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# Anti-pre-S antibodies in different groups of patients with hepatitis B virus infection

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**Abstract** The anti-pre-S antibody in the samples of sera from normal healthy persons and patients with different clinical types of liver diseases due to hepatitis B virus (HBV) infection was detected by a newly established enzyme-linked immunosorbent assay technique. This test is a blocking assay where anti-pre-S antibody in the patient's serum blocks subsequent addition of horse radish peroxidase-labelled polymerized human serum albumin (pHSA) to the pHSA-receptor site of HBsAg molecules fixed on a solid surface. Anti-pre-S activity was not detected in any from 95 healthy persons who were negative for all HBV-markers or from 105 healthy HBV carriers. In 12 sera from HBV vaccine recipients, anti-pre-S activity was noted in higher proportions compared with anti-HBs, after both the second and third doses of vaccine. Anti-pre-S activity was detected in small proportions of HBsAg positive sera from acute viral hepatitis (4.2%) and chronic active hepatitis (10%). In subacute viral hepatitis patients, the anti-pre-S antibody was totally absent. However, anti-pre-S activity was recorded in high proportions of HBsAg-positive sera from patients with cirrhosis of liver (57.2%) and fulminant hepatitis (41.6%). The anti-pre-S antibodies were assumed to be implicated in the clearance of HBV particles from circulation without causing tissue damage.

**Key words:** antibody, anti-pre-S antibody, hepatitis B virus, polymerized human serum albumin.

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## INTRODUCTION

Recent investigations have improved significantly knowledge of the molecular structure of hepatitis B virus (HBV) and its replication. The genetic structure of HBV-DNA has been characterized by molecular cloning and sequence analysis of the HBV genome.[1,2] Four major open reading frames (S, C, P and X) located on the long or minus (−) strand of the HBV genome were found to carry the total protein coding capacity of the virus. The S-region that encodes proteins of viral envelope is preceded by a small region, called the pre-S gene. The pre-S gene comprises two subregions, pre-S1 and pre-S2, which encode the polypeptides pre-S1 protein and pre-S2 protein.[3] Recent reports have demonstrated that the pre-S1 protein is a marker of HBV replication[4] and that the pre-S2 protein contains a binding site for polymerized human serum albumin (pHSA) which assists HBV particles to attach to the membrane of hepatocytes.[5,6]

Preliminary data suggest that the host immune response against pre-S proteins is protective against HBV infections.[3] Antibodies against pre-S proteins are considered to play an important role in the clearance of HBV particles from the circulation.[7] Limited data on anti-pre-S antibodies in different clinical types of hepatitis due to HBV infection are available. This paper describes a new enzyme-linked immunosorbent assay (ELISA) technique developed to test anti-pre-S antibodies in serum and reports their prevalence in different clinical types of liver disease due to HBV infection.

## METHODS

### Human sera

Serum specimens were collected from 95 healthy subjects, 12 patients with fulminant hepatitis (FH), 24 patients with acute viral hepatitis (AVH), four patients with subacute hepatic failure (SAHF), 10 patients with chronic active hepatitis (CAH) and 14 patients with cirrhosis of the liver (C). Sera from 105 healthy HBV carriers and 12 HBV vaccine recipients (after their second and third doses of vaccine; HB-Vax, Merck, Sharp and Dohme, USA), that is, after 1 month and 6 months, were also screened. The sera samples were stored at −70°C. The diagnosis of each group of patients was confirmed clinically, serologically and, where necessary, by histology as described previously.[8]

### Serological assays

Serum specimens were tested for HBsAg by the micro-ELISA method.[9] IgM anti-HBc and anti-HBs were determined by the enzyme immunoassay kits from Abbots, USA. HBV-specific DNA-polymerase was tested by the method of Fang *et al.*[10]

### HBsAg particles

HBsAg-particles enriched in pHSA-receptors were purified from the pooled sera of HBeAg positive HBV carriers according to the method described by Vyas *et al.*[11] In order to deprive HBsAg particles of pHSA-receptors without affecting their antigenicity, the proteolytic digestion of HBsAg was performed by mixing HBsAg solution (800 ng/ml) with Trypsin enzyme (20 μg/ml; Sigma) solution in 0.1 mol/l Tris NaCl buffer, pH 7.5, and incubating the mixture for 3 h at 37°C as described by Budkowska *et al.*[5]

