J Tokyo Wom Med Coll
67 (8) 519～525 (1997)

CLINICAL IMPLICATIONS OF HEPATITIS B VIRUS pre-S2 TYPE: A POSSIBLE ROLE FOR pre-S2 PEPTIDE PRESENTATION BY HLA-A24 IN CHRONIC HEPATITIS B PATHOGENESIS

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(Received March 26, 1997)

The pre-S2 region of the hepatitis B virus (HBV) was classified into three types according to their nucleotide sequences. We attempted to determine these pre-S2 types by polymerase chain reaction (PCR), using specific primers prepared from the first 39 nucleotides of the pre-S2 region and found that quite similar results were obtained for sequencing studies and PCR. Subsequently, we examined the pre-S2 type in 45 HBV-infected chronic hepatitis B patients together with HLA class I phenotype and disease activity. The HLA-A24 antigen was found in 27 of 32 type I pre-S2 HBV-infected patients with active hepatitis, whereas none of 6 patients with inactive disease had HLA-A24. Serial studies in 13 patients with HLA-A24 showed that with remission of hepatitis, clearance of type I pre-S2 HBV was seen in all patients. These results suggest that HLA-A24 expression in the setting of type I pre-S2 HBV infection may play a role in the pathogenesis of chronic hepatitis B.

Introduction

The clinical spectrum caused by chronic infection with hepatitis B virus (HBV) was different among infected individuals, from asymptomatic carriers at one end, to chronic active hepatitis that may progress to cirrhosis and hepatocellular carcinoma at the other[1]. Although the immune response, particularly cell-mediated immunity, to HBV-derived antigens is believed to play an important role in the pathogenesis of chronic hepatitis B (CH-B)[2)~6)], it is not clear in what way the immune response might differ between patients with distinct clinical outcomes. Recently, accumulating evidences indicate[7)~14)] that many genetic variants of HBV exist; some of these have been reported to be tightly associated with hepatocyte injury[9)10)]. We recently reported[7)8)] that the first 39 nucleotides of the HBV pre-S2 region (nt 3205-3228) was variable among different viral isolates. We classified this region into three types: type I, II and III pre-S2. In these studies, in patients with high serum ALT concentrations, HLA-A24 was predominant in type I pre-S2 HBV infected patients, whereas HLA-A2 was most common in both type II and III pre-S2 HBV infected patients. We therefore hypothesized that a molecular complex composed of these variable pre-S2 region encoded peptides and HLA class I antigens might have an important role in the pathogenesis of CH-B. In this study, to extend these observations, we first established a polymerase chain reaction (PCR) assay for determining pre-S2 type and then studied whether pre-S2 type contributed to elucidation of the mechanisms underlying the onset and remission of chronic hepatitis.

Materials and Methods

1. Patients

Forty-five CH-B patients were studied. The diagnosis of CH-B was based on biochemical and histological findings. No subjects had a history of hepatitis C virus or hepatitis delta virus infection. Among the CH-B patients who were HLA-A24 positive, 10 were examined for pre-S2 type at two time points, in an active hepatitis phase (ALT>100 IU/*l*) and in remission (ALT<31 IU/*l*); three others were examined serially over periods of 3 to 4 years. The remaining CH-B patients were examined at a single time point during their clinical course. Their HLA phenotype was determined by an ordinaly microcytotoxic test using Terasaki's plate.

2. Amplification and the sequencing of the pre-S2 region

HBV DNA was extracted from serum samples as previously described[15]. The pre-S2 region (nt 2853-3182) was amplified by PCR using 5′TCTTGGGAACAAGAGCTACAGC3′ (nt 2832-2853) and 5′GGAATCCTGATGTTGTGTTCTC3′ (nt 161-182) as primers. PCR was performed with a slight modification of the procedure described by Saiki et al[16]. In brief, target sequences were amplified using a Dane Amp DNA amplification kit (Perkin-Elmer Cetus, Norwalk, CT, USA). The reaction was allowed to proceed for 40 cycles (94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min). If the amounts of PCR products were insufficient for sequencing, a second round of PCR was carried out by the same procedure using 5′CCAGATTGGGACTTAACCCCA3′ (nt 2974-2995) and 5′TCCCCAGTCCTCGAGGAGATTG3′ (nt 120-141) as inner primers. To determine the nucleotide sequences, PCR products were eluted from a 5% polyacrylamide gel and then sequenced by the dideoxy-chain termination method using PCR primers as sequence primers.

