Article

The Study of IgG Subclass Profiles of Anti-HBc in Populations with Different Status of HBV Infection

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To study IgG subclasses for the hepatitis B virus (HBV) core antigen (anti-HBc) in different populations, a comparison was made between 104 chronic carriers (60 male and 44 female) and 434 recovered individuals (247 male and 192 female). Biochemistry analyses of AST (aspartate aminotransferase) and ALT (alanine aminotransferase) were also performed. Among the 104 chronic carriers, 21 patients were found to be ALT and AST abnormal (> 25 IU/ml). After comparing these ALT and AST abnormal patients with other ALT and AST normal chronic carriers, no statistical difference was observed in the OD values of the anti-HBe ($p > 0.05$). The ELISA results showed the anti-HBc IgG subclass pattern was IgG1 > IgG3 > IgG4 in chronic carriers and IgG3 > IgG1 > IgG4 in recovered individuals ($p < 0.05$). This result suggests the IgG1/IgG3 ratio may be related with HBV status. However, in spite of the different anti-HBc IgG1/IgG3 patterns demonstrated in different populations, both anti-HBc IgG1 and IgG3 concentrations were significantly higher in chronic carriers ($p < 0.05$). Therefore, both the anti-HBc IgG1/IgG3 ratio and their amounts differed. They may play a significant role in chronic carriers and recovered individuals. The anti-HBc IgG subclass profiles of chronic carriers were not changed regardless of liver inflammation, and were independent of sex and age. *Cellular & Molecular Immunology*. 2005;2(5):393-398.

Key Words: anti-HBs, anti-HBe, anti-HBc, ELISA, HBV

Introduction

The hepatitis B virus (HBV) causes a wide spectrum of symptoms, ranging from acute hepatitis to liver cirrhosis. The genome of the hepatitis B virus contains four open reading frames encoding the surface antigen (HBsAg), core antigen (HBcAg), e antigen (HBeAg), DNA polymerase, and protein X. HBsAg, HBeAg, and anti-HBc antibody can be detected in individuals infected with HBV for more than six months. In cured patients, both HBsAg and HBeAg were cleared after anti-HBs and anti-HBe antibodies were produced. Patients who fail to mount a vigorous immune response to acute HBV infection develop chronic infection, and both surface and e antigens as well as the anti-HBc antibody can be detected in these individuals (1). In addition to the direct elimination performed by killer T cells, the cell-mediated immune response also needs assistance from helper T (Th) cells, which work in two ways. First, the Th1 cells stimulate macrophages, which then clear virus particles. Second, the Th2 cells stimulate B cells to generate immunoglobulins, which adhere to the surface of virus particles and induce opsonization. In other words, immunoglobulins act through the humoral immune response to inhibit viral reproduction (2). Differences in secreted immunoglobulins, manifested in classes, subclasses, and subclass patterns, may be the result of the conformational binding of different antigenic structures to MHC class I or class II molecules (3). However,

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the specific role that each Ig plays in the clearance of HBV remains unclear.

It was previously documented that the elimination of HBV particles had a close relationship with immune response. The different protein compositions of the hepatitis B virus can evoke different immune responses, inducing the generation of different IgG subclass patterns. The three structural forms of the viral proteins, the HBsAg, the particulate HBcAg, and the nonparticulate HBeAg, may preferentially elicit different Th cell subsets. The different specific IgG subclasses against these antigens reflect the difference between stimulating antigens, immune response, and the stages of viral disease.

According to previous reports, the anti-HBs immuno-globulin for vaccinees immunized with cDNA HBsAg consisted mainly of IgG1 and IgG2 (4), while that of individuals vaccinated with plasma-purified HBsAg consisted of IgG1 (5). In individuals naturally infected, the anti-HBs IgG consisted mainly of IgG3 and IgG1 (6). Our study also suggested that anti-HBs subclass IgG1 was predominant in cured patients, chronic carriers and vaccines. The samples from both chronic carriers and vaccinees exhibited a significantly higher concentration of total IgG and IgG1 than samples in recovered individuals ($p < 0.05$) (7). Evaluation of the differential effects of vaccines demonstrated that the resulting anti-HBs immunoglobulin consisted predominantly of IgG1 and IgG4 (8), for the vaccines produced by Recombivax H-B, Merck Sharp, and Dohme. The anti-HBc immunoglobulin consisted mainly of IgG1 in carriers; whereas in patients with liver cirrhosis the IgG subclass titers were IgG3 > IgG1 (9, 10). Our study also showed that the anti-HBc IgG subclass pattern was IgG1 > IgG3 > IgG4 in hepatitis chronic carriers, while in HBV cured individuals, it was IgG3 > IgG1 > IgG4 (11). The pattern for chronic carriers was in accordance with the data reported by Sallberg et al. (12).

