WESTERN BLOT ANALYSIS OF THE REACTIVITY BETWEEN ENVELOPE PROTEINS OF HEPATITIS B VIRUSES FROM BRAZILIAN CARRIERS AND ANTIBODIES RAISED AGAINST RECOMBINANT HEPATITIS B VACCINES

L. DE CASTRO, C.F.T. YOSHIDA, A.M.C. GASPAR, S.A. GOMES*

Department of Virology, Oswaldo Cruz Foundation, Av. Brasil 4365, 21045-900 Rio de Janeiro, Brazil

Received September 2, 1996

Summary. – A Western blot assay was standardized to evaluate the antigenic reactivity of hepatitis B virus (HBV) strains circulating in Brazilian population with antibodies raised against recombinant hepatitis B (HB) vaccines. In this assay, HBV envelope proteins from infected human blood were detected by antibodies from rabbits immunized with either of two recombinant vaccines. These were Engerix B (Smith Kline Beecham, Belgium) containing exclusively S protein particles and TGP-943 (Takeda Chemical Industries, Japan) containing M protein particles. Forty-seven serum samples, presenting HB surface antigen. (HBsAg) reverse passive haemagglutination assay (RPHA) titers ranging from 1:32 to ≥1:4096 after HBV particles concentration, were tested. Twenty-seven samples were from acute hepatitis cases and 20 were from chronic cases (11 from cirrhotic patients and 9 from asymptomatic carriers). Four HBV serotypes, *adw2, adw4, ayw2* and *ayw3*, were identified in these samples. Infectivity of these sera was evaluated by HBV DNA detection by polymerase chain reaction (PCR). HBV DNA was present in 62% of samples from acute cases and in all samples from chronic cases. Despite the differences between serotypes, genotypes, forms of infection, and infectivity of the samples, antibodies against both vaccines reacted with HBV envelope proteins from all but one sample. In one sample from cirrhotic patient, only a small protein of unexpected size reacted with TGP-943 antibodies.

Key words: hepatitis B, hepatitis B surface antigen; env proteins; vaccine; polymerase chain reaction; Brazil

Introduction

HBV is an enveloped DNA virus that causes acute and chronic liver disease including cirrhosis and hepatocellular carcinoma. HBV DNA encodes three envelope (env) proteins within a single open reading frame (ORF) (for review see Ganem, 1996). Each of the three proteins encoded by pre-S/S region arise from separate translation initiations at each of three in-frame start codons. The smallest (S) of the env proteins, encoded by S gene, contains 226 amino acids (aa) and occurs in glycosylated (gp27) and unglycosylated (p24) forms. The middle (M) protein encoded by pre-S2/S domains contains a hydrophilic N-terminal extension of 55 aa arising from translation of pre-S2 domain. M protein exists in mono- (gp33) and diglycosylated (gp36) forms. Initiation of translation at the start codon in pre-S1 domain results in production of the large (L) protein. L protein has 108 or 119 additional aa from pre-S1 region (depending on viral subtype), and occurs in glycosylated (gp42) and unglycosylated forms (p39). These six proteins generally named HBsAg are present in different quantities in HB viral particles. Blood of infected persons reveals large quan-

*Corresponding author.

Abbreviations: aa = amino acid; DTT = dithiothreitol; EDTA = ethylenediamine tetraacetic acid; ELISA = enzyme-linked immunosorbent assay; env = envelope; HB = hepatitis B; HBV = hepatitis B virus; HBsAg = hepatitis B surface antigen; MoAb = monoclonal antibody; nt = nucleotide; ORF = open reading frame; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; RPHA = reverse passive haemagglutination assay; RT = room temperature SDS = sodium dodecyl sulphate; SDS-PAGE = polyacrylamide electrophoresis in the presence of SDS

tities of spherical particles of 20 nm diameter present in 10^3 – 10^6-fold excess over 43 nm virions and smaller quantities of filaments of 20 nm diameter with variable length (Ganem, 1996). S protein is the major component of all three forms of particles. L protein is preferentially located in virions and filamentous particles and contributes to 10 – 20% of total envelope proteins (Heerman *et al.*, 1984). M protein, preferentially located in virions and 20 nm spherical particles, may contribute to 5 – 15% of total particle proteins (Stibbe and Gerlich, 1982; Gerlich *et al.*, 1987).

The serological heterogeneity of HBsAg has long been established and the HBV strains are classified into nine serotypes, *ayw1, ayw2, ayw3, ayw4, ayr, adw2, adw4, adrq*$^+$ and *adrq*$^-$, according to the antigenic determinants and subdeterminants of their HBsAg (Couroucé *et al.*, 1976; Couroucé-Pauty *et al.*, 1978). In all serotypes, the common *a* antigenic determinant is located within domains bordered by aa 120 and 147 of the major S protein. The expression of *d/y* and *w/r* subdeterminants has been localized at aa 122 and 160, respectively. A shift from Lys to Arg at these sites is responsible for subtype changes (Okamoto *et al.*, 1987; Okamoto *et al.*, 1989). Antibodies to the *a* determinant confer protection to all subtypes of HBV while antibodies to the subtype determinant do not.

