GENOTYPES AND SUBTYPES OF HEPATITIS B VIRUS IN THAILAND

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Abstract. Hepatitis B virus exhibits considerable variability evident in its various antigenic subtypes, which complicates the characterization of epidemiological factors, particularly in areas endemic for hepatitis B. Our group investigated the genotypes and subtypes prevalent in Thailand employing nested PCR and sequencing of the *a* determinant, as well as the sub-determinants located on the S gene. The sera examined originated from a mixed range of HBV-infected individuals. The results were mostly consistent with those reported for Southeast Asia in that genotype C (54.4%) dominates over genotypes A (22.1%) and B1 (23.5%). Regarding the subtypes, we have exclusively found *adw*2 (45.6%) and *adr* (54.4%) as expected for this area, with one case of subtype *adw* representing the exception. While genotype and/or subtype of HBV do not predispose to clinical disease, they nevertheless may account for those few cases reported in which a mutation, particularly within the *a* determinant of the S gene, causes evasion of routine detection by commercial kits, particularly as long as the respective individuals remain asymptomatic carriers solely expressing anti-HBc.

INTRODUCTION

The variability of hepatitis B virus (HBV) is estimated to range between that of DNA and RNA viruses since its replication includes reverse transcription of an RNA intermediate into DNA thereby employing reverse transcriptase devoid of a proofreading capacity. The resulting variability becomes evident in the antigenic subtypes which are defined by monospecific, polyclonal antisera against hepatitis B surface antigen (HBsAg). The presence of one common antigenic determinant, designated *a* , and two mutually exclusive determinant pairs, *d/y* (Le Bouvier, 1971). and *w/r* (Bancroft *et al,* 1972) has provided the means to differentiate between four major subtypes of HBsAg, *adw, adr, ayw,* and *ayr.* Additional sub-specificities assigned to the *w* determinant have been described, resulting in a nine-member classification as *adw*2, *adw*4, *ayw*1, *ayw*2, *ayw*3, *ayw*4, *adrq*$^+$, *adrq*$^-$, and *ayr,* respectively (Courouce-Pauty *et al,* 1976). The common as well as the subtype-specific epitopes are defined by different amino acid (aa) residues (Norder *et al,* 1992). Particularly important are amino acid positions 122 *(d/y)* and 160 *(w/r),* as changes at these positions can either cause the total loss of subtype determinant expression or lead to an antigenic switch from one subtype to another (Okamoto *et al,* 1987). Moreover, not only samples containing a mix of *adw* and *adr* strains, but even complex subtypes such as *adywr* can occur (Kanagawa *et al,* 1992). Yet the majority of neutralizing antibodies elicited against HBsAg (anti-HBs) during the normal immune response are anti-*a* specific; thus, there is cross-reactivity among different subtypes.

A change in the environment exerting selective pressure, as for example administration of vaccines, monoclonal or polyclonal antibodies, can cause the emergence and eventual dominance of minor strains, as for example the escape variants which are caused by point mutations in the *a* determinant (Carman *et al,* 1993). Point mutations, not only in the key residues for the subtype determinants but also in positions within the common a determinant, can lead to other variants which apparently lack subtype expression (Tachibana *et al,* 1989 ; Okamoto *et al,* 1989). This suggests the *a* determinant to be a highly conformational structure. Based on the comparison of complete genomes, four genomic groups of HBV were genetically classified and thus defined. They were subsequently referred upon as

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genotypes designated A-D. Sequencing the S-gene of HBV provided the molecular basis for assessing the serological variations of HBsAg within the major four subtypes. This molecular approach also led to the identification of two new genotypes of HBV designated E and F (Magnius *et al,* 1995).

