Journal of Medical Virology 64:312–319 (2001)

Hepatitis B Virus Markers in Anti-HBc Only Positive Individuals

Bernard Weber,[1,2]* Walter Melchior,[3] Ralph Gehrke,[3] Hans Wilhelm Doerr,[2] Annemarie Berger,[2] and Holger Rabenau[2]

[1]*Laboratoires Réunis Kutter-Lieners-Hastert, Junglinster, Luxembourg*
[2]*Institut für Med. Virologie, Universitätskliniken Frankfurt, Germany*
[3]*Roche Diagnostics, Penzberg, Germany*

Isolated reactivity to hepatitis B virus (HBV) core antigen (anti-HBc) is observed relatively frequently in immunocompromised individuals, intravenous drug abusers (IVDA), and in the presence of HCV infection. The reason for the lack of HBsAg is not clear. The aim of the present study was to investigate which factors (genetic variability of S gene, low-level HBsAg, and immune complexes may be responsible for the failure of HBsAg detection with commercial HBsAg screening assays. Dilution series of two recombinant HBsAg escape mutants and dilutions of serum samples from chronic HBV carriers with multiple insertions in the *a* determinant and different HBsAg subtypes were tested with a highly sensitive assay that detects wild-type HBsAg (Elecsys HBsAg, Roche Diagnostics, Penzberg, Germany) and two assays that detect HBV wild-type and escape mutants (Murex HBsAg Version 3, Murex and Enzygnost HBsAg 5.0, Dade Behring, Marburg, Germany). Elecsys HBsAg showed in comparison to Murex HBsAg Version 3 and Enzygnost HBsAg 5.0 a reduced sensitivity for escape mutant detection. On the other hand, the best performance for HBsAg subtype detection was obtained with Elecsys HBsAg. In the second part of the study, a selected panel of isolated anti-HBc reactive (n = 104) serum samples (AxSYM Core) was submitted to testing by Elecsys HBsAg, Murex HBsAg Version 3, Enzygnost HBsAg 5.0, and HBsAg detection after immune complex dissociation (ICD) and anti-HBs determination with two different assays (AxSYM Ausab and Elecsys Anti-HBs). To assess the specificity of anti-HBc test results, all the samples were tested by a second anti-HBc assay (Elecsys Anti-HBc). Quantitative HBV DNA detection was undertaken with a commercially available HBV PCR assay (Amplicor HBV Monitor). HCV infection was present in 65.4% of anti-HBc only reactive individuals. Five AxSYM Core positive samples were negative by Elecsys Anti-HBc. Overall, 15 (14.4%) AxSYM Ausab negative samples gave positive results with Elecsys Anti-HBs (median value: 21 IU/ml). No low-level HBsAg carrier was detected among the isolated anti-HBc reactive individuals with Elecsys HBsAg. There was no evidence for the presence of immune complexes. Only one sample was repeatedly reactive by the Murex HBsAg, suggesting that the a mutant form of HBsAg was responsible for the isolated anti-HBc reactivity, however neutralisation assay was not interpretable and HBV DNA PCR was negative. Fifteen (14.4%) anti-HBc only positive individuals were HBV DNA carriers with concentrations ranging from 800 to more than > 4,000,000 copies of viral DNA/ml. In conclusion, the most probable explanations for isolated anti-HBc reactivity in our study group are a possible interference of HBsAg synthesis by HCV infection (65.4%) and divergence of results of anti-HBs assays (14.4%). There is no evidence for the presence of low-level HBsAg carriers and immune complexes. HBsAg mutants cannot be excluded definitively by the test strategy used in the present evaluation. ***J. Med. Virol. 64:312–319, 2001.***

© 2001 Wiley-Liss, Inc.

KEY WORDS: HBsAg mutants; low-level HBsAg carriers; immune complexes; viral load; anti-HBs test variability

Elecsys and Amplicor are trademarks of a member of the Roche Group. Enzygnost is a trademark of Dade Behring Diagnostics GmbH. AxSYM is a trademark of Abbott Laboratories Corporation. Murex is a trademark of International Murex Technologies Corporation.

*Correspondence to: Dr. Bernard Weber, Laboratoires Réunis Kutter-Lieners-Hastert, Centre Langwies, L-6131 Junglinster, Luxembourg. E-mail: laborklh@pt.lu

Accepted 16 October 2000

© 2001 WILEY-LISS, INC.

INTRODUCTION

Detection of antibody against hepatitis B virus core antigen (anti-HBc) by enzyme immunoassay (EIA) is used solely or in combination with HBV surface antigen (HBsAg) screening for the diagnosis of hepatitis B. Anti-HBc detection is used as a screening method prior to hepatitis B vaccination in high prevalence groups [Lok et al., 1988]. The detection of anti-HBc by routine EIAs in a competitive test format is subject to frequent non-specific reactions causing false-positive results [Sherertz et al., 1983; Kessler et al., 1985; Schmidt et al., 1988; Caspary et al., 1989; Schifan et al., 1993]. Isolated false-positive anti-HBc reactivity (presence of anti-HBc; HBsAg and anti-HBs negative) has been attributed to cross-reactive antibodies or interfering substances in serum; this effect was also observed for anti-HBs [Sherertz et al., 1983; Kessler et al., 1985]. One of the major reasons for false-positive reactivity is the non-specific activation of premature B-lymphocytes resulting in the production of IgA or IgM-related molecules without previous exposure to HBV [Sällberg and Magnius, 1989; Robertson et al., 1991]. Pretreatment of serum samples with reducing agents, i.e., dithiothreitol (DTT) or potassium bisulfite (MBS) improves significantly the specificity of anti-HBc determination [Weare et al., 1991; Weber et al., 1998].

