DETECTION OF ANTIBODIES AGAINST DNA POLYMERASE OF HEPATITIS B VIRUS IN HBsAg-POSITIVE SERA USING ELISA

Li Xiang Rui, Ph.D., Young Min Park*, M.D., Jong Yong Choi*, M.D., Boo Sung Kim*, M.D., Guhung Jung, Ph.D.

Department of Biology Education, Seoul National University; *Department of Internal Medicine, Kangnam St. Mary's Hospital; Molecular Hepatology Laboratory, Institute of Human Molecular Genetics, WHO Collaborating Center for Reference and Research on Viral Hepatitis, College of Medicine, The Catholic University of Korea, Seoul, Korea

Objectives] DNA polymerase (pol) of Hepatitis B virus (HBV) includes 3 different domains such as terminal protein (TP), reverse transcriptase (RT) and RNase H. Humoral immune responses to each of these proteins have not been well documented previously, although antibody to pol was detected in serum of patients with chronic hepatitis B. We have constructed TP (amino acids 1-182), RT (amino acids 346-685) and RNase H (amino acids 690-832).

Methods] By ELISA using each protein expressed in E. coli as antigens, the corresponding antibodies were tested in serum from 40 patients with type B viral chronic liver diseases. (20 HBeAg-positive and 20 HBeAg-negative). As negative controls, sera from 3 healthy young men were used. With the mean values of the OD, which were tested 4 times per each test sample and 3 times per each control sample, we considered to be positive if the mean OD of each test sample is 2-fold or higher than that of controls.

Results] Five of 40 sera (12.5%) contained one or two different antibodies detectable by this method: 4 of 20 HBeAg-positive sera (20%) and 1 of 20 HBeAg-negative sera (5%). Anti-TP, anti-RT and anti-RNase H antibodies were detected in 2.5% (1/40), 10% (4/40) and 7.5% (3/40), respectively. Among 4/20 HBeAg-positive ELISA-positive sera, anti-TP, anti-RT and anti-RNase H were positive in 5% (1/20), 20% (4/20) and 10% (2/20), respectively, while 1 HBeAg-negative ELISA-positive sera were positive only for anti-RNase H.

Conclusions] These results suggest that the corresponding antibody responses to individual recombinant peptides derived from 3 domains of DNA polymerase may tend to be detected more frequently in HBeAg-positive sera than in HBeAg-negative sera from various patients with type B viral chronic liver diseases.

Key words] Hepatitis B Virus, DNA polymerase, anti-DNA polymerase antibody, Terminal protein, Reverse transcriptase, RNase H

INTRODUCTION

Hepatitis B virus (HBV) causes various liver diseases, including acute hepatitis, fulminating hepatic failure, chronic hepatitis, cirrhosis and hepatocellular carcinoma]. HBV genome is a 3.2kb partially double stranded DNA which consists of 4 open reading frames, S, C, X and P.

Characteristically, this virus replicates through pregenomic RNA intermediate after the reverse transcription and produces various peptides, including hepatitis B surface antigen (HBsAg), hepatitis X protein (HBxAg), hepatitis B core antigen (HBcAg), hepatitis B e antigen (HBeAg) and DNA polymerase. Because humoral
immunity can produce corresponding antibodies to each of them. HBV infection is evaluated using antigen-antibody detection system, such as HBsAg/anti-HBs, HBeAg/anti-HBe and IgM/IgG anti-HBc antibody tests. DNA polymerase which is encoded by P gene is essential for HBV replication and contains at least three different functions. Each of the corresponding domains was named as terminal protein (TP), DNA polymerase/reverse transcriptase (RT) and RNase H, respectively. Reportedly, using synthetic or in vitro translated peptides, antibodies against DNA polymerase (anti-pol) and its domains were detected in sera from patients infected acutely and chronically with HBV, and was suggested as a new diagnostic marker for HBV infection.

Recently, we have constructed each of those 3 functional domains of DNA polymerase by recombinant technology, and expressed each of them in E. coli. By ELISA using these recombinant peptides, TP, RT and RNase H as antigens, the corresponding antibodies were tested in sera from subjects with chronic liver diseases to evaluate their usefulness.

**MATERIALS AND METHODS**

**Human sera:** Total of 40 sera were obtained from patients with HBV infection who visited the Department of Internal Medicine, Kangnam St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul, Korea, during 1997. They consisted of 20 HBeAg-positive and 20 HBeAg-negative. Male and female patients were 30 and 10, respectively. Mean age was 38±11 years old (range, 21-62 years old). Number of cases of chronic hepatitis B and hepatocellular carcinoma were 32 and 8, respectively. Three sera from healthy young men, who were negative for all HBV markers, were also obtained as negative controls.

**Serologic examination:** HBsAg, anti-HBs antibody, HBeAg, anti-HBe antibody and total anti-HBc antibody were measured by commercially available radioimmunoassay kits (RIA, Abbott Laboratories, Chicago, Ill, USA). Anti-hepatitis C virus (anti-HCV) antibody measured by a third generation enzyme immunoassay kit (ELIA, Abbott Laboratories) were all negative. All cases had HBV DNA in serum detectable by a multi-target polymerase chain reaction (PCR) with two primer sets derived from Pre/C region and S region of HBV genome.