Some time ago, it was noted that the different subtypes of HBV reflect the geographical distribution of the different genotypes (Mazzur *et al,* 1974; Courouce-Pauty *et al,* 1983). Yet, studies to that end have been hindered by the genetic heterogeneity of some subtypes as for example *adw*2 which has been found to belong to three different genomic groups (Norder *et al,* 1992). The genomic groups of HBV approximately indicate the geographical area of origin. Within the established genomic groups a number of geographical clusters were found which share amino acid substitutions in the S gene product (Norder *et al,* 1993). Therefore, further research is required to determine which of the genetic alterations noted only once represent epidemiologically stable variants and which on the other hand are mutations not propagated as epidemiological entities.

Particularly in areas endemic for hepatitis B virus infection, *eg* Thailand with 6.4 - 6.9% of all pregnant women screened in the course of a one-year period diagnosed HBV-positive (Poovorawan *et al,* 1989), elucidation of the predominant viral strains might well prove crucial regarding the development and implementation of preventive measures so that in the long run hepatitis B along with its fatal sequelae such as liver cirrhosis and hepatocellular carcinoma might be eradicated. Hence, our group investigated the HBsAg subtype and genotype distribution in Thailand, emphasizing the correlation between the antigenic and molecular characteristics of the HBV isolates examined.

MATERIALS AND METHODS

Population study

Asymptomatic HBV carriers

Our study included 20 HBV-positive sera derived from asymptomatic blood donors obtained by the National Blood Center, Thai Red Cross, Bangkok, Thailand, as well as 14 HBV-positive sera taken from mothers diagnosed as HBV carriers, who attended the Viral Hepatitis Research Unit at Chulalongkorn Hospital.

Hepatoma patients

We examined 8 specimens of HBV-positive sera diagnosed as hepatocellular carcinoma by histopathology at Chulalongkorn Hospital and Ramathibodi Hospital, Bangkok.

Chronic hepatitis patients

Seventeen specimens of HBV-positive sera obtained from chronic hepatitis patients at Chulalongkorn Hospital and Ramathibodi Hospital, Bangkok, were included in the study. The diagnosis of chronic hepatitis was based on persistent elevation of ALT for more than 6 months with, as well as without histological diagnosis.

Children or infants infected after vaccination

Our study included 9 infants who had been born to mothers diagnosed as HBV carriers and who developed HBV infection after having received the complete vaccine regimen.

Subtype and genotype determination

DNA extraction

DNA was extracted from serum applying the alkaline extraction method (Kaneko *et al,* 1989). Briefly, a 10 (µl aliquot of serum was incubated with NaOH at a final concentration of 0.1 M in a 1.5 ml micro-centrifuge tube at 37°C for 60 minutes. The mixture was subsequently centrifuged in a micro-centrifuge for 15 seconds and neutralized with HCl at a final concentration of 0.1 M.

HBV DNA detection

The selection of primer sets for HBV DNA amplification was based on sequence data provided by Dr M Yano at the WHO Collaborating Center, Nagasaki, Japan.

HBV DNA was amplified by nested PCR in an automated thermocycler (Perkin Elmer Cetus) as described elsewhere (Saiki *et al,* 1988). Briefly, 5 (µl of the respective DNA sample were added to a reaction mixture containing 1 U of Taq polymerase (Perkin Elmer Cetus), each of four deoxynucleotide triphosphates (Promega) at a concentration of 200 µM, primer pair F4 with the sequence 5'-GTC CTC CAA TTT GTC CTG G-3' (nt no. 348-366) and R5

with the sequence 5'-AGC CCA AAA GAC CCA CAA TTC-3' (nt no. 1015-995) for the first amplification round, and primer pair S2-1 with the sequence 5'-CAA GGT ATG TTG CCC GTT TG-3' (nt no. 455-474) and S1-2 with the sequence 5'-CGA ACC ACT GAA CAA ATG GC-3' (nt no. 704-684) for the second amplification round, respectively, each primer at a 1 μM concentration, 10 mM Tris buffer and 1.5 mM $MgCl_2$ at a final volume of 50 μl. The first amplification round consisted of one cycle at 94°C, 55°C and 72°C for 1 minute each, followed by 30 cycles comprising a 30 second denaturation step at 94°C, a 30 second annealing step at 55°C, and a 1 minute extension step at 72°C, each. The amplification was concluded by one cycle at 94°C for 1 minute, 55°C for 2 minutes and 72°C for 10 minutes. For the second amplification round 5 μl of the first PCR product were added to the reaction mixture and amplification was performed in a manner identical to the first round. 10μl of each amplified DNA sample were loaded on a 2% Nusieve agarose gel stained with ethidium bromide on preparation. Electrophoresis was performed at 120 V for 45 minutes and the product band of 249 base pairs was visualized on a UV-light box.

