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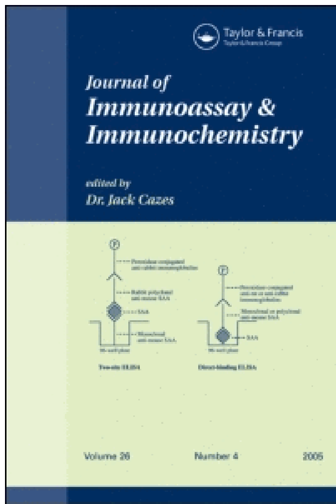

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ANTI-HBC IgG SUBCLASSES IN DIFFERENT POPULATIONS BY COMPARING A VARIETY OF ELISA PLATES

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ABSTRACT

The number of IgG subclasses for hepatitis B virus (HBV) core antigen (anti-HBc), demonstrated for HBV-infected individuals, was measured by enzyme-linked immunosorbent assay (ELISA). Four commercially available hepatitis B core antigen (HBcAg) plates and one prepared plate were tested for ELISA sensitivity by the detection of 14 serum samples drawn from HBV chronic carriers, cured patients, vaccinees, and non-infected individuals.

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Differences in optical density (OD) values were obtained by comparing data gathered from the five plate types, suggesting that different plates may have different binding capabilities for each anti-HBc IgG subclass and, thus, contribute to the different ELISA sensitivities. Of these plates, the GB plate showed the most obvious absorbance changes for anti-HBc subclasses in different populations. These data also indicated different patterns for IgG-specific subclasses for various populations. For HBsAg+ carriers, the OD for IgG_1 was greater than for IgG_3. By contrast, the OD for IgG_3 was higher than that for IgG_1 in those subjects who were negative for HBsAg.

INTRODUCTION

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Hepatitis B virus (HBV) infection is one of the most prevailing diseases in Taiwan.(1) During the course of infection, the surface antigen (HBsAg) and the *e* antigen (HBeAg) of the virus are released into the blood stream and circulate through the body, with both antigens detected in the blood. Core antigen cannot be detected, however, because it is not released from hepatic cells during HBV reproduction. Even if cells are ruptured, the released core antigen is soon neutralized and hydrolyzed to HBeAg.(2)

Generally, HBs and HBe antigen, and anti-HBc antibody, can be detected in infected individuals during the acute phase. In addition to the anti-HBc antibody, anti-HBs and/or anti-HBe antibody will be demonstrated in cured individuals.(3) If the infection persists over six months, the infected individual is deemed a chronic carrier. In the sera of these chronic carriers, only anti-HBc antibody and HBsAg, produced and secreted long term, are detected. During the course of infection, the anti-HBc IgG will develop and the subclass profile will also change to reflect different types of HBsAg+ liver diseases.(4-8)

Hepatitis B-virus structural proteins consist of various antigenic epitopes which stimulate different immune responses and result in the production of versatile immunoglobulins reflecting different classes and subclasses.(9) Therefore, it seems reasonable to suggest that different hepatitis B vaccines, with different sources of HBs antigen, will generate different subclasses of immunoglobulin.

For example, the anti-HBs IgG for vaccinees immunized with recombinant hepatitis B vaccine consists mainly of IgG_1 and IgG_4,(10,11) while that of individuals vaccinated with plasma-purified HBsAg consists of IgG_1.(12) 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.(13) This variation in binding evokes either the Th_1 or Th_2 immune response and results in the production of different subclass patterns.(14-16)

The aim of this study was to measure and evaluate the titers of anti-HBc IgG subclasses, as detected using different ELISA plates which were constructed with HBc antigens that were generated and applied using different methods. This data was also used in the selection of test plates for further large-scale analysis of anti-HBc immunoglobulin-subclass development.

EXPERIMENTAL

Serum Samples

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Blood samples were obtained from twelve outpatients attending the Chung Shan Medical and Dental College hospital in April, 1997, and from two employees at the Institute of Biomedical Sciences, Academia Sinica in May, 1996. The positive and negative control sera were also included in this study. The age, sex, sample-collection time and results of serum markers for HBV, including HBsAg, anti-HBs, anti-HBc, HBe Ag and anti-Hbe, were tested using both Axsym EIA (Abbott Laboratories, USA) and Dainabot RIA (Dainabot Co., Japan) and recorded (Table 1). Sera were separated from blood samples by centrifugation at 3,500 x *g* for 15 min, and aliquoted and stored at -70C for future use.

