

## IgG SUBCLASS ANALYSIS OF ANTI-HBs ANTIBODIES PRODUCED IN RESPONSE TO VACCINATION WITH RECOMBINANT HBsAg AND INFECTION WITH THE WILD HEPATITIS B VIRUS

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### • ABSTRACT

**Background:** Viral hepatitis, particularly hepatitis B virus (HBV) infection remains a major public health problem worldwide and different profiles of IgG subclasses of anti-HBs antibody have been reported to be produced in individuals infected with HBV and those vaccinated with HBsAg.

**Objective:** To evaluate the difference among various profiles of IgG subclasses produced in response to HBV infection and HBsAg observed in HBV positive patients and those vaccinated with HBsAg.

**Methods:** Antibody to hepatitis B surface antigen (HBsAg) was purified by affinity chromatography from sera of 24 normal individuals vaccinated with recombinant HBsAg (rHBsAg) and 18 persons infected with hepatitis B virus (HBV). The isotype and IgG subclass profiles of the purified anti-HBs antibodies were determined by enzyme-linked immunosorbent assay (ELISA) using plates pre-coated with rHBsAg and isotype specific monoclonal antibodies.

**Results:** IgG2 was the predominant subclass of anti-HBs antibody in both groups of individuals followed by IgG4 and IgG1. IgG3 was hardly detectable in the majority of samples tested.

**Conclusion:** No significant differences were observed between the two subject groups with regards to the levels of IgG subclasses and isotypes, suggesting similar antibody responses to rHBsAg and the wild type HBV.

**Irn J Med Sci 2000; 25(1&2):42-49**

**Key Words • Hepatitis B virus • vaccination • hepatitis B antibodies • IgG**

### Introduction

Viral hepatitis, particularly hepatitis B virus (HBV) infection remains a major public health problem worldwide. Exposure of the adult normal population to HBV predominantly results in clinically symptomatic or asymptomatic acute infection with subsequent development of a protective antibody response to the surface components of the virus.<sup>1,2</sup> Antibody to HBsAg is usually detectable at the late convalescent period. Appearance of this antibody indicates recovery from infection and acquisition of a long-lasting immunity.<sup>3</sup>

Vaccination with recombinant or plasma-derived HBsAg has also been demonstrated to induce a protective antibody response in similar proportions to that of the normal population.<sup>4-6</sup> However, different profiles of IgG subclasses of anti-HBs antibody have been reported to be produced in individuals infected with HBV and those vaccinated with HBsAg.<sup>7-10</sup> In this study, anti-HBs antibody was purified by affinity chromatography from hyperimmune sera of normal individuals vaccinated with recombinant HBsAg and individuals naturally infected with HBV and subsequently, the isotype and IgG subclass profiles of the purified antibody were determined.

### Materials and Methods

#### Clinical samples and subjects:

Sera were collected from the peripheral blood of 30 vaccinated healthy individuals (M/F: 20/10, mean

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age?SD:27?4.7) and 18 non-vaccinated persons (M/F: 8/10, mean age?SD:36?16.6) who had naturally acquired anti-HBs antibodies. The subjects were vaccinated with three 20 ?g doses of recombinant HBs vaccine (Engerix-B, SmithKline Beechman, Belgium) administered intramuscularly at 0, 1 and 6 month intervals. All sera were screened for HBsAg and antibody to HBs and HBe antigens, using commercial kits. All sera from infected individuals were positive for both antibodies, whereas of the vaccinated subjects, only six samples were found to be anti-HBe positive and were therefore excluded from the study. HBsAg was negative in all samples.

**Screening of HBV markers:**

HBsAg and anti-HBs antibody were detected by sandwich ELISA, and anti-HBe antibody by competitive ELISA, using commercial kits (Organon Teknika, Boxtel, Holland).