HBV serotypes are not uniformly distributed around the world. In Brazil, a country possessing a miscigenated population, serotypes *ayw2, ayw3, adw2* and *adw4* are commonly found (Gaspar and Yoshida, 1987).

Besides S protein, which is the major component of most HB recombinant vaccines, pre-S1 and pre-S2 antigens have been shown to play an important role in the immunological responses at B- and T-cell levels (Chisari and Ferrari, 1995). For this reason, pre-S1 and pre-S2 peptides have been included in some HB vaccines. Engerix B (Smith Kline Beecham) is a recombinant yeast-derived vaccine, consisting exclusively of S particles from an *adw2* HBV strain (De Wilde *et al.*, 1985; Andre and Safary, 1987). The expression of S gene in yeast cells results in the synthesis of p24 protein, which is assembled into particles closely resembling the 20 nm particles found in human serum (Valenzuela *et al.*, 1982). TGP-943 (Takeda Chemical Industries) is a recombinant yeast-derived vaccine expressing preS-2/S gene of an *adr* serotype; M protein particles secreted from transfected yeast cells are composed of two major glycoproteins gp34 and gp37 (Fujisawa *et al.*, 1990; Kuroda *et al.*, 1991).

Some specific point mutations in S gene resulting in a significant change of the structure of the *a* antigenic determinant are well documented (Magnius and Norder, 1995). Escape mutants may arise in persons infected with HBV after vaccination or after treatment with immunoglobulins (Carman *et al.*, 1990; Fujii *et al.*, 1992; Harrison and Zuckerman, 1992). This type of mutation has also been reported in a carrier who received neither HBV vaccine nor immunoglobulins (Moriyawa *et al.*, 1991). The most common mutation found in escape mutants is a substitution of Arg by Gly at aa 145 in S protein. This mutation has a marked effect on the antigenicity, as shown by the failure of yeast-expressed mutated HBsAg to bind monoclonal anti-*a* antibodies, vaccine and covalescent antisera (Waters *et al.*, 1991).

In a previous work, we reported a large diversity of HBV strains isolated in Brazil (Niel *et al.*, 1994). The aa sequences of pre-S/S proteins of some of these strains revealed variations rarely or not found in strains isolated in other parts of the world (Moraes *et al.*, 1996). Furthermore, the only Brazilian strain completely sequenced (subtype *adw4*), revealed the greatest known divergence of the S protein concerning strains used for vaccine preparation (Naumann *et al.*, 1993). It is important to know whether diverse strains can be recognized by antibodies elicited by standard recombinant HBV vaccines. Here we evaluate the reactivity between antibodies, generated in rabbits by recombinant vaccines Engerix B and TGP-943, and env proteins of HBV strains circulating in Brazil.

Materials and Methods

Immunization of rabbits. Two rabbits were immunized with Engerix B vaccine (20 μg), two with TGP-943 vaccine (20 μg), and one with HB viral particles (25 μg) purified from a human serum infected with an *adw2* strain as described previously (Yoshida *et al.*, 1986). All rabbits were inoculated intramuscularly 3 times, on days 0, 15 and 30. Antisera were collected 45 days after the first immunization. Anti-HBs antibodies were titrated by enzyme-linked immunosorbent assay (ELISA, Monolisa anti-HBs, Sanofi Pasteur) after 10-fold serial dilution (1:10 to 1:100,000). The calibration was performed using anti-HBs standards containing 10, 50, 100 and 150 mIU/ml of anti-HBs of human origin (Monolisa anti-HBs standards, Sanofi Pasteur).

Serological studies. Forty-seven HBsAg-positive sera (27 from acute cases, 11 from cirrhotic cases and 9 from asymptomatic carriers) were selected by ELISA (Auszyme Monoclonal diagnostic kit, Abbott Laboratories) and subtyped by immunodiffusion or by using MoAbs as described previously (Niel *et al.*, 1994). HBsAg titration was done by reverse passive haemagglutination assay (RPHA) (Biomanguinhos, Fiocruz, Brazil).

Concentration of HBV particles from sera was performed as described previously (Fernholz *et al.*, 1993) with some modifications. One ml of serum was mixed with 1 ml of TNE buffer (10 mmol/l Tris-HCl, pH 7.4, 0.15 mol/l NaCl, 1 mmol/l ethylenediamine tetraacetic acid (EDTA)). The mixture was layered on 3 ml of 20% sucrose in TNE and centrifuged at 45,000 rpm in a SW 50.1 rotor (Beckman) for 4 hrs at 4°C. The pellet was resuspended in 100 μl of TNE containing 0.1% Tween 20.

