Research paper

Quantification, epitope mapping and genotype cross-reactivity of hepatitis B preS-specific antibodies in subjects vaccinated with different dosage regimens of BM32

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ABSTRACT

Background: Chronic hepatitis B virus (HBV) infections are a global health problem. There is a need for therapeutic strategies blocking continuous infection of liver cells. The grass pollen allergy vaccine BM32 containing the preS domain of the large HBV surface protein (LHBs) as immunogenic carrier induced IgG antibodies in human subjects inhibiting HBV infection in vitro. Aim of this study was the quantification, epitope mapping and investigation of HBV genotype cross-reactivity of preS-specific antibodies in subjects treated with different dosage regimens of BM32.

Methods: Hundred twenty eight grass pollen allergic patients received in a double-blind, placebo-controlled trial five monthly injections of placebo (aluminum hydroxide, \(n = 34\)) or different courses of BM32 (2 placebo + 3 BM32, \(n = 33\); 1 placebo + 4 BM32, \(n = 30\); 5 BM32, \(n = 31\)). Recombinant \textit{Escherichia coli}-expressed preS was purified. Overlapping peptides spanning preS and the receptor-binding sites from consensus sequences of genotypes A–H were synthesized and purified. Isotype (IgM, IgG, IgA, IgE) and IgG subclass (IgG1–IgG4) responses to preS and peptides were determined by ELISA at baseline, one and four months after the last injection. IgG1 and IgG4 subclass concentrations specific for preS and the receptor-binding site were measured by quantitative ELISA.

Findings: Five monthly injections induced the highest levels of preS-specific IgG consisting mainly of IgG1 and IgG4, with a sum of median preS-specific IgG levels reaching up to 1.8 mg/ml. More than 20% of preS-specific IgG was directed against the receptor-binding site. BM32-induced IgG cross-reacted with the receptor-binding domains from all eight HBV genotypes A–H.

Interpretation: BM32 induces high levels of IgG1 and IgG4 antibodies against the receptor binding sites of all eight HBV genotypes and hence might be suitable for therapeutic HBV vaccination.

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1. Introduction

Hepatitis B virus (HBV) infections are a major health threat. It is estimated that more than 250 million people are chronically infected with HBV but only a relatively small portion (i.e., approximately 10%) know of their condition and even less receive treatment [1].
Chronic hepatitis B virus (HBV) infections are one public health challenge recognized by the World Health Organization as a priority. There is an urgent need for therapeutic strategies block virus entry into liver cells. The preS domain of the large HBV surface protein (LHBs) contains the binding site for the sodium taurocholate co-transporting polypeptide (NTCP) receptor for HBV and hence is an important target for treatment as well as for prophylactic immunization. In particular, there is a need for therapeutic vaccines which can induce sufficient levels of IgG antibodies blocking the NTCP binding site of common HBV genotypes. We have found that the recombinant grass pollen allergy vaccine BM32 which contains fusion proteins consisting of preS as carrier protein and hypoallergenic B cell epitope-derived grass pollen allergen peptides can induce IgG antibodies which inhibit HBV infection of liver cells in vitro.

**Evidence before the study**

Chronic hepatitis B virus (HBV) infections are a major public health challenge and recognized by the World Health Organization as a priority. There is an urgent need for therapeutic strategies blocking virus entry into liver cells. The preS domain of the large HBV surface protein (LHBs) contains the binding site for the sodium taurocholate co-transporting polypeptide (NTCP) receptor for HBV and hence is an important target for treatment as well as for prophylactic immunization. In particular, there is a need for therapeutic vaccines which can induce sufficient levels of IgG antibodies blocking the NTCP binding site of common HBV genotypes. We have found that the recombinant grass pollen allergy vaccine BM32 which contains fusion proteins consisting of preS as carrier protein and hypoallergenic B cell epitope-derived grass pollen allergen peptides can induce IgG antibodies which inhibit HBV infection of liver cells in vitro.