DNA purification and sequencing

The PCR product was purified for sequencing using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's specifications and subsequently subjected to 2 % agarose gel electrophoresis in order to ascertain its purity. For determining the concentration of the amplified DNA, we measured the absorption at 260 nm of every sample in a UV spectrophotometer (Shimadzu UV 160 A). The concentration was calculated according to the formula that 1 OD 260 = 50 μg double-stranded DNA. Between 10 and 30 ng/μl (3-6 μl) of every respective DNA were subjected to cycle sequencing using dye-labelled terminators (8 μl and 3.2 pmole of specific primer at a final reaction volume of 20 μl) which represents a rapid and convenient method for performing enzymatic extension reactions for subsequent DNA sequencing on the Abi Prism 310 Genetic Analyser (Perkin Elmer Cetus). This round of amplifications was performed according to the manufacturer's specifications using primer S2-1 to amplify the particular DNA strand of interest for further sequencing. Cycle sequencing consisted of 25 cycles at 96°C for 10 seconds (denaturation), 50°C for 5 seconds (annealing), and 60°C for 4 minutes (extension). The reaction was concluded by cooling the thermal ramp to 4°C. The extension products were subsequently purified from excess un-incorporated dye terminators by ethanol precipitation according to the manufacturer's specifications (Perkin Elmer Cetus) and subsequently prepared for loading on the Abi Prism 310 Genetic Analyser.

For all the subsequent steps we referred to the Abi Prism 310 Genetic Analyser user's manual (Perkin Elmer Cetus).

The nucleotide sequences obtained were then translated into the corresponding amino acid sequences applying the universally valid triplet code. The results were compared with the amino acid residues specifying determinants of HBsAg (Magnius *et al,* 1995), in particular relating to HBV S-protein variation between amino acids 120-160 and serological subtypes (Howard *et al,* 1995).

The subtypes and genotypes were determined according to the classification provided by Helene Norder (Magnius *et al,* 1995).

RESULTS

Using the primer set selected for nested PCR most HBV-positive sera gave the identification band of the predicted size, 249 bp, on agarose gel. Those primers had been chosen for amplification of the a determinant of the S gene in particular, as this sequence has been shown to code for the antigenic epitope of the virus and thus is most prone to be subjected to environmental pressure and resulting mutation. Subsequent sequencing revealed the distribution of HBV genotypes and subtypes among the Thai population as shown in Tables 1 and 2. One isolated case expressed subtype *adw* rather than *adw*2, the sole difference between them, according to Norder *et al,* (1993) residing in amino acid position 126 which is isoleucine in *adw*, rather than threonine in the *adw*2 subtype.

DISCUSSION

Various techniques have been employed in order to determine the genotype and subtype of hepatitis B virus isolates derived from different sources. Among those are mainly either enzyme-linked

immunosorbent assays using monoclonal antibodies or radio-immunoassays (Swenson *et al,* 1991; Arauz-Ruiz *et al,* 1997). The group of Magnus Lindh has recently applied PCR with subsequent restriction enzyme treatment in order to genotype hepatitis B virus based on the generated RFLPs (Lindh *et al,* 1997). Helene Norder's group performed HBV subtyping at the genomic level by site-directed amplification thereby refining previous subtyping results of HBsAg (Norder *et al,* 1991).

