Mapping of immunodominant B-cell epitopes and the human serum albumin-binding site in natural hepatitis B virus surface antigen of defined genosubtype

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Twelve MAb s were generated by immunization of BALB/c mice with plasma-derived hepatitis B virus surface spherical antigen particles subtype ayw2 (HBsAg/ayw2 genotype D). Their epitopes were mapped by analysis of reactivity with plasma-derived HBsAg/ayw2 and HBsAg/adw2 (genotype A) in enzyme immunoassays and blots. Mapping was supported by nested sets of truncated preS2 proteins and preS2 peptides. Five antibodies were S domain-specific, seven were preS2-specific and 11 had a preference for genotype D. According to our data, group I of the three known epitope groups of preS2 has to be divided into IA and IB. Three preS2-specific MAb s forming the new group IA reacted with genotype D residues 3–15 which have not yet been described as an epitope region. IA antibodies strongly inhibited the binding of polymerized human serum albumin. Two antibodies (group II) reacted with the glycosylated N-terminal region of preS2 in plasma-derived HBsAg, but not with a preparation from transfected murine cells. One group III antibody was subtype-specific and reacted with the highly variable preS2 sequence 38–48. Only one antibody (group IB) mapped to the region (old group I) which was believed to be immunodominant and genotype-independent. Geno(sub)type-specific epitopes of preS2 are obviously the immunodominant components of natural HBsAg in BALB/c mice, but these epitopes may be masked by serum albumins in humans. The data may explain why it is difficult to detect anti-preS2 antibodies in human recipients of preS2-containing vaccines, in spite of the preS2 immunodominance in mice.

Introduction

Hepatitis B virus (HBV)-infected liver cells produce large amounts of non-infectious subviral particles termed hepatitis B surface (HBs) protein spheres or filaments, which are secreted into the blood. HBs particles consist of three co-terminal proteins (large LHBs, middle MHBs and small SHBs). All share a common C-terminal domain, but differ in their N termini. The small HBs protein (SHBs) is most abundant on the surface of virions and in HBs particles. The SHBs protein consists of 226 aa with at least two and probably four transmembrane-spanning alpha helices (Berting et al., 1995). Numerous cross-linked cysteines all lead to a complex tertiary and quaternary structure of this protein. Protective antibodies raised against the SHBs, therefore, are usually directed against conformational epitopes and are not reactive in Western blots (WB). Position Asn-146 is an N-glycosylation site which is linked to a glycan in half of the proteins. Amino acids 120–160 are most exposed on the surface of the particles and determine HBs antigenicity (Ganem, 1996; Kann & Gerlich, 1998). HBsAg exists in different sero-subtypes, which are encoded by six HBV genotypes A–F (Magnius & Norder, 1995; Naumann et al., 1993). SHBs particles of subtype adw2 (genotype A) produced in yeast cells are currently being used as an HBV vaccine in hundreds of millions of people (Ellis et al., 1991).

The MHBs protein has a 55 aa N-terminal extension named the preS2 domain and an S domain which is identical to SHBs. The preS2 domain is hydrophilic and does not contain
HBV genotypes and, in addition, recombinant proteins and isolated MAbs using antigens from the same and from different composition (Schmitt et al., 1999) for immunization. We (Meisel et al., 1994) and others (Howard et al., 1991; Neurath et al., 1986c; Milich et al., 1985; Mimms et al., 1990) have previously mapped several mouse-derived MAbs against the preS2 domain of natural HBsAg particles, but the immunization and selection procedure favoured generation of MAbs against group-specific epitopes (Meisel et al., 1994) or was not well-specified in this respect. Here, we used HBs spheres from one HBV carrier with a well-defined protein composition (Schmitt et al., 1999) for immunization. We analysed the domain and the genotype specificities of the isolated MAbs using antigens from the same and from different HBV genotypes and, in addition, recombinant proteins and peptides. We could assign some preS2 MAbs to the previously defined epitope groups I to III, but the immunodominant preS2 region identified in our study did not map to these groups. The new MAbs may be used for testing the quality of HBsAg preparations and for fine analysis of the immune response against HBs proteins in recipients of preS-containing vaccines.