**Added value of the study**

This study demonstrates in a clinical dose-finding study, performed in 128 patients that 5 monthly injections of BM32 induce high levels of preS-specific IgG antibodies consisting mainly of IgG1 and IgG4 subclass responses which are directed against the NTCP binding site of HBV. Importantly, BM32-induced IgG antibodies cross-react with all common HBV genotypes and hence should be cross-protective.

**Implications of all the available evidence**

The findings of our study suggest that BM32 may further be studied as a vaccine candidate for the treatment and prevention of HBV infections covering the most common HBV genotypes. BM32-induced antibodies have the correct epitope specificity to prevent virus entry into liver cells and their levels seem to be high enough to inhibit infection even in the presence of circulating preS antigen. The BM32-induced IgG subclass response consists mainly of IgG1 and IgG4 antibodies which may mediate non-inflammatory neutralization and, at the same time, are accompanied by mild activation of antibody-dependent cellular cytotoxicity (ADCC) and complement as recommended for therapeutic vaccines. This study therefore may pave the road for further clinical studies evaluating BM32 for therapeutic HBV vaccination.

Accordingly, more than 800,000 people die from HBV infections each year and HBV infections cause a series of severe liver diseases such as chronic hepatitis, cirrhosis and hepatocellular carcinoma. Hepatocellular carcinoma develops in approximately 40% of patients suffering from chronic hepatitis B and it is one of the leading causes of cancer-related death worldwide. It is therefore a goal of the World Health Organization (WHO) to eliminate HBV as a public health threat until 2030. This goal is vigorously pursued also by the International Coalition to Eliminate HBV (ICE-HBV), an international, independent research-based forum which was established in 2016 to foster research dedicated to the cure of HBV [2].

One of the possible strategies for therapy and eventually cure of chronic HBV infections is the inhibition of continuous infection of liver cells [3]. In this context it is of note that considerable progress has been made regarding the understanding of how HBV infects hepatocytes. The preS domain of LHBs which consists of the preS1 and preS2 domains contains an epitope which was identified as host cell receptor binding site on HBV [4] and antibodies directed to this epitope were found to neutralize HBV infections [5]. Subsequently it was shown that the preS1 domain plays an important role in the infection of liver cells [6] and that it is possible to inhibit HBV infection with preS1-derived peptides [7]. Finally, it could be demonstrated that preS1 contains the binding site for the HBV receptor on liver cells which was identified as sodium taurocholate co-transporting polypeptide (NTCP) [8].

Therefore, the inhibition of viral entry via the NTCP receptor has been considered as a promising strategy for therapy of chronic HBV infections [9]. Besides the use of viral entry inhibitors therapeutic vaccination has been considered [10]. In particular, vaccines containing preS1 are possible candidates for therapeutic vaccination against HBV because antibodies induced by such vaccines would inhibit continuous infection in patients suffering from chronic hepatitis by blocking the NTCP-binding site of HBV and will not be absorbed by circulating hepatitis B surface antigen (HBsAg) [11].

We have used preS as a carrier protein for the construction of therapeutic vaccines for the treatment of immunoglobulin E (IgE)-associated allergy [12]. The preS-based allergy vaccines are fusion proteins consisting of allergen-derived peptides from the IgE binding sites of major allergens and preS. These vaccines are constructed to induce in allergic patients upon vaccination focused IgG antibody responses towards the IgE binding sites of allergens in order to block allergen recognition by IgE and IgE-mediated allergic inflammation [13]. In this new generation of hypoallergenic allergy vaccines, preS serves as a carrier protein to provide T cell help for the allergen-specific IgG antibody production avoiding stimulation of allergen-specific T cells and thus the induction of T cell-mediated allergic inflammation in the patients [14]. Interestingly, the analysis of HBV-specific immune responses in allergic patients treated with the preS-containing grass pollen allergy vaccine, BM32 [15,16], revealed that BM32 induces also preS-specific antibody responses against the N-terminus of preS. These antibodies inhibited HBV infection of liver cells in vitro, which was accompanied by a preS-specific CD4+ and to a lower extent a CD8+-T cell response [17]. Thus results obtained for BM32 indicated that preS-containing allergy vaccines may eventually not only protect against allergy but also against HBV infections.