Western blot assay. Five μl of the concentrated HBV particle samples was denaturated by boiling in 5 μl of a buffer containing 0.125 mol/l Tris-HCl pH 6.8, 6% sodium dodecyl sulphate (SDS),

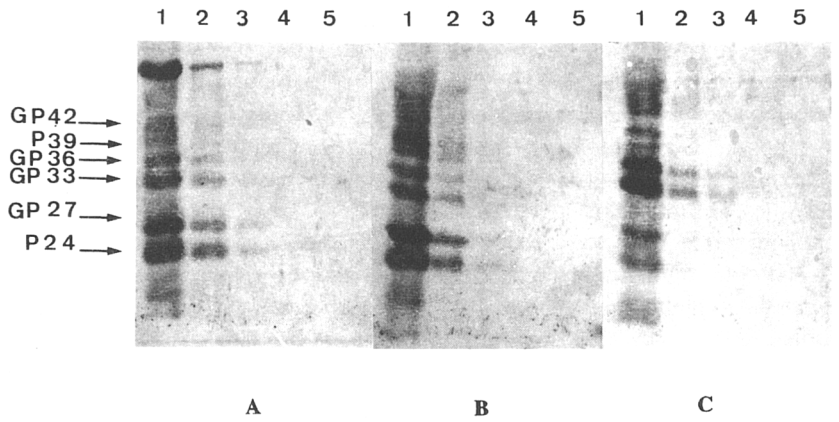

Fig. 1
Standardization of Western blot assay using env proteins of a serum containing high titer of HBsAg
Anti-Engerix B (A), anti-native particle (B), and anti-TGP-943 (C) antibodies were used for detection. Concentrated HBV particles undiluted (lanes 1) or diluted 10-, 100-, and 1000-fold (lanes 2-4). Negative control (lanes 5).

20% glycerol, 10% dithiothreitol (DTT), 0.1% bromophenol blue, and separated by 12% polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE). After electroblotting of proteins to nitocellulose membranes, non-specific binding sites were blocked in phosphate-buffered saline (PBS) containing 5% non- fat milk and 0.05% Tween 20. For detection of HBV env proteins, filters were allowed to react in the same buffer for 2 hrs at room temperature (RT) with 1:100 dilution of rabbit antibodies raised against (i) Engerix B vaccine, (ii) native HBV particles, (iii) TGP-943 vaccine. After three washings with PBS, the blots were incubated for 1 hr at RT with goat anti-rabbit IgG conjugated with peroxidase at a dilution of 1:1000. The blots were washed 3 times with PBS and the bound peroxidase was detected by diaminobenzidine and hydrogen peroxide.

DNA extraction and polymerase chain reaction (PCR). DNA extraction method has been previously described (Niel *et al.*, 1994). Pre-S region DNA was amplified by using primers PS1 (5'CCATATTCTTGGGAACAAGA3') and PS2 (5'GTCCCCAGTCCTCGAGAAG3'), localized at nucleotides (nt) 2826-2845and 143-124, respectively. Primers PS4 (5'ACACTCATCCTCAGGCCATGCAGTG3') and S2 (5'GGGTTTAAATGTATACCCAAAGA3'), localized at nt 3194-3218 and 841-819, respectively, were used for amplification of Pre-S2/S region. Primers S1 (5'CTTCTCGAGGACTGGGGACC3'), localized at nt 124-143, and S2 were used for amplification of S region. PCR was done in the following conditions: 95°C, 30 secs; 55°C, 1 min; 72°C, 2 mins; 30 cycles, followed by a final elongation at 72°C for 7 mins. Total volume 100 µl.

Results

Standardization of Western blot assay

Rabbit sera that presented the highest titers of anti-HBs antibodies were used for detection of env proteins in Western blot assay. The highest titers were 660,000 mIU/ml for rabbits inoculated with Engerix B vaccine and with native HBV particles, and 750,000 mIU/ml for a rabbit inoculated with TGP-943 vaccine. Standardization of the assay was performed with viral particles concentrated from a human serum containing high levels of HBsAg (RPHA titer of 1:4096). This serum sample was obtained from an individual with acute hepatitis and was used as a positive antigen control in all subsequent assays. A serum from an individual who did not present HBV markers served as a negative control. To evaluate the limit of detection of env proteins, the positive antigen control was 10-fold serially diluted. Fig. 1 shows the reactivity of the positive antigen control (lanes 1-4), against anti-Engerix B (A), anti-native particle (B), and anti-TGP-943 (C) antibodies. The anti-Engerix B antibodies detected non-glycosylated and glycosylated forms of the three env proteins of the undiluted and 1:10 diluted positive antigen control (Fig. 1A, lanes 1 and 2). At a dilution of 1:100 only S protein was revealed (lane 3). Anti-native particle antibodies presented a similar reaction pattern (Fig. 1B, lanes 1-3). In contrast, a serum from TGP-943-vaccinated rabbit showed strong bands with M protein and weaker ones with L and S proteins (Fig. 1C, lanes 1-3). All three antisera detected env proteins in positive antigen control up to dilution of 1:100. No specific band could be seen with the negative control (lanes 5).