Methods

HBsAg preparations. For most experiments, HBsAg was isolated from plasma donations from one HBV carrier. Part of the HBsAg/ayw2 preparation and all of the HBsAg/adw2 preparation were made according to Pontisso et al. (1989); later, we used a preparation of HBsAg/ayw2 from another HBV carrier which was purified by a similar but not identical procedure (Tolle et al., 1998). In both cases, combinations of zonal sedimentation and isopycnic centrifugation in CsCl or KBr were used to obtain HBsAg particles which were virtually free of soluble contaminants. The protein composition of the preparations was analysed by SDS–PAGE and silver staining (Fig. 1a). The samples contained ca. 10% HBsAg and 90% LHBs. The sequence of the HBV genome from the donor of HBsAg/adw2 was determined in our laboratory (S. Schaefer; GenBank accession no. X02763). The preS2 and S amino acid sequence was exactly as described by Valenzuela et al. (1981). The preS sequence of the HBsAg/ayw2 isolate was almost identical to the sequence reported by Bichko et al. (1985). In some experiments, we used a preparation of HBsAg particles containing HBsAg/ayw3, which were expressed in transfected C127 mouse cells (Thoma et al., 1990). This antigen was provided by Colin Love, Medeva Pharma, Liverpool, UK, and is being developed as a new hepatitis B vaccine, Hepagene.

Immunization of BALB/c mice and generation of MAbs. Five female BALB/c mice were injected subcutaneously with 100 µg HBsAg/ayw2 in 100 µl PBS mixed with the same volume of complete Freund’s adjuvant. After 6 weeks, each mouse was boosted with 50 µg HBsAg/ayw2 mixed with incomplete Freund’s adjuvant. A second booster was applied 2 weeks later. After another week (3 days before splenectomy), 50 µg HBsAg was injected into the intraperitoneal cavity. Splenocytes were fused 1:1 with the SP 2/0 myeloma line (Köhler & Milstein, 1975) using standard protocols (Köhler & Milstein, 1975). Following HAT selection, the hybridoma supernatants were tested by enzyme immunoassay (EIA) and positive cells were subcloned to obtain MAbs.

Peptides. The peptides covering preS2 sequence 1–26 ayw2, adw2, 1–11 D, ayw2, 1–12 ayw2 and 11–22 ayw2 were kindly provided by M. Bienert (Humboldt University, Berlin, Germany). PreS2 peptides 8–25 ayw2, adw2 and 13–30 adw were synthesized by Promega and A. Tsianis (Institute of Biomedical Research, Riga, Latvia) by the bead-bag solid-phase method using FMOC-protected amino acids (Bachem). Peptides were dissolved in water to a concentration of 2 mg/ml without further purification. Tryptic peptides from the preS2 domain of natural HBsAg/ayw2 particles were prepared as described by Schmitt et al. (1999).

Enzyme immunoassay (EIA). Polysorb microwells with flat bottoms (Nunc) were incubated with 100 ng highly purified HBsAg in 100 µl PBS per well for 12–20 h at 4 °C. After removal of the liquid, 200 µl of 1% BSA/PBS was added to each well for 2 h at 37 °C. Synthetic peptides were coated onto Nunc maxisorb microwells at 10 µg/ml in 0.005 M sodium phosphate buffer, pH 9.6. Tryptic peptides from natural HBsAg particles were diluted 1:50 in PBS and coated onto Nunc polysorb plates. Washing was usually done twice with 0.25% Tween 20/PBS and three times with PBS; for synthetic peptides, 0.05% Tween 20.

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(a) Silver-stained proteins of HBs spheres with genosubtype D/ayw2 (D) and genosubtype A/adw2 (A) after SDS–PAGE. (b) Reaction of MAbs generated in this study and of reference MAbs for LHBs, MHBs and SHBs with the HBs proteins of genotype D or A using WB.