In this study we have analyzed the preS-specific antibody response in a clinical study performed in grass pollen allergic patients who had been treated with different dosage regimens of BM32 (i.e., three, four or five monthly injections) to quantify the concentrations of BM32-induced preS-specific antibodies by the different dose regimens. We also performed a detailed epitope mapping of the preS-specific antibody responses using synthetic peptides comprising the NTCP-binding site and the accessory domain involved in the inhibition of infection and quantified the concentrations of IgG antibodies directed to the NTCP-binding site. Furthermore, we synthesized a set of peptides comprising the NTCP-binding site and the accessory domain involved in the inhibition of infection and the consensus sequences established for eight different common HBV genotypes (A–H) to study if vaccination with BM32 induces antibodies which may cross-protect against different HBV genotypes.

### 2. Materials and methods

#### 2.1. Study design, medication and patients

In this study we analyzed serum samples from 128 grass pollen allergic patients who had participated in a double-blind, placebo-controlled clinical trial CS-BM32-004 (Clinicaltrials.gov ID: NCT02643641, EudraCT No.: 2015-004531-43). The latter study had been approved by the Clinical Pharmacology Ethics Committee, Vienna, Austria and by the Austrian Regulatory Agency (AGES). Written informed consent was obtained from each patient and the study was conducted according to the principles of the declaration of Helsinki and the International Conference on Harmonization of Good
Clinical Practice guidelines. The study was intended to evaluate the effect of different pre-seasonal dosing schedules (three, four, five pre-seasonal subcutaneous injections) of the recombinant B cell epitope-based grass pollen allergy vaccine BM32 (i.e. mix of 20 μg of each BM32 component) versus placebo on the development of grass pollen allergen-specific IgG levels (Fig. 1). Here we analyzed the preS-specific antibody responses of the patients from this study.

BM32 is a vaccine for immunotherapy of grass pollen allergy which is composed of four different recombinant fusion proteins (BM321, BM322, BM325 and BM326) adsorbed onto aluminum hydroxide. Each of the fusion proteins consists of HBV surface protein preS as a carrier and hypoallergenic peptides derived from the IgE-binding sites of the four major timothy grass pollen allergens (Phl p 1: BM321; Phl p 2: BM322; Phl p 5: BM325 and Phl p 6: BM326) (Fig. 1: BM321, BM322, BM325 and BM326) adsorbed onto aluminum hydroxide. Each of the fusion proteins consists of HBV surface protein preS as a carrier and hypoallergenic peptides derived from the IgE-binding sites of the four major timothy grass pollen allergens (Phl p 1: BM321; Phl p 2: BM322; Phl p 5: BM325 and Phl p 6: BM326) (Fig. 2a). A detailed description of the structure and peptide sequences of BM32 has been reported [15]. Subjects had been randomized in a 1:1:1:1 ratio to subcutaneous treatment with three, four or five BM32 injections or placebo (Fig. 1). The demographic characteristics of the patients are summarized in Table S1. Sera were collected from visit 3 (January) until visit 12 (October) (Fig. 1) and stored until use at −20 °C.

Fig. 1. Overview of the CS-BM32-004 study. Study subjects (n = 128) were divided into a group receiving five injections of placebo (n = 34), two placebo plus three injections of BM32 (n = 33), one placebo and four BM32 injections (n = 30) or five BM32 injections (n = 31) before the grass pollen season. Serum samples from visits 3, 8 and 11 were analyzed.

Additionally, eight serum samples obtained in the course of clinical trials CS-BM32-002 (NCT01445002) and CS-BM32-003 (NCT01538979) which had been tested earlier for in vitro virus neutralization [17] were analyzed.