3. Determination of HBV pre-S2 type by PCR

The nucleotide sequences coding the first 13 amino acids of the pre-S2 region were classified types I, II, and III according to homology with the pre-S2 regions of the prototype adr, adw, and ayw strains[17], respectively (Fig. 1).

The sequenced samples were further examined by specific PCR analysis to determine pre-S2 type. One hundred microliters of serum were extracted at 65°C for 3 h with 1 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 0.5% sodium dodecyl sulfate, and 100 μl/ml proteinase K. DNA was extracted with phenol-

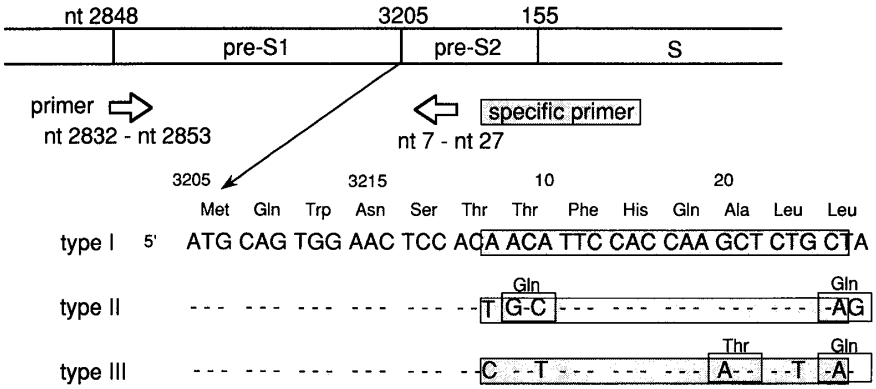

Fig. 1 Variation in the sequences coding for the first 13 amino acids of the pre-S2 region

Heterogeneity in this region was classified into three types (type I, type II, and type III) according to similarities with prototype HBV sequences (adr, adw, and ayw, respectively). Dashes denote nucleotide identity with type I. Amino acids are boxed at areas of sequence difference. The sequences for specific primers to distinguish between the pre-S2 types are shaded.

chloroform, precipitated with sodium acetate-ethanol, and washed with 70% ethanol. The pellet was dissolved in 32 μl of 10 mM Tris-HCl/1 mM EDTA pH 8.0. Ten microliters of the DNA were used for PCR. The primers, purchased from Japan Bioservice Co. Tokyo, Japan, had the following sequences: sense primer: 5′TCTTGGGAACAAGAGCTACAG-C3′ (nt 2823-2853), anti-sense (specific primers): 5′AGCAGAGCTTGGTGGAATGTT3′ (nt 7-27) for the type I primer, expected amplified fragment 417 bp; 5′TGCAGAGCTTGGTGGAAGG-CA3′ (nt 7-27) for the type II primer, expected amplified fragment 396 bp; 5′TGAAGAGTTT-GGTGGAAAGTG3′ (nt 7-27) for the type III primer; expected amplified fragment 384 bp. The final concentration of primers was 1 μM. Then, 1.25 U of Taq polymerase (Toyobo Co., Tokyo, Japan) were added to give a final reaction volume of 50 μl. Forty cycles of amplification were performed by denaturing at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min. The PCR products (10 μl of a 50 μl reaction mix) were fractionated on 1% agarose gels and visualized by ethidium bromide staining.

Results

1. Determination of pre-S2 type by PCR

To examine whether PCR efficiently detects the three distinct pre-S2 types, three standard DNA samples from isolates of each pre-S2 type were analyzed by PCR (Fig. 2). Type I pre-S2 was detected only by a primer specific for type I, but type II or III primers. Similarly, amplification signals were detectable only with a type II specific primer with type II pre-S2 samples, and DNA from type III pre-S2 samples was amplified only by a type III specific primer.

Subsequently, available 31 samples, were analyzed in the same manner, and we compared PCR results with those obtained by direct sequencing (Table 1). Of the 23 type I pre-S2 samples, 21 were classified as type I by PCR, while 5 of 6 type II pre-S2 samples were also determined to be type II by PCR. In 2 type I samples, a specific band was detected not only with the type I primer but also with the type II primer, though the type II signals were weak. One type II sample gave a clear band with the type II primer and a weak signal with the type I primer. The results for a type III samples