Detection of HBV env proteins from acute cases

Fig. 2 exemplifies Western blot assay of HBV env proteins with 9 samples from acute cases with anti-Engerix B (A), anti-native particle (B), and anti-TGP-943 (C) antibod-

ies. In all samples, reactive bands corresponding to S proteins were the most intensive provided anti-Engerix B and anti-native particle sera were used. When anti-TGP-943 antibodies were employed, the strongest bands were for M protein. In samples in lanes 3, 8, and 11, where only S proteins were detected by anti-Engerix B (A) and by anti-native particle (B) antibodies, M proteins reacted strongly with anti-TGP-943 antibodies (C). The two forms of M protein, detected by anti-TGP-943 antibodies in the sample in lane 3 (Fig. 2C), presented a slightly higher mobility as compared to other samples. The higher electrophoretic mobility of M proteins of the strain under study was repeatedly observed. It could be due to differences in conformation or charge, or to a deletion in pre-S2 region. To test the latter possibility DNA from this sample was extracted and pre-S2/S region was amplified by PCR. The PCR product migrated at the same position as the amplified pre-S2/S products from other samples indicating the absence of a deletion (data not shown).

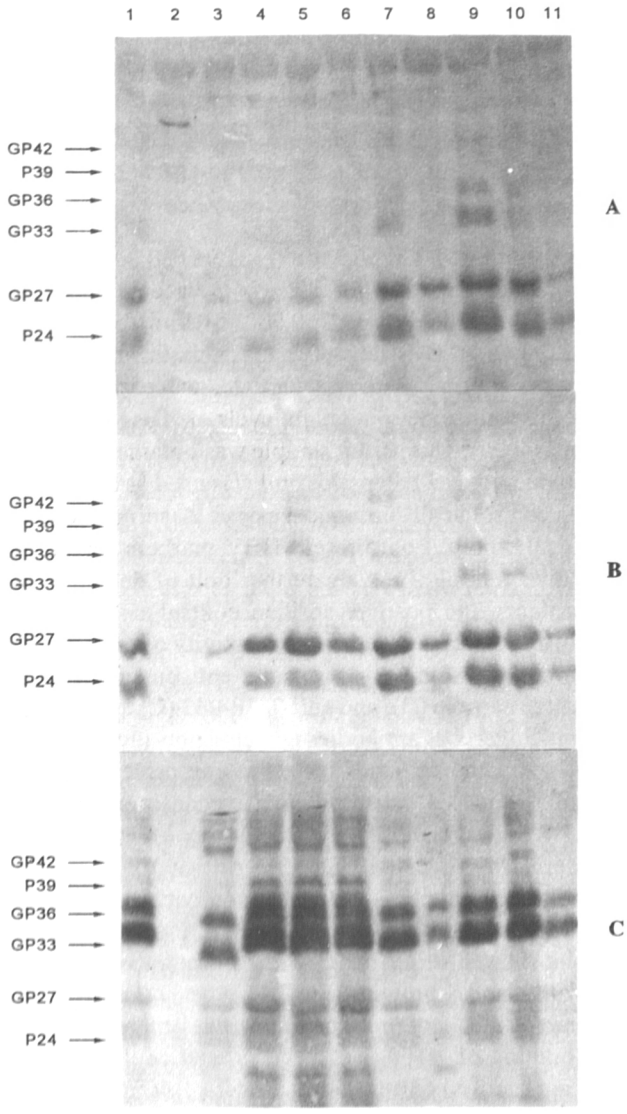

Fig. 2
Detection of env proteins from acute HB cases with anti-Engerix B (A), anti-native particle (B) and anti-TGP-943 (C) antibodies
Positive control (lanes 1), negative control (lanes 2), samples No. 17 (lanes 3), 22 (lanes 4), 21 (lanes 5), 18 (lanes 6), 6 (lanes 7), 1 (lanes 8), 12 (lanes 9), 3 (lanes 10) and 2 (lanes 11).

Results of serotyping, HBV DNA PCR amplification, RPHA titration and HBV env protein reactivity of 38 samples from hepatitis cases are summarized in Table 1. The serotype distribution of 24 of the 27 samples from acute cases was as follows: Fifteen (63%) samples were *adw2,* six (25%) were *ayw3,* two (8%) were *adw4* and one (4%) was *ayw2.* All PCR assays were carried out using two different pairs of oligonucleotides from conserved sequences from pre-S and S regions of HBV genome. HBV DNA could be detected in 15 (63%) of 24 samples. With the PS1-PS2 oligonucleotides pair, isolates of *adw* serotypes gave a PCR product of M_r higher than that of the isolates of *ayw* serotypes. Pre-S region DNA from all 7 *ayw* samples (six *ayw3,* one *ayw2*) was successfully amplified. In contrast, pre-S region of only 6 (five *adw2,* one *adw4*) of 17 *adw* isolates, could be amplified. Amplification with S1-S2 primers allowed to detect DNA from 17 samples, i.e. from two more than with PS1-PS2 pair.

