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Anti-pre-S(2) analysis after hepatitis B vaccination in haemodialysis patients

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Summary

We investigated the development of anti-pre-S(2) antibodies by enzyme immunoassay and by Western blot analysis in a vaccination study in haemodialysis patients; both the Pasteur plasma vaccine retaining the pre-S(2) epitopes and the Merck, Sharp and Dohme (MSD) plasma vaccine containing only HBsAg were used. By enzyme immunoassay (EIA), one anti-pre-S(2) response at month 7 was registered in 23 patients after 5 injections with the 5 μg dose of Pasteur vaccine (PS group), whereas 6 responses were seen in 20 patients after 4 injections with the 10 μg dose (PD group). None of the 22 vaccinees injected with MSD vaccine (40 μg) (4 injections, MD group) showed an anti-pre-S(2) response at month 7. In the Western blot an anti-pre-S(2) response was seen in 12 PS patients, in 8 PD patients and in none of the MD patients. Anti-pre-S(2) responses were predominantly observed in patients with a high anti-HBs response but exceptions occurred. Prevaccination anti-pre-S(2) positivity, in the absence of anti-HBc and anti-HBs, was detected in dialysis patients with EIA as well as Western blot, in 10.8 and 21.6%, respectively; similar findings were made in health care personnel. The possible nature of this phenomenon is discussed. In this study the Western blot technique has been shown to be a suitable test system for qualitative and semi-quantitative analysis of anti-pre-S(2) antibodies after hepatitis B vaccination with a higher sensitivity, but probably also a different specificity than the EIA.

Hepatitis B vaccination; Dialysis; Anti-pre-S(2); EIA; Western blot

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Introduction

Hepatitis B vaccination is the most powerful means of preventing hepatitis B infection. However, in one group at risk of hepatitis B infection, i.e. the haemodialysis patients, vaccination proved only to be effective with the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB) vaccine and the Pasteur vaccine (Crosnier et al., 1981; Desmyter et al., 1983) but not with the most widely used Merck, Sharp and Dohme (MSD) vaccine (Stevens et al., 1984).

The purification and inactivation processes differ for these three vaccines (Adamowicz et al., 1981; Reerink-Brongers et al., 1982; Tabor et al., 1983), particularly the purification process for the two vaccines inducing protective immunity does not comprise a pepsin treatment. Therefore, the CLB vaccine and the Pasteur vaccine may elicit antibodies against epitopes not present in the MSD vaccine (Neurath et al., 1986a).

The hepatitis B virus coat consists of three proteins, the major protein (S-protein, 24/27 kDa), the middle protein (pre-S(2) protein, 33/36 kDa) and the large protein (pre-S(1) protein, 39/42 kDa) (Heermann et al., 1984; Neurath et al., 1985a). Both pre-S(1) and pre-S(2) proteins may play a role in the attachment of the virus to the HBV-specific receptor on the hepatocyte membrane (Neurath et al., 1986b). Therefore, the production of anti-pre-S(2) antibodies by the pre-S(2) antigens in the CLB and the Pasteur vaccine might have supplemented the anti-S response and might have added to the efficacy of these vaccines in haemodialysis patients.

This theory has not been well substantiated by facts, due to the limited experience with anti-pre-S(2) measurements in haemodialysis patients and the uncertainty about the influence of the test principle on the outcome of the tests.

We have evaluated the immune response to the pre-S(2) protein in the Pasteur vaccine in sera from dialysis patients by a sandwich enzyme immunoassay (EIA) and by Western blot analysis. Sera from haemodialysis patients injected with MSD vaccine were tested for comparison.

Materials and Methods

Haemodialysis patients (32 male, 33 female) were stratified for age and randomized to receive 5 μg doses of Pasteur vaccine (N=23) at month 0,1,2,4,6 and 12, 10 μg doses of Pasteur vaccine (N=20) at month 0, 1, 2 and 6, or 40 μg doses of the Merck, Sharp and Dohme (MSD) vaccine (N=22) at month 0,1,2 and 6. The mean age of the participants was 57.3±14.2 years.