Depending on the prevalence of HBV infection and patient group investigated, 1 to 32% of positive anti-HBc are isolated positive findings results [Hadler et al., 1984]. Isolated anti-HBc readings are observed frequently in intravenous drug abusers, HIV infected individuals [Scheitlin and Grob, 1992; Sanchez-Quijano et al., 1993], HBV and hepatitis C virus (HCV) co-infected patients [Jilg et al., 1995], and pregnant women [Gross et al., 1993]. The presence of anti-HBc alone is often interpreted as evidence of past hepatitis B where anti-HBs has fallen below the level of detection. However, between 10% and 40% of individuals with this serological pattern are chronic carriers of HBV [for a review, see Weinberger et al., 1997].

The reason for the lack of HBsAg in anti-HBc only positive individuals is not clear, but several explanations have been suggested. HBsAg may be hidden in circulating immune complexes [Ackerman et al., 1994; Joller-Jemelka et al., 1994]. HBsAg synthesis may be downregulated by coinfection with HCV [Sheen et al., 1992; Shih et al., 1993]. Variations in the pre-S region, or mutations in the surface antigen itself and especially in the *a* determinant, which is recognised by anti-HBs, may render HBsAg undetectable by conventional assays [Melegari et al., 1994, Carman and Mimms, 1997; Seddigh-Tonekaboni et al., 2000; Zuckerman, 2000]. A certain proportion of anti-HBc only positive individuals may be low-level HBsAg carriers, with antigen concentrations below the detection limit of available serological tests [Carman et al., 1997].

The aim of the present study was to examine these hypotheses with assays that may be used under routine diagnostic conditions. The sensitivity of screening assays for the detection of HBsAg mutants and different HBV subtypes was assessed by testing dilution series of well characterised, recombinant mutants and subtypes. A selected panel of isolated anti-HBc reactive serum samples (n = 104) was submitted to testing with i) a new highly sensitive HBsAg assay, ii) two assays (Murex HBsAg Version 3 and Enzygnost HBsAg 5.0) that detect HBsAg mutants, iii) HBsAg detection after immune complex dissociation (ICD), and iv) anti-HBs detection with two different assays. To assess the specificity of anti-HBc test results, all the samples were tested by a second anti-HBc assay. Quantitative HBV DNA detection was carried out with a commercially available HBV PCR assay.

MATERIAL AND METHODS

HBsAg Mutants and Subtypes

To assess the sensitivity of screening assays for the detection of HBsAg mutants and different HBV subtypes, dilution series of four well-characterised mutants and HBV subtypes in pooled HBsAg and anti-HBc negative serum were tested. Included were the following.

Crude yeast extracts of two recombinant surface antigen mutants F134Y/G145R and P142S/G145R/N146D. Mutant F134Y/G145R was obtained from a HBsAg positive liver transplant recipient treated with anti-HBs antibody. Mutant 142S/G145R/N146D was from a patient suffering from chronic hepatitis B who showed HBsAg/anti-HBs seroconversion under immune globulin therapy [McMahon et al., 1992; Carman et al., 1996].

DNA extraction from serum, amplification, and sequencing was carried out as described by Hou et al. [1994]. Briefly, 5 μl of extracted DNA were submitted to nested PCR. For the first round PCR, external primers Pol.1 and Pre-S2 which correspond to nucleotides 895–912 and 3171–3188 were used. With the internal primers S1-EcoR1 and S4-EcoR1, a 695 bp sequence was amplified. Amplification products were sequenced using Sequenase Version 2 Kit (Amersham, Arlington Heights, IL) and external primers Pre-S2 and Pol.1 and primer S3, which corresponds to nucleotides 457–476. Amplified HBV DNA was cloned into pCRII vector of TA cloning kit (Invitrogen, La Jolla, CA). Cloned DNA was submitted to EcoRI restriction enzyme cleavage and inserted into EcoRI restriction site of pHILD2 vector (kindly donated by Professor Howard Thomas, London).

HBsAg genes cloned with pHILD2 were amplified with touch-down PCR using primers FS_HA-EcoRI and RS_KpnI (Fig. 1). Amplification was carried out with Perkin Elmer 9600 thermocycler and involved 30 cycles in which primers were denatured at 94°C (1 minute), annealed at 70 to 40°C (1 minute), and extended at 72°C (1 minute). By using primer FS_HA-EcoRI, an N-terminal haemagglutinin Tag (HA-Tag) was incorporated into HBsAg (Fig. 2).

pGAPZ and amplification products (pGAPZ-145, mutant 145 (134 F→Y/145 G→R), subtype *adr* and

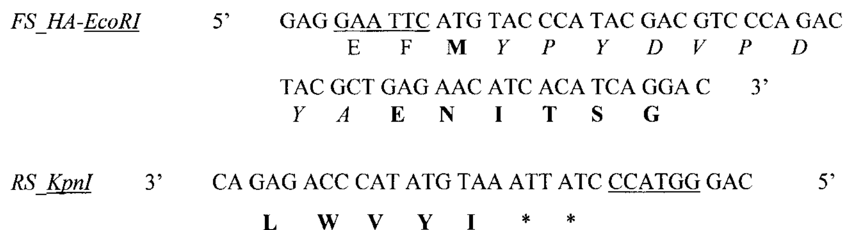

Fig. 1. Nucleotid- and amino acid sequence of PCR primers FS_HA-EcoRI and RS_KpnI. Restriction enzyme sites are underlined. Aminoacids deduced from HBV genome are printed bold. Aminoacids corresponding tohemagglutinin-Tag are printed in italics.

pGAPZ-146 (triple-mutant 146 (142 P→S/145 G→R/ 146 N→D), subtype *adw*) were submitted to E*co*RI and K*pn*I restriction enzyme digestion. Constructs were verified after ligation, selection and amplification by double strand sequencing.

Expression of HBV antigens was undertaken in *Pichia pastoris*. HBV DNA genes coding for surface protein were cloned into yeast expression vector pGAPZ (Invitrogen). Expression of HBV genes is regulated by the constitutive glycerinaldehyde promotor. *Pichia pastoris* Strain X-33 was cultured in YPD-Medium and made competent with "*Pichia* EasyComp Kit" (Invitrogen). Transformation of competent cells was carried out in accordance with the manufacturers' recommendations (Invitrogen). Plasmids pGAPZ-145 and -146 were linearised with restriction enzyme R*ca*I prior to transformation. Selection was performed on culture medium (YPD agar) supplemented with Zeocin (150 μg/ml). Single colonies were subcultured in liquid broth medium (2–3 ml) and yeast cells were disrupted with glass beads (400–600 μm, Sigma Chemical Co., St. Louis, MO).