### Labelling of horse radish peroxidase with pHSA

The preparation of pHSA was carried out by gluteraldehyde treatment as described previously.[12,13] In brief, 50 mg of human serum albumin (Sigma), dissolved in 2 ml 0.1 mol/l phosphate buffer, pH 6.8, was mixed on an end-to-end mixer with 0.2 ml 2.5 % glutaraldehyde at room temperature (20°C) for 4 h. The mixture was extensively dialysed against 0.1 mol/l phosphate buffered saline (PBS),

pH 7.2, and then certrifuged. The clear supernatant was chromatographed on a Sephadex G-200 column (2 × 85 cm) followed by monitoring of eluant fractions for protein content by the method of Lowry *et al.*[14] The leading protein peak was pooled, concentrated and dialysed against 0.1 mol/l carbonate buffer, pH 9.6, before storing it as pHSA at −20°C. The labelling of horse radish peroxidase enzyme (HRPO) with pHSA was carried out by the two-step glutaraldehyde method described by Engvall.[15] The HRPO–pHSA conjugate was frozen and stored in small aliquots after adding equal amounts of glycerol to it. The final dilution of the conjugate used in the present ELISA was determined by checker-board titrations.

### ELISA procedure

The detection of anti-pre-S antibodies by the present ELISA technique is based on the principle that pretreatment of HBsAg with serum that is positive for anti-pre-S reduces the subsequent binding of HRPO-labelled pHSA to pHSA-receptor-bearing HBsAg fixed on a solid surface. Reduction in binding reflects the measurement of anti-pre-S activity in serum. In the assay procedure, the antigen (HBsAg enriched in pHSA-receptors) was bound to the wells of polystyrene plate (Nunc, Denmark) by adding 50 µl HBsAg solution (1 ng/ml) in carbonate buffer, pH 9.6, to each well. The plate was incubated overnight (18–20 h) at room temperature (20°C) and then washed three or four times with 0.1 mol/l PBS, pH 7.2, containing 0.5% Tween-20 (PBS-T). Additional protein binding sites were saturated by adding 200 µl 0.5% gelatin in PBS, pH 7.2, to each well and incubating the plate overnight at 20°C followed by washing. Before addition to plate, the test serum was treated with receptor-free HBsAg to remove interfering anti-HBs. Serum (100 µl) was mixed with 10 µl of a solution of HBsAg (800 ng/ml) already deprived of pHSA-receptor by proteolytic digestion. This mixture was incubated at 37°C for 4 h as described elsewhere.[16] Finally, 50 µl of this mixture was added to the well and incubated at 37°C for 2 h. The plate was washed as usual followed by the addition of 50 µl of HRPO–pHSA conjugate to each well. After an incubation of 2 h at 37°C, the washing cycle was repeated and colour was produced by adding 50 µl of a freshly prepared solution of *o*-phenylenediamine (0.4 mg/ml) in 0.1 mol/l phosphate citrate buffer, pH 5.0, containing 0.06% $H_2O_2$ to each well and incubating the plate in darkness at 20°C for 15 min. The enzyme reaction was stopped by adding 50 µl of 3 N $H_2SO_4$. The absorbance was measured at 492 nm with an ELISA reader. Serum samples from healthy persons without markers of HBV infection served as controls for the test. The result was expressed by the percent inhibition:

$$I\,(\%) = \frac{(D_{\text{control}}) - (D_{\text{sample}})}{(D_{\text{control}})} \times 100$$

where $D$ is the optical density.

The test serum causing more than 50% inhibition was considered to be positive for anti-pre-S. Where necessary, serum anti-HBs interfering in the assay system was neutralized as described by Okamoto *et al.*[16] before testing serum for anti-pre-S activity.