Sera were obtained at month 0,1,2,3,4,6,7,11 and 13 and denoted as PS (single dose: 5 μg dose, Pasteur vaccine), PD (double dose: 10 μg dose, Pasteur vaccine) or MD (double dose: 40 μg dose, MSD).

All sera were initially tested for HBsAg, anti-HBc and anti-HBs by radioimmunoassays (AUSRIA II, CORAB, AUSAB, Abbott Laboratories). All sera were negative for these markers at month 0. Anti-HBs was quantitated with the aid of

a reference panel derived from the WHO standard reference serum (CLB, Amsterdam, The Netherlands). Details on patients and anti-HBs response after vaccination will be published elsewhere (Smit-Leijs et al., 1989).

Prevaccination sera from thirty health care workers, negative for anti-HBc and anti-HBs, served as a control for anti-pre-S(2) detection by EIA and Western blot analysis in prevaccination sera.

Anti-pre-S(2) enzyme immunoassay

All sera from month 0 and 7 were tested in a sandwich enzyme immunoassay provided by Dr. Ph. Adamowicz, Pasteur Vaccins, France (Alberti et al., 1988; Coursaget et al., 1988a). The solid phase consisted of a synthetic peptide (pre-S(2), residue 120–150), with a lysine as residue 150 in stead of threonine in the protein). Captured anti-pre-S(2) antibodies from serum were recognized by a monoclonal antibody (anti-human IgGl, covalently linked to horse-radish peroxidase). *O*-phenylenediamine (OPD) was used as a substrate. Absorbance was read in a photometric strip reader at 492 nm. A reference panel was added to the test. The cut-off level was calculated at 4/30 of the absorbance value at 300 mU/ml derived from the linear regression equation for the reference panel sera. This cut-off level calculation was based on the negative control value + 2.1 SD for a large number of sera (> 200) without hepatitis B parameters. Sera were tested undiluted. Results above the cut-off level were denoted as anti-pre-S(2) positive. An anti-pre-S(2) response was defined as seroconversion from month 0 to month 7 or a more than twofold increase in titre. Sera from vaccinees with an anti-pre-S(2) positivity at month 0 and/or month 7 were investigated longitudinally.

Western blot analysis

HBsAg purified from the cell culture supernatant of the Chinese Hamster Ovary (CHO) cell line transfected with HBV/ayw (Michel and Tiollais, 1984), kindly provided by Dr. Ph. Adamowicz (Institut Pasteur, Paris, France), was used in SDS-polyacrylamide gel electrophoresis (12.6% acrylamide; 16 μg HBsAg/cm gel), according to Laemmli (1970). The same batch of antigen was used in all experiments. Electrophoretic separation was followed by Western blotting, essentially according to Towbin et al. (1979). The nitrocellulose membrane was cut into 0.5 cm strips. To limit the amount of serum to be used (standard dilution 1:3), the relevant part of the strip was cut out and used for antibody detection. Antibody binding was demonstrated by horse-radish peroxidase-labeled polyvalent goat anti-human IgG and 4-chloro-1-naphtol (Biorad Laboratories, Richmond, CA) as a substrate. Rabbit anti-HBs (CLB) was used to mark the position of the S protein bands (22/26 kDa) and the anti-pre-S(2) band (34 kDa). A minor band at 30 kDa was present which may have been a partially glycosylated pre-S(2) protein (Michel and Tiollais, 1984). An anti-pre-S(2) response upon vaccination was defined as a seroconversion at the 34 kDa position from month 0 to month 7 or a marked increase in stain at the 34 kDa position on the blot (Fig. 1) in the absence of sero-

conversion/titre increase at the 22/26 kDa position (antibodies against linear S-epitopes; (see 'Specificity determination (Western blot)' in Results). From a limited number of vaccinees with a positive pre-S(2) response (34 kDa band) at month 7 sera were investigated longitudinally.