Detection of recombinant proteins was carried out by using a sandwich ELISA. Owing to the high affinity of rat monoclonal anti HA-Tag 3F10-IgG (MAK-3F10-IgG, Roche Biochemicals), recombinant proteins can be detected in crude yeast extracts without any further purification. HA-Tag labelled recombinant protein bind to biotinylated MAK<HA>Ra-3F10-IgG-Bi coupled to the solid phase via a streptavidine bridge and peroxydase labelled MAK<HA>Ra-3F10-IgG-POD. Of the subcultures with the highest HBsAg yield, cultivation in 10 l fermenters was carried out. Also tested were two serum samples from chronic HBV carriers with 2 (mutant 122-RA), respectively, 3 (mutant 123-RGA) amino acid insertions [Yamamoto et al., 1994; Hou et al., 1994].

For the assessment of the influence of genetic variability (HBV subtypes) on HBsAg detection, HBsAg performance panel (Roche Diagnostics, Penzberg, Germany), consisting of dilution series of serum samples from patients infected with different subtypes (*adw2*, *ayw4*, *ayw2*, *ayw3*, *adr*, *adr*/ayr) in HBV negative serum, and one sample with low HBsAg concentration (0.05 IU/ml), were tested with different assays.

Analytical Specificity

The specificity was assessed by testing potentially interfering sera (rheumatoid factor positive (>500 U/ml) and serum samples spiked with bilirubin (0.4 mg/ml and haemoglobin (15 mg/ml).

Isolated anti-HBc Positive Individuals

A total of 104 anti-HBc only positive individuals were selected for the present evaluation. Inclusion criteria in the study were a negative HBsAg (AxSYM HBsAg), anti-HBs (AxSYM Ausab), and a positive anti-HBc result by AxSYM Core (inhibition value >90%) and availability of anti-HIV and anti-HCV results from routine serological testing at the Institute of Medical Virology, University of Frankfurt. The mean age of the anti-HBc only positive individuals was 47.3 years (range 25–83 years). Of the anti-HBc only reactive individuals, 13 were suffering from terminal renal failure undergoing nephrodialysis or organ transplantation, 14 were intravenous drug addicts, and 34 were treated or under follow-up at the gastroenterology outpatient clinic for chronic hepatitis C virus infection. A total of 16 patients were undergoing cardiothoracic surgery, 6 HIV-infected individuals were treated at the infectious diseases outpatient clinic. Two anti-HBc only positive individuals were haemophiliacs. The remaining 19 patients were treated at the different institutions of the University of Frankfurt, including clinics for gynaecology, cardiology, and neurology. Median values for ALT, AST and γGT were 14 U/l (range 3–365 U/l), 5 U/l (range 1–385 U/l), and 18.5 U/l (range 4–347 U/l), respectively. Elevated ALT, AST, and γGT values were observed in 15 (14.4%), 5 (4.8%), and 37 (35.6%) of the patients, respectively.

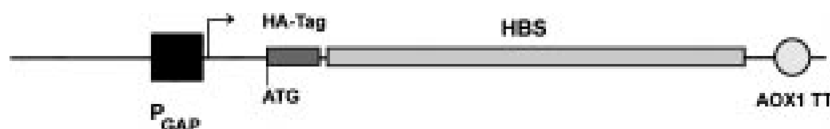

Fig. 2. Schematic organisation of constructs pGAPZ-145 and -146. Hemagglutinin -Tag of 9 aa is inserted after HBs-ATG. Transcription is terminated at AOX1 TT of yeast alcohol-oxidase1-gene.

Seven individuals were HIV-1 antibody positive. HCV antibodies were present in 68 patients. HCV and HIV co-infection was observed in 4 patients.

Anti-HBc and Anti-HBs Detection

Antibody to hepatitis B virus core was determined by using two automated competitive assays, AxSYM Core (Abbott, Delkenheim, Germany) and Elecsys Anti-HBc (Roche Diagnostics, Penzberg, Germany). Both tests include a pretreatment step with a reducing agent dithiothreite (DTT) in order to eliminate unspecific IgA or IgM-related molecules. Quantitative anti-HBs determination was carried out by the AxSYM Ausab and Elecsys Anti-HBs.

Elecsys HBsAg

Elecsys HBsAg is a one-step sandwich assay for the qualitative detection of HBsAg in human serum or plasma with a total incubation time of 18 minutes. All the reaction steps of Elecsys HBsAg are carried out automatically by the Elecsys 2010 system. The assay can be performed directly from the primary patient blood collection tube and random access is possible.

Results are calculated by the Elecsys software by comparing the chemiluminescence signal obtained from the sample with the cut-off value previously determined by HBsAg calibration. Samples with a signal/cut-off (s/co) ≥ 1 are considered as positive and have to be repeated in double determination. If the s/co is <1, the sample is negative. The detection limit is 0.014 PEIU/ml and 0.017 PEIU/ml for Paul Ehrlich Institute (PEI, Langen, Germany) standards *ad* and *ay*, respectively [Weber et al., 1999].

Murex HBsAg Version 3

Murex HBsAg Version 3 (Abbott-Murex, Dartford, England) is a microtitre-based two-step sandwich assay for the qualitative detection of HBsAg in human serum or plasma. The sample is preincubated in microwells coated with a mixture of mouse monoclonals specific for different epitopes of the *a* determinant and mutant forms of HBsAg. Affinity purified goat antibody to HBsAg conjugated to horseradish peroxidase is added to the sample in the well. After washing, a solution containing 3,3′,5,5′-tetramethylbenzidine (TMB) and hydrogen peroxyde is added. The cut-off value is calculated by adding 0.05 OD to the mean of the negative control replicates. Samples giving an absorbance equal or greater than the cut-off value are considered initially reactive and should be retested in duplicate. Samples that are repeatedly reactive are presumed to contain HBsAg and should be confirmed using the Murex HBsAg Confirmatory kit.