Anti-Engerix B antibodies revealed L, M, and S proteins in 10 (37%) acute samples (Table 1). In 17 (63%) samples, only S proteins were detected. Anti-native particle antibodies detected all env proteins in 13 samples and S proteins alone in 14 samples. All 27 acute samples showed reactive bands against M proteins with anti-TGP-943 antibodies. In addition, S and L proteins could be detected in 13 and 22 samples, respectively (Table 1, the last column). No correlation could be established between the RPHA titers and the pattern of detection of env proteins with the three anti-HBs antibodies.

Detection of HBV env proteins from chronic cases

Eleven samples from cirrhotic patients were analyzed (Table 1). When subjected to PCR amplification with primers PS1-PS2, only five samples were successfully amplified. PCR with S1-S2 primers detected HBV DNA in all 11 samples. Titers of HBsAg in cirrhotic patients were generally lower than those in acute cases and only S proteins from these patients were revealed by anti-Engerix B and anti-native particle antibodies in 10 of 11 samples. Anti-TGP-943 antibodies revealed M and S proteins in 4 samples and M protein alone in 6 samples. Sample No. 38 did not present bands of expected size with any rabbit antiserum, but a band of approximately 20 K with anti-TGP-943 antibodies (Fig. 3B, lane 12).

Table 1. Analysis of sera from HB cases: Serotyping, DNA PCR amplification, HBsAg RPHA titration, and Western blot detection of env proteins

Sample No.	Serotype	HBV DNA	HBsAg RPHA[a]	Detection of HBV env proteins by antibodies against		
				Engerix B[b] vaccine	Native particles	TGP-943 vaccine
Acute cases						
1	*adw2*	–	128	S	S	M,S
2	*adw2*	–	128	S	S	M,S
3	*adw2*	+(higher)[c]	512	S,M,L	S,M,L	M,S,L
4	*adw2*	–	1024	S	S	M
5	*adw2*	–	1024	S	S	M,S
6	*adw2*	+(higher)	1024	S,M,L	S,M,L	M,S,L
7	*adw2*	–	2048	S	S	M,S
8	*adw2*	–	≥ 4096	S	S	M,S
9	*adw2*	–	≥ 4096	S	S	M
10	*adw2*	–	≥ 4096	S	S	M,S
11	*adw2*	–	≥ 4096	S,M,L	S,M,L	M,S,L
12	*adw2*	+(S)[d]	≥ 4096	S,M,L	S,M,L	M,S,L
13	*adw2*	+(higher)	≥ 4096	S	S	M,S
14	*adw2*	+(higher)	≥ 4096	S,M,L	S,M,L	M,S,L
15	*adw2*	+(higher)	≥ 4096	S,M,L	S,M,L	M,S,L
16	*adw4*	+(higher)	512	S S M		
17	*adw4*	+(S)	≥ 4096	S	S	M,S
18	*ayw2*	+(lower)[c]	≥ 4096	S,M,L	S,M,L	M,S,L
19	*ayw3*	+(lower)	≥ 4096	S	S	M
20	*ayw3*	+(lower)	≥ 4096	S	S	M
21	*ayw3*	+(lower)	≥ 4096	S	S,M,L	M,S,L
22	*ayw3*	+(lower)	≥ 4096	S	S,M,L	M,S,L
23	*ayw3*	+(lower)	≥ 4096	S	S,M,L	M,S,L
24	*ayw3*	+(lower)	≥ 4096	S,M,L	S,M,L	M,S,L
25	ND	ND	≥ 4096	S	S	M,S
26	ND	ND	≥ 4096	S,M,L	S,M,L	M,S,L
27	ND	S,M,L	S,M,L	M,S,L		
Cirrhotic cases						
28	*adw2*	+(higher)	32	S	S	M,S
29	*adw2*	+(higher)	128	S	S	M
30	*adw2*	+(higher+lower)	256	S	S	M
31	*ayw3*	+(S)	64	S	S	M,S
32	*ayw3*	+(S)	256	S	S	M
33	*ayw3*	+(S)	512	S	S	M
34	*ayw3*	+(S)	1024	S	S	M
35	*ayw3*	+(S)	1024	S	S	M,S
36	*ayw3*	+(lower)	≥ 4096	S	S	M,S
37	ND	+(lower)	64	S	S	M
38	ND	+(lower)	32	–	–	–

[a]Reciprocal titer of HBsAg after concentration of HBV particles.
[b]The sequence of the letters defines the decreasing band intensity of the corresponding proteins.
[c]Depending on the serotype, PCR with PS1-PS2 primers gave a band of 539 bp (higher) or 506 bp (lower).
[d]+(S): HBV DNA not detected by PS1-PS2 primers but by S1-S2 primers only.
ND = not done.