The aim of this study was to measure and evaluate the anti-HBc IgG subclass distribution patterns between HBV chronic carriers and recovered individuals, and to compare the immunoglobulin production in chronic hepatitis patients with or without ALT and AST abnormality. In the present study, samples from different groups of patients were used to evaluate the anti-HBc IgG patterns, reflecting the immune status of a HBV-infected person from acute infection to recovery (i.e., ALT and AST normal) or chronic infection (i.e. ALT and AST abnormal). This evaluation would be helpful in understanding the immune modulating mechanism for HBV infection. The study of IgG-subclass patterns promotes a deeper understanding of the relationship between these patterns and immunity in virus clearance.

Materials and Methods

Serum samples
This study was approved by the institutional review board for medical ethics and human investigation at Chung Shan Medical University. Blood samples were obtained from outpatients attending Chung Shan Medical University Hospital. All the patients' gender, age, and past history of

medication information were recorded for this study. Both positive and negative control sera were also included in this study. The results of serum markers for HBV, including HBsAg, anti-HBs, anti-HBc, HBeAg, and anti-HBe, were detected using enzyme immunoassay (AXSYM system, Abbott Laboratories, Abbott Park, IL, USA) and radio immunoassay (Dainabot Co. Ltd., Japan) and recorded. Sera were separated from the blood samples by centrifugation at $3,500 \times g$ at $25^{o}C$ for 15 min, then aliquoted and stored at $-70^{o}C$ for future use.

According to the results generated from HBV serum markers, 104 (60 male and 44 female) samples were defined as HBV chronic carriers (testing HBsAg, anti-HBc, and anti-HBe positive), and 439 (247 male and 192 female) samples were classified as recovered individuals (testing both HBsAg and HBeAg negative; anti-HBs, anti-HBc, and anti-HBe all positive). Of the 543 samples, 14 samples were randomly chosen and divided into two groups for evaluation by the ELISA plates used in this study. Subjects 1-8 were defined as HBV chronic carriers and constituted group A. Group B (subjects 9-14) consisted of individuals who had been infected but were clear of surface antigen at the time of our study. Positive and negative controls were commercially provided by General Biological (Taiwan). Biochemistry analysis of AST (aspartate aminotransferase) and ALT (alanine aminotransferase) were also routinely performed by an automatic analyzer (ARCO, Biotechnica Instruments, Italy).

ELISA plates
As with our previous study (11), the ELISA plates used for detecting the anti-HBc IgG subclass were also purchased from GB. The blocking phosphate buffer containing 1% bovine serum albumin (100 μl) was added to each well of the plates. The plates were incubated at $37^{o}C$ for 30 min, and were then washed again in the same manner. After the last washing, the plates were stored at $-20^{o}C$ until their later use. The plates were defrosted 30 min before testing. The antigens used for these commercial plates were derived from HBV core gene recombinants.