Antigen, Antibody, and ELISA Plate

Total anti-HBc immunoglobulin (IgG total) G and all its subclasses (IgG_1, IgG_2, IgG_3 and IgG_4) were detected using ELISA. Anti-HBc plates used in this study included Wellcozyme (Wellcome, United Kingdom; WC), Enzygnost (Behring, Germany; BR) and two domestically sourced plates, EverNew (EverNew, Taiwan; EN) and Anticorase B-96 (General Biological, Taiwan; GB). The antigens used for Wellcozyme and Enzygnost plates were generated from recombinant.

The purpose-made test plate (SC) was prepared using core antigen purchased from BIOTEC (U.K.). Test sera were diluted 1:100 in ELISA-buffer diluent which was made from 0.05 % Tween-20 and 1 % bovine-serum albumin in phosphate buffer. To the 96-well HBc plates, 100 μL of

Table 1. The Age, Sex, Collection Time, and Results for Serium HBV Markers for Samples

Population Group[a]	Sample No.	Collection Date	Sex[b]	Age	HBS Ag	Anti-HBS	Anti-HBc	HBe Ag	Anti-HBe
A	1	970408	M	25	+	–	+	+	–
A	2	970408	F	19	+	–	+	–	–
A	3	970408	M	60	+	–	+	+	–
A	4	870410	M	40	+	–	+	+	–
A	5	970407	M	26	+	–	+	+	–
A	6	960523	M	36	+	–	+	+	–
B	7	970409	F	22	–	+	+	–	–
B	8	970409	F	24	–	+	+	–	–
C	9	970409	F	21	–	+	–	–	–
C	10	960516	F	45	–	+	–	–	–
D	11	970409	M	40	–	–	–	–	–
D	12	970409	M	31	–	–	–	–	–
D	13	970403	F	29	–	–	–	–	–
D	14	970402	F	20	–	–	–	–	–

[a]Population group: A, HBV carriers; B, HBV cured individuals; C, vaccines; D, non-infected individuals.
[b]Sex: M, Male; F, Female.

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diluted sera was added, and plates were incubated at 37C for 90 min. After incubation, plates were washed three times with ELISA washing solution, then 100 L of diluted different mouse antihuman monoclonal antibody was added. Mouse monoclonal antibodies to total IgG subclasses (Clone MK1A6), IgG_1 (clone NL16), IgG_2 (clone GOM2), IgG_3 (clone ZG4) and IgG_4 (clone RJ4), respectively, all of IgG1 isotypes, were obtained from BIONOSTICS (U.K.) and used at 1/2000 dilution.

Plates were incubated at 37°C for 90 min, and the wells were washed another three times with ELISA-washing solution using an ELISA washer (BIO-RAD, USA), and then 100 μL of enzyme-antibody conjugate (rabbit anti-mouse conjugate 1:5000 diluted in ELISA diluent) was added to each of the wells which were then incubated at 37C for 90 min and then washed again in the same manner. Substrate solution (ortho-phenyldiamine, OPD) was added and plates were incubated at 37C for 15 min. Absorbance was read at 492 nm (Spectrophotometer Model 550, Bio-Rad).

RESULTS

In this study, we utilize the ELISA technique to evaluate the total anti-HBc IgG levels for all of the subclasses derived for 14 samples, using four commercial and one purpose-made ELISA plates. For ELISA, HBc antigens were immobilized onto the plates, then test sera were added and incubated at an appropriate temperature to allow the specific binding of antigen and antibody. Unbound antibodies were washed off and monoclonal antibodies added against total and individual subclasses (mouse anti-human IgG_1, IgG_2, IgG_3, IgG_4).

After washing, these immobilized antigen- antibody-secondary antibody complexes were incubated with an excess of enzyme-labeled tertiary antibodies (rabbit anti-mouse conjugate). Finally, enzyme substrates were added and absorptions recorded.

According to results generated for HBsAg, anti-HBs, anti-HBc, HBeAg, and anti-HBe (Table 1), the 14 samples were divided into four populations. Subjects 1-5, defined as HBsAg carriers (testing both HBsAg and anti-HBc positive), made up Population A. Population B (subjects 6-8, testing HBsAg negative and anti-HBc positive) consisted of persons who had been infected but were clear for core antigen at time of study. Subjects 9 and 10, tested HBsAg negative but anti-HBs positive induced by vaccination (plasma purified HBsAg), were classified as Population C. Population D consisted of subjects 11-14, testing negative for all markers and considered non-infected.

