ORIGINAL ARTICLE

Hepatitis B Surface Antigen Subtypes in Hepatitis B Seropositive Subjects in University Hospital, Kuala Lumpur

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Summary

Hepatitis B surface antigen can be serologically defined as *ayw*1, *ayw*2, *ayw*3, *ayw*4, *ayr*, *adw*2, *adw*4 and *adrq+* or *adrq-*. A study of common HBsAg subtypes in 44 HBsAg reactive sera in University Hospital was conducted using a solid-phase sandwich EIA. Eleven samples were found not typable and among the 33 typable HBsAg reactive sera, 3 HBsAg subtypes: *adw*, *adr* and *ayw* were identified. Subtype *adw* was found in 66.7% (22/33) of the typable HBsAg reactive sera; 24.2% (8/33) was of subtype *adr* and 6.0% (2/33) of subtype *ayw*. One sample was found to be reactive to both *adw* and *adr*. HBsAg subtype *adw* was found more commonly in Chinese but among the Malays, HBsAg subtype *adr* appeared to predominate. However, the small sample size precludes firm conclusions on the predominant subtype among the Malays.

Key Words: Hepatitis B surface antigen, Subspecificities, Subtypes, Monoclonal antibodies

Introduction

Hepatitis B virus (HBV) is a DNA virus and is found in 3 morphologically distinct forms: as a sphere of about 22nm, as a filamentous particle of the same diameter and several hundred nm in length and as a more complex structure known as a Dane particle. A sphere or filamentous particle is an incomplete virus and does not contain HBV DNA. The Dane particle is a complete virus consisting of a nucleocapsid and an envelope. The envelope is made up of hepatitis B surface antigen (HBsAg) and this antigenic determinant is found in both the Dane and incomplete particles.

The envelope of HBV consists of a host-derived phospholipid bilayer membrane encoded by the S gene. Biochemical analyses of the envelope of HBV reveal 3 polypeptides, termed major, middle and large protein. The large envelope protein consists of pre-S1, pre-S2 and HBsAg; the middle envelope protein has pre-S2 and HBsAg; the major protein is composed of HBsAg alone.

The existence of subspecificities of HBsAg was first demonstrated by Levene and Blumberg[1] in 1969 and further confirmed by La Bouvier[2]. All known serotypes of HBV contain the common *a* determinant and one of each of the mutually exclusive determinants *d/y* and *w/r*. Additional serological specificities, originally designated as subdeterminants of *a* and subsequently as subdeterminants of *w*, have allowed the identification of 4 serotypes of *ayw* and 2 of *adw*, thus, the subtypes of HBsAg is serologically defined as *ayw*1, *ayw*2, *ayw*3, *ayw*4, *ayr*, *adw*2, *adw*4 and *adr* and also designated as P1 to P8[3]. The *q* determinant was originally found to be expressed on all HBsAg subtypes except *adw*4[4]. Subsequently, lack of *q* was also demonstrated in some *adr* subtype, thus, *adr* subtype can be defined as either *adrq+* or *adrq-*[5].

A genetic classification of HBV genome, based on the nucleotide divergences of 8% or more between the HBV strains can be classified into 6 genetic groups designated as A to F[6,7]. Strains specifying *adw* are found in groups A, B, C and F, and those specifying *ayw* in groups A, B, D and E[8]. Strains specifying *r* have so far only been found in group C[6]. The different serotypes have distinct geographical distribution throughout the world. The serotype *adw*4 is widespread in French Polynesia and Argentina and also found in Brazil[9]. The *adw*4 serotype is found in the inhabitants of Amazona State[10], Chile and the Marquesas Island, but rarely found in Europe[9]. Subtypes *adw*2, *ayw*3, and *ayw*2 are prevalent in North and South America, Europe and much of Asia. Subtypes *ayw*2, *ayw*4 and *adw*2 are commonly found in Africa, *adrq+* is widespread in Southeast Asia, and *adrq-* is prevalent in Australia. *ayw*1 and *ayr* subtypes are mostly found in Vietnam[9]. Group A strain can be divided into 2 geographical groups, one in western Europe and the other found mainly in South Africa; Group D is found mainly in the Mediterranean area, Middle East and in South Asia; the genomic groups B and C are confined mainly to populations with origins in South-East Asia, the Far East and the Pacific area[11]. Genotype E has so far been found only in sub-Saharan Africa. Genomic group F contains the most divergent of all HBV strains, and is found in aboriginal populations of the Americas, Polynesia and rarely in Europe[9].