20 in PBS was used. Hybridoma supernatants (100 µl) containing RPMI-1640 medium with 10% foetal calf serum were added to the wells and incubated for 1 h at room temperature. If appropriate, dilutions were made in 0–1% BSA/PBS. After five washings as before, 100 µl peroxidase (POD)-labelled antibody against mouse IgG (affinity-purified from goat or rabbit serum; both from Sigma) diluted 1:1000 in 0–1% BSA/PBS was added to each well for 1 h at room temperature. After five washings, as described above, an o-phenylenediamine/H₂O₂ substrate (tablets from Abbott Laboratories) was added for 15 min at room temperature and the amount of coloured product was measured by A₅₇₀.

**Recombinant preS2 proteins.** For WB, we used frCP-preS2 expression protein libraries constructed on the basis of plasmid pFRd8 and its derivatives. As a source of preS2 fragments, we used plasmids pHB320 and PAE10 containing cloned HBV genomes subtypes ayw2 (Bichko et al., 1985) and adw2 (Meisel et al., 1993), respectively. Fragments of different size were cloned within polylinkers inserted into the frCP gene. Libraries of recombinant frCP-preS2 plasmids were expressed in *Escherichia coli* K802 cells. Bacteria were pelleted, suspended in SDS–PAGE electrophoresis buffer containing 2% SDS and 2% β-mercaptoethanol, and lysed at 100 °C for 5 min.

**Western blotting (WB).** SDS–PAGE was done by standard procedures using a Tall-Mighty-Small chamber (Hoefer). The stacking and separation gels contained 3–25% and 12.5% polyacrylamide, respectively. Purified HBsAg (50 µg) in 50 µl solution was mixed with 5 × Laemmli’s sample buffer and stacking buffer to yield 1% SDS/DTT in 250 µl. Samples were then boiled for 5 min and subjected to electrophoresis in one wide slot for 1.5 h at 100–150 V until bromophenol blue was close to the lower edge of the gel. The separated proteins were blotted onto a PVDF membrane (Millipore) in a semi-dry transversal electroblotting chamber at 8 mA/cm² for 1 h. Every run included one narrow slot with protein markers (Bio-Rad low range). The strip with the protein marker was cut off and stained with Coomassie blue; the membrane with the separated HBs proteins was shaken overnight with 5% milk powder in PBS at 4 °C (Gülden & Heermann, 1988). After three washings with 0.25% Tween 20/PBS, the membrane was cut into 4 mm wide strips. The strips were incubated with 200 µl antibody solution and 25 µl 10% BSA/PBS for 2 h at room temperature. After washing three times with 0–0.5% Tween 20/PBS, the strips were incubated with 500 µl anti-mouse IgG F(ab') fragment conjugated with alkaline phosphatase (Boehringer Mannheim) diluted 1:1000 in 0–1% BSA/PBS. Bound conjugate was detected either with NBT/BCIP substrate (Sigma) or by ECL (Amersham). Reference MAbs were: MA18/7 for LHBs (Heermann et al., 1984); Q19/10 for MHBs (Heermann et al., 1988); and H166 for SHBs (Peterson et al., 1984). WB for frCP-preS2 fusion protein was done as described previously (Meisel et al., 1994). The WB shown in Fig. 5 were not cut into strips and bound antibody was revealed using POD-labelled anti-mouse antibody (Dianova) diluted 1:2000 in 0–1% BSA/PBS and ECL.