Results

Anti-pre-S(2) by enzyme immunoassay

Before vaccination with the plasma-derived vaccines, 7 of the 65 patients in this study (10.8%, 95% confidence interval (CI): 3.3–18.3) were anti-pre-S(2) positive. In prevaccination sera from thirty health care workers anti-pre-S(2) positivity was detected in ten cases (33.3%, 95% CI: 17.6–49.0).

Only one of the 23 patients receiving the 5 μg dose (PS group) of Pasteur vaccine seroconverted from month 0 to month 7 (Table 1). In all four patients with anti-pre-S(2) positivity at month 0 the level of anti-pre-S(2) positivity remained constant from month 0 to month 13. Among the patients vaccinated with the 10 μg dose (PD group) five patients seroconverted: two at month 1, two at month 2 and one at month 3. In serum from one of the two patients with anti-pre-S(2) positivity at month 0, an increase (2.2 ×) of anti-pre-S(2) positivity was seen at month 7. After month 7 anti-pre-S(2) positivity decreased rapidly in the four patients who could be tested at month 11 and 13.

Anti-HBs (by AUSAB) was not detected in month 1 sera but occasionally in month 2 sera (PS: 3/23; PD: 7/20). In the PD group all anti-pre-S(2) responders as registered at month 7 already had anti-HBs at month 2. At month 7 all sera with an anti-HBs level above 2500 IU/l (range 2745-19970 IU/l) were anti-pre-S(2) positive. In the PS group no anti-HBs levels above 1800 IU/l were observed at month 7.

None of the patients vaccinated with MSD vaccine showed anti-pre-S(2) positivity at month 7.

TABLE 1

Anti-pre-S(2) response in EIA for dialysis patients vaccinated with Pasteur vaccine (5 or 10 μg) and MSD vaccine (40 μg)

Vaccine	Anti-pre-S(2) positive		Response	
	Time after vaccination		Seroconversion	Increase titer (>2×)
	Month 0	Month 7		
Pasteur, 5 μg[a]	4/23	5/23	1/19	0/4
Pasteur, 10 μg[b]	2/20	7/20	5/18	1/2
MSD, 40 μg[b]	1/22	0/22	0/21	0/1

[a]Vaccination at month 0,1,2,4,6 and 12.
[b]Vaccination at month 0,1,2 and 6.

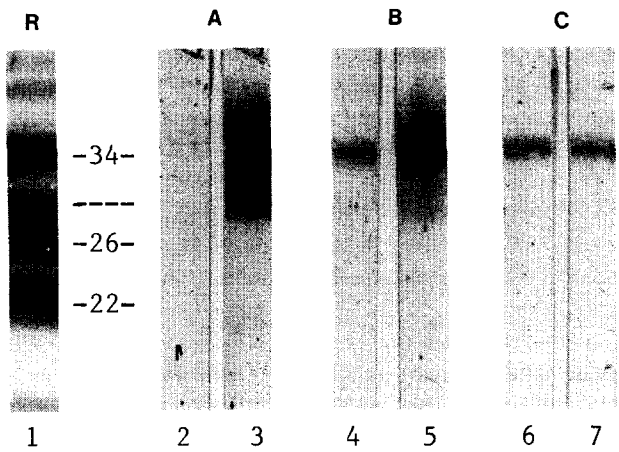

Fig. 1. Western blot analysis with CHO cell line HBsAg in sera from haemodialysis patients vaccinated with hepatitis B vaccine (Pasteur, PS group). Lane 1: Rabbit anti-HBs; lanes 2–7: patient's sera at month 0 (lanes 2, 4 and 6) and month 7, (lanes 3, 5 and 7). Three types of response: seroconversion (A), titre increase (B), and status quo (C).

Anti-pre-S(2) by Western blot analysis

In the PS group eight patients had seroconverted at month 7 (Fig. 1, lanes 2 and 3) and 4 of the 5 patients with an anti-pre-S(2) positivity at month 0 showed an increase of stain at the 34 kDa position in the Western blot at month 7 (Fig. 1, lanes 4 and 5). In one patient the level of anti-pre-S(2) positivity remained constant (Fig. 1, lanes 6 and 7).