To estimate the effect of particular aa variations in the detection of env proteins by antibodies raised against vaccines, 9 samples from asymptomatic HBV carriers were included in this study. Seven (strains BrI, BrK, BrL, BrF, BrN, BrB and BrJ from *ayw2, ayw3,* and *adw2* serotypes) presented at least one rare or unique variation in pre-S2/S aa sequence (Moraes *et al.,* 1996). In addition, two *adw4* strains, w4B (Naumann *et al.,* 1993) and BrC (Moraes *et*

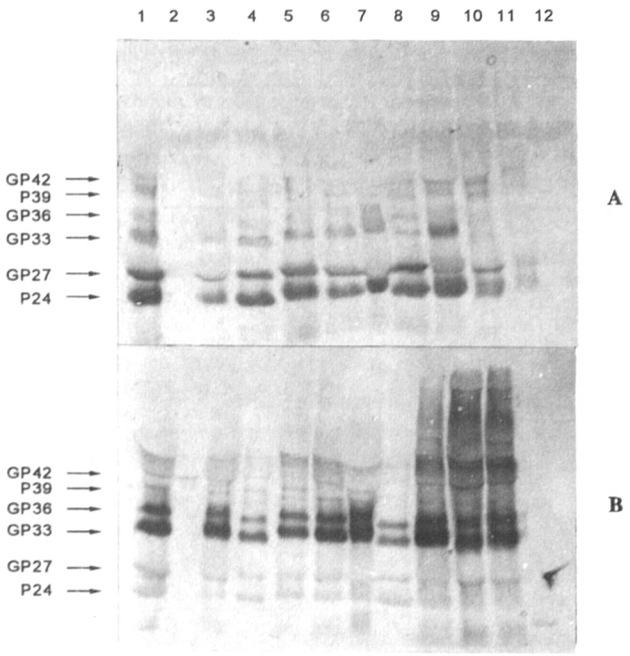

Fig. 3
Detection of env proteins from chronic HB cases with anti-Engerix B (A) and anti-TGP-943 (B) antibodies
Positive control (lanes 1), negative control (lanes 2), strains BrI (lanes 3), BrK (lanes 4), BrL (lanes 5), BrF (lanes 6), BrN (lanes 7), BrB (lanes 8), BrJ (lanes 9), BrC (lanes 10) and w4B (lanes 11), and sample No. 38 from cirrhotic case (lanes 12). Strains Br (Moraes *et al.*, 1966) and isolate w4B (Naumann *et al.*, 1993) from asymptomatic cases have been described previously.

al., 1996), phylogenetically distant from other HBVs, were also analyzed. All these samples, originated from Brazilian carriers, presented titers ≥1:4096 in RPHA. Env proteins (S, M and L) in these samples were detected by vaccinal antibodies as shown in Fig. 3 (lanes 3-11).

Discussion

Most epitopes of S protein are thought to be conformational and disulfide-dependent (Brown *et al.*, 1984; Stirk *et al.*, 1992). For this reason, it has been suggested that antibodies against S antigens are mostly non-reactive in Western blot analysis (Heermann *et al.*, 1988). However, the present work shows that rabbit antibodies raised against HBV particles (purified from human sera) and Engerix B vaccine detected S protein more intensively than M and L proteins in Western blot assay. In contrast, anti-TGP-943 antibodies detected preferentially M protein, suggesting that TGP-943 vaccine favours the appearance in rabbits of antibodies against epitopes present in the pre-S2 domain of M protein. The induction by TGP-943 vaccine of higher amounts of anti-pre-S2 antibodies than anti-S antibodies, has also been observed in humans and chimpanzees (Kuroda *et al.*, 1991).

Most of the human sera in which all env (S, M, L) proteins were detected by anti-HBs antibodies possessed HBsAg RPHA titers ≥1:4096. However, in more than a half of the infectious sera with high titers of HBsAg, only one protein, S or M, depending on anti-HBs antibodies, could be revealed. It has been shown that the ratio of the three env proteins in HBV particles is variable in different HBV-containing sera (Stibbe and Gerlich, 1982; Heermann *et al.*, 1984; Gerlich *et al.*, 1987). This individual variation may explain why HBsAg titers did not correlate with the Western blot pattern of the three env proteins. Env proteins from concentrated particles with HBsAg titers lower than 1:32 were rarely detected with the anti-HBs antibodies used (data not shown).

The infectivity of sera in hepatitis cases was evaluated by HBV DNA PCR amplification using two different pairs of oligonucleotide primers. The PS1-PS2 pair was less efficient in HBV DNA detection than the S1-S2 pair. Products amplified from pre-S region presented differences in length between serotypes *adw* and *ayw*. Alignment of 48 complete HBV genome sequences available in Genbank confirmed that *ayw* strains possessed a deletion of 11 codons in the 5'-end of pre-S1 region with respect to other strains (data not shown). In sample No. 30, two pre-S region DNA products of M_r corresponding to *adw* and *ayw* serotypes were observed. It is possible that a mixture of strains (one *adw* and another *ayw*) was present in this serum. Whereas HBV DNA was detected in all samples from patients with cirrhosis, the frequency of detection in samples from acute cases was only 62%. The presence of HBV DNA in sera therefore correlated with the form of infection ($P < 0.002$) but not with HBsAg levels evaluated by RPHA titers.