Enzyme-linked immunosorbent assay (ELISA)
In this study, 1:100 diluted sera and 0.1 μg antigen per well were used (11). Test sera were diluted with an ELISA buffer diluent, made from 0.05% Tween-20 and 1% bovine serum albumin in a phosphate buffer. Aliquots of 100 μl of diluted sera or positive/negative controls were added to the 96-well plates and incubated at $37^{o}C$ for 180 min. After incubation, the plates were washed 3 times with washing solution (consisting of 0.05% Tween-20 in normal saline). Then 100 μl diluted mouse anti-human monoclonal antibodies, purchased from Bionostics, Inc. (Devens, MA, USA), against IgG1 (Clone NL16), IgG2 (Clone GOM2), IgG3 (Clone ZG4), IgG4 (Clone RJ4), and total IgG subclasses (Clone MK1A6), were diluted 1:1,000 with a ELISA buffer diluent and added to the plates, respectively. Plates were incubated at $37^{o}C$ for 60 min, and the wells were washed again with three changes of washing solution. Then, 100 μl of diluted enzyme-antibody, a rabbit
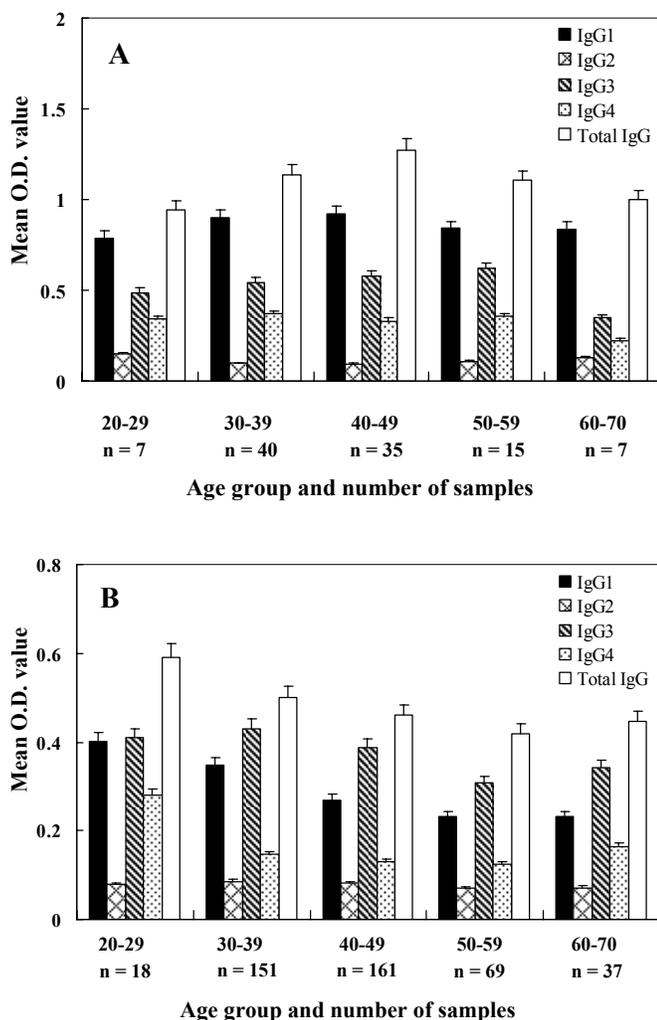
Figure 1. Profile of the anti-HBc IgG subclasses produced in (A) HBV chronic carriers and (B) recovered individuals with different years of age.

anti-mouse antibody conjugated with horseradish peroxidase (DAKO Ltd, Cambridgeshire, U.K.), was added and diluted at 1:5,000 in an ELISA diluent. The plates were then incubated at 37°C for another 60 min, and washed again in the same manner. Substrate solution (ortho-phenyl-diamine) was added, and the plates were left at 37°C for 15 min. Absorbance was read at 495 nm (Spectrophotometer Model 550, Bio-Rad, Hercules, CA, USA) and recorded. The variations among the plates were standardized by comparing the positive control of each plate with each other. The adjusted optical density (O.D.) value of the sample = the original O.D. times the mean O.D. of the positive control, then divided by the O.D. of the positive control of the tested plate. The cutoff value for each plate was set at three times the O.D. for each negative control.

Statistical analysis
The Kruskal-Wallis test was used to analyze the different amounts of antigen for coating among the ELISA plates. The

Wilcoxson rank sum test was used to test the O.D. of the IgG subclasses between sets of two different groups. The Chi-Square test was utilized to compare the population number percentages of different IgG subclasses in a group. *p* values < 0.05 were taken to be significant.

Results

The selection of test plates for large-scale analysis
A variety of reagents including Murex (England), Behring (Germany), GB (Taiwan), EverNew (Taiwan) commercial kits as well as one self-prepared (SP) reagent set were used in our previous anti-HBc (11) study to check sensitivity and specificity. Based on the evaluation data of 100% specificity and sensitivity, we chose the commercial GB plates for our anti-HBc study.

The different IgG subclass profiles of male and female populations with different ages
Of the 543 samples, 104 (60 male and 44 female) samples were defined as HBV chronic carriers, and 439 (247 male and 192 female) samples were classified as recovered individuals. For anti-HBc detection (Figure 1 and Table 1), the mean O.D. values of the IgG2 subclass in both populations were the lowest with different years of age. The mean O.D. values of total IgG, IgG1, IgG3, and IgG4 in chronic carriers were significantly higher than that in recovered individuals, with differences of 0.684, 0.592, 0.161, and 0.212, respectively ($p < 0.05$) (Table 2). Moreover, the O.D. for IgG1 was greater than that for IgG3 in chronic carriers. By contrast, the O.D. for IgG3 was greater than that for IgG1 in recovered individuals ($p < 0.05$). The O.D. values for the IgG1 and IgG3 subclasses were clearly different between the two populations. In chronic carriers, 100 (96.2%) individuals had O.D. values for the IgG1 subclass higher than that for IgG3, and 96 (92.3%) and 82 (78.8%) individuals had O.D. values for the IgG1 and IgG3 subclasses, respectively, that were higher than 0.8 and 0.5. In recovered individuals, there were 93 (89.4%) individuals whose O.D. value for the IgG3 subclass was higher than that for IgG1, and 102 (98.1%) and 101 (97.1%) individuals whose O.D. values for IgG1 and IgG3, respectively, were below 0.5.