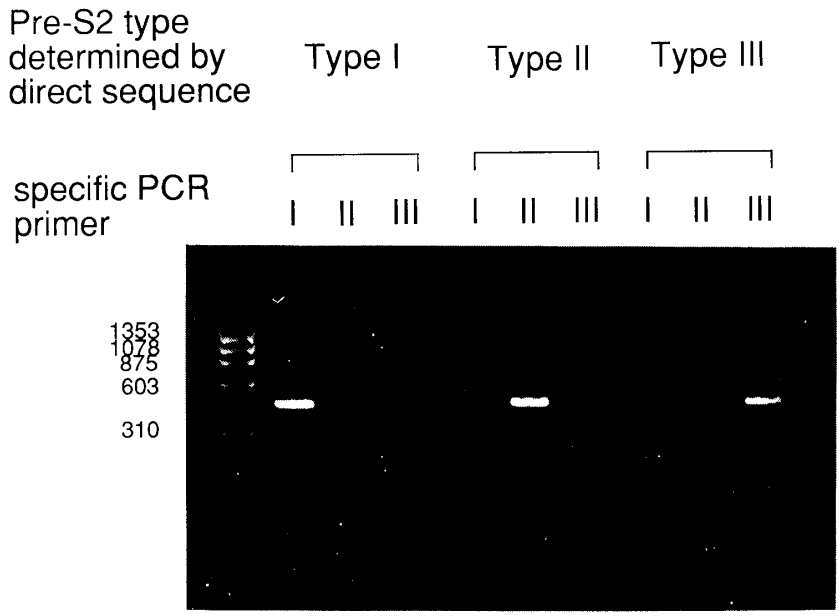

Fig. 2 Representative PCR analysis for determination of HBV pre-S2 types
Note that each pre-S2 type sample has an appropriate signal for each specific primer. PCR products obtained using specific primers were 417, 396, and 384 bp.

Table 1 Sensitivity and specificity of PCR for determination of pre-S2 type

pre-S2 type by sequencing	pre S2 type by PCR		
	type I	type II	type III
type I (n=23)	23*	0	0
type II (n=6)	0	6**	0
type III (n=2)	0	0	2

* : 2 of 23 had a trace type II pre-S2 signal, ** : 1 of 6 had a trace type I pre-S2 signal.

were identical to those obtained with direct sequencing. These results showed that, including the 3 samples having additional weak bands, the pre-S2 type of all samples was accurately determined by PCR.

2. The prevalence of HLA-A2 and -A24 phenotypes in CH-B patients infected with each pre-S2 type HBV

To evaluate of the clinical usefulness of pre-S2 type determination by PCR, we examined the prevalences of both the HLA-A24 and -A2 phenotype in patients infected with each pre-S2 type of HBV (Table 2). Among theose infected with type I pre-S2 HBV, HLA-A24 was found in 27 of 32 patients (84%) with high serum ALT concentrations, but none (0%) of 6 with normal ALT concentrations. The difference in the prevalence of HLA-A24 between these two groups was statistically significant ($p=0.0002$).

On the other hand, HLA-A2 was found in all patients with high serum ALT concentrations infected by either type II or type III pre-S2 HBV. However, it was also found that 8 of 12 patients infected with type II HBV with normal ALT concentrations.

3. Serial study of the pre-S2 type in patients with HLA-A24

To extend the above observations, 10 HLA-A24 positive patients who have been carefully followed in our institute were studied during different clinical phases (Table 3). All patients with elevated serum ALT concentrations had type I pre-S2 HBV. However, the pre-S2 type was either converted from type I to type II (7 patients) or became undetectable (3 patients) with declining serum ALT concentrations.

Furthermore, the pre-S2 type of 3 other patients was determined on several occasions during their clinical courses (Fig. 3a, b, and c). In case 1, two pre-S2 types (type I and III) of HBV were present during exacerbation. However, type I pre-S2 HBV disappeared with the decline in serum ALT concentration. In case 2, type I pre-S2 HBV was present while the serum ALT concentration was high (from Feb. '90 to Dec. '91), whereas it became undetectable following serum ALT normalization. However, it reappeared with re-elevation of serum ALT in May '94. Case 3 was infected with type I pre-S2 HBV in Sept. '91, which was present continuously until Oct. '93. However, clearance of type I pre-S2 HBV coincided with hepatitis remission.