The ELISA techniques were performed according to the manuals and the OD readings for total IgG, IgG_1, IgG_2, IgG_3 and IgG_4 were taken (Table 2). The OD values for anti-HBc total IgG positive/negative control were 0.260/0.020 for WC, 0.027/0.090 for BR, 0.365/0.037 for EN, 0.367/0.044 for GB, and 0.206/0.047 for SC. The cutoff value for each plate was set at three times the OD for each negative control. Using this standard, four subjects, detected by WC, were false positives,(9, 10, 11, 13) five cases, detected by BR, were false negatives,(4-8) and one case(11) was false positive, detected by SC. Both the sensitivity and specificity for EN and GB plates were 100 %.

For total IgG, the mean OD detected was 0.293 using WC, but only 0.080 for BR (Table 3). The GB plates demonstrated the highest OD for every IgG subclass compared to the other plates, moreover, the mean OD for the total IgG also reached the highest figure, 0.779 (Table 2). Of these different plates, GB demonstrated the most obvious absorbance changes for anti-HBc subclasses for different populations. Hence, the GB plate was chosen for a further large-scale epidemiological study for different

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Table 2. The O.D. Values for Samples Using Different ELISA Plates*

Plate		Wellcome (WC)					Behring (BR)					Ever New (EN)					General	
No.	Group	Total	IgG1	IgG2	IgG3	IgG4	Total	IgG1	IgG2	IgG3	IgG4	Total	IgG1	IgG2	IgG3	IgG4	Total	IgG1
1	A	0.503	0.148	0.042	0.113	0.210	0.113	0.058	0.048	0.062	0.059	0.507	0.250	0.038	0.282	0.034	1.005	0.715
2	A	0.362	0.171	0.042	0.079	0.178	0.095	0.063	0.042	0.046	0.043	0.410	0.301	0.042	0.184	0.034	0.872	0.675
3	A	0.245	0.120	0.046	0.041	0.117	0.088	0.064	0.039	0.039	0.041	0.546	0.293	0.042	0.046	0.042	0.734	0.654
4	A	0.229	0.118	0.041	0.052	0.132	0.063	0.041	0.028	0.038	0.036	0.708	0.372	0.043	0.095	0.039	0.584	0.761
5	A	0.193	0.131	0.035	0.029	0.122	0.051	0.045	0.028	0.029	0.034	0.675	0.438	0.046	0.040	0.040	0.739	0.652
6	A	0.224	0.093	0.041	0.031	0.034	0.071	0.055	0.028	0.030	0.031	0.548	0.368	0.052	0.047	0.048	0.741	0.508
7	B	0.147	0.059	0.040	0.061	0.045	0.055	0.039	0.039	0.053	0.032	0.152	0.043	0.039	0.042	0.048	0.174	0.109
8	B	0.109	0.051	0.047	0.063	0.046	0.040	0.038	0.040	0.055	0.041	0.143	0.040	0.037	0.048	0.043	0.144	0.085
9	C	0.077	0.056	0.040	0.047	0.043	0.041	0.039	0.041	0.039	0.038	0.052	0.042	0.042	0.045	0.042	0.071	0.161
10	C	0.063	0.061	0.043	0.044	0.046	0.057	0.044	0.041	0.040	0.040	0.064	0.047	0.040	0.041	0.045	0.057	0.140
11	D	0.107	0.064	0.038	0.030	0.093	0.036	0.036	0.033	0.028	0.029	0.062	0.062	0.048	0.049	0.044	0.082	0.055
12	D	0.056	0.043	0.041	0.041	0.042	0.039	0.056	0.066	0.045	0.041	0.041	0.041	0.041	0.039	0.039	0.053	0.043
13	D	0.068	0.046	0.042	0.043	0.059	0.041	0.043	0.047	0.046	0.042	0.053	0.040	0.041	0.039	0.043	0.114	0.152
14	D	0.056	0.046	0.041	0.042	0.045	0.032	0.039	0.041	0.043	0.044	0.042	0.039	0.045	0.039	0.040	0.044	0.038
15	N	0.020	0.036	0.041	0.033	0.049	0.027	0.026	0.026	0.033	0.028	0.037	0.042	0.053	0.037	0.044	0.044	0.055
16	P	0.260	0.124	0.030	0.030	0.032	0.090	0.059	0.029	0.029	0.030	0.365	0.237	0.048	0.046	0.042	0.367	0.416

* Population group: A, HBV carriers; B, HBV cured individuals; C, vaccines; D, non-infected individuals. N, negative; P, positive.