In chronic carriers, the IgG1 subclass was higher than the IgG3 subclass, and reached its highest level at 40-49 years of age, then was decreased with the increase of age. But in recovered individuals, the IgG3 subclass was higher than the IgG1 subclass, except in the 20-29 year-old male group (Table 1). However, both increasing and decreasing trends were not significant ($p > 0.05$). In both males and females chronic carriers, the distribution pattern of the mean anti-HBc subclasses was IgG1 > IgG3 ($p < 0.05$); while in the recovered individuals, it was IgG3 > IgG1 ($p < 0.05$).

The association of ALT and AST abnormality with anti-HBc IgG subclasses
ALT and AST have been documented as the best markers for liver inflammation (13). Among the 104 chronic carriers

Table 1. The mean O.D. values of different anti-HBc IgG subclasses produced in HBV chronic carriers and recovered individuals of male and female

Sex/Age	Chronic carriers						Recovered individuals					
	Number	IgG1	IgG2	IgG3	IgG4	Total	Number	IgG1	IgG2	IgG3	IgG4	Total
Male												
20-29	4	0.714	0.154	0.381	0.362	0.842	8	0.405	0.083	0.362	0.344	0.545
30-39	22	0.914	0.089	0.553	0.369	1.241	88	0.334	0.077	0.424	0.146	0.483
40-49	21	0.933	0.127	0.631	0.351	1.366	92	0.281	0.074	0.388	0.125	0.446
50-59	9	0.854	0.108	0.645	0.341	1.185	36	0.177	0.071	0.264	0.133	0.367
60-70	4	0.618	0.103	0.371	0.202	0.804	23	0.229	0.072	0.293	0.157	0.413
Female												
20-29	3	0.886	0.150	0.631	0.317	1.084	10	0.399	0.076	0.447	0.229	0.548
30-39	18	0.879	0.110	0.526	0.367	1.012	63	0.368	0.097	0.441	0.146	0.526
40-49	14	0.897	0.107	0.493	0.298	1.126	72	0.253	0.088	0.389	0.134	0.481
50-59	6	0.818	0.111	0.585	0.379	0.982	33	0.291	0.068	0.353	0.117	0.476
60-70	3	1.133	0.166	0.316	0.256	1.267	14	0.237	0.074	0.419	0.176	0.505

Table 2. Mean O.D. values ± SD of the different anti-HBs, anti-HBe, and anti-HBc IgG subclasses produced in HBV chronic carriers with ALT & AST normal and abnormal and recovered individuals

Groups	Sample No.	Anti-HBc antibodies				
		IgG1	IgG2	IgG3	IgG4	Total
Chronic carriers	104	0.885 ± 0.056	0.105 ± 0.016	0.548 ± 0.046	0.342 ± 0.036	1.156 ± 0.073
ALT & AST normal	83	0.871 ± 0.054	0.102 ± 0.020	0.536 ± 0.049	0.338 ± 0.034	1.139 ± 0.086
ALT & AST abnormal	21	0.939 ± 0.061	0.117 ± 0.018	0.596 ± 0.041	0.356 ± 0.037	1.221 ± 0.071
Recovered individuals	439	0.293 ± 0.054	0.080 ± 0.024	0.387 ± 0.042	0.130 ± 0.039	0.472 ± 0.065

(mean years of age = 42.6), 21 patients (mean years of age = 45.2) were found to be ALT and AST abnormal (> 25 IU/ml). The mean O.D. values ± SD of anti-HBc total IgG, IgG1, IgG2, IgG3, and IgG4 of both ALT and AST normal and abnormal chronic carriers were listed in Table 2. After comparing these ALT and AST abnormal patients with other ALT and AST normal chronic carriers, no statistical difference was observed in the O.D. values of the anti-HBc IgG subclasses.

