvant was used in the latter three injections. Blood samples were obtained before each booster injection for determination of serum antibody titer. Serum was separated from blood

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samples. The anti-PreS sera were purified using Protein A Sepharose 4B Fast Flow affinity adsorption column and was stored at  $-20^{\circ}\text{C}$  for later use.

#### Preparation of ELISA plate for detection of PreS antigen

The ELISA plate was coated with purified rabbit anti-PreS polyclonal antibody at a concentration of  $10\text{ mg/L}$  at  $4^{\circ}\text{C}$  stored overnight. After blockage with 1% BSA and dilution, the plate was vacuum-dried. then it was stored at  $4^{\circ}\text{C}$  for later use. When it is used, test blood sample ( $50\text{ }\mu\text{L}$ ) was added and incubated at  $37^{\circ}\text{C}$  for 30min. After washing, the enzyme-labelled anti-HBs monoclonal antibody was added and incubated at  $37^{\circ}\text{C}$  for 30 min after washing, OPD color developer was added to develop the color by protecting from light at  $37^{\circ}\text{C}$  for 10 min, then  $2\text{ mol/L H}_2\text{SO}_4$  was added to terminate the reaction. The OD value was determined using DG3022 Enzyme Labelling Instrument. When the D value of the test sample was  $\geq$  the average positive value  $\times 0.08$ , it was taken as positive, otherwise, as negative.

#### Competitive inhibition test

The PreS-positive sera were diluted with serum-diluting solution containing purified rabbit anti-PreS antigen polyclonal antibody ( $20\text{ mg/L}$ ). After incubation at  $37^{\circ}\text{C}$  for 1 h, the diluted sera were placed at  $4^{\circ}\text{C}$  overnight. The  $100\text{ }\mu\text{L}$  of the sera were taken out and were added to the ELISA plate coated with rabbit anti-PreS1 antibody. After that the plate was incubated at  $37^{\circ}\text{C}$  for 30 min. The rest steps were carried out by following the routine. The neutralizing inhibition rate  $\geq 50\%$  was taken as positive.

$$\text{Competitive inhibition rate} = \frac{\text{Average non-competitive D value} - \text{Average competitive D value}}{\text{Average non-competitive D value}} \times 100\%$$

## RESULTS

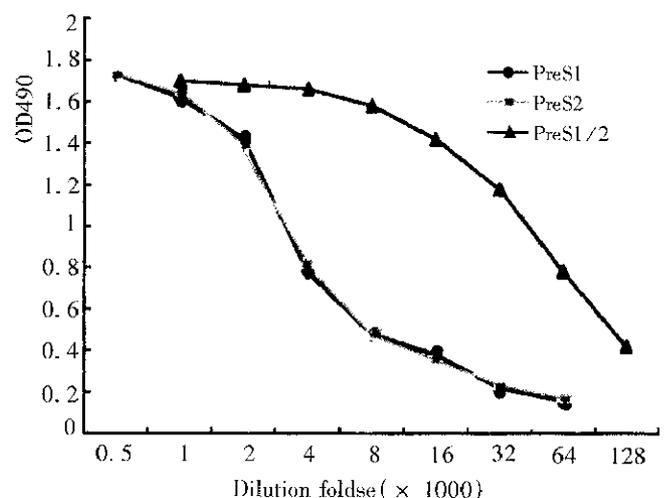
#### Purification of recombinant antigen

Using pQe8 expression vector, the vector pQePreS1/2 expressing HBV ayw subtype including PreS1 and PreS2 gene fragments was constructed. After incubation, PreS1/2 protein with the relative molecular mass of 22000 (including three parts-PreS1:10-108/preS2:1-55/S1-22) was expressed. There were six histidine residues binding to the N-terminal of the product, the quantity of the recombinant protein expressed amounted to about 10% of the whole *bacteria*. The PreS1/2 existed mainly in inclusion bodies. The inclusion bodies were dissolved by denaturant and were purified on Ni-NTA agarose column. A purity over 95% was achieved. From 1 L cultured bacteria, 10 mg

purified protein could be obtained. Then the denaturant was removed from the purified protein in the course of renaturation.