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Table 3. The Mean O.D. Values for Each Population Using Different ELISA Plates

		Mean O.D. ± 2SD				
Plate	Population Group	Total	IgG_1	IgG_2	IgG_3	IgG_4
WC	A	0.293 ± 0.237	0.130 ± 0.054	0.041 ± 0.007	0.058 ± 0.065	0.132 ± 0.120
	B	0.128 ± 0.054	0.055 ± 0.011	0.044 ± 0.010	0.062 ± 0.003	0.046 ± 0.014
	C	0.070 ± 0.020	0.059 ± 0.007	0.042 ± 0.004	0.046 ± 0.004	0.045 ± 0.004
	D	0.072 ± 0.048	0.050 ± 0.019	0.041 ± 0.003	0.039 ± 0.012	0.060 ± 0.047
BR	A	0.080 ± 0.046	0.054 ± 0.019	0.036 ± 0.017	0.041 ± 0.024	0.041 ± 0.020
	B	0.048 ± 0.021	0.039 ± 0.014	0.040 ± 0.014	0.054 ± 0.003	0.037 ± 0.013
	C	0.049 ± 0.023	0.042 ± 0.007	0.041 ± 0.000	0.040 ± 0.014	0.039 ± 0.003
	D	0.037 ± 0.008	0.044 ± 0.018	0.047 ± 0.028	0.041 ± 0.017	0.039 ± 0.013
EN	A	0.566 ± 0.220	0.337 ± 0.136	0.044 ± 0.009	0.116 ± 0.196	0.040 ± 0.011
	B	0.148 ± 0.013	0.042 ± 0.004	0.038 ± 0.003	0.045 ± 0.008	0.046 ± 0.007
	C	0.058 ± 0.017	0.045 ± 0.007	0.041 ± 0.003	0.043 ± 0.006	0.044 ± 0.004
	D	0.050 ± 0.020	0.046 ± 0.022	0.044 ± 0.007	0.042 ± 0.010	0.042 ± 0.005
GB	A	0.779 ± 0.287	0.661 ± 0.171	0.098 ± 0.112	0.346 ± 0.663	0.114 ± 0.116
	B	0.159 ± 0.042	0.097 ± 0.171	0.098 ± 0.112	0.143 ± 0.055	0.053 ± 0.000
	C	0.064 ± 0.020	0.151 ± 0.029	0.133 ± 0.100	0.135 ± 0.025	0.048 ± 0.008
	D	0.073 ± 0.063	0.072 ± 0.108	0.091 ± 0.109	0.053 ± 0.013	0.052 ± 0.014
SC	A	0.552 ± 0.338	0.245 ± 0.118	0.044 ± 0.010	0.075 ± 0.076	0.040 ± 0.006
	B	0.180 ± 0.013	0.053 ± 0.023	0.041 ± 0.006	0.082 ± 0.021	0.039 ± 0.014
	C	0.103 ± 0.048	0.061 ± 0.047	0.041 ± 0.004	0.046 ± 0.013	0.043 ± 0.014
	D	0.124 ± 0.107	0.045 ± 0.012	0.042 ± 0.006	0.043 ± 0.005	0.042 ± 0.002

Population group: A, HBV carriers; B, HBV cured individuals; C, vacines; D, non-infected individuals.

populations (unpublished data). The mean OD values for each IgG subclass for different populations are revealed in Table 3.

Notably, under the same assay conditions, the anti-HBc IgG-subclass patterns were consistent for different populations, though they demonstrated different OD values with different plates. It was found that the samples from population A exhibited a higher concentration for IgG1 than for IgG3, however, this pattern was reversed for population B, where IgG3 concentration was higher.

DISCUSSION

These findings demonstrate that different sources of HBc antigens produce different results. For plate GB, the mean OD values for IgG_1 and IgG_2 were 0.61 and 0.346, respectively and for EN, 0.337 and 0.116, respectively, while for the other two plates (WC and BR), both values were below 0.13. These differences may be due to the source of antigen, different epitopes and/or the method used for conjugation.

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Interestingly, all the samples in population A revealed higher concentrations for IgG_1 than for IgG_3, a subclass pattern consistent with that reported by Sllberg (1989) for cured individuals ($IgG_1 > IgG_3 > IgG_4$; 17). The results for population B in this study, however, suggested the reverse was the case for HBV carriers ($IgG_3 > IgG_1$).