**Assessment of reactivity of the monoclonal antibodies:**

Murine monoclonal antibodies (MAbs) specific for human IgG (8a4), IgA (2D7), IgM (AF6), IgG1 (JL512 and NL16), IgG2 (GOM2), IgG3 (ZG4) and IgG4 (RJ4 and HP6023) were used for the detection of isotypes and IgG subclasses of the purified antibodies. All monoclonal antibodies were of the IgG1 isotype.<sup>11</sup> The antibody HP6023 was purchased from Sigma. Reactivity of MAbs were assessed using the ELISA method. Anti- $\gamma$  1 (20 ?g/ml), anti- $\gamma$  2 (10 ?g/ml), anti- $\gamma$  3 (5 ?g/ml), anti- $\gamma$  4 (20 ?g/ml), 8a4, 2D7 and AF6 (10 ?g/ml) were added in duplicate to microtitre ELISA plates (Nunc, Denmark) pre-sensitized with serial concentrations of purified  $\gamma$  1,  $\gamma$  2,  $\gamma$  3,  $\gamma$  4, IgA, IgM and IgG paraproteins and incubated for 1 hour at 37 ?C. After washing (3 $\times$ BS/0.05% Tween 20) the plates were incubated with a 1/2000 dilution of horseradish peroxidase-conjugated sheep anti-mouse Ig (prepared in our laboratory) in PBS/Tween for 1 hour at 37 ?C. Washed plates (3 $\times$ BS/Tween) were subsequently developed with o-phenylenediamine tetrachloride (OPD) (Sigma) and the reaction stopped after 20 minutes with 20% H<sub>2</sub>SO<sub>4</sub>. Optical density (OD) was measured at 492 nm using a multiscan ELISA reader (Organon Teknika, Boxtel, Holland).

**Production and purification of polyclonal rabbit anti-HBs Antibody:**

White New Zealand rabbits were given 5 im injections with 10 ?g of recombinant HBsAg (Engerix-B, SmithKline Beechman, Belgium) at 2 week intervals. The first dose was given in complete Freund's adjuvant, whereas other injections were administered in incomplete Freund's adjuvant. Following serum titration of anti HBs antibody from immunized rabbits, hyperimmune sera were collected and subjected to ion-exchange chromatography on DE-52 column (Whatman, U.K), equilibrated and eluted with 0.01M phosphate buffer pH 7.2. IgG was collected as a breakthrough fraction and subsequently coupled to CNBr-activated Sepharose 4B (Pharmacia, Sweden) according to the manufacturer's instructions.

**Affinity purification of HBsAg and anti-HBs antibody from human serum:**

HBsAg was purified from pooled serum of carrier individuals containing a high titer of the antigen, using rabbit anti HBs-Sepharose 4B column. Bound HBsAg was eluted with 0.1M Glycine/HCl pH 2.5 and immediately dialyzed against carbonate/bicarbonate buffer (0.1M, pH 8.3) containing 0.5M NaCl and coupled to CNBr-activated Sepharose 4B as described above. Coupled Sepharose 4B was then packed into a small column (10 ml) and used to purify anti-HBs antibody from serum collected from the subjects included in the study. Five mls of serum from each individual, pre dialyzed with 0.1M Tris HCl, pH 8.1, was passed through the column. Following washing with the same buffer, bound protein was eluted with 0.1M Glycine/HCl pH 2.5 and immediately dialyzed against PBS. The purity of the eluted anti-HBs antibody was evaluated by SDS-PAGE, using a 10% gel as previously described.<sup>12</sup>

*Archive of SID***Immunoblotting of purified anti-HBs antibody:**

Western blot analysis of affinity purified anti-HBs antibody was performed as basically described elsewhere. Briefly, purified anti-HBs in disruption buffer containing 2% SDS, 1% bromophenol blue and 3% sucrose in 0.2 mM Tris/HCl pH 7 was loaded onto a 10% polyacrylamide slab gel containing 0.1% SDS. Proteins were subjected to electrophoresis at 150 V for 3.5 hours and then transferred to nitrocellulose membrane (Schleicher & Schuell, Germany) at 600 mA for 2 hours. The membrane was then blocked for 2 hours with 2% BSA. Following extensive washing with PBS, bound proteins were revealed with horseradish peroxidase-conjugated HBsAg or anti-human IgG (as control) and developed in 0.05% diaminobenzidine tetrahydrochloride substrate (Sigma).

**Determination of isotype and IgG subclasses of anti-HBs preparations:**

ELISA plates pre-sensitized with 5 µg/ml of rHBsAg were incubated with purified anti-HBs antibody at adequate concentration in duplicates. The plates were incubated with isotype or subclass specific MAbs at the concentrations given above. Bound IgG, IgM, IgA or IgG subclasses were revealed using horseradish peroxidase conjugated sheep anti-mouse Ig pre-adsorbed with human Ig (prepared in our laboratory) and developed with OPD. Optical density was finally measured at 429 nm.

**Statistical analysis:**

Comparison between groups was analyzed by t-test using the SPSS statistical package and p values of less than 0.05 were considered significant.