#### Results

Using routine methods, rabbits were immunized with renatured recombinant PreS1/2 antigen and the titers of their immune sera against the recombinant protein were determined by ELISA. Rabbit sera of blood samples taken before immunization served as the negative control (D value  $< 0.01$ ). The titer of antibody directing against PreS1/2 antigen in rabbit sera reached 1:128 000, 1:512000, respectively on 40 d, 60 d after first immunization. In order to identify the immunogenicity of different components of the recombinant PreS1/2 antigen, recombinant PreS2 (PreS2: 1-55/S:1-9) and PreS121-47 synthetic polypeptides were also used in determination of the serum titer on d40 (Figure 1), and the result indicated in the immune sera the titers of antibodies directing against PreS1 polypeptide and PreS2 antigen were similar.



**Figure 1** The immunogenicity of different components of the recombinant PreS1/2 antigen

#### Establishment of a detection method for PreS antigen and determination of its sensitivity, specificity and preciseness

Using purified anti-PreS1/2 polyclonal antibody a double antibody sandwich ELISA method for detecting PreS1/2 antigen was established. The anti-sera of rabbits immunized with PreS1/2 were purified by using Protein A Sepharose 4B Fast Flow affinity adsorption gel column. Then  $10\text{ mg/L}$  of the purified antibody was used to coat ELISA plate to adsorb HBV viral particles present in the sera and PreS1/2 antigen on the subunit particles. The specifically absorbed particles were detected with enzyme labeled anti-HBs antibody. This method was used in determination of purified Dane particle

sera. Along with multiple proportional dilution, the D values of reaction wells decreased successively and the dilution titer of the sera could be up to 1:2048, corresponding to detection of 5000 HBV viral particles per mL. Purified Dane particle sera and 99 hepatitis C patients' positive sera were detected and the result of the former was positive, whereas that of the latter was negative. Neutralization of PreS antigen-positive patients' sera with purified rabbit anti-PreS antibody resulted in competitive inhibition all rates reached over 80%.

Judged by intra-and inter-assay coefficients of variation, the precision of the detection method for PreS/2 antigen was evaluated. The intra-assay coefficient of variation was revealed by choosing PreS antigen-positive sera with high, medium and low values one each and by paralleled determination of 10 wells simultaneously each. The inter-assay coefficient of variation was demonstrated by determination of the above three sera for 10 times each at different time. The intra-assay coefficients of variation of the high, medium, low PreS antigen-positive sera were 3.8%, 4.5% and 13.3%, respectively; while the inter-assay coefficients of variation were 2.56%, 3.17% and 10.5%, respectively. These results indicate that precision has reached the requirement of Ministry of Public Health of China for intra and inter-assay coefficients of variation for diagnostic reagents (the national standard is  $\leq 15\%$ ). Detection rates of PreS1/2 antigen in various populations of people, in sera of 200 normal blood donors, PreS1/2 was not positively detected. The detection rates of PreS1/2 antigen in sera of patients at different phases of HBV infection were listed in Table 1.

**Table 1 Positive rates in sera at different phases of HBV infection**

Phase of infection	Number of cases			Positive rate(%)
	Negative PreS1/2	Positive PreS1/2	Total	
HBsAg(+)HBeAg(+)Anti-HBc(+)	40	260	300	87
HBsAg(+)Anti-HBe(+)Anti-HBc(+)	50	142	192	74
Anti-HBs(+)	24	0	24	0

## DISCUSSION

The PreS domain of HBV protein locates on the

surface of HBV particle and can induce protective antibody in human body and in mouse. In the literature it is reported that the elimination of PreS antigen from serum patients with acute hepatitis B can foresee the disappearance of clinical symptoms. For patients with chronic hepatitis B, persistent existence of PreS in serum indicates progression of the disease course. In the meantime, monitoring PreS1/2 antigen has also important significance for evaluation of curative effect of antiviral therapy in the se patients.

Most investigators have established detection methods for PreS antigen by using anti-PreS monoclonal antibody. This study used highly expressed in *Escherichia coli* and purified HBV PreS antigens, which cover the whole PreS2 domain and the most parts of PreS1 domain. Three experimental results indicate that the re comb inant PreS antigen is also a good immunogen in rabbit. In rabbit anti-PreS sera, the titer of antibody directing against PreS1:21-47 basically equal to the o f antibody directing against PreS2: 1 - 55. A method of detecting PreS antigen established by using purified rabbit polyclonal antibody is predicted to be able to determine simultaneously PreS1 and PreS2 antigens in serum of patient. By using this method, we have detected preliminarily PreS antigen in HBeAg positive and anti-HBeAg positive and the results show that the detection frequency of PreS antigen is relatively high in both situations. Statistical analysis indicates that the presence of PreS antigen in serum is independent of the existence of HBeAg ( $\chi^2 = 12.59, P < 0.01$ ).

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