After the 10 μg dose Pasteur vaccine injections, 8 dialysis patients seroconverted between month 0 and month 7. Three patients in this group (PD) showed anti-pre-S(2) positivity at month 0, which was unchanged at month 7 (Table 2). From the patients receiving MSD vaccine six were anti-pre-S(2) positive at month 0 and month 7 and no anti-pre-S(2) response was observed.

In the control group of thirty health care workers four cases (13.3%, 95% CI:

TABLE 2

Anti-pre-S(2) response in Western blot assay for dialysis patients vaccinated with Pasteur vaccine (5 and 10 μg) and MSD vaccine (40 μg)

Vaccine	Anti-pre-S(2) positive		Response	
	Time after vaccination		Seroconversion	Increase titer
	Month 0	Month 7		
Pasteur, 5 μg[a]	5/23	13/23	8/18	4/5
Pasteur, 10 μg[b]	3/20	11/20	8/17	0/3
MSD, 40 μg[b]	6/22	6/22	0/16	0/6

[a]Vaccination at month 0,1,2,4,6, and 12.
[b]Vaccination at month 0,1,2 and 6.

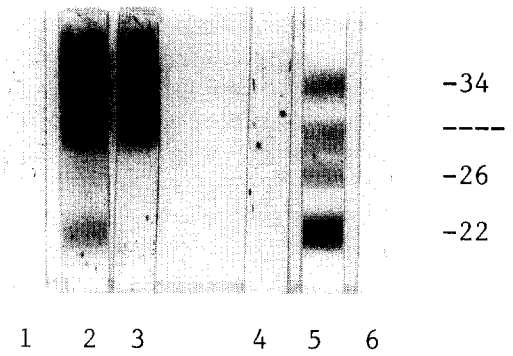

Fig. 2. Determination of the S protein specificity in sera from haemodialysis patients after hepatitis B vaccination. Lanes 1–3: PD group, lanes 4–6: MD group). Lanes 1 and 4, month 0 sera; lanes 2 and 5, month 7 sera; lanes 3 and 6, month 7 sera after pre-incubation with pepsin-treated HBsAg (devoid of pre-S(2) sequences).

1.2–25.4) of low-titre anti-pre-S(2) positivity were registered as compared to 14 cases (21.5%, 95% CI: 11.5–31.5) in month 0 sera from the 65 dialysis patients.

Specificity determination (Western blot)

Patients with high levels of anti-HBs (>2000 IU/l) sometimes showed a 24 and/or 27 kDa reaction in the Western blot. This immune reactivity was presumably directed against the linear epitopes of the S-protein and could also add to staining at the 34 kDa position. After preincubation with HBsAg/ad without pre-S(2) antigen (pepsin-digested HBsAg, kindly provided by Dr. G. Wolters, Organon, Oss, The Netherlands) the reactivity at the position of the S-protein disappeared without detectable reduction of the reactivity at the 34 kDa position (Fig. 2, lanes 2 and 3). This illustrates the non-S-protein character of the immune reaction at the 34 kDa position. Preincubation of the sera with CHO cell line HBsAg (not pepsin-digested) resulted in the disappearance of immune reactivity at the 22/26 kDa as well as the 30/34 kDa position (results not shown).

Sera from the MD group with high levels of anti-HBs were characterized by an immunoreactivity at the 22/26 kDa as well as the 30/34 kDa position with emphasis on staining at the 22 kDa position on the blot. After preincubation of these sera with HBsAg/S-protein, the immunoreactivity at the 22/26 kDa as well as the 30/34 kDa position completely disappeared (Fig. 2, lane 5 and 6). This confirms the S protein character of the immune reactivity at the 30/34 kDa position after MSD vaccine injections.

In one experiment we found that preincubation of the blot with a 'normal' sheep serum could prevent the appearance of prevaccination anti-pre-S(2) positivity in the three cases that could be investigated. The 'normal' sheep serum which was weakly anti-HBs positive by AUSAB, did not prevent the binding of anti-pre-S(2) after vaccination. Further testing was impossible because of lack of this 'normal' sheep serum.