**Generation of labelled and unlabelled pHSA.** HSA (400 mg; Behringwerke, puriss. crystallized) was dissolved in 10 ml 0–1 M sodium phosphate buffer, pH 6–8, and dialysed against this buffer. To half of this solution, 28 mg horseradish peroxidase (Sigma) was added and dissolved. To the other half, 0.66 ml of a 2% glutardialdehyde solution was added for 3 h at room temperature. Thereafter, the pH was increased to 9.5 by addition of 0.5 M sodium carbonate. This solution was chilled in ice and 0.3 ml of 30% sodium borohydride in 0.01 M sodium carbonate was added under a hood for 2 h on ice. Thereafter, the solution was dialysed overnight against 0–1 M sodium phosphate buffer, pH 6–8, under the hood. The samples were passed through a Biogel A 0.5 (Bio-Rad) column of 70 cm length and 1.6 cm diameter using PBS at 10 ml/h. The eluate was scanned with a flow-through photometer at 280 nm and fractionated into 1.8 ml portions. The peak at the void volume was attributed to polymers; the composition of following fractions was analysed by SDS–PAGE using 8% polyacrylamide and Coomassie blue staining. Pools of approx. 10–15 ml containing dimers, oligomers and polymers were collected separately, divided into aliquots and stored at −20 °C. In order to determine binding capacity, pHSA–POD was diluted in ten-fold steps to 1:1000 and 100 µl was added to HBsAg/ayw2 coated microplates for 1 h at room temperature, washed and assayed for bound POD as described for the EIA.

**Binding of pHSA and inhibition of binding to HBsAg.** Microplates were coated with HBsAg as described before and post-coated with foetal calf serum for 2 h at 37 °C. Fifty microlitre samples of MAbs or dilutions thereof were mixed with 50 µl 1:100 pHSA–POD oligomers in 0–1% BSA/PBS. The mixture was transferred to the HBsAg-coated microplates and incubated for 1.5 h at room temperature. The
plates were washed as before and bound POD was detected also as before. Positive controls for inhibitory activity contained unlabelled pHSAn in 1:2 dilution and a well-studied preS2-specific MAb S26 (ascites diluted 1:1000; described by Sominskaya et al., 1992), which inhibits pHSAn binding to preS2 very strongly. Each run included at least four negative controls with BSA/PBS which yielded absorbance values around 3. Values below 50% of the negative controls were considered positive. Very strong inhibition yielded absorbance values below 0.1.

**Tryptic cleavage of preS2 protein.** For partial digestion of HBsAg at the solid phase of microplates, 1 µg trypsin (Boehringer Mannheim) in 100 µl Tris–HCl, pH 8.0, was added to each coated well for various time periods, removed and the reaction was stopped by addition of the trypsin inhibitor Pefabloc (Boehringer Mannheim). Thereafter, EIA was carried out with the various MAbs as described above. By this treatment, the N-terminal fragments are stepwise removed from the protein, and epitopes on these fragments can no longer be detected. For WB of the above. By this treatment, the N-terminal fragments are stepwise removed from the protein, and epitopes on these fragments can no longer be detected. For WB of SHBsAg, trypsin digestion was carried out as above with the addition of the trypsin inhibitor Pefabloc (Boehringer Mannheim) in 100 µl Tris–HCl, pH 8.0, which yielded absorbance values around 3. Values below 50% of the negative controls were considered positive. Very strong inhibition yielded absorbance values below 0.1.

**Results**

Characterization of S-specific MAbs by EIA and WB

Screening of 258 hybridoma clones from the spleens of five BALB/c mice immunized with HBsAg/ayw2 yielded 39 clones which bound to HBsAg/ayw2. Twelve antibodies which produced strong and consistent results in EIA using the HBsAg/ayw2 preparation were subcloned and characterized completely (Table 1). MAb 1-8H1 is not shown in Table 1 because it seemed to be identical to MAb 1-8H1. Five MAbs were found to be S-specific. The epitopes of MAbs 1-9C1 and D30-6 were identified within the S domain, staining SHBs and MHBs in WB (Fig. 1b). In contrast to the sequence-specific S reference MAb H166, MAb 1-9C1 reacted more strongly with HBsAg/ayw2 than with HBsAg/adw2 in EIA and WB. MAb D30-6 reacted in WB only with SHBs/ayw2 but not with SHBs/adw2 (data not shown). This specificity for determinant ‘y’ was also present in EIA. MAb D30-4 was also ‘y’-specific in EIA, but it did not react in WB.