Our group has applied amplification by nested PCR using a set of primers especially selected for the amino acids of the S-gene coded for by nucleotides 476 to 684 which cover the *a* determinant, as well as the sub-determinants, followed by direct sequencing of the obtained stretch of amplified DNA. We have found this method exceeded those mentioned above in sensitivity, as well as in specificity.

The results of our study are mostly consistent

Table 1

Subtypes of hepatitis B virus in Thailand.

Groups	No.	Subtype	
		*adw*2 (%)	*adr* (%)
Asymptomatic HBV carriers *	34	12 (35.3)	21 (61.8)
Hepatoma	8	4 (50)	4 (50)
Chronic hepatitis	17	10 (58.8)	7 (41.2)
Children of carrier mothers with vaccine failure	9	5 (55.6)	4 (44.4)
Total	68	31 (45.6)	37 (54.4)

* In this particular case, the amino acid sequence was compatible with subtype *adw* detected in an isolate from Indonesia (Norder *et al,* 1993).

Table 2

Genotypes of hepatitis B virus in Thailand.

Groups	No.	A	B1	C
		No. (%)	No. (%)	No. (%)
Asymptomatic HBV carriers	34	5 (14.7)	7 (20.6)	22 (64.7)
Hepatoma	8	3 (37.5)	1 (12.5)	4 (50)
Chronic hepatitis	17	4 (23.5)	6 (35.3)	7 (41.2)
Children or infants infected after vaccination	9	3 (33.3)	2 (22.2)	4 (44.4)
Total	68	15 (22.1)	16 (23.5)	37 (54.4)

with those previously reported regarding the distribution of HBsAg subtypes and genotypes in Southeast Asia (Swenson *et al,* 1991) in that we detected genotypes A and B1, distinct from genotype B2 by the presence of lysine instead of arginine at position 122 (Howard *et al,* 1995), at a frequency of approximately 20 %, whereas genotype C amounted to 54.4 %. As to the subtypes, we have exclusively found *adw*2 which can belong to either genotype A or B1, and *adr* belonging to genotype C. Hence, we have established the *ad* sub-determinant as the one prevailing among the Thai population. Since the *a* determinant of the S-gene product, located between amino acid position 139 and 147, has been recognised to be the sequence crucial for antigenic epitope structure and conformation and thus for the immune response the fact that the hepatitis B vaccine in use in Thailand has *ay* specificity need not cause worry as its efficacy against *ad* subtypes is not impeded. As to the question whether any given genotype and/or subtype of HBV might predispose to clinical disease, HBV's pathogenicity is not based exclusively on its genotype and/or subtype. On the one hand, mutations in the *a* determinant of HBsAg, especially one mutation from Gly to Arg at amino acid residue 145, have been reported to lead to so-called escape mutants in vaccinees (Magnius *et al,* 1995). Yet on the other hand, the S protein of HBV the genetic make-up of which determines the genotype and subtype of the virus has never been shown to cause transformations observed in cirrhosis and particularly in hepatocellular carcinoma. Rather, the two candidates responsible for severe clinical disease are the core gene, especially with its pre-core mutations leading to fulminant hepatic failure (Hasegawa *et al,* 1994 ; Ogata *et al,* 1993), and the X-gene product which has been ascribed miscellaneous transactivating capacity (Colgrove *et al,* 1989 ; Siddiqui *et al,* 1989).

Yet, genotype and/or subtype may account for the few cases reported to date, who were serologically negative but PCR-positive for HBV (Paterlini *et al,* 1991; Carman 1995), especially individuals expressing anti-HBc as the only serological marker in whom the prevalence of amino acid substitutions in the *a* determinant of HBsAg is significantly raised (Weinberger *et al,* 1996). In other words, those which due to altered genotype and/or subtype might escape routine detection by monoclonal antibody kits. Moreover, these few individuals' carrier status may well remain undetected especially as long as they do not develop any symptoms indicative of HBV infection.

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