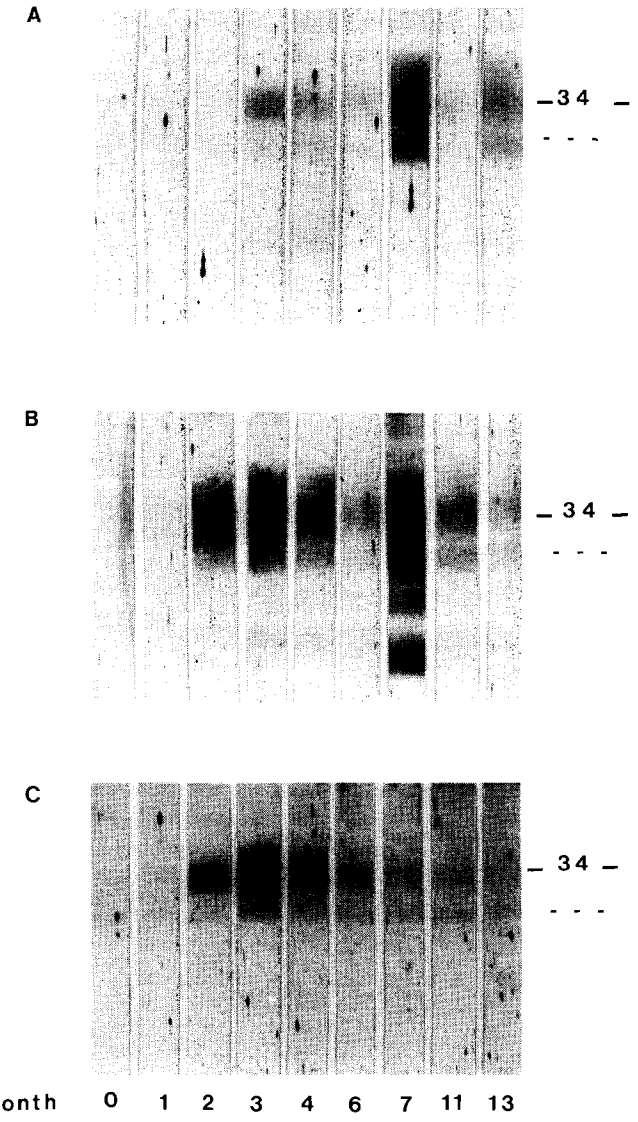

Fig. 3. Anti-pre-S(2) analysis by Western blotting of sequential sera during and after hepatitis B vaccination from groups PS (A), PD (B) and PS (C), respectively. Vaccine injections were at months 0,1,2,4,6 and 12 for PS and at months 0,1,2 and 6 for PD. Serum samples were taken at months 0,1,2,3,4,6,7,11 and 13. Anti-HBs (by AUSAB) in these sera was 0, 0, 2.7, 31, 124, 94, 668, 309 and 8177 IU/l in case A; 0, 0, 3.4, 106, 105, 119, 19972, 2015 and 2163 IU/l in case B; all samples from case C were anti-HBs negative.

Comparison of anti-S and anti-pre-S(2) response (Western blot)

Among the 23 patients in group PS (Table 2) 16 had an anti-HBs and 12 an anti-pre-S(2) response at month 7. Eleven patients were simultaneously anti-HBs and anti-pre-S(2) positive. From the 7 anti-HBs negative patients only one showed an anti-pre-S(2) response. In the PD group 14 anti-HBs and 8 anti-pre-S(2) responses were seen. The eight anti-pre-S(2) positive sera were also anti-HBs positive.

In anti-HBs+/anti-pre-S(2)+ sera, groups PS and PD combined, a significantly higher geometric mean titre of anti-HBs (486.0 IU/l, 95% CI 163.4–1445.6) was observed than in anti-HBs+/anti-pre-S(2)− sera (23.7 IU/l, 95% CI 10.1–55.3).