Discussion

In the current study, PCR analysis was used to determine HBV pre-S2 type. Using specific primers corresponding the sequence spanning approximately nt 7-27 of the HBV genome

Table 2 Prevalences of HLA-A2 and HLA-A24 phenotypes in CH-B patients infected with respective pre-S2 type HBV

pre-S2 type	ALT level	Prevalence of HLA-A24		Prevalence of HLA-A2	
type I	High	84% (27/32)	0.0002	41% (13/32)	<0.05
	Low	0% (0/6)		100% (6/6)	
type II	High	25% (1/4)	n.s.	100% (4/4)	n.s.
	Low	67% (8/12)		67% (8/12)	
type III	High	33% (1/3)	n.s.	100% (3/3)	n.s.
	Low	0% (0/3)		0% (0/3)	

Table 3 Clearance of type I pre-S2 with the decline of serum ALT level in HLA-A24+CH-B

Patient	HLA class I phenotype	pre-S2 type	
		High ALT	Low ALT
1. 26M	A24 B52/46	Type I	Type II
2. 46M	A24/11 B52/55 Cw3	Type I	Type II
3. 24M	A24/2 B52/55 Cw1/7	Type I	Type II
4. 38M	A24/31 B35/61 Cw3/4	Type I	Type II
5. 38M	A24/2 B52/39 Cw7	Type I	Type II
6. 50M	A24/2 B51/46	Type I	Type II
7. 45M	A24 B62/39 Cw7	Type I	Type II
8. 60F	A24/33 B54/60	Type I	–
9. 24M	A24/2 B54/60	Type I	–
10. 40F	A24/11 B52/39 Cw7	Type I	–

(Fig. 1), we distinguished three pre-S2 types by PCR. The PCR results were in general agreement with those of the direct sequencing study, though weak extra bands were present in 3 out of 31 samples (Table 3). These extra bands are thought to represent a small amount of DNA undetectable by sequencing but none the less present in the sera, in addition to the DNA giving rise to the major band. Therefore, these results indicate that these pre-S2 types can be detected by this PCR method.

In our previous studies[7][8], we hypothesized that a suitable combination of HBV pre-S2 type and HLA class I phenotype, such as type I pre-S2 with HLA-A24, or either type II or III pre-S2 HBV with HLA-A2, might be required for hepatocyte injury. To assess this hypothesis, we examined the prevalence of both HLA-A24 and -A2 in patients with different pre-S2 types. As shown in Table 2, an HLA-A24 phenotype was frequently found in type I pre-S2 HBV infected patients with high serum ALT concentrations, but was absent in all 6 patients with normal serum ALT concentrations (84% vs. 0%; $p=0.0002$). It was noteworthy that the pre-S2 type I HBV observed in 10 patients with active hepatitis was converted to type II (7 patients) or became undetectable (3 patients) with recovery from hepatocyte injury (Table 3). These findings suggest that type I pre-S2 binds to HLA-A24, allowing efficient recognition by cytotoxic T cells (CTL), while other types of pre-S2 do not. Furthermore, recovery from hepatocyte injury can be explained by either a clearance of type I pre-S2 HBV or a mutation in the pre-S2 sequence producing a viral peptide which no longer binds to HLA-A24.

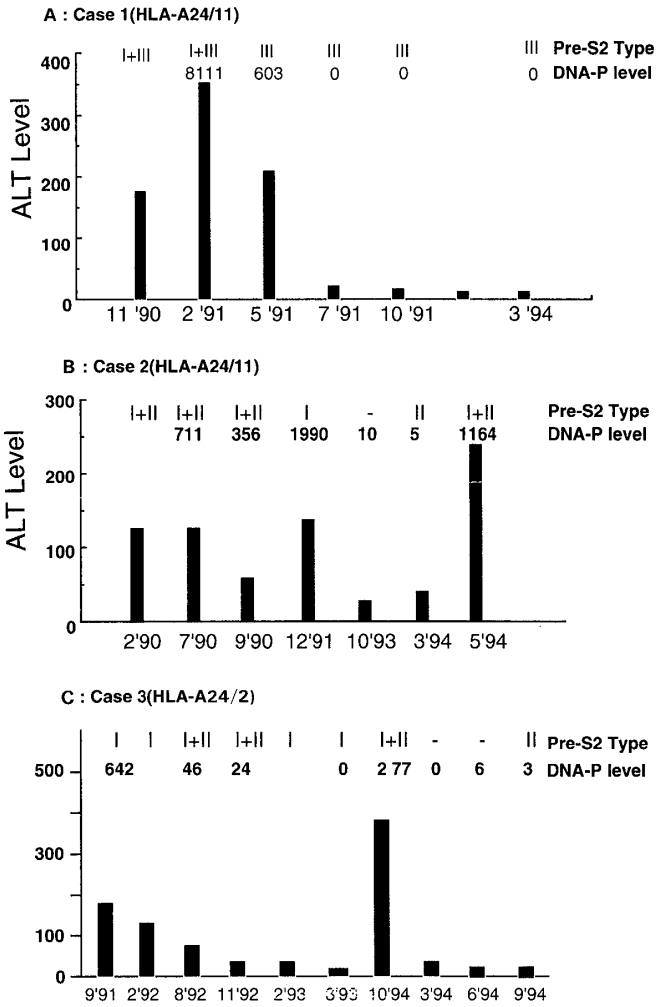

Fig. 3 Determination of pre-S2 type at different clinical phases

A: In case 1 (HLA-A24/11), both type I and type III pre-S2 were present during exacerbation, but only type III pre-S2 was detectable after the remission of hepatitis.