**Results****Affinity purification of anti-HBs antibody:**

Using a small HBsAg column, anti-HBs antibody was purified from serum of the vaccinated and infected individuals and then subjected to SDS-PAGE to determine their purity. The purified protein was largely concentrated in the 150 KD region (IgG), though low levels of contamination with other proteins (mainly transferrin with MW of 80 KD) was also detectable (data not presented). Reactivity of the purified antibody was then evaluated by immunoblotting using enzyme-conjugated HBsAg. The results obtained for 2 samples have been shown in Figure 1. The efficiency of the HBsAg column was tested by titration of the bound and unbound proteins in ELISA plates pre-coated with HBsAg. Anti-HBs reactivity was concentrated in the bound fraction and almost devoid of the unbound proteins (data not presented).

**Assessment of reactivity of the isotype and subclass specific monoclonal antibodies:**

To be able to compare the ELISA measurements for the isotypes and subclasses of anti-HBs antibody in each subjects groups, reactivity of the monoclonal antibodies was determined by titration of the antibodies over serial concentrations of the related isotype or IgG subclass within a standard sample. The detection limit of all MAbs was less than 60 ng/ml. Of the Anti-isotype MAbs, anti-IgA (2D7) displayed higher reactivity, whereas anti-IgM (AF6) and anti-IgG (8a4) were found to have similar reactivities (Fig. 2). Different profiles of reactivity were observed for anti-IgG subclass MAbs. MAbs specific for IgG3 and IgG4 displayed overlapping titration curves and higher reactivities than those reacting with IgG1 and IgG2 (Fig. 3). The latter MAbs were also found to overlap along all points of the titration curves.

**Determination of isotypes and IgG subclasses of the purified anti-HBs antibody:**

Of the three major human immunoglobulin isotypes, IgG was the predominant isotype within the purified anti-HBs antibody isolated from both subject groups, followed by IgM (Fig. 4). IgA was hardly detectable in the majority of the samples. IgG2 was found to be the major IgG subclass in purified anti-HBs preparations isolated from both groups of subjects (Fig. 5). IgG4 and IgG1 came in the next order, whereas IgG3 was either negative or detectable at very low levels in most cases. No significant differences were found between the two subject groups with regards to the levels of isotypes or IgG subclasses. However, there appears to be significant differences between the levels of IgG subclasses within each subject group ([Table 1](#)).

**Discussion**

In the present study, we have demonstrated that IgG and IgG2 are the predominant isotype and subclass of anti-HBs antibody produced in both groups of vaccinated and infected individuals.

No significant differences were evident between the two subject groups with regard to the levels of the anti-HBs IgG subclasses or isotypes.

Our findings are not consistent with some previous reports. While IgG1 has been found to be the major subclass of anti-HBs antibody induced following vaccination, the published data with regard to the other IgG subclasses is highly controversial.<sup>8,9,13-16</sup> Either IgG4<sup>8,9,13</sup> or IgG2<sup>14,16</sup> has been reported to sub-dominate the anti-HBs antibody response, by different groups. IgG3, however, was hardly detectable in most reports. Our results also confirm the previous findings regarding the diminished response of IgG3. Production of high levels of IgG1 was also observed in our subjects, however, predominance of IgG2 has not been previously reported. Considering the fact that IgG2 is the major IgG subclass produced to polysaccharide antigens, whereas HBsAg is a proteinaceous antigen to which IgG1 and IgG3 are expected to be induced predominantly,<sup>7-10</sup> our results are not comprehensible according to the present dogma. One possible explanation which could be marshalled to account for the discrepancy may be related to the source of the vaccine. Plasma-derived HBsAg has been employed in the majority of the aforementioned studies. Taking into account that plasma-derived HBsAg is highly heterogeneous in many respects, such as the methods of purification, source of serum, serotype of the antigen and the chemical formulations used for viral neutralization and vaccine preservation, this might have resulted in some of the differences mentioned above. To the best of our knowledge, analysis of the profile of IgG subclasses of anti-HBs antibody following vaccination with recombinant HBsAg has not been studied extensively. So far, there has been only one published report regarding the anti-HBs IgG subclass response to recombinant HBsAg.<sup>16</sup> In parallel with our results, IgG2 was more represented than IgG4 or IgG3, though IgG1 dominated the anti-HBs antibody response. The recombinant HBsAg employed in the latter study was similar to that of our study, consisting mainly of the non-glycosylated form (P24) of HBsAg. This rules out contribution of the carbohydrate moiety of the glycosylated molecule in production of the IgG2 subclass, as was previously suggested for the plasma-derived HB vaccine.<sup>14</sup> The use of recombinant HBsAg, however, may only explain the predominance of IgG2 following vaccination, but not during infection. Apart from the possible effects of the type of HBsAg, there remains two other possibilities which may contribute to the shift of anti-HBs antibody response towards the IgG2 subclass. Both of these possibilities are associated with the methodology of measurement of the anti-HBs antibody. Since, no standard anti-HBs preparation with known concentrations of all IgG subclasses is available, quantitation of different subclasses of anti-HBs is almost impossible. This has prompted most investigators to use crude absorbance data, titration values or values extrapolated from irrelevant dose-response curves for measurement.<sup>8,9,14-16</sup> Person and colleagues<sup>8</sup> developed an assay to