A shift in the prevailing HBV genotypes has been reported in Sweden[12] and in Japan[13]. Information on such genotype shift is important in understanding the failure to obtain protection with current HBV vaccines in some countries[14,15]. This paper presents the results of a study to investigate the common hepatitis B surface antigen subtypes in hepatitis B seropositive subjects in University Hospital, Kuala Lumpur.

Materials and Methods

Blood samples

Forty-four known HBsAg positive frozen sera were used in this study. The samples consisted of 25 females and 19 males. There were 30 Chinese, 11 Malays and 3 Indians. The youngest patient was 7 years and the oldest 90 years with a mean age of 31.5 years. Thirteen patients were admitted to wards with acute hepatitis, 5 were drug addicts who were also anti-HIV antibodies reactive; 26 patients were asymptomatic chronic hepatitis B carriers attending the Hepatitis B Carrier Clinic.

Detection of HBsAg and HBeAg

The HBsAg was diagnosed by using AxYSM HBsAg 3rd generation microparticle enzyme immunoassay (MEIA) (Abbott Laboratories, Abbott Park, IL 60064, USA) according to the procedures recommended by the manufacturer. Initially reactive sample was repeated using the same method and only repeatedly reactive sample was considered as HBsAg reactive.

Qualitative determination of HBeAg in human plasma or serum was performed using AxYSM HBe MEIA (Abbott Laboratories, Abbott Park, IL 60064, USA) according to the manufacturer's instructions.

HBsAg subtyping

HBsAg subtype EIA kit (Institute of Immunology Co., Ltd, Tokyo, Japan) kindly donated by Abbott Laboratories was used in the study. The kit was developed for research purposes to detect respective subtypic determinant *d, y, w* and *r* in HBsAg reactive samples for identifying HBsAg subtypes: *adw, adr, ayw* and *ayr*. The assay was based on solid-phase sandwich EIA and 96 well microplate was coated with monoclonal antibody against the common determinant *a* of HBsAg. HBsAg in positive samples captured on the solid phase and their subtypic determinants *d, y, w,* or *r* were detected by peroxidase-labelled monoclonal antibody against corresponding determinant. 50µl of patient sera and control sera were dispensed to respective wells recommended by the manufacturer. Four blank wells were set up for each assay. The assay procedures consisted of primary reaction and secondary reaction step. The primary reaction step included incubation of the plate after dispensing at room temperature for 16 - 24 hours, the plate was washed 5 times manually using the aspirator. In the secondary reaction step, 50µl each of labelled monoclonal antibody against determinant *d, y, w,* and *r* were dispensed to wells for detecting determinant *d, y, w* and *r* respectively. The plate was incubated at 37°C for 2 hours. After washing 5 times

manually using an aspirator, 100µl of color developer containing enzyme substrate was added to each wells and the plate incubated in the dark at room temperature for 30 minutes. The color developer containing enzyme substrate must be prepared fresh and used up within 1 hour. 50µl of reaction stopper was added to all wells after incubation and the absorbance measured at 490nm using a microplate reader. The absorbance must be measured within 2 hours after coloring reaction was stopped. Positive samples had absorbance value > cut off value; the samples with absorbance value < cut off value were considered as negative.

Results

Eleven samples (25%) were non-typable using the present method. Seven samples were not reactive to all the enzyme-labelled monoclonal antibody *d, y, w* and *r*. One sample each reacted to enzyme-labelled monoclonal antibody *w* and *y*. Two samples reacted to only enzyme-labelled monoclonal antibody *y*.