Although studies with recombinant HB vaccines produced in yeast cells have proved that they are safe, immunogenic and as effective as human plasma-derived vaccines (Andre and Safary, 1987; Panda *et al.*, 1991; Soyletir *et al.*, 1992), a few data are available concerning the reactivity of antibodies raised against these vaccines with various HBV strains. Recently, strains belonging to *adw4* serotype (originated from aboriginal population of the New World) have been clustered into a separate genomic group on the basis of S gene variability (Norder *et al.*, 1992; 1994). In all sequenced *adw4* strains, S protein has Ser at position 140 instead of Thr present in the vaccine protein (Magnius and Norder, 1995). Here we show that *adw4* strains react as well as isolates of other serotypes with antibodies elicited by both Engerix B and TGP-943 vaccines.

Variations within HBV subtypes in Brazilian population have been detected by ELISA using a set of MoAbs. With this technique, strains of the same serotype (*ayw3, ayw2,* or *adw4*) presented different well characterized reactive patterns, while *adw2* strains did not show any variation

(AMC Gaspar, personal communication). Recently, restriction map analysis (Niel *et al.,* 1994) and sequencing (Moraes *et al.,* 1996) have confirmed the diversity of Brazilian HBV isolates. In addition to *adw4* strains, previously sequenced isolates presenting rare or unique aa substitutions in pre-S2/S regions (Moraes *et al.,* 1996) were tested for env reactivity with vaccinal antibodies. These aa substitutions did not interfere with the Western blot detection. Finally, all but one HBsAg-positive samples randomly selected from acute and cirrhotic cases showed bands of env proteins reactive with antibodies against both vaccines, although differences in their reaction patterns were observed. A sample in which only a band of approximately 20 K was detected with anti-TGP-943 antibodies presented DNA from pre-S and S regions of expected length. One possible explanation for the absence of anti-env proteins antibodies reactivity is that this sample might not contain sufficient amounts of env proteins to be detected in our experimental conditions. Another possibility is that this strain presented aa changes that would affect the binding affinity of the antibodies to env proteins. The sequencing of pre-S/S region should clarify this point.

Acknowledgements. We are grateful to Drs. C. Niel and L. Lewis for critical reading of the manuscript and to Ms M. Sidoni for technical assistance in rabbit immunization. The TGP-943 vaccine was a gift from the Institute of Immunology Co. Ltd., Tokyo, Japan. This work was supported by grant No. 10/4662-0 from the Banco do Brasil and by CNPq.

References

Andre FE, Safary A (1987): Summary of clinical findings of Engerix-B, a genetically engineering yeast-derived hepatitis B vaccine. *Postgrad. Med. J.* **63** (Suppl. 2),169–178.

Brown SE, Howard CR, Zuckerman AJ, Steward MW (1984): The affinity of antibody responses in man to hepatitis B vaccine determined with synthetic peptides. *Lancet* **ii**, 184–187.

Carman WF, Zanetti AR, Karayiannis P, Waters J, Manzillo G, Tanzi E, Zuckerman AJ, Thomas HC (1990): Vaccine-induced escape mutant of hepatitis B virus. *Lancet* **336**, 325–329.

Chisari FV, Ferrari C (1995): Hepatitis B virus immunopathogenesis. *Ann. Rev. Immunol.* **13**, 29–60.

Couroucé AM, Holland P, Muller PV, Soulier JP (1976): HBs antigen subtypes: Proceedings of the International Workshop on HBs antigen subtypes. *Bibl. Haematol.* **42**, 1–158.

Couroucé-Pauty AM, Lemaire JM, Roux JF (1978): New hepatitis B surface antigen subtypes inside the *ad* category. *Vox Sang.* **35**, 304–308.

De Wilde M, Cabezon T, Harford N, Rutgers T, Simoen E, Van Wijnendaele F (1985): Production in yeast of hepatitis B surface antigen by R-DNA technology. *Dev. Biol. Standard.* **59**, 99–107.

Fernholz D, Galle R, Stemler M, Brunetto M, Bonino F, Will H (1993): Infectious hepatitis B virus variant defective in pre-S2 protein expression in a chronic carrier. *Virology* **194**, 137–148.

Fujii H, Moriyama K, Sakamoto N, Kondo T, Yasuda K, Hiraizumi Y, Yamazaki M, Sakaki Y, Okochi K, Nakajima E (1992): Gly145 to Arg substitution in HBs antigen of immune escape mutant of hepatitis B virus. *Biochem. Biophys. Res. Commun.* **184**, 1152–1157.

Fujisawa Y, Kuroda S, Van Eerd PMCA, Schellekens H, Kaninuma A (1990): Protective efficacy of a novel hepatitis B vaccine consisting of M (pre-S2+S) protein particles (a third generation vaccine). *Vaccine* **8**, 192–198.