Two further MAbs, 2-3A2 and 2-3A3, reacted in EIA weakly with HBsAg/ayw2 and HBsAg/adw2 but strongly with the HBsAg of the hepatitis B vaccine. Hepagene (data not shown). Hepagene contains both SHBs genosubtype A/adw2 and MHBs/SHBs genosubtype D/ayw3. A major difference between ayw2 and ayw3 is a sequence 125–127, which is MTT in ayw3 but TTP in ayw2 and adw2. Since these two MAbs did not react in WB, a discontinuous epitope with these and other unidentified sequence elements of SHBs must be assumed.

**PreS2-specific MAbs**

The remaining seven MAbs were preS2-specific according to WB (Fig. 1b; Table 1). They reacted with MHBs and partially with LHBs, but not with SHBs. Epitopes of MAbs 1-8H1 and 1-8H2 seemed to require the glycoside bound to Asn-30 of MHBs due to the fact that only GP33 and GP36, but no LHBs could be detected in WB. Removal of the glycan with PNGase F led to a complete loss of reactivity of MAb 1-8H1 in EIA (data not shown) as was previously described for MAb Q19/10 (Heermann et al., 1988).

**Table 1. Reactivity of MAbs raised against natural HBsAg/ayw2 spheres with two different genotypes of HBsAg in EIA or WB**

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<th>HBsAg/ayw2</th>
<th>HBsAg/adw2</th>
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<tr>
<td></td>
<td>EIA</td>
<td>WB</td>
</tr>
<tr>
<td>1-9C1</td>
<td>3+</td>
<td>2+ (S, M, L)</td>
</tr>
<tr>
<td>D30-4</td>
<td>3+</td>
<td>0</td>
</tr>
<tr>
<td>D30-6</td>
<td>3+</td>
<td>0</td>
</tr>
<tr>
<td>2-3A2</td>
<td>1+</td>
<td>0</td>
</tr>
<tr>
<td>2-3A3</td>
<td>2+</td>
<td>0</td>
</tr>
<tr>
<td>1-8H1</td>
<td>3+</td>
<td>3+ (M)</td>
</tr>
<tr>
<td>2-11B1</td>
<td>3+</td>
<td>3+ (M, L)</td>
</tr>
<tr>
<td>3-11C1</td>
<td>3+</td>
<td>3+ (M, L)</td>
</tr>
<tr>
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<td>3+</td>
<td>3+ (M, L)</td>
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<tr>
<td>2-12F2</td>
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<td>3+ (M, L)</td>
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<tr>
<td>1-9D1</td>
<td>3+</td>
<td>2+ (M, L)</td>
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3+, Strong reactivity; A ayw3 > 1·5; 2+, moderate; A ayw3 > 0·8; 1+, weak reactivity; A ayw3 > 0·3; 0, negative; (0), borderline detectable. ayw2 and adw2 are SHBsAg determinants.

* conf., Conformational; seq., sequential. ND, Data not shown.
Mapping of B-cell epitopes on HBV surface antigen

MAbs 2-11B1, 3-11C1 and 3-11C2 were similar to each other in that they reacted strongly with HBsAg/ayw2, but reacted only very weakly or not at all with HBsAg/adw2.

MAb 2-12F2 seems to be universally reactive with preS2 both in EIA and WB and with both HBsAg genotypes used here. MAb 1-9D1 reacted in EIA very well with HBsAg/ayw2 and rather weakly with HBsAg/adw2. In WB, a moderately strong reaction was only found with HBsAg/ayw2 showing MHBs and LHBs.

Mapping of preS2-epitopes

PreS2-specific MAbs have been divided into three subgroups (see Fig. 2) as suggested by Mimms et al. (1990) and by Meisel et al. (1994). Group II antibodies, according to Mimms et al. (1990), bind to epitopes at the very N-terminal-most region of MHBs. Such antibodies react in WB only with MHBs where the Asn-4 of preS2 is glycosylated, but not with LHBs where Asn-4 of preS2 is not glycosylated. Reference MAb Q19/10 is such an antibody. A similar pattern is shown by our MAbs 1-8H1 and 1-8H2. Surprisingly, these antibodies show, in contrast to Q19/10, a stronger reactivity with HBsAg/ayw2 than with HBsAg/adw2 in EIA (data not shown) and in WB (Fig. 1b). HBsAg/ayw3 expressed in C127 mouse cell line (Hepagene) did not react at all with MAb 1-8H1 for unknown reasons.