Longitudinal analysis of anti-pre-S(2) response during vaccination (Western blot)

Six patients (PS:3; PD:3) with an anti-pre-S(2) response at month 7 were selected for a longitudinal study. Anti-pre-S(2) was first detected at month 1 (PD:1), month 2 (PS:1; PD:1).

Fig. 3 exemplifies the first appearance of anti-pre-S(2) at month 3 after three doses of 5 μg Pasteur vaccine, a booster reaction at month 7 and a second booster reaction at month 13. The second booster reaction seems to be stronger for anti-HBs than that for anti-pre-S(2) antibodies. Fig. 3 shows in a case of the 10 μg dose (PD) vaccination a rapid decrease of anti-pre-S(2) from month 3 to month 6 and from month 7 to month 13.

Maximum intensity of anti-pre-S(2) was seen at month 7. Another patient from the PS group (anti-HBs non-responder) had his maximum anti-pre-S(2) response at month 3 without any further response to the booster doses (Fig. 3).

Comparison of EIA and Western blot analysis

The only patient in the PS group with seroconversion for anti-pre-S(2) in EIA was anti-pre-S(2) negative in Western blotting. The six patients from the PD group with an anti-pre-S(2) response in EIA were also anti-pre-S(2) positive in Western blotting. Prevaccination anti-pre-S(2) positivity in EIA ($N=7$) and Western blotting ($N=14$) was seen in different sera, with two exceptions. In the control group in one case only the anti-pre-S(2) reactivity in EIA ($N=10$) and Western blotting ($N=4$) was observed simultaneously.

Discussion

With a synthetic peptide pre-S(120–150) as a solid phase and a monoclonal second antibody, we registered one (4.3%) anti-pre-S(2) response with the regular 5 μg dose Pasteur vaccine and six (30.0%) responses with the double dose (10 μg) vaccination in haemodialysis patients. No anti-pre-S(2) responses were registered after vaccination with a double dose (40 μg) of MSD vaccine.

The presence of anti-pre-S(2) antibodies after vaccination was also reported by

Neurath et al. (1986a) for medical personnel and haemodialysis patients with Pasteur vaccine and CLB vaccine but not with MSD vaccine. The anti-pre-S(2) antibodies were detected with a synthetic pre-S(120–145) peptide covalently linked to β-lactamase conjugate, the level of which varied by lot, manufacturer and dose of the vaccine. By inhibition enzyme immunoassay Budkowska et al. (1987) found anti-pre-S(2) antibodies in 43.7% of Pasteur-vaccinated adults in France. In theory, this inhibition immunoassay using CHO-HBsAg may be cross-reactive for anti-HBs. Alberti et al. (1988) reported a 50% (10/20) anti-pre-S(2) response after Hevac-B (Pasteur) vaccination in healthy subjects with the same EIA we used. In children and infants anti-pre-S(2) responses to Pasteur vaccine were seen in 2–10% after primary immunization and in 15–71% after the booster dose, again with the same test system we used (Coursaget et al., 1988a).

We extended our search for anti-pre-S(2) antibodies with Western blot analysis since the amino acid sequence of the synthetic peptides in enzyme immunoassays is restricted and may give rise to an underestimation of the anti-pre-S(2) response after natural infection (Alberti et al., 1986) as well as after vaccination (Alberti et al., 1988). In contrast to anti-S and anti-pre-S(1) antibodies, which are largely directed against conformational epitopes, the anti-pre-S(2) antibodies are evoked by linear epitopes (Milich et al., 1985; Heermann et al., 1987). The use of Western blotting appeared to be favourable since it not only confirmed the results from our enzyme immunoassay experiments after the 10 μg dose vaccination but extended the seroconversion rate for anti-pre-S(2) antibodies after the 5 μg dose vaccination [EIA: 4.3% (1/23); Western blotting: 34.8% (8/23)]. An increase of the anti-pre-S(2) titre was observed in four more cases, which brings the response rate up to 52.2%.