Among the 33 (75%) typable sera, 69.6% (23/33) of the HBsAg reactive sera were subtype *adw*, 24.2% (8/33) were subtype *adr*, 6.0% (2/33) were subtype *ayw*. One serum was found reactive to both *adw* and *adr* (Table I). No subtype *ayr* was detected in this study.

In asymptomatic chronic hepatitis B carrier, 10 out of 26 samples were non-typable (Table I). The 2 HBsAg subtypes found in this group of patients were *adw* and *adr*. Fourteen (87.5%) of the typable sera were HBsAg subtype *adw* and only 2 (12.5%) were subtype *adr*. Among the HBsAg reactive patients with acute hepatitis, 50% (6/12) of the typable sera were found to be subtype *adw*, *adr* subtype made up 41.7% (5/12) and 8.3% (1/12) were ayw subtype. For patients with co-HIV infection, 3 HBsAg subtypes were found and *adw* (3/5) was the most common, followed by one case of *adr* and *ayw*.

Among the typable samples (Table II), the distribution of HBsAg subtypes was found to be different in different ethnic groups. Although only 9 Malays HBsAg positive samples were typable, 44% (4/9) were HBsAg subtype

Table I
Distribution of HBsAg Subtypes in Hepatitis B Carriers

Samples	HBsAg Subtypes				
	adw	*adr*	*ayw*	*ayr*	NT*
Acute hepatitis (n=13)	6	5	1	0	1
IDU with HIV-1 positive (n=5)	3	1	1	0	0
Chronic hepatitis B carriers (n=26)	14	2	0	0	10

**non-typable*

Table II
The Distribution of HBsAg Subtypes in Different Ethnic Groups

Ethnic Groups	HBsAg Subtypes(%)			
	adw	*adr*	*ayw*	*ayr*
Chinese (N=23)	20 (86.9)	3 (13.0)	0	0
Malays (N=9)	3 (33.3)	4 (44.4)	2 (22.2)	0
Indians (N=1)	0	1 (100)	0	0

adr, 33% (3/9) were HBsAg subtype *adw* and 22% (2/9) were subtype *ayw*. The only Indians patient was found to have HBsAg subtype *adr*. The majority of Chinese HBsAg reactive samples (86.9% i.e. 20/23) was found to have HBsAg subtype *adw*, 13.0% (3/23) were found to have HBsAg subtype *adr* and there was no HBsAg *ayw* subtype.

HBeAg was detected in 24 (54.5%) of the 44 HBsAg reactive patients exhibiting enhanced infectivity in this group of patients. The HBsAg subtype was not affected by the presence or absence of this marker.

Discussion

This study of HBsAg subtypes in HBsAg reactive sera indicated that 3 HBsAg subtypes: *adw, adr* and *ayw* could be found in University Hospital. Subtype *adw* constituted 69.7% of the typable HBsAg reactive sera, 24.2% were found to be of subtype *adr* and subtype *ayw* made up 6.0% of the cases. Subtype *ayr* was not found in the HBsAg reactive sera in this study. Since our panel of monoclonal antibodies could not distinguish between *adw*2 or *adw*4 and *ayw*1 or *ayw*2 at the subdeterminant levels, comparison of the HBsAg subtypes to reported cases of HBsAg subtypes in other Southeast Asian countries was only made at the common determinant level.

The common HBsAg subtypes found in Vietnam was *ayw*1 (51%), *adw*2 (29%), *ayw*2 (1%) and *ayr* (3%)[9]. Swenson et al[16] reported that 88% of HBsAg-positive sera from Laos were *adr*, the remainders were *ayw*1 (8%) and *adw*2 (4%). Snitbhan et al[17] also reported that HBsAg/*adr* to HBsAg/*adw* was approximately 10:1 in Thailand, suggesting that *ad* was the predominant combination in South East Asia and determinants *w* and *r* are more useful epidemiological markers than *y* and *d*. In our study, *adw* appeared to be the predominant HBsAg subtype, indicating that the distribution of HBsAg subtypes in Malaysia may be different to that in Vietnam, Thailand and Laos.