Enzygnost HBsAg 5.0

Enzygnost HBsAg 5.0 (Dade Behring, Marburg, Germany) is a microtitre-based two-step sandwich assay for the qualitative detection of HBsAg in human serum or plasma. In the first step, the HBsAg contained in the sample reacts simultaneously with the polyclonal anti-HBs antibodies attached to the wells of the microtitre plate and with mouse monoclonal anti-HBs antibody, which is biotin conjugated (conjugate 1). In the second step, after removing the unbound reactants, peroxidase-labelled streptavidin (conjugate 2) is added. After removing unbound reactants, the enzyme activity of the bound conjugate 2 is determined by the addition of TMB chromogen. The cut-off value is calculated by adding 0.05 OD to the mean of the negative control replicates. Samples giving an absorbance equal to or greater than the cut-off value are considered initially reactive and should be retested in duplicate.

Immune Complex Dissociation (ICD)

Immune complex dissociation was performed according to the protocol published by Rabenau et al. [1996]. Briefly, 100 μl of sample were mixed with 50 μl of 0.5 N HCl and incubated for 1 hour at 37°C. Afterwards, 50 μl of 0.5 N NaOH was added and samples were tested for the presence of HBsAg with the Elecsys HBsAg assay.

Amplicor HBV Monitor Test

Amplicor HBV Monitor Test (Roche Diagnostic Systems, Branchburg, NJ) is a quantitative PCR kit for the amplification of HBV DNA in clinical specimens. The amplification target is defined by two primers HBV-104UB and HBV-104D. These primers amplify a highly conserved 104 base pair sequence in the pre-Core/core region of the HBV genome. DNA is amplified using one biotinylated and one non-biotinylated oligonucleotide primer. Amplification of HBV target and internal standard (IS) occur simultaneously. The quantification of HBV DNA is performed by using an external standard curve generated with every run. Six HBV standards (0–1,000,000 copy standard) are included in each run. The detection limit of HBV Monitor test is 400 copies HBV DNA/ml. The PCR assay quantitates virus titres from 400 to 4×10^7 HBV DNA copies/ml of serum or plasma.

HIV and HCV Serology

HIV and HCV antibody detection were performed by using AxSYM HIV-1/HIV-2 and AxSYM HCV, respectively. All tests were performed and interpreted in accordance with the manufacturers' recommendations.

RESULTS

Sensitivity and Specificity of HBsAg Screening Assays

Murex HBsAg Version 3 assay showed the highest sensitivity for the detection of recombinant mutants F134Y/G145R and P142S/G145R/N146D. Both mutants were detected undiluted by the Enzygnost HBsAg 5.0 (Table I). Elecsys HBsAg detected only mutant F134Y/G145R in undiluted crude yeast extract.

TABLE I. Results Obtained With HBsAg Screening Assays by Testing HBV Surface Gene mutants, Different Subtypes, and Potentially Interfering Sera

	Highest reciprocal dilution with a positive result (dilution series)/ index (signal/cut-off value) potentially interfering samples			
	Murex HBsAg Version 3	Enzygnost HBsAg 5.0	Elecsys ® HBsAg	AxSYM HBsAg
HBV surface gene mutants				
F134Y/G145R	1,000	1[a]	1[a]	n.d.
P142S/G145R/ N146D	1,000	1[a]	Not detected	n.d.
122-RA	100	1,000	1,000	n.d.
123-RGA	10,000	10,000	1,000	n.d.
HBV subtypes				
adw2	100,000	100,000	400,000	400,000
ayw4	6,400	6,400	440,000	440,000
ayw2	200,000	200,000	1,000,000	1,000,000
ayw3	128,000	32,000	128,000	128,000
adr	6,000	30,000	30,000	30,000
adr / ayr	4,000	4,000	32,000	32,000
adr / ayr	32,000	4,000	32,000	32,000
Potentially interfering sera spiked with (results are expressed as index signal/cutoff-value)				
Bilirubin 0.4 mg/ml	2.24	0.48	0.58	0.97
Rheumatoid factor (>500 U/ml)	0.62	0.637	0.39	9.46
Hemoglobin (15 mg/ml)	1.46	0.706	0.50	1.02

[a]Positive result with undiluted serum.

Insertion mutants (human serum) were detected by all the three assays with a variable sensitivity (Table I).

Elecsys HBsAg and AxSYM HBsAg showed a comparable sensitivity for the detection of HBV subtypes (Table I). In contrast, Murex HBsAg Version 3 and Enzygnost HBsAg 5.0 were one dilution step less sensitive than the comparative assays for different subtypes. False-positive results were observed for potentially interfering samples by the Murex HBsAg and AxSYM HBsAg (Table I).

Results of Anti-HBc and Anti-HBs Testing in Anti-HBc Only Positive Samples

Five AxSYM Core anti-HBc positive samples were negative by the Elecsys Anti-HBc (Fig. 3). One of these samples which was anti-HBs negative by the AxSYM anti-HBs was positive for antibody against HBV surface antigen by the Elecsys Anti-HBs (100 IU/ml). Overall 15 (14.4%) AxSYM anti-HBs negative samples gave positive results with Elecsys Anti-HBs. Anti-HBs concentrations ranged between 11 and 100 IU/ml (median value: 21 IU/ml).