## RESULTS

The specificity of binding between HBsAg enriched in pHSA-receptor and pHSA in the present ELISA technique was demonstrated by the blocking of the binding after pretreating HBsAg with unlabelled pHSA (Fig. 1). Presence of anti-HBs in serum was found to inhibit the interaction between HBsAg and pHSA–HRPO conjugate. A relation between the anti-HBs level in serum and its inhibitory action is shown (Table 1). Anti-HBs was inhibitory only when present in serum at a titre value of more than 1 : 3200. Furthermore, the inhibition was reversed by treating sera with receptor-free HBsAg, without an apparent effect on anti-pre-S activity. Pre-

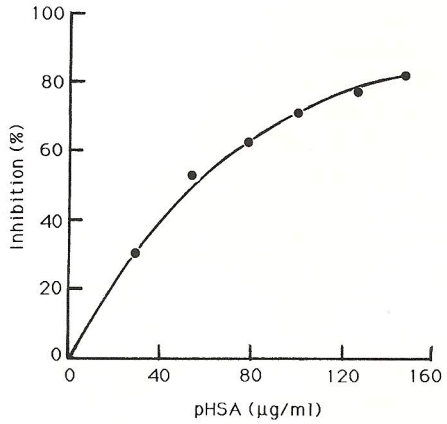


**Figure 1** pHSA solution (50 µl) of varying concentrations was pre-incubated with HBsAg coated on microtitre plate before the addition of pHSA-HRPO conjugate. The *D* value in well without added pHSA was used as control to calculate inhibition (%).

**Table 1** Effect of serum anti-HBs level on the binding between HBsAg and pHSA-HRPO conjugate

| Serum level of anti-HBs (titre) | Inhibition of binding (%) |
|---|---|
| 1 : 400 | 0 |
| 1 : 800 | 0 |
| 1 : 1600 | 0 |
| 1 : 3200 | 0 |
| 1 : 6400 | 20 |
| 1 : 12 800 | 53 |

Six sera samples with above titres values of anti-HBs and free from anti-pre-S activity were pre-incubated with HBsAg fixed on a solid surface before adding pHSA-HRPO conjugate and after washing the rest of experiment was performed as described above. Inhibition (%) was calculated in reference to the *D* values obtained with conjugate only, without pretreatment with anti-HBs positive sera.

treatment of four sera having more than 1 : 3200 titre of anti-HBs (1 : 4800, 1 : 6400, 1 : 6400 and 1 : 12 800 titres, respectively) and positive for anti-pre-S, with receptor-free HBsAg abolished anti-HBs activity completely without reducing anti-pre-S activity. It also indicates that this concentration of receptor-free HBsAg is sufficient for complete neutralization of anti-HBs with a 1 : 12 800 titre value rarely observed in patients' sera. Since anti-HBs sometimes interferes even at low titre values, however, each serum in the present study was essentially preabsorbed with receptor-free HBsAg before analysing for anti-pre-S activity.

An analysis of anti-pre-S antibodies in the sera from healthy persons and patients groups of HBV infections are shown (Table 2). None of the sera from healthy persons was positive for anti-pre-S antibody. Similarly, anti-pre-S antibody was not detected in any of the 105 samples of sera from healthy HBV carriers. In HBV vaccine recipients, a good immune response against pre-S protein was observed. Of 12 HBV vaccine recipients, anti-pre-S could be detected in nine after the second dose (1 month) and in all the 12 cases after the third dose (6 months) of vaccine. In all vaccine recipients, immune response against pre-S proteins was stronger than HBsAg, as indicated by the comparatively low positivity of anti-HBs both after the second dose as well as the third dose of vaccine.

The prevalence rate of anti-pre-S antibody in HBsAg positive sera from subacute AVH (SAVH), AVH, and CAH was quite low (Table 2). However, in C patients positive for HBsAg, anti-pre-S could be detected in significantly high proportions, that is, eight of 14 sera. Similarly, in HBsAg positive sera from FH patients, anti-pre-S positivity was observed in a comparatively high proportion (41.6%). Except for AVH and FH, in each one of the disease groups anti-HBs was present in a higher percentage of cases.