Group I antibodies have been reported by several authors (Milich et al., 1987; Mimms et al., 1990; Meisel et al., 1994) to be most prevalent, group-specific and inhibitors of pHSA binding. They bind to preS2 sequence 13–25. Only MAb 2-12F2 had these properties, as did the second preS2 reference antibody S26 (Sominskaya et al., 1992). MAbs 2-11B1, 3-11C1 and 3-11C2 inhibited pHSA binding very efficiently (Fig. 3) but were clearly subtype-specific in that they reacted with HBsAg/ayw2 but not at all (2-11B1) or very weakly (3-11C1 and 3-11C2) with HBsAg/adw2 (Table 1; Fig. 1b). Certain DNA isolates of genotype A in Germany (Kochel et al., 1990; GenBank accession no. X51970) and the USA (Valenzuela et al., 1981) have an identical sequence to genotype D (Bichko et al., 1985; Galibert et al., 1979) within the preS2 amino acid sequence (7–34), but our genotype A isolate encoded an Ala instead of Thr-7 in genotype D. Cross inhibition in EIA of the three MAbs with S26, which binds to preS2 (13–16), was only weak (data not shown). Thus, we conclude that the epitopes of 2-11B1, 3-11C1 and 3-11C2 are placed between aa 1 and 12 and involve Thr-7 (Fig. 2).
Furthermore, we concluded that pHSA binding requires amino acids from region 1–12 in addition to those in region 13–16. The group-specific MAb 2-12F2 inhibited binding of pHSA (Fig. 3) and S26 (data not shown) only weakly. Thus, its epitope extends proximally to the epitope QDPR (13–16) of S26 into the conserved region. The weak inhibition of pHSA binding by MAb 2-12F2 suggested, furthermore, that the pHSA-binding site does not extend beyond Arg-16 as shown in Fig. 2. MAb 1-9D1 was not glycoside-dependent, it did not inhibit pHSA binding and it was subtype-specific. This suggested that it belonged to group III of the preS2 MAbs. This assumption was confirmed by EIA's using partial digests of HBsAg/ayw2. Previous work (Heermann et al., 1987) has shown that N-terminal preS2 peptide sequences can be removed from solid-phase bound HBs particles by trypsin, leaving the S domain and C-terminal preS2 sequences. HBsAg/ayw2 was coated onto an ELISA plate and trypsin was added for different time intervals. The epitopes of 1-8H1, 1-8H2, 3-11C1 and 3-11C2 were removed most rapidly after 5–15 min of digestion. Removal of the epitope of 2-12F2 required approximately twice as much time. The epitope of 1-9D1 was resistant for 90 min under these conditions (Fig. 4), which was the case with the S epitopes of 1-9C1, D30-4 and D30-6 (data not shown).

The more rapid removal of epitopes of 1-8H1, 1-8H2, 2-11B1, 3-11C1 and 3-11C2 by trypsin than of the epitope of 2-12F2 confirmed the mapping suggested above. Cleavage at Arg-16 was most rapid and removed the N-terminal epitopes from the solid phase. MAb 2-12F2 obviously did not include Arg-16, because its epitope would then be destroyed equally rapidly.

Detection of the larger MHBs fragments generated by trypsin in WB using either MAb 2-12F2 or 1-9D1 confirmed these results. A protein fragment containing the epitope of MAb 2-12F2 appeared after 1 min digestion in the liquid phase indicating cleavage at position Arg-16 and the epitope disappeared within 40 min, due to complete cleavage at Arg-18 (Fig. 5). In comparison, the protein fragment containing the epitope of MAb 1-9D1 also appeared after 1 min digestion but then remained stable for 60 min indicating slow cleavage at Arg-48.