Results of Murex HBsAg Version 3, Elecsys HBsAg, Immune Complex Dissociation and Viral Load Determination

No low-level HBsAg carrier was detected among the isolated anti-HBc reactive individuals by the Elecsys HBsAg (Fig. 3). There was no evidence for the presence of immune complexes (Fig. 3). Only one sample was repeatedly reactive with the Murex HBsAg, suggesting that a mutant form of HBsAg was responsible for the anti-HBc only reactivity (Fig. 1). However, HBV DNA could not be amplified with Amplicor HBV Monitor and a nested "in house" PCR (Professor R. Tedder, London) so that a further characterisation of that sample was not possible. The result of neutralisation assay was not interpretable. Fifteen (14.4%) anti-HBc only positive individuals were HBV DNA carriers (Fig. 1) with concentrations ranging from 800 to more than >4,000,000 copies of viral DNA/ml (median value 3,200 copies/ml). HBV DNA was present at low copy number in three anti-HBs positive individuals. Anti-HBs titres ranged from 25 to 100 IU/ml. All three patients were immunosuppressed (two anti-HIV positive individuals and one renal transplant recipient).

DISCUSSION

In the first part of this study, different HBsAg screening assays were tested for their ability to detect HBsAg variants and different HBV subtypes. Modification of commercial assays is necessary to increase the sensitivity of detection of S gene variants. Polyclonal-antibody-based assays do not guarantee full sensitivity [Waters et al., 1992]. By using monoclonal antibodies directed against different S gene mutants in the Murex HBsAg Version 3 assay, it was possible to achieve a higher sensitivity in dilution series of recombinant expressed HBsAg variants than with the alternative assays (Table I). Screening assays, such as the Elecsys HBsAg which uses monoclonal antibodies obtained by the immunisation with wild-type HBsAg showed in comparison to Murex HBsAg Version 3 a reduced sensitivity for escape mutant detection. No material

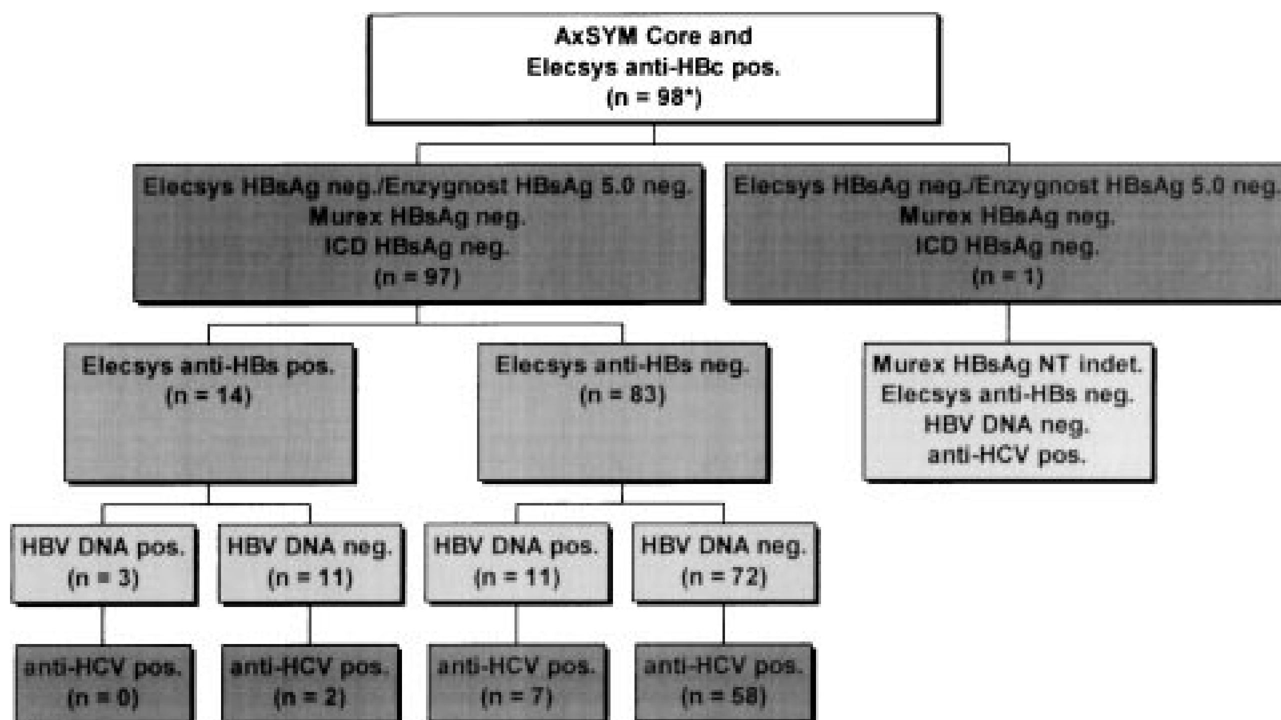

Fig. 3. Results of anti-HBc, HBsAg, anti-HBs detection, and viral load determination in isolated anti-HBc positive individuals. *Six Elecsys Anti-HBc negative/Abbott Core samples were deleted from analysis.

was available to compare detection of recombinant and native HBsAg. On the other hand, the best performance for HBsAg subtype detection was obtained by the Elecsys HBsAg. Enzygnost HBsAg and Elecsys HBsAg showed a higher analytical specificity than the Murex HBsAg Version 3.

Among 104 anti-HBc positive individuals, only one sample was repeatedly positive by the Murex HBsAg Version 3 assay, which detects different HBsAg mutants. Since the assay is based on a mixture of monoclonal antibodies raised against a limited number of mutants it probably cannot detect the whole repertoire of possible HBsAg mutants. A further explanation for the lack of HBsAg is that there are variants in other parts of the genome that down-regulate the production of antigens [Feitelson et al., 1995; Fukuda et al., 1995].

Although anti-HBc assays with reducing agents are specific, the validity of a result can be enhanced by retesting the initially reactive sample and by distinguishing between strong- and weak-positive samples. The latter are frequently nonspecific and give mostly a negative result by an alternative assay [Weber et al., 1998]. For these reasons, only samples that present inhibition values $> 90\%$ by the AxSYM Core assay were included in the present study. However, 5 (4.8%) AxSYM Core positive samples gave negative results with the Elecsys Anti-HBc. One of the five discrepant samples was anti-HBs (100 IU/ml) and HBV DNA (3,200 copies/ml) positive, suggesting that the AxSYM Core result was true positive. For the other four samples a definitive classification as anti-HBc positive or negative was not possible.