## DISCUSSION

The ELISA technique presently discussed is a simple and specific assay system that detects anti-pre-S antibody in the blood. The specific binding between HBsAg and the pHSA-HRPO conjugate is evident from the blocking of binding after pretreatment of HBsAg with unlabelled pHSA. The high specificity of this technique to detect anti-pre-S is reflected by the absence of anti-pre-S

**Table 2** HBV markers in sera from groups of healthy persons, vaccine recipients and different HBV infected patients

| Groups | No. | HBsAg positive | IgM anti-HBc positive | Anti-HBs positive | Anti-pre-S positive |
|---|---|---|---|---|---|
| Healthy controls (Indians) | 60 | 0 | ND | 0 | 0 |
| Healthy controls (Norwegian) | 35 | 0 | ND | 0 | 0 |
| Healthy HBV carriers | 105 | 105 | ND | 1(1%) | 0 |
| HBV vaccine recipients | | | | | |
| after second dose | 12 | 0 | 0 | 4(33%) | 9(75%) |
| after third dose | 12 | 0 | 0 | 10(83.3%) | 12(100%) |
| SAHF | 4 | 4 | 4 | 1(25%) | 0(0%) |
| AVH | 24 | 24 | 24 | 2(8.4%) | 1(4.2%) |
| CAH* | 10 | 10 | 7 | 6(60%) | 1(10%) |
| C | 14 | 14 | 9 | 7(50%) | 8(57.2%) |
| FH | 12 | 12 | 12 | 1(8.3%) | 5(41.6%) |

*All 10 patients with CAH were cases of hepatitis B proved by histology and serology.
ND: not done.

antibodies in normal samples of sera from healthy persons.

The present ELISA method is based on the principle that anti-pre-S blocks the pHSA-receptor on HBsAg and thus reduces the binding of pHSA–HRPO to that site. On analysing the effect of anti-HBs on the binding between pHSA and HBV particles, it was observed that anti-HBs inhibits the binding of pHSA–HRPO to HBsAg at relatively high concentrations (over a titre value of 1 : 3200). This inhibition by anti-HBs was reversed by pre-incubating it with HBsAg free of pHSA-receptor. However, anti-pre-S activity remained unchanged when sera positive for anti-pre-S but negative for anti-HBs were pre-incubated with pHSA-receptor-free HBsAg, before assaying for anti-pre-S activity. This stresses the high specificity of the assay system. In fact, anti-HBs has its site on the polypeptide region encoded by the S-gene, which is away from the pHSA-receptor encoded by the pre-S gene. The inhibitory effect of anti-HBs only at higher levels has also been demonstrated by Okamoto *et al.*[16] However, it is always advisable to treat sera with pHSA-receptor-free HBsAg before analysing for anti-pre-S, as in certain cases traces of anti-HBs may interfere in the assay system. In this study each serum was first treated with receptor-free HBsAg before assaying for anti-pre-S activity.

The results of the present study show that anti-pre-S antibody appears earlier than anti-HBs after HBV infection. This is supported by the findings of Milich *et al.* that immune response to the pre-S region and S-region are regulated independently.[17] In HBV vaccine recipients, anti-pre-S was detected in 75%, compared with 33% of cases of anti-HBs after the second dose of vaccine and in 100% compared with 83.3% of cases of anti-HBs after the third dose of vaccine. The overall prevalence of anti-pre-S in these recipients remained unchanged on treating their sera with HBsAg devoid of pHSA receptor before assaying for anti-pre-S activity. The early appearance of anti-pre-S is an agreement with the findings of Okamoto *et al.*[16] However, Okamoto *et al* did not find a host immune response in the vaccine recipients, which was recorded by the present study. The HBV vaccine used by them was purified from the HBeAg positive sera of HBV carriers. The total lack of pHSA-receptor or loss of antigenicity of pHSA-receptor during the preparation of this vaccine appears to be the probable reason for the absence of host immune response. The present study used HB vaccine (HB-Vax) from Merck, Sharp and

Dohme, USA. This was found to bind efficiently with pHSA-HRPO conjugate when used to coat the plate, thus showing the presence of intact pHSA-receptors in the vaccine. A strong immune response in HB-Vax recipients and the presence of intact pHSA-receptor in this vaccine confirm the findings of Sansonno *et al.* that HB-Vax contains receptor for pHSA despite treatment with protease, urea, and formaline during manufacture of the vaccine.[18]