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quantitate anti-HBs titre, based on OD values obtained from a standard curve constructed using known input of IgG1 human monoclonal anti-HBs antibody. This measurement, however, was confined to the predominant IgG1 subclass.

Considering this limitation in quantitation of the isotype or subclass of anti-HBs antibody, and the fact that a variety of isotype specific MAbs are used in different studies, determination of reactivity of the MAbs is crucial to validation of the results. This important point has not been taken seriously in most other studies. Of the anti isotype antibodies employed in this study, anti-IgA (2D7) displayed better reactivity than anti-IgM or anti-IgG. Therefore, inability to detect anti-HBs antibody of the IgA isotype in both subject groups is not related to the binding activity of the MAb. Similarly, low level expression of IgG3 within the purified anti-HBs preparations does not reflect lower reactivity of the corresponding antibody. Indeed, the monoclonal anti-IgG3 and anti-IgG4 (ZG4 and RJ4+HP6023, respectively) displayed much better binding activities than the anti-IgG2 or anti-IgG1 antibodies employed in this study (Fig. 4). The latter two MAbs overlapped along all points of the titration curves. Thus, predominance of IgG2 is actually magnified, taking into consideration the fact that the anti-IgG2 antibody (Gom2) displayed lower binding activity than the anti-IgG3 or anti-IgG4 antibodies. The second possibility lies in the use of affinity purified anti HBs preparations, rather than the crude serum. HBsAg has long been known to bind to some serum proteins, including, albumin, transferrin, lactoferrin and immunoglobulin.<sup>17,18</sup> In recent years this list has been extended to other proteins, such as apolipoprotein or  $\gamma$  2-glycoprotein and Annexin V.<sup>19-22</sup> Such a diverse binding activity, may facilitate non-specific binding of a variety of serum proteins and particularly non-immune Ig to HBsAg, resulting in variations in measurement of IgG subclasses, depending on the serum samples being tested. Obviously, affinity purification of anti-HBs antibody will eliminate a large proportion of serum impurities and concentrate the anti-HBs content, giving a more clean assay with less interferences. To our knowledge, the present study is the first to use affinity purified anti-HBs antibody for isotypic and subclass analyses. Some environmental factors such as nutrition may also influence the IgG subclass profile of the anti-HBs antibody response. For example, human milk has been shown to modulate the IgG subclass response to hepatitis B vaccine in breast fed infants towards the IgG2 subclass,<sup>15</sup> Though such factors may not be applicable in justifying the predominance of IgG2 in adult infected individuals.

Our results regarding the similarity of the IgG subclass response between the naturally infected and vaccinated individuals, also differ from previous reports. While IgG1 and IgG3 have been demonstrated to dominate the anti-HBs response following infection<sup>7,10,23</sup> IgG1 and IgG4<sup>8,9,13,23</sup> or IgG1 and IgG2<sup>14,16</sup> have been reported as the major subclasses of anti-HBs following vaccination with HBsAg. This discrepancy could also be attributed to the problems described above.

Finally, precise quantitation of the anti-HBs isotype or subclass may only be achieved if human monoclonal anti-HBs antibodies of different isotypes and subclasses are available. Some of these monoclonal antibodies, though of limited isotypes and subclasses have recently been produced.<sup>24</sup> We are currently embarking on the production of such antibodies, using both EBV immortalization and hybridoma production (unpublished data).

### Acknowledgements

*We are grateful to Dr. M. Goodall from Birmingham University, U.K., for providing some of the monoclonal anti-isotype antibodies. Thanks are also due to S. Gharagozloo for technical assistance. This study was supported in part by a grant from the Blood Transfusion Center of Iran*

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