Using the HPLC-purified preS2 fragments from complete tryptic digestion of HBsAg/ayw2, MAbs 2-11B1, 3-11C1 and 3-11C2 bound to fragment 1–16, and MAb 1-9D1 bound to fragment 19–48 in EIA. MAb 2-12F2 did not react with any fragment due to the fact that its epitope contains Arg-18.

The binding sites of MAbs 2-11B1, 3-11C2 and 2-12F2 were also fine-mapped using recombinant frCP-preS2 proteins in WB (Table 2). The epitopes of MAbs 3-11C2 and 2-12F2 were further narrowed down by using various synthetic peptides as shown in Table 3. The data contributed to the mapping as shown in Fig. 2. A reaction was also obtained for MAb 1-9D1 with frCP-preS2 (1–48) genotype D. The reaction can only be due to sequence 36–48 because sequence 19–35 is identical in HBsAg/ayw2 and ayw3. Since MAb 1-9D1 bound to the O-glycosylated and non-glycosylated tryptic preS2 fragment 19–48 from natural HBsAg, it is suggested that Thr-37, which is conjugated with an O-glycan, is not part of the epitope. Thus, the epitope of 1-9D1 is narrowed down to residues 38–48 which carry the subtype-specific group III epitopes. Phe-46 is part of the epitope since digestion of HBsAg with chymotrypsin gave rise to a stable MHBs
Fig. 5. Kinetics of trypsin digestion of MHBs protein subtype ayw2 in liquid phase and detection of blotted fragments with MAbs 1-9D1 (top) and 2-12F2 (bottom). The protein doublet of GP33/36 is cleaved at Arg-16 generating the doublet of the large C-terminal MHBs fragment. The rapidly following cleavage at Arg-18 destroys the epitope of MAb 2-12F2 but not that of 1-9D1. The decrease of 1-9D1 staining intensity over time indicates slow cleavage at Arg-48 and removal of this epitope.

Table 2. Reaction of three group I MAbs with coat proteins of fr phage fused with preS2 sequences in WB

<table>
<thead>
<tr>
<th>preS2 sequence/genotype</th>
<th>Reaction with MAb:</th>
<th>3-11C2</th>
<th>2-12F2</th>
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<tr>
<td>1–48/D</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4–55/A</td>
<td>–</td>
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<td>+</td>
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<tr>
<td>1–19/D</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>1–15/D</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>1–13/D</td>
<td>–</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>13–22/AD</td>
<td>ND</td>
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<td>–</td>
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ND, Data not shown.

intermediate at this cleavage site which was no longer reactive in EIA (data not shown).

Discussion

Our study was initiated to identify novel B-cell epitopes of natural HBsAg with defined genosubtypes in highly reactive individuals such as the inbred mouse strain BALB/c. In spite of being characterized as good responders to SHBs protein and its group-specific epitopes (Milich, 1997), these mice generated only five of 12 MAbs which were directed against SHBs. Furthermore, all of the five SHBs MAbs showed an exclusive (D30-4 and D30-6) or strong (1-9C1, 2-3A2 and 2-3A3) HBsAg subtype preference. A high proportion of subtype-specific anti-SHbs was also found in human recipients of plasma-derived HBsAg vaccine (Legler et al., 1983). Although it is generally accepted that immunization with one HBsAg subtype protects against HBV genotypes with other HBsAg subtypes, it is plausible that subtype-specific anti-SHbs antibodies may also contribute to protection. A larger multitude of immunizing epitopes may also help to prevent selection of antibody-resistant escape mutants (Carman et al., 1989).