The results of anti-pre-S analysis in healthy normal controls, asymptomatic HBV-carriers and different groups of patients are shown (Table 2). Anti-pre-S was totally absent in 105 HBV carriers. These results are in agreement with those of Okamoto *et al.* and show a very low prevalence of anti-pre-S in carriers.[16] Since anti-pre-S antibodies are assumed to be implicated in the immunological clearance of HBV particles from circulation,[19,20] the lack of these antibodies in carriers is one of the possible reasons for the persistence of HBV particles with continuous multiplication. This is evident by the presence of HBV specific DNA-polymerase in more than 50% cases of these carriers (unpubl. data). The low prevalence of both anti-HBs as well as anti-pre-S in HBV carriers may be due to the poor host immune response against HBV envelope proteins. The test of anti-pre-S in the HBsAg positive sera from acute viral hepatitis again shows a very low prevalence of this antibody in these groups. Since anti-pre-S antibodies usually develop after the disappearance of pre-S proteins[5] and only rarely do pre-S antigen and anti-pre-S antibodies occur together, there is a high possibility of the presence of pre-S proteins in these sera from AVH, SAHF and CAH, negative for anti-pre-S antibody. The presence of pre-S proteins is further confirmed by the positive pHSA binding activity in significantly higher proportions of these sera, as reported in earlier studies.[6,13] Thus, low anti-pre-S positivity in these sera can be explained as being due to a high prevalence of pre-S proteins. In cirrhosis patients, the presence of anti-pre-S in higher group is possibly the sequela of the typical reported pattern of anti-pre-S appearance where anti-pre-S appear in a biphasic pattern, that is, early in the course of acute type B hepatitis and then during the convalescence phase.[16] Thus, anti-pre-S present during cirrhosis is the persistent antibody produced in the late phase of acute infection.

An analysis of 12 serum samples from patients with FH demonstrated a low prevalence of anti-HBs (8.3%). However, the prevalence rate of anti-pre-S antibody in these patients was 41.6%. The low anti-HBs positivity in these patients contradicts the earlier reports where the high prevalence of anti-HBs was demonstrated in FH patients.[21,22] The results of high anti-pre-S positivity, however, are similar to those reported by Theilmann *et al.*[23] These findings indicate that patients with FH show a good immune response to pre-S proteins resulting in an early appearance of anti-pre-S antibodies. Low anti-HBs positivity shows that in this group of FH patients, a good immune response is present exclusively to pre-S proteins and not to HBsAg. This is further supported by the findings of Milich *et al.* that immune responses against pre-S proteins and HBsAg are regulated independently.[17]

It has been suggested that, because it appears late, often weeks after clearance of HBV virions, anti-HBs is involved in the pathogenesis of liver and not the clearance of HBV particles from circulation.[24] On the other hand, anti-pre-S plays a role in the clearance of HBsAg and not the pathogenesis of liver. Therefore, an hypothesis can be made about the roles of these antibodies in immune mechanism during HBV infection. Anti-pre-S appears very early and forms a complex with HBsAg. This complex is cleared by the type III hypersensitivity reactions without damaging tissue. In contrast, anti-HBs forms a complex that is deposited on tissue surface leading to its damage by type II–IV hypersensitivity reactions. Thus, clear-

ance of HBsAg by anti-HBs takes place by the damage of infected tissue. This hypothesis needs experimental investigation.

## CONCLUSIONS

It is concluded that in all those patients or vaccine recipients who show a normal immune response to pre-S proteins, anti-pre-S antibody is developed earlier than anti-HBs. Anti-pre-S clears HBV particles from circulation by direct neutralization of HBV particles and thus its action appears different from that of anti-HBs which is involved in the pathogenesis of liver damage and thus removes HBV from circulation by damaging HBV infected liver cells by an immune mechanism. The absence of anti-pre-S in the patients allows HBV particles to persist uninterrupted. For effective protection against HBV infection, the HB vaccine used must be enriched in the pre-S proteins to induce anti-pre-S antibodies.

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