B: In case 2 (HLA-A24/11), the type I pre-S2 was detectable at two different clinical phases (Feb, '90 to Dec. '91) and May '94) in where it showed high ALT level.

C: In case 3 (HLA-A24/2), type I pre-S2 was detected continuously during the exacerbation phase. However, clearance of type I pre-S2 coincided with the hepatitis remission.

A recent study shows that peptides with an HLA-A24 binding motif contain methionine at position 2 and phenylalanine at position 9[18]. A peptide containing the last residue of pre-S1 and the first eight residues of type I pre-S2 (Gly-Met-Gln-Trp-Asn-Ser-Thr-Thr-Phe) contains this binding motif. Therefore, we propose that type I pre-S2 peptides can bind to HLA-A24 thereby making them recognizable to CTL. This hypothesis is now being tested by cytotoxicity assays. HLA-A2 was the predominant phenotype in both type II and III pre-S2 HBV infected patients with high serum ALT concentrations. However, these types also were found in patients with normal ALT concentrations. Furthermore, HLA-A2 binding motifs[18] are not present in this region. Therefore, this region appears not to be a CTL epitope, although we cannot completely exclude this possibility. Alternatively, peptides binding to HLA-A2 may exist in other regions.

Genomic variations exist in several regions of the HBV genome[7)~14)], and some of these are associated closely with hepatocyte injury[9)10)]. Therefore, the clinical spectrum of chronic hepatitis may not be due just to genetic events in a limited region of pre-S2. In fact, we have demonstrated previously[6] that CTL epitopes were present in the HBV core antigen as well as the surface antigen, using HBV-DNA transfected human myeloma cells as targets for *in vitro* cytotoxicity assays. However, the importance of pre-S2 peptides in the pathogenesis of CH-B is also supported by the finding that the titer of pre-S2 antigen is related to the degree of liver injury[19] and by the observation that genetic alterations in this region are linked to the development of CH-B[11)~14)]. Moreover, Barnaba et al demonstrated[20] that a pre-S2 peptide fragment, amino acid residues nt 120-134, can be a target epitope for liver infiltrating CTLs in CH-B patients. This peptide includes the region which we hypothesized to be a CTL-recognition epitope. All of these results support our hypothesis.

In conclusion, PCR proved to be an effective method for the determination of HBV pre-S2 type. Furthermore, pre-S2 type appears to be important for further understanding of the pathogenesis of CH-B.

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B型肝炎ウイルス pre-S2 領域の分類とその臨床応用 —HLA-A24 により提示される pre-S2 ペプチドの慢性肝炎の発症における役割—

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B型肝炎ウイルス（HBV）は，その持続感染にも関わらず肝細胞障害を起こすことのない無症候性キャリアが存在することから，宿主の免疫反応，とりわけキラーT細胞（CTL）が肝障害の主役を担っていると考えられているが，その詳細についてはよくわかっていない．

我々は，pre-S2領域の開始より39塩基にわたる部分は変化に富む領域で，これらの変異のパターンをHBs抗原のサブタイプ別のpre-S2配列との類似性より大きくI型，II型，III型pre-S2の3型に分類できることを明らかにした．今回は，これらのpre-S2型の分類に有用な新しいPCR法を開発し，以下の事実を明らかにできた．血清トランスアミナーゼ値が高値でI型pre-S2ウイルス感染患者の32例中27例がHLA-A24保持者であるのに対し，血清トランスアミナーゼ値が半年以上正常な患者では上記のような相関は見られなかった．また，HLA-A24を持つ患者において，血清トランスアミナーゼ値が高い時に見られたI型 pre-S2 HBVがALT値の低下に伴い異なった型のpre-S2を持つHBVへ転換する現象が多く見られた．

この事実は，少なくとも，HLA-A24を有する患者において，肝細胞障害が発現するためには，I型pre-S2 HBVの存在が重要な意味を持っていることを示している．