Discussion

According to the ELISA results analyzed by the Chi-Square test, the anti-HBc IgG subclass pattern was IgG1 > IgG3 > IgG4 in chronic carriers, and IgG3 > IgG1 > IgG4 in recovered individuals ($p < 0.05$). This was in accordance with the data reported by other groups (9, 10, 12) and the findings in our previous study (11). The IgG2 level of anti-HBc was the lowest IgG isotype both in carriers and recovered individuals. The IgG subclass profiles of anti-HBc in the ELISA results showed that the IgG subclass pattern of the relative anti-HBs IgG subclass titers was IgG1 > IgG3 > IgG4 in both chronic carriers and recovered individuals ($p <$

0.05). Chronic carriers were not changed regardless of liver inflammation (ALT and AST abnormal), and were independent on sex and age. Furthermore, the mean O.D. values of anti-HBc total IgG, and all IgG subclasses except for IgG2, of either males or females, were significantly higher in chronic carriers than that in recovered individuals. For anti-HBc subclass profiles, IgG3 > IgG1 in recovered individuals and IgG1 > IgG3 in chronic carriers also suggest the IgG1/IgG3 ratio may be related to HBV status. However, in spite of the different anti-HBc IgG1/IgG3 patterns demonstrated in the different populations, both anti-HBc IgG1 and IgG3 concentrations were significantly higher in chronic carriers ($p < 0.05$). Therefore, the anti-HBc IgG1/IgG3 ratio and their amounts were different and may play significant roles in chronic carriers and recovered individuals. The role of the HBc antigen in HBV infection was more complicated than expected.

The different immune responses that stimulate different productions of IgG subclasses have been investigated in a murine model by Milich et al. (14-16). The induction of different cytokines was found to correlate with the pattern of evoked IgG subclasses (15, 16). HBcAg and HBeAg share all the characterized T-cell epitopes and were important targets of antiviral immunity, although HBcAg and HBeAg were

serologically distinct (17). Many previous studies have provided evidence that HBcAg was extremely immunogenic (18), and functioned as both a T-cell-dependent and T-cell-independent antigen (15). Milich et al. (15, 16) suggested HBcAg tended to evoke the Th1 pathway of immune response; while HBeAg tended to evoke the Th2 (or Th0) pathway. Interestingly, the studies in HCV also showed similar findings. Gramenzi et al. (19) reported that (i) subjects who cleared HCV showed a cytokine profile similar to healthy controls; (ii) a preferential shift towards a Th1 profile was associated with a more favourable clinical outcome in chronic hepatitis C patients; and (iii) a prevalent Th2 profile was implicated in HCV pathogenesis and severity of liver disease. The study by Raghuraman et al. (20) also suggested that plasma levels of sFas in patients with chronic HCV infection showed significant positive correlations with ALT and TNF-α levels, and TNF-α levels showed a significant positive correlation with ALT levels.

Recently, Cao et al. (21) revealed that HBcAg-specific CD4$^+$ Th1-type T cells can modulate function and exert a regulatory role in deleting HBcAg-binding, or -specific human B cells *in vivo*, which may be important in controlling infection. HBcAg was also an effective carrier for heterologous peptide epitopes. Mice immunized with hybrid HBcAg-circumsporozoite protein particles were shown to produce a highly titered serum IgG antibody against the rodent malaria agent *Plasmodium yoelii* challenge (17). The report by Liu et al. suggested that recombinant adenoviruses containing mutant HBV genomes infected mice produced anti-HBc efficiently to comparable levels; the IgG1 and IgG2a specific for HBcAg were present in mice sera, and the response was dominated by IgG2a (22). Moreover, the L60V and I97L substitutions had no influence on humoral immune responses, but could down-regulate T-cell responses to HBcAg (22). More recently, Kim et al. (23) suggested that TNF-α promoter polymorphisms were associated with the clearance of HBV infection. Kobayashi et al. reported that HBV, with the wild-type sequences of the precore region and core promoter, prevails in patients with acute self-limited hepatitis, unlike patients with chronic hepatitis (24). The alteration of IgG subclass patterns which are induced by Th1 cytokine treatment (e.g., IL-2 and IFN-γ) (14) or by a specific antigen (e.g., HBcAg) (18) to modulate the immune response from Th2 towards Th1 pathway, may instruct the treatment or vaccine development, respectively, for HBV and other viral infections similar to HSV gD (25) and HIV gp120 (26) being proposed to exert immunomodulatory effects on Th1/Th2 cell subset distribution. HBc was suggested as a promising candidate for a therapeutic vaccine for the control of chronic HBV infection (27). However, as this study revealed both anti-HBc IgG1 and IgG3 were significantly higher in chronic carriers than that in recovered individuals, it would be a difficult task to evoke a desired IgG pattern without elevating IgG concentration when using HBc as part of the HBV vaccine subunits. Further study is needed to test if Th1 cytokine treatment is a better choice than using HBcAg with HBsAg for HBV vaccine and therapy.

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