HBsAg circulating immune complexes are a common feature of chronic hepatitis B and correlate with HBV replication [Tsai et al., 1998]. Joller-Jemelka et al. [1994] demonstrated that complexed HBsAg was present in more than 30% of anti-HBc only positive sera, highest rates were observed in individuals with hepatopathies (up to 80%), in i.v. drug users (up to 63%), and in haemodialysis patients (39%). HBV DNA was detected by nested PCR in 39% of the patients. The absence of immune complexes in our sample may be attributed to different test methods. While in most of the previous studies [Ackerman et al., 1994, Tsai et al., 1998] immune complexes were removed by passage through C1q affinity column, we used ICD by acid pretreatment of serum samples. This technique proved to dissociate immune complexes even in the presence of a large excess of anti-HBs [Rabenau et al., 1996]. Another possible explanation is the fact that a relatively low percentage of selected samples (14.4%) were viraemic.

Similar to HBsAg tests, anti-HBs assays detect a variety of antibodies to HBsAg, including antibodies to the *a* and subtype specific determinants of HBsAg. Comparative studies using anti-HBs tests from different manufacturers clearly indicated that anti-HBs concentrations differ considerably from assay to assay [Hess et al., 1997]. In unselected blood donors, 3.4% showed discrepant results among three anti-HBs tests. One test gave a positive result up to 400 IU/ml, while

two other tests scored the same sample negative. In our sample of selected anti-HBc positive individuals, 14.4% showed discrepant results between two anti-HBs assays.

Three anti-HBc and anti-HBs positive individuals were HBV DNA carriers. In HIV infected patients who have recovered completely from an acute hepatitis B infection and became anti-HBs positive, hepatitis B infection may be reactivated after progression to AIDS [Altfeld et al., 1998].

The Elecsys HBsAg was used in the present study in order to test the hypothesis that a highly sensitive HBsAg assay may detect low-level HBsAg in HBV carriers. However, none of the anti-HBc only reactive samples that were negative by the AxSYM HBsAg was positive (Fig. 1). Of 2,000 antenatal clinic attendees in Papua New Guinea, 5% of HBsAg positive subjects were negative by a widely used monoclonal assay but PCR positive. The monoclonal assay had a sensitivity of 0.5–1 ng/ml. These samples were reactive by another assay with a sensitivity of 0.1 ng/ml. Over 50% of these discordant samples had rare or unique variants of the major hydrophilic region of HBsAg [Carman et al., 1997]. These data also demonstrate that the sensitivity of current screening HBsAg assays should be increased arguing for implementation of detection of HBV DNA in blood donors. By using the Amplicor HBV Monitor with a detection limit of 400 copies/ml, viral DNA was detected in 14.4% of anti-HBc only positive individuals. Other investigators who used nested PCR with a detection limit of less than 50 genome equivalents per ml observed prevalences of HBV DNA up to 40% in anti-HBc only reactive individuals [Jilg et al., 1995]. These individuals are mostly chronic HBV carriers and show very low virus concentrations in their sera but nevertheless can transmit the infection [Hoofnagle et al., 1978; Rakela et al., 1980; Dodson et al., 1997]. In the clinical diagnostic laboratory, individuals with active infection may be detected by using highly sensitive anti-HBc-IgM assays [Brunetto et al., 1993, Weber and Doerr, 1996].

As expected, a high HCV seroprevalence was observed in our sample of anti-HBc only positive individuals since most of them were from high risk groups, i.e., haemodialysis patients, organ transplant recipients, intravenous drug addicts, HIV- infected, and one third of the patients were treated or under follow-up at the gastroenterology outpatient clinic for chronic hepatitis C virus infection. HCV suppresses the replication of HBV and also suppresses substantially the expression of HBV surface proteins in vitro and in vivo [Shih et al., 1993; Chu et al., 1998]. Concurrent HCV infection can enhance the termination of the HBsAg carrier state in chronic HBsAg carriers [Sheen et al., 1994].

In conclusion, the most probable explanations for isolated anti-HBc reactivity in our study patients are a possible interference of HBsAg synthesis by HCV infection (65.4%) and variability of anti-HBs assays (14.4%). Isolated Anti-HBc is observed in resolved HBV infection without detectable anti-HBs. An anamnestic response (anti-HBs $\geq$20 mIU/ml) to vaccination may serve to identify these individuals [Hollinger, 1996; Silver et al., 1998]. There is no evidence for the presence of low-level HBsAg carriers and immune complexes. However, HBsAg mutants cannot be excluded definitively by the test strategy used in the present evaluation, since rare or unique variants may not be necessarily detected. Weinberger et al. [1997] observed that the exchange rate per amino acid in the *a* determinant in isolates from anti-HBc only positive individuals was significantly higher than in the residual parts of the molecule and even higher than in the same region of the HBsAg positive controls. On the other hand, sequences were found from solely anti-HBc positive individuals, which were considered previously to stem from "normal" HBsAg positive HBV carriers. Besides, no single position seems to be predominantly mutated. Cacciola et al. [1999] did not observe changes in sequenced HBV genomes of HBsAg negative infections that are known to interfere with viral activity and gene expression. It is possible that structural alterations of HBsAg prevent the recognition by serological assays in anti-HBc alone positive individuals. Therefore, the immunological properties of recombinant antigens should be examined.

ACKNOWLEDGMENTS

We are very grateful to Professor Howard Thomas, London, for providing us HBsAg mutants and to Prof. Tedder for performing PCR in the Murex HBsAg Version 3 reactive sample.

REFERENCES

Ackerman Z, Wands JR, Gazitt Y, Brechot C, Kew MC, Shouval D. 1994. Enhancement of HBsAg detection in serum of patients with chronic liver disease following removal of circulating immune complexes. J Hepatol 3:398–404.