Ganem D (1996): Hepadnaviridae and their replication. In Fields BN, Knipe DM, Howley PM (Eds): *Virology*. Vol. 2, Lippincott-Raven Publishers, Philadelphia, pp. 2703–2737.

Gaspar AMC, Yoshida CFT (1987): Geographic distribution of HBsAg subtypes in Brazil. *Mem. Inst. Oswaldo Cruz* **82**, 253–258.

Gerlich WH, Heerman KH, Thomssen R (1987): The envelope proteins of hepatitis B virus and their potential role in vaccination against hepatitis B virus-induced liver diseases. *Contr. Oncl.* **24**, 200–210.

Harrison TJ, Zuckerman AJ (1992): Variants of hepatitis B virus. *Vox Sang.* **63**, 161–167.

Heermann KH, Goldman U, Schwartz W, Seyffarth T, Baumgarten H, Gerlich WH (1984): Large surface proteins of hepatitis B virus containing the pre-S sequence. *J. Virol.* **52**, 396–402.

Heermann KH, Gultekin H, Gerlich WH (1988): Protein blotting: techniques and application in virus hepatitis research. *La Ricerca Clin. Lab.* **18**, 193–221.

Kuroda S, Fujisawa Y, Iino S, Akahane Y, Suzuki H (1991): Induction of protection level of anti-pre-S2 antibodies in humans immunized with a novel hepatitis B vaccine consisting of M (pre-S2+S) protein particles (a third generation vaccine). *Vaccine* **9**, 163–169.

Magnius LO, Norder H (1995): Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. *Intervirology* **38**, 24–34.

Moraes MTB, Gomes SA, Niel C (1996): Sequence analysis of pre-S/S of hepatitis B virus strains of genotypes A, D, and F isolated in Brazil. *Arch. Virol.* **141**, 1767–1773.

Moriyawa K, Nakajima E, Hohjoh H, Asayama R, Okochi (1991): Immunoselected hepatitis B virus mutant. *Lancet* **337**, 125.

Naumann H, Schaefer S, Yoshida CF, Gaspar AMC, Repp R, Gerlich WH (1993): Identification of a new hepatitis B virus (HBV) genotype from Brazil that expresses HBV surface antigen subtype *adw4*. *J. Gen. Virol.* **74**, 1627–1632.

Niel C, Moraes MT, Gaspar AMC, Yoshida CF, Gomes SA (1994): Genetic diversity of hepatitis B virus strains isolated in Rio de Janeiro, Brazil. *J. Med. Virol.* **44**, 180–186.

Norder H, Hammas B, Löfdahl S, Couroucé AM, Magnius LO (1992): Comparison of the aminoacid sequences of nine different serotypes of hepatitis B surface antigen and

genomic classification of the corresponding hepatitis B virus strains. *J. Gen. Virol.* **73**, 1201–1208.

Norder H, Couroucé AM, Magnius LO (1994): Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology* **198**, 489–503.

Okamoto H, Imai M, Tsuda F, Tanaka T, Miyakawa Y, Mayumi M (1987): Point mutation in the S gene of hepatitis B virus for a *d/y* or *w/r* subtypic change in two blood donors carrying a surface antigen of compound subtype *adyr* or *adwr*. *J. Virol.* **61**, 3030–3034.

Okamoto H, Omi S, Wang Y, Itoh Y, Tsuda F, Tanaka Akahane Y, Miyakawa Y, Mayumi M (1989): The loss of subtypic determinants in alleles, *d/y* or *w/r*, on hepatitis B surface antigen. *Mol. Immunol.* **26**, 197–205.

Panda SK, Ramesh R, Rao KV, Gupta A, Zuckerman AJ, Nayak NC (1991): Comparative evaluation of the immunogenicity of yeast-derived (recombinant) and plasma-derived hepatitis B vaccine in infants. *J. Med. Virol.* **35**, 297–302.

Soyletir G, Bahceci E, Akoglu E, Soyogul U, Lawrance RA (1992): Clinical evaluation of low dose intradermally administered hepatitis B vaccine: a comparison of plasma-derived and recombinant yeast-derived vaccines. *Vaccine* **10**, 301–304.

Stibbe W, Gerlich WH (1982): Variable protein composition of hepatitis B surface antigen from different donors. *Virology* **123**, 436–442.

Stirk HJ, Thornton JM, Howard CR (1992): A topological model for hepatitis B surface antigen. *Intervirology* **33**, 148–158.

Valenzuela P, Medina A, Rutter WJ (1982): Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature* **298**, 347–351.

Waters JS, Brown SE, Steward MW, Howard CR, Thomas HC (1991): Analysis of the antigenic involved in the induction of a protective antibody response. *Virus. Res.* **22**, 1–12.

Yoshida CFT, Camargo IF, Mercadante LAC, Gaspar AMC, Gomes DFDL, Shatzmayr HG (1986): Hepatitis B serological patterns of asymptomatic carriers in an endemic region and evaluation of blood plasma as a source of hepatitis B vaccine. *Vaccine* **4**, 253–256.