The relationship in our study between the prevalence of anti-pre-S(2) in high-titred anti-HBs-positive sera confirms the results of others (Neurath et al., 1986a; Coursaget et al.,1988a). Although the anti-pre-S(2) response and the anti-S response may be regulated separately (Milich et al., 1985; Neurath et al., 1985b) we found among the 14 anti-HBs negative cases (PS+PD) only one example of anti-pre-S(2) positivity by Western blot. The advantage of the pre-S(2) protein to circumvent the anti-HBs unresponsiveness will be a matter of debate (Coursaget et al., 1988b), until the results with the recombinant hepatitis B vaccine based on the CHO cell line with a relative concentration of 35% pre-S(2) protein (Michel and Tiollais, 1984) as compared to the 1–2% in the plasma-derived vaccine become available. Subsequently, evaluation of the anti-pre-S(2) response should be done by a test system which enables detection of the immune response on the full extent of the epitopes specific for the pre-S(2) protein, since our study confirms the limited usefulness of a peptide-based EIA for this purpose. The inhibition immunoassay as described by Budkowska et al. (1987) as well as the Western blot analysis rely on a relatively low anti-HBs titre, otherwise pre-absorption with S protein has to be executed.

In prevaccination sera from dialysis patients and health care workers, an anti-pre-S(2)-like activity was registered in EIA and in Western blot although largely in different sera for the two techniques. These sera were anti-HBs and anti-HBc

negative and none of the individuals concerned developed anti-HBc within the next thirteen months. In our EIA the cut-off level was approximately 40 mU/ml. If we adopt the value of 75 mU/ml from the work of Coursaget et al. (1988a), using the same test system, none of the 30 health care workers will be positive. However, in the dialysis group (N=65) 4 out of the 7 sera with anti-pre-S(2) positivity at month 0 had titres > 75 mU/ml. The use of Senegalese sera in the control group by Coursaget et al. (1988a) is doubtful (Budkowska et al., 1988) and may have raised the cut-off level by including sera with anti-pre-S(2)-like activities.

Hoofnagle et al. (1983) detected naturally occurring IgM-anti-HBs activity in 48% of the sera from 33 species and they suggested a similar reactivity in man. A naturally occurring reactivity directed against the pre-S(2) protein has not been described so far, but it may well exist, since a 'normal' sheep serum in our study could block the anti-pre-S(2) reactivity in prevaccination sera but was not competitive for anti-pre-S(2) antibodies evoked by vaccination in Western blotting.

Budkowska et al. (1988) reported the presence of anti-pre-S(2) as a sole marker of hepatitis B infection in sera from the Ivory Coast and suggested cross-reactivity between the classical HBV and the recently described HBV-2 (Coursaget et al., 1987). Until the anti-pre-S(2) positivity in the sera from the Ivory Coast has been validated, it may be suggested that Budkowska et al. (1988) found a similar type of anti-pre-S(2)-like activity as we did. An anti-pre-S(2)-like activity has also been ascribed to antibodies which interact with the (polymerized) human serum albumin (pHSA) (Hellström et al., 1986). Cross-reactivity between anti-pre-S(2) antibodies and anti-nHSA (natural HSA) seems not likely (Neurath et al., 1988), but a role for anti-polymerized human serum albumin auto-antibodies cannot be excluded (Milich et al., 1981; Nardiello et al., 1985). Finally, a protein similarity search has revealed a probably significant homology between IgA and pre-S(2) sequences (Neurath et al., 1986b).

In summary, several hypotheses on the existence of anti-pre-S(2) like antibodies, with affinity for pre-S(2)-epitopes but not necessarily the same epitopes which evoke virus neutralizing antibodies are still open to investigation.

To circumvent the pitfalls in assessing an anti-pre-S(2) response, we have compared paired sera but we are aware that even this precaution will not exclude a cross-linking anamnestic response to epitopes not related to the hepatitis B virus. The need for further characterization of the pre-S(2)-protein directed antibody response with the aim to detect antibodies with virus neutralizing activity is evident.

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