**Antigens:** Recombinant TP, RT and RNase H are maltase binding protein-fusion proteins expressed in E. coli and purified by affinity column chromatography. TP was encoded by P gene sequence from start codon to nucleotide 546 (amino acids 1-182), RT from nucleotides 1040 to 2056 (amino acids 346-685) and RNase H from 2072 to 2528 (amino acids 690-832) (Fig 1).

**Second antibody:** Horseradish peroxidase conjugated goat anti-human IgG (Sigma, product No. A-0293).

**Chemical reagents:** Enzyme linked immunosorbent assay (ELISA) enzyme substrate 3,3',5,5'-tetramethylbenzidine, dihydrochloride (TME), (Sigma, No. T-8768). Bovine serum albumin (BSA), (Sigma, No. A-7030).

**ELISA procedure:** The antigens were diluted to 2µg/ml in PBS, pH7.4 and dispensed to 96-well plate 50 µl (100 ng protein) per well. After coated at 37°C overnight, the plate was washed 3 times with PBS and blocked with 1% BSA in PBS for 1 h at room temperature. Then washing the plate again. The human sera were diluted with PBS to 1:400, and 5 µl was added to the washed plate wells. Each sample had 4 repeats. After being incubated at room temperature for 1 h, the plate was washed as before. After that, 50 µl diluted second
antibody (18000 in PBS) was added to each well and incubated at room temperature for 1 h. Following 3 times wash of the plate, 50 μl 1 substrate solution (13 mg TMB in 25ml 0.1 M sodium acetate buffer, pH5.6, warmed at 40°C for 30 min, and 33 μl of 30% hydrogen peroxide was added immediately before use) was added to each well. The color reaction was developed at room temperature for 30 min and stopped by the adding of 50 μl 2M H₂SO₄. Then the optical density was read at 450nm using a Maxline Microplate Reader (Emax, Molecular Devices).

**Interpretation:** The mean OD, which was calculated from ODs of 3 healthy human sera (total 12 repeats), was used as a negative control in each experiment. The mean OD of each test sample was calculated from the ODs measured 4 times. We considered to be positive if the mean OD of each test sample is 2-fold or higher than that of the negative control.

**RESULTS**

Five of 40 sera (12.5%) had anti-pol antibodies detectable by ELISA. They consisted of 4/20 HBeAg-positive sera (20%) and 1/20 HBeAg-negative sera (5%). Three of 4 HBeAg-positive ELISA-positive samples showed the positivity to 2 different antibodies: 1 positive for both anti-TP and anti-RT, 2 positive for both anti-RT and anti-RNase H (Table 1).

Anti-TP, anti-RT and anti-RNase H antibodies were detected in 2.5% (1/40), 10% (4/40), and 7.5% (3/40), respectively. Among 4/20 HBeAg-positive ELISA-positive sera, anti-TP, anti-RT and anti-RNase H were positive in 5% (1/20), 20% (4/20), and 10% (2/20), respectively, while 1 HBeAg-negative ELISA-positive sera were positive only for anti-RNase H.

**DISCUSSION**

In the ELISA system, the connection of antibody and antigen is a kinetic matter. There are two methods that can be used to determine optimal concentration of antigen and antibody. One is to fix the antigen concentration and to use serial dilution of antibody. Another is to fix the antibody concentration and to change the antigen. The antibody titer may be different from sample to sample. To avoid any neglect of positive samples, we used uniform antigen concentration and different dilution of sera (from 1:50 to 1:500). In the lowest (1:50) and highest dilution (1:500), no positive samples were detected (data not shown). In the dilution of 1:100 to 1:300, some samples were positive, but the dilution of 1:400 was optimal.

In the previous reports, with in vitro translated radiolabeled peptides as antigens using immunoprecipitation gel assay (RIPGA), antibodies against pol and its domains were detectable in 73% of acute hepatitis B and 87% of chronic hepatitis B. Among patients with hepatocellular carcinoma, only 27% were positive. Those antibodies were also detected in 80% of serum from HBsAg-positive patients with chronic renal failure treated with hemodialysis. However, by a Western blot analysis, only 13% of sera with HBV infection had

| Table 1. Mean optical densities of samples positive for anti-DNA pol. |
|---|---|---|---|---|---|---|---|---|---|
| Antigen | HBeAg-positive (N=20) | HBeAg-negative (N=20) |
| Patient No. | Mean O.D. | Mean O.D. | Ratio* | Patient No. | Mean O.D. | Mean O.D. | Ratio* |
| --- | --- | --- | --- | --- | --- | --- | --- |
| TP | #11 | 0.49±0.01 | 0.21±0.025 | 2.33 |
| RT | #2 | 0.52±0.029 | 0.169±0.013 | 3.09 |
| RNase H | #11 | 0.42±0.018 | 2.51 |
| #12 | 0.34±0.018 | 2.01 |
| #3 | 0.58±0.029 | 2.68 |
| #12 | 0.44±0.013 | 0.28±0.026 | 2.04 |

TP: terminal protein, RT: reverse transcriptase, O.D.: optical density.