Eleven (25%) HBsAg reactive sera were not determined by the current method. These undetermined samples were found mainly in asymptomatic chronic hepatitis B carriers (Table I). The low HBsAg titers in these samples may be an important contributing factor. The enzyme-labelled monoclonal antibodies *d, y, w* and *r* coated on the plate can detect subtypic determinants with HBsAg ≥3ng/ml. If the HBsAg titer of sample is 2^6 or higher by reverse passive hemagglutination method, the HBsAg subtype can be easily detected. In any sample with HBsAg titer lower than 2^6, the subtypic determinants may not be detected by the enzyme-labelled monoclonal antibodies.

This study involved a relatively small number of patients. However, it is interesting to note that common HBsAg subtypes in University Hospital found in this study are the same as the results reported by Kamath in 1975[18]. The three common hepatitis B surface antigen subtypes found in Malaysia are *adr*, *adw* and *ayw*. Among all the typable sera in this study, *adw* was found predominantly in Chinese (86.5%) but not among the Malays. *Adr* (44.4%) appeared to be a common subtype in Malays, and 22.2% of the HBsAg reactive sera of Malays were found to be subtype *ayw*. The preponderance of the *adr* in the Malays and *adw* in Chinese suggest that Malaysian Chinese and Malays could have acquired the subtypes from their country of origin and subsequently maintained the subtype by intrafamilial transmission[19]. Further study involving a larger sample size is needed to demonstrate the basis and clinical significance of racial differences in HBsAg subtypes among the two major ethnic groups in Malaysia.

In studying the HBsAg subtype in high-risk groups, Swenson et al[16] reported that *adw*2 was the most common subtype in homosexual men in USA, and among HBsAg reactive prison inmates with a history of intravenous drug use, *ayw*3 subtype accounted for 52.5%, *ayw*2 and *adw*2 subtype were each found in 22% and 3.4% were subtype *ayw*1-2. In our study, although the number is small, among the HIV reactive sera, HBsAg subtype *adw*(3/5) was the most common, subtype *adr*(1/5) and *ayw* subtype (1/5) were also found.

One chronic hepatitis B carrier was found to have HBsAg subtype *adw* and *adr*. This could be due to exposure to HBV of different subtype species[20]. It is generally believed that anti-HBs produced after HBV infection can confer protection against infection with either homologous or heterologous HBV subtypes. However, the development of acute hepatitis B in a patient with pre-existing anti-HBs has been documented. Koziol et

al[21] reported that the reinfection of HBV as the result of pre-existing of anti-HBs consisted of anti-*w* antibody of restricted subspecificity which permitted reinfection by HBV with a heterologous *w* subdeterminant. Swenson et al[22] also reported that the preexisting anti-HBs of anti-d did not confer protection against reinfection of HBV subtype *ayw*.

It is important to study the geographical distribution of HBsAg subtypes as well as anti-HBs subtypes so as to understand the protective efficacy of HBV vaccine. The current hepatitis B vaccines confer protection against both homologous and heterologous subtypes of HBV presumably by the development of anti-*a* antibody[23]. The development of monospecific anti-d in the absence of anti-*a* response after HBV vaccination has been documented[24]. This can result in the re-infection of patient with HBV despite HBV vaccination.

Conclusion

Subtype *adw* is the predominant HBsAg subtype found in hepatitis B seropositive subjects in University Hospital. The monoclonal antibodies with restricted reactivity for HBsAg subtypes and anti-HBs could be used to determine the HBsAg subtypes in hepatitis B carriers and the anti-HBs subtypes in the patients with immunity to hepatitis B virus either through naturally acquired infection or vaccination in future study.

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