Altfeld M, Rockstroh JK, Addo M, Kupfer B, Pult I, Will H, Spengler U. 1998. Reactivation of hepatitis B in a long-term anti-HBs positive patient with AIDS following lamivudine withdrawal. J Hepatol 29:306–309.

Brunetto MR, Cerenzia MT, Oliveri F, Piantino P, Randone A, Calvo PL, Rocca G, Galli C, Bonino F. 1993. Monitoring the natural course and response to therapy of chronic hepatitis B with a semi-quantitative assay for IgM anti-HBc. J Hepatol 19:431–436.

Cacciola I, Pollicino T, Squadrito G, Cerenzia G, Orlando ME, Raimondo G. 1999. Occult hepatitis B virus infection in patients with chronic hepatitis C liver disease. N Engl J Med 341:22–26.

Carman WF, Trautwein C, van Deursen FJ, Colman K, Dornan E, McIntyre G, Waters J, Kleim V, Muller R, Thomas HC, Manns MP. 1996. Hepatitis B virus envelope variation after transplantation with and without hepatitis B immune globulin prophylaxis. Hepatology 24: 489–493.

Carman WF, Mimms LT. 1997. Pre-S/S gene variants of hepatitis B virus. In: Rizetto M, Purcell RH, Gerin JL, Verne G, editors. Viral hepatitis and liver disease. Torino: Edizioni Minerva Medica. p 108–115.

Carman WF, Van Deursen FJ, Mimms LT, Hardie D, Coppola R, Decker R, Sanders R. 1997. The prevalence of surface antigen variants of hepatitis B virus in Papua New Guinea, South Africa, and Sardinia. Hepatology 26:1658–1666.

Caspary G, Beyer HJ, Elbert G, Koerner K, Muss P, Schunter FW, Uy A, Gerlich W, Thomssen R, Schmitt H. 1989. Unsatisfactory specificities and sensitivities of six enzyme immunoassays for antibodies to hepatitis B core antigen. J Clin Microbiol 27:2067–2072.

Chu CM, Yeh CT, Liaw YF. 1998. Low-level viremia and intracellular expression of hepatitis B surface antigen (HBsAg) in HBsAg carriers with concurrent hepatitis C infection. J Clin Microbiol 36:2084–2086.

Dodson SF, Issa S, Araya V, Gayowski T, Pinna A, Eghtesad B, Iwatsuki S, Montalvo E, Rakela J, Fung JJ. 1997. Infectivity of hepatic allografts with antibodies to hepatitis B virus. Transplantation 64:1582–1584.

Feitelson MA, Duan LX, Guo J, Horiike N, McIntyre G, Blumberg BS, Thomas HC, Carman W. 1995. Precore and X region mutants in hepatitis B virus infections among renal dialysis patients. J Viral Hepatitis 2:19–31.

Fukuda R, Xuan-Tanh N, Ishimura N, Ishihara S, Chowdhury A, Kohge N, Akagi S, et al. 1995. X gene and precore region mutations in the hepatitis B virus genome in persons positive for antibody to hepatitis B e antigen: comparison between asymptomatic "healthy" carriers and patients with severe chronic active hepatitis. J Infect Dis 172:1197–1197.

Gross A, Joller-Jemelka HI, Wick AN, Grob PJ. 1993. Der Hepatitis-serologische Befund "Anti-HBc allein", zirkulierende virale DNS und Befund-Interpretation. Schweizer Medizinische Wochenschrift 123:1193–1202.

Hadler SC, Murphy BL, Schable CA, Heyward WL, Francis DP, Kane MA. 1984. Epidemiological analysis of the significance of low-positive test results for antibody to hepatitis B surface and core antigens. J Clin Microbiol 19:521–525.

Hess G, Karayiannis P, Babiel R, Thomas HC. 1997. Variants of the hepatitis B virus: a diagnostic and vaccine challenge. In: Rizetto M, Purcell RH, Gerin JL, Verne G, editors. Viral hepatitis and liver disease. Torino: Edizioni Minerva Medica. p 974–976.

Hollinger FB. 1996. Hepatitis B virus. In: Fields BN, Knipe DM, Howley PM, editors. Virology, 3rd edition. Philadelphia-New York: Lippincott-Raven. p 2739–2807.

Hoofnagle JH, Seeff LB, Bales ZB, Zimmermann HJ. 1978. Type B hepatitis after transfusion with blood containing antibody to hepatitis B core antigen. N Engl J Med 298:1379–1383.

Hou J, Karayiannis P, Waters J, Luo K, Liang C, Thomas HC. 1995. A unique insertion in the S gene surface antigen-negative hepatitis B immune globulin prophylaxis. Hepatology 21:273–278.

Jilg W, Sieger E, Zachoval R, Schätzl H. 1995. Individuals with antibodies against hepatitis B core antigen as the only serological marker for hepatitis B infection: high percentage of carriers of hepatitis B and C virus. J Hepatol 23:14–20.

Joller-Jemelka HI, Wicki AN, Grob PJ. 1994. Detection of HBs antigen in "anti-HBc alone" positive sera. J Hepatol 21:269–272.

Kessler HA, Harris AA, Payne JA, Hudson E, Potkin B, Levin S. 1985. Antibodies to hepatitis B surface antigen as the sole hepatitis B marker in hospital personnel. Ann Intern Med 103: 21–26.

Lok ASF, Lai CL, Wu PC. 1988. Prevalence of isolated antibody to hepatitis B in an area endemic for hepatitis B. Hepatology 8:766–770.

McMahon G, Ehrlich PH, Moustafa ZA, McCarthy LA, Dottavio D, Tolpin MD, Nadler PI, Ostberg L. 1992. Genetic alterations in gene encoding the major HBsAg DNA and immunological analysis of recurrent HBsAg derived from monoclonal antibody-treated liver transplant patients. Hepatology 15:757–766.

Melegari M, Bruno S, Wands JR. 1994. Properties of hepatitis B virus pre-S1 deletion mutants. Virology 199:292–300.

