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New broadly reactive neutralizing antibodies against hepatitis B virus surface antigen

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ABSTRACT

Hepatitis B virus (HBV) surface antigen (HBsAg) is considered to be the most important target for the diagnosis and immune prophylaxis of HBV infection. HBsAg-specific monoclonal antibodies (MAbs) are extensively used for studying the complex structure of the HBsAg, mapping the neutralizing epitopes and development of HBV diagnostic tests. However, the efficiency of anti-HBV binding strongly depends on the epitope structure and MAb capability to recognize different HBV variants. In the current study, 9 MAbs against yeast-expressed HBsAg of ayw2 serotype were generated and 7 of them were shown to recognize a linear epitope comprising amino acid (aa) residues 119-GPCRTCT-125 within the main antigenic "a" determinant of HBsAg. One MAb of the highest affinity (clone HB1) was selected for detailed cross-reactivity studies, generation of recombinant single-chain antibody (scFv) and molecular modelling of antibody-epitope interaction. The importance of each aa residue within the identified MAb epitope was determined by alanine substitution study that revealed aa residues C(121), T(123), C(124) and T(125) as essential for binding. These aa residues are highly conserved among HBV variants. In contrast, alanine substitution of G119, P120 and R122 had no or minor influence on the reactivity with the MAb. Certain aa residues at position 122 (either R or K) define different HBV serotypes (either d or y), therefore, the affinity of the MAb HB1 for the epitope with R122K substitution was determined to evaluate its diagnostic potential. The MAb recognized both epitope variants with high affinity. Sequence alignment of the MAb epitope within different HBV strains demonstrated that the shortest peptide recognized by the MAb 121-CR(K)TCT-125 is identical among different human HBV genotypes (HBV A-F, H) and monkey HBV species (HBVCP, HBVGO, HBVGB, WMHBV). In line with these data, the MAb HB1 was cross-reactive in Western blot with a large panel of antigens derived from different HBV genotypes. Recombinant scFv consisting of immunoglobulin VH and VL regions joined by a 20 aa-long linker was generated by cloning the respective cDNA sequences from hybridoma HB1. The recombinant scFv generated in *Escherichia coli* recognized the same epitope as the parental MAb HB1. Cloning of HB1 VH and VL regions allowed determination of their primary structure and subsequent computer modeling of antibody–epitope interaction. The generated molecular models of HB1 variable region with its target peptides were in accordance with experimental data showing the importance of certain aa residues in antibody binding. In conclusion, the current study describes new HBsAg-specific antibodies with HBV-neutralizing potency and a broad cross-reactivity against different HBV strains. The generated MAb HB1 will be of great value in diagnostic and research settings, while the recombinant HB1-derived scFv represents a promising "building block" for producing anti-HBV tools with a potential biopharmaceutical application.

1. Introduction

Human hepatitis B virus (HBV) is a small, 42 nm sized virus of the Hepadnaviridae family causing acute and chronic hepatitis that may lead to development of liver cirrhosis and even-

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tually to hepatocellular carcinoma (Seeger and Mason, 2000). HBV outer lipid envelope contains three forms of the viral surface proteins, small (SHBs), middle (MHBs = PreS2 + S) and large (LHBs = preS1 + preS2 + S), surrounding the viral nucleocapsid. Besides virions, HBV-infected hepatocytes also produce a surplus of subviral particles (SVPs), consisting of different amounts of three HBV surface proteins. The viral surface proteins share the same C-terminal S-domain containing the 226 amino acid (aa)-long SHBs, the most abundant protein in virions and SVPs (Block et al., 2007). The term "HBsAg" defines the antigenic properties of HBV surface proteins. Within the S-domain, the region between aa 99 and 169 is defined as the major hydrophilic determinant. The anti-HBsAg response following natural HBV infection or vaccination is comprised mainly of antibodies that recognize this HBsAg region (Zanetti et al., 2008). The major antigenic determinant of HBsAg contains a highly conformational cluster of epitopes located between aa 120 and 150 in the extracellular antigenic loop termed as the "a" determinant. This immunodominant region of the HBsAg is common among 8HBV genotypes (A to H or putative I) and subgenotypes A1-7, B1-9, C1-16, D1-9, F1-4 (Norder et al., 2004; Shi et al., 2012). The "a" determinant is extremely rich in cysteine residues forming disulfide bonds that are essential for HBsAg conformation and antigenicity. The exposed part of the "a" determinant comprises three loops (mini-loop aa 121–124 (Qiu et al., 1996)), the first-loop aa 124–137 (Dreesman et al., 1982) and the second loop aa 139–147 (Bhatnagar et al., 1982; Brown et al., 1984) held together by disulfide bonds. The HBV surface proteins bear cellular receptor-binding sites located within the amino-terminal preS1 domain of the LHBs interacting with hepatic bile acid transporter, NTCP (Yan et al., 2012) and the HBsAg "a" determinant interacting with heparin sulfate proteoglycans (Leistner et al., 2008; Sureau and Salisse, 2013). The activity of the receptor-binding site within the "a" determinant depends on cysteine residues that are essential for its conformation (Mangold et al., 1995). Artificial aa substitution of R122A in HBsAg affects the infectivity of HBV, indicating that this aa position is important for binding of HBV particles to hepatocytes (Abou-Jaoudé and Sureau, 2007, 2005). A conformation-dependent determinant of HBV entry consists also of P105, V106, P108, I110, T118, G119, P120, C121, R122, T123, M133, K141, P142, N146,T148, C149, I150, P153, W156 (Salisse and Sureau, 2009). Mutagenesis of S113, R122, Y134 (Ashton-Rickardt and Murray, 1989), C121, C124, C137 (El Chaar et al., 2010), N146 (Julithe et al., 2014), P120, T123 (Tian et al., 2007) aa residues has revealed their importance for immunological recognition and conformation of HBsAg.