The relatively greater abundance of preS2-specific MAbs in our study is consistent with the conclusion of Neurath et al. (1984) that the preS2 domain may be ‘immunodominant’ in BALB/c mice with MHC genotype H2d. Within the preS2 sequence, two overlapping sequences, 14–20 and 18–24, were previously described as immunodominant and subtype-independent (Milich et al., 1987; Milich, 1997). Our own
previous study on preS2-specific MAbs (Meisel et al., 1994) seemed to confirm these reports. However, the mice used for generation of these MAbs were immunized with one genotype and boosted with another, preferably stimulating group-specific antibody production. Using the same genotype for priming and boosting, the present study generated predominantly geno-type-specific preS2 MAbs, some of which even distinguished genosubtypes. We found only one of seven MAbs (2-12F2) which belonged to group I and had an epitope located within the so-called immunodominant conserved preS2 sequence, aa 14–24. All the other epitopes, overlapping with this region, reached from position 3–15 and were genotype D-specific. We suggest epitopes ranging from aa 3–15 and 13–24 to distinguish groups IA and IB, respectively.

The only known function of the sequence covering group IA epitopes is the binding of HSA. The binding is species-specific and does not occur with non-primate serum albumin (Machida et al., 1984). The binding is strongly enhanced by polymerization of albumin with glutaraldehyde but not with other reagents (Yu et al., 1985). Polymerization with glutaraldehyde possibly mimics a naturally occurring modification because a subfraction of HSA also binds to preS2 (Krone et al., 1990). This albumin dissociates from preS2 during purification in high molarities of salt as used in this study. Thus, the albumin-binding sites in preS2 were fully accessible to the antigens used for immunization in this study and can stimulate antibody production against epitopes within the albumin-binding site. Our findings suggest that the albumin-binding site is preferentially recognized by the B-cells of BALB/c mice. Possibly, these epitopes can also be recognized in human recipients of preS2-containing HBsAg vaccines. Previous studies have shown that 10 μg/ml MHBs-containing HBs particles are sufficient to exhaust the fraction of serum albumin which binds to preS2 (Krone et al., 1990). However, in vaccine recipients, 10–40 μg HBsAg encounter ca. 3000 ml human serum. The binding site of modified HSA seems to be completely within sequence 3–16, because neither glycoside-dependent group II MAbs 1-8H1 and Q19/10 nor the group IB MAb 2-12F2 significantly inhibited pHSA binding. Genotype-specific preS2 MAbs like 1-9D1 have been previously described as group III (Milich et al., 1985; Mimmn et al., 1990; Meisel et al., 1995). Both in our previous work and in this study, only one of six or seven preS2 MAbs belonged to this group. In agreement with the previous mapping of group III MAbs, the epitope of MAb 1-9D1 is between aa 38–48. Positions 35–54 of preS2 are most variable between and even within HBV genotypes. This suggests that this region may be under heavy pressure from the immune system. The relatively low number of MAbs against this region would, however, mean that it is not immunodominant in mice, possibly because it is less flexible than region 5–23. This presumption is consistent with the rapid cleavage of MHBs by trypsin at Arg-16 and Arg-18 but the slow cleavage at Arg-48. Nevertheless, region 35–54 may be the more accessible B-cell target in humans because region 3–15 may be covered by HSA. Another masking of preS2 epitopes occurs at Thr-37 of MHBs genotype D in natural HBsAg by O-glycosylation (Schmitt et al., 1999). In fact, preS2 epitopes involving Thr-37 have not yet been described. The reason that the highly hydrophilic and highly conserved sequence 24–34 has also not yet been identified as a target for antibodies remains unknown.

In summary, the preS2 domain of natural HBsAg particles is immunodominant in mice in spite of being a minor polypeptide component. However, its immunodominant group IA epitopes overlap with a binding site for HSA and may thus, not be available in humans. This could explain the weak and transient anti-preS2 response in most HBV patients or recipients of preS2-containing vaccines. Other potential epitopes of preS2 are less accessible, modified by glycans and/or highly variable. This makes recognition of antibodies against these other regions difficult and unreliable. While preS2 would still probably be a valuable addition to HBV vaccines due to its T-cell epitopes (Cupps et al., 1993), the evaluation of such vaccines may be more complex than anticipated. The predominantly subtype-specific antibody response against both SHBs and preS2 epitopes suggests that HBV vaccines containing more than one genotype may be useful. It should be noted that the first HBV escape mutant was found in recipients of a vaccine containing SHBs genotype A while the escape virus had genotype D (Carman et al., 1989).

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