The elimination of HBV depends mainly on cell-mediated immune response, particularly the killer T cell. The helper T cell (Th) assists the process in two ways. First, the Th1 cells stimulate macrophages, and, in turn, these macrophages clear virus particles and virus-infected cells. Second, the Th2 cells stimulate B cells to generate immunoglobulins (antibodies) that act through the humoral immune response to neutralize the virus. Different protein compositions for hepatitis B virus can evoke different immune responses. For example, the e protein triggers a T cell-dependent immune response while the core protein triggers both T cell-independent and T cell-dependent immune responses.(18)

Further, it has been reported that some viral proteins may function by modulating the immune response during viral infection. The mechanism for antigen stimulation for different subclasses of immunoglobulin has been reported for the mouse,(16) however, this mechanism remains unclear for the human immune-response system, though similar studies exploring immunoglobulin patterns for different viral infections have been published.(19)

More recently, the study performed by Milich et al., (1998), has suggested that the presence of serum HBe Ag ablated the expected Th_1-

mediated anti-HBc antibody response, shifting it toward a Th_2 phenotype.(19) For acute hepatitis B infection, the number of T cells (CD4) was higher than for chronic hepatitis (20), however, the expressed CD molecules associated with increased T-cell numbers were not analysed. Since the number of IgG subclasses that can be detected during the course of hepatitis infection changes, the detection and analysis of IgG-subclass patterns may promote understanding of the relationship between these patterns and T cell-mediated immunity for virus clearance, and also used as a marker for the progression of this disease.

Of all the IgG subclasses, IgG_1 is predominant during HBV infection, and for Rubella infection(21) as well.(4-7) For Varicella infection, however, IgG_3 is prevalent, as is IgG_1 for Zoster.(22) While the role of IgG_2 appears less important for virus infection,(10,21) it is the most frequently induced immunoglobulin G during bacterial infection.(23,24) It has been demonstrated that IgG_2 is induced in people immunized with recombinant hepatitis subunit vaccine (without glycosylation),(11) despite reports that IgG_2 may indicate a specific response to carbohydrate.(25,26) For bacterial infection, e.g., *Peudomonas aeruginosa*, IgG_2 and IgG_3 predominate.(23) In parasitic *Leishmania infatum* infection in dogs, the main types of immunoglobulin G present were IgG_1 and IgG_2.(27) A previous report has suggested that IgG_3 can be constructed from the heavy chain of IgM.(28) The immune response for IgG_1 and IgG_3 may be triggered by glycoprotein.(29) Shakib et al. (1980) have demonstrated the presence of IgG_4 for allergic reactions, and it did not bind complement.(30)

Some studies have reported that the predominant anti-HBc antibody for acute hepatitis B-infected patients was IgG_1 followed in order of prevalence by IgG_3 and IgG_4.(17,31) During the acute phase, the subclasses IgG_1 and IgG_3 appear first, while persisting IgG_3 indicates the potential for continuing reproduction of the antigen in the body of the host, resulting in antibody production.(4,17) Sällberg et al. (1990, 1991) have suggested that the relative strength of each IgG-subclass pattern for anti-HBc antibody production in chronic-hepatitis infected patients is $IgG_1 >> IgG_3 > IgG_4$.(6,7) Of all the hepatitis patients, the primary IgG subclass for Anti-HBc anbody in asymptomatic carrier patients was IgG_1. For liver cirrhosis patients, however, titers for IgG_3 were higher than for IgG_1,(31) with IgG_3 predominance also indicated by histoimmunostain techniques.(32)

The results reported by Akbar et al., (1991) suggest that the anti-HBc IgG_1 subclass predominates initially, and as the disease progresses, anti-HBc IgG_3 subclass numbers increase.(31) This study supports the proposition of different patterns for anti-HBc IgG subclasses in HBV carriers and cured individuals, suggesting different epitopes present on viral antigens may con-

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dition the kinetics of production, and the release of immunoglobulins, so that the resulting IgG-subclass profile may be modulated by T cell-mediated immunity, to a certain extent. Moreover, it seems reasonable to suggest that the change in anti-HBc IgG-subclass patterns reflects the transfer of disease progression from carriers to cured individuals during infection, i.e., from group A to B in this instance. Further studies are required to clarify these phenomena. To this end, we have analyzed the anti-HBc IgG subclasses for 96 HBV carriers and 434 HBV-cured individuals (unpublished data), and we hope this will help to enhance our understanding of the relationship of immune modulation with immunoglobulin G during hepatitis B infection.

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