Rabenau H, Schütz R, Berger A, Doerr HW, Weber B. 1996. How accurate is serologic testing of plasma pools for hepatitis B virus surface antigen, anti-human immunodeficiency virus 1 and 2, and anti-Hepatitis C virus? Infusionstherapie Transfusionsmedizin 23:124–130.

Rakela J, Mosley JW, Aach RD, Gitnick GL, Hollinger FB, Stevens CE, Szmuness W. 1980. Viral hepatitis after transfusion with blood containing antibody to hepatitis B core antigen. Gastroenterology 78:1318.

Robertson EF, Weare JA, Randell R, Holland PV, Madsen G, Decker RH. 1991. Characterisation of a reduction-sensitive factor from human plasma responsible for false activity in competitive assays for antibody to hepatitis B core antigen. J Clin Microbiol 29: 605–610.

Sällberg M, Magnius LO. 1989. Enzyme immunoassay for anti-hepatitis core (HBc) immunoglobulin G1 and significance of low-level results in competitive assays for anti-HBc. J Clin Microbiol 27:849–853.

Sanchez-Quijano A, Jauregui JI, Leal M, Pineda JA, Castilla A, Abad MA, Civeira MP, Garcia de Pesquera F, Prieto J, Lissen E. 1993. Hepatitis B virus occult infection in subjects with persistent isolated anti-HBc reactivity. J Hepatol 17:288–293.

Scheitlin TH, Grob PJ. 1992. Infektionen mit den Hepatitisviren HAV, HBV und HCV sowie mit dem AIDS Virus HIV bei Drogenabhängigen der Cassenszene Zürichs: eine Prävalenzstudie. Schweizer Medizinische Wochenschrift 120:621–629.

Schifan RB, Shirley MD, Rivers SL, Sampliner RE, Krammes JE. 1993. Significance of isolated hepatitis B core antibody in blood donors. Arch Intern Med 153:2261–2266.

Seddigh-Tonekaboni S, Waters JA, Jeffers S, Gehrke R, Ofenloch B, Horsch A, Hess G, Thomas HC, Karayiannis P. 2000. Effect of variation in the common, "a" determinant on the antigenicity of hepatitis B surface antigen. J Med Virol 60:113–121.

Sherertz RJ, Spindel E, Hoofnagle JH. 1983. Antibody to hepatitis B surface antigen may not always indicate immunity to hepatitis B virus infection. N Engl J Med 309:1519.

Schmidt PJ, Leparc GF, Samla CT. 1988. Comparison of assays for anti-HBc in blood donors. Transfusion 28:389–391.

Sheen IS, Liaw YF, Chu CM, Pao CC. 1992. Role of hepatitis C virus infection in spontaneous hepatitis B surface antigen clearance during chronic hepatitis B virus infection. J Infect Dis 165:831–834.

Sheen IS, Liawe YF, Lin DY, Chu CM. 1994. Role of hepatitis C and delta viruses in the termination of chronic hepatitis B surface antigen carrier state: a multivariate analysis in a longitudinal follow-up study. J Infect Dis 170:358–361.

Shih CM, Lo SJ, Miyamura T, Chen SY, Wu LYH. 1993. Suppression of hepatitis B virus expression and replication by hepatitis C virus core protein in HuH-7 cells. J Virol 67:5823–5832.

Silva AE, McMahon BJ, Parkinson AJ, Sjogren MH, Hoofnagle JH, Bisceglie AM. 1998. Hepatitis B virus DNA in persons with isolated antibody to hepatitis B core antigen who subsequently received hepatitis B vaccine. Clin Infect Dis 26:895–897.

Tsai JF, Margolis HS, Jeng JE, Ho MS, Chang WY, Hsieh MY, Lin ZY, Tsai JH. 1998. Immunoglobulin- and hepatitis B surface antigen-specific circulating immune complexes in chronic hepatitis B virus infection. Clin Immunol Immunopathol 86:246–251.

Waters J, Kennedy M, Voet P, Hauser P, Petre J, Carman W, Thomas HC. 1992. Loss of the common "a" determinant of hepatitis B surface antigen by a vaccine induced escape mutant. J Clin Invest 90:2543–2547.

Weare JA, Robertson EF, Madsen G, Hu R, Decker RH. 1991. Improvement in the specificity of assays for the detection of antibody to hepatitis B core antigen. J Clin Microbiol 29:600–604.

Weber B, Doerr HW. 1996. Bedeutung von anti-HBc-IgM für die Diagnose und Verlaufskontrolle der Hepatitis B: aktuelle Entwicklungen. Laboratoriumsmedizin 20:390–394.

Weber B, Michl U, Mühlbacher A, Paggi G, Bossi V. 1998. Evaluation of the new automated Enzymun-Test® Anti-HBc Plus for the detection of hepatitis B virus (HBV) core antibody. Intervirology 41:17–23.

Weber B, Bayer A, Kirch P, Schlüter V, Schlieper D, Melchior W. 1999. Improved detection of Hepatitis B surface antigen (HBsAg) with a new rapid automated assay. J Clin Microbiol 37:2639–2647.

Weinberger KM, Kreuzpaintner EA, Hottenträger B, Neifer S, Jilg W. 1997. Mutations in the S-gene of hepatitis B virus isolates from chronic carriers with anti-HBc as the only serological marker of HBV infection. In: Rizetto M, Purcell RH, Gerin JL, Verne G, editors. Viral hepatitis and liver disease. Torino: Edizioni Minerva Medica. p 138–143.

Yamamoto K, Horikita M, Tsuda F, Itoh K, Akahane Y, Yotsumoto S, Okamoto H, Miyakawa Y, Mayumi M. 1994. Naturally occuring escape mutants of hepatitis B virus with various mutations in the S gene in carriers seropositive for antibody to hepatitis B surface antigen. J Virol 68:2671–2676.

Zuckerman AJ. 2000. Effect of hepatitis B virus mutants on efficacy of vaccination. Lancet 355:1382–1383.