Currently available HBV vaccines are recombinant purified SVPs that confer long-term protective immunity by eliciting antibodies against the "a" determinant. Neutralizing antibodies induced by immunization are targeted principally to the conformational epitopes, and there is evidence that mutations within this HBsAg region can allow replication of HBV in vaccinated persons, since the induced antibodies do not recognize critical changes in the HBsAg domain (Zuckerman and Zuckerman, 2000). Moreover, mutations within the "a" determinant may decrease the efficiency of HBV diagnostic assays based on HBsAg detection (Carman et al., 1995; Coleman et al.,1999). The analytical relevance of HBsAg and anti-HBsAg assays may be also dependent on HBV genotype and serotype (Ashton-Rickardt and Murray, 1989). Thus, although a wide variety of immunochemical commercial tests is available for HBsAg detection, the sensitivity of these assays is dependent on the anti-HBsAg usage. The selection of suitable antibodies capable to recognize different HBV variants is crucial for HBV diagnostics. In addition, broadly-reactive neutralizing antibodies directed to HBsAg may represent a promising tool for a post-exposure treatment of HBV infection (Kim et al., 2008; Tajiri et al., 2010; Tan et al., 2013).

In the current study, we report the development and detailed characterization of HBsAg-specific monoclonal and recombinant antibodies with HBV-neutralizing capability and a broad reactivity against different HBV strains.

2. Materials and methods

2.1. Recombinant antigens for immunization and epitope mapping

The HBV320 genome (GenBank X02496.1, genotype D, subtype *ayw2*) (Bichko et al., 1985), kindly provided by Prof. Paul Pumpens (Riga, Latvia), was used as a template for PCR reaction to amplify the SHBs sequence. Recombinant HBsAg was generated in yeast *Saccharomyces cerevisiae* and purified by density-gradient centrifugation as described previously (Glebe et al., 2005). The purified HBsAg ayw2 was used for immunization of BALB/c mice and generation of hybridomas.

Chimeric proteins for MAb epitope mapping were generated by insertion of HBsAg segment (aa 101–169) into murine polyomavirus (MPyV) major capsid protein VP1 either at BC loop (aa 83–84) or HI loop (aa 293–294). The expression of chimeric proteins in yeast *S. cerevisiae* and their purification was performed as described previously for murine polyomavirus VP1 protein harbouring HBV preS1 sequence (Skrastina et al., 2008).

2.2. SHBs from different HBV strains

The SHBs expressed in HeLa cells were used to test MAb reactivity with different HBV strains. The genes encoding SHBs of different HBV genotypes (obtained from the HBV genotype reference panel, now serving as a WHO genotype standard (Chudy et al., 2012) were cloned into pCEP-Puro vector, a pCEP4-vector (Life technologies, Darmstadt, Germany) where the hygromycin B resistance gene was substituted by a puromycin resistance gene. The constructs were stably transfected into HeLa cells. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and transfected in 10 cm dishes with FuGene HD (Roche, Mannheim, Germany) according to the manufacturer's protocol. For selection of clones with stable SHBs expression, cells were cultivated in the presence of 3 μg/mL puromycin for 2 to 3 weeks. Supernatants of the stable transfectants were collected after 6 days of cultivation. For Western blot analysis and ELISA, the supernatants were concentrated by factor 20 and for Western blot analysis additionally precipitated by 12.5% PEG-6000. The SHBs were quantified by a quantitative immunoassay using Abbott Architect analyzer (Abbott Laboratories, Abbott Park, IL, USA) according to manufacturer's recommendations.

2.3. Generation of hybridomas

Hybridomas were generated essentially as described previously (Zvirbliene et al., 2014) by fusing spleen cells of BALB/c mice immunized with recombinant yeast-expressed purified HBsAg ayw2 with mouse myeloma Sp 2/0-Ag14. Positive hybridoma clones were screened by an indirect ELISA and then stabilized by limiting dilution cloning on a macrophage feeder layer. Hybridoma cells were cultivated in complete DMEM containing 15% FBS (Merck Millipore, Darmstadt, Germany). The isotypes and subtypes of the monoclonal antibodies (MAbs) were determined by ELISA using Monoclonal Antibody Isotyping Kit (BD Biosciences, NJ, USA).

All procedures involving laboratory mice were performed at the breeding colony of the Center for Innovative Medicine (Vilnius, Lithuania) by FELASA-certified personnel under controlled