*Ratio of Mean O.D. of individual samples to Mean O.D. of negative controls.
anti-pol antibody. Moreover, in the present experiment, 12.5% of the samples had one or two antibodies against each peptide derived from 3 different function domains of P gene. So, it is possible that the sensitivity to detect antibodies against pol and its domains depends on the phases of disease, the detection method or the design for the recombinant antigen construction.

Reportedly, the detection of anti-pol antibody was correlated with viral replication such as HBeAg positivity\(^9\), \(^{12}\), but was less often in serum samples without HBeAg\(^9\). Similar to these observation, anti-pol antibodies in our study were identified in 20% of HBeAg-positive subjects, while in 5% of HBeAg-negative subjects, respectively.

It has been known that RNase H domain in vitro translated amino acids 622-832 is strong in immunogenicity, compared to other domains\(^9\), \(^{12}\). So, antibodies to this peptide and another protein including this domain (amino acids 463-832) were detected in more than 80% of the patients’ sera. However, antibodies against the peptides from amino acids 463-648 and the upstream were detectable only in about 27%\(^9\), suggesting that the main immunogenic domain is located in amino acids 648-832.

We have used two peptides, consistent with RT (amino acids 346-685) and RNase H (amino acids 690-832), which were almost the same as the peptides reported previously\(^9\). Additionally, one peptide derived from the upstream of those two peptides (amino acids 1-182) was used to detect anti-TP. However, sensitivities to detect each corresponding antibody was very low (10%, 7.5% and 2.5%, respectively), compared to those of the previous study\(^9\). So, it is possible, in order to get more efficient results, that a piece of domain, amino acid 648-689, may be necessary in addition to the RNase H protein used in this study (amino acid 690-832).

ACKNOWLEDGMENT

We wish to acknowledge Miss Sun-Young Oh and Mr. Ginam Cho, Young Bin Hong and Sung Gyoo Park for their helpful discussion and assistance. This study was supported partly by the 1997 Sungyi Medical Research Foundation.

REFERENCES

1. Desmet VJ. Liver lesions in hepatitis B viral infection. Yale J. Biol. Med. 1988; 61:61-83.
2. Schodel F, Weimer T, Will H. HBV molecular biology and immunology. Biotest Bull. 1999; 4:63-83.
3. Battenschlager R, Schaller H. The amino-terminal domain of the hepadnaviral P-gene encodes the terminal protein (genomic linked protein) believed to prime reverse transcription. EMBO J. 1988; 7:485-492.
4. Toh H, Hayashide H, Mijata T. Sequence homology between retroviral reverse transcriptase and putative polymerase of hepatitis B virus and cauliflower mosaic virus. Nature (London) 1983; 305:827-829.
5. Khudjakov YE, Malkov AM. Prediction of terminal protein and 3′-mononucleoside H domains in the gene P product of hepadnaviruses. FEBS Lett 1989; 243:115-118.
6. Routhell G, Tucker W, Schaller H. Mutational analysis of the hepatitis B virus P gene product: domain structure and RNase H activity. J Virol 1990; 64:613-620.
7. Schodel F, Weimer T, Will H. Sprenger R. Amino acid sequence similarity between retroviral and E. coli RNAse II and hepadnaviral gene product. AIDS Res Hum Retroviruses 1988; 4:9-11.
8. Feltison MA. Millman L, Duncan GD, Blumberg BS. Presence of antibodies to the polymerase gene product(s) of hepatitis B and woodchuck hepatitis virus in natural and experimental infections. J Med Virol 1988; 24:121-136.
9. Weimer T, Weimer K, Tu Z-X, Jung MC, Pape GR, Will H. Immunogenicity of human hepatitis B virus P-gene derived proteins. J Immunol 1989; 143:3750-3756.
10. Chang L, Dinagst J, Gamend J, Varnum H. Detection of antibodies against hepatitis B virus polymerase antigen in hepatitis B virus-infected patients. Hepatology 1989; 10:332-335.
11. Stemler M, Hess J, Braun R, Will H, Schroder CH. Serological evidence for expression of polymerase gene of human hepatitis B virus in vivo. J Gen Virol 1988; 9:689-693.
12. Weimer T, Schodel F, Jung MC, Pape GR, Abert A, Fattovich G, Beerans H, Vaneerd PMCA, Will H. Antibodies to the RNase H domain of hepatitis B virus P protein are associated with ongoing viral replication. J Virol 1990; 64:5665-5668.
13. Yuki N, Hayashi N, Kasahara A, Katayama K, Ueda K, Fushimoto H, Kamada T. Detection of antibodies against the polymerase gene product in hepatitis B virus infection. Hepatology 1990; 2:883-888.
14. Crowther JR. ELISA, Theory and Practice. Humana Press Inc. Totowa, New Jersey, 1995. P:131-137.
15. Horikke N, Blumberg BS, Feltison MA. Characteristics of hepatitis B X antigen, antibodies to X antigen, and antibodies to the viral polymerase during hepatitis B virus infection. J Infect Dis 1989; 159:1104-1111.