2.2. Expression and purification of recombinant preS; synthesis, purification and characterization of preS-derived peptides

Recombinant preS protein comprising preS1 + preS2 from HBV genotype A2, (Fig. 2) (GenBank: AAT28735) and a C-terminal hexahistidine tag was expressed in E. coli BL21 (DE3) (Agilent Technologies, USA) as described [18]. The purification protocol was refined: after harvesting E. coli cells, the cell pellet was solubilized in 6 M Guanidine-HCl, 100 mM NaH2PO4, 10 mM Tris–HCl [pH = 8] by stirring overnight at room temperature. The lysate was cleared of cellular debris by ultracentrifugation at 42,200 g at 4 °C for 20 min and the recovered supernatant was incubated with 2 ml of pre-equilibrated Ni-NTA resin (Qiagen, Hilden, Germany) for 4 h at room temperature. The mixture was then loaded onto a column, washed with 8 M Urea, 100 mM NaH2PO4, 10 mM Tris–HCl [pH = 6.3] and eluted with 8 M Urea, 100 mM NaH2PO4, 10 mM Tris–HCl [pH = 3.5]. Elution fractions containing the recombinant preS protein were dialysed against 10 mM NaH2PO4 [pH = 4.2] to remove urea. The purity and characteristics of recombinant preS were assessed by SDS-PAGE, mass spectrometry and circular dichroism.

The following preS-derived peptides were produced: Eight overlapping peptides spanning the whole preS sequence (P1–P8, 30 amino acids length and 10 amino acids overlap) as depicted in Fig. 2b [17], three peptides A, B and C comprising the NTCP binding site and the accessory domain involved in the inhibition of infection [6,19] of HBV-genotype A as indicated in boxes in Fig. 2c with one cysteine residue added to the N-terminus of each peptide to facilitate coupling to carrier molecules and eight peptides comprising the consensus preS amino acid 13–51 sequences of HBV genotypes A–H as shown in Fig. 3a, b. The preS-derived synthetic peptides were produced using 9-fluorenylmethoxy carbonyl (Fmoc)-strategy on the Liberty peptide synthesizer (Liberty Microwave Peptide Synthesis, CEM corporation, Matthews, NC) using PEG-PS (polyethylenglycolpolystyrene) preloaded resins of loading capacity 0.46–0.59 mmol/g as

Fig. 2. Schematic representation of the BM32 vaccine components. (a) Each of the four BM32 vaccine components (BM321, BM322, BM325, BM326) are recombinant fusion proteins consisting of allergen-derived peptides (pink) fused to the N- and C-terminus of preS, the N-terminal domain of LHBs protein, consisting of preS1 and preS2. In the LHBs the division between preS1 and preS2 does not exist, whereas preS2 domain forms the N-terminal domain of the non-essential middle MHBs protein. Certain HBV genotypes contain a deletion of 11 codons at the N-terminus of preS1. (b) Synthetic peptides (P1–P8) spanning preS1 and preS2 are indicated. The NTCP binding site is indicated in orange and the accessory domain involved in the inhibition of infection is marked in blue. (c) Sequences of three peptides A (green), B (violet) and C (red) used for mapping of antibody responses to the N-terminus are indicated in boxes.
Fig. 3. Identification of consensus sequences representing the preS1 region of HBV genotypes A–H containing the NTCP binding site and the accessory domain involved in the inhibition of infection. (a) Algorithm of the definition of the consensus sequences (AliView software). (b) Alignment of preS1-derived peptide sequences (aa 13–51) of genotypes A–H. Hydrophobic amino acids (A, L, M, F, W, V) are shown in blue, positively charged (K, R) in red, negatively charged (E, D) in magenta, polar (N, Q, S, T) in green, aromatic (H, Y) in cyan, cysteines (C) in pink, glycines (G) in orange, prolines (P) in yellow. (c) Percentages of identity to the consensus sequence of region aa 13–51 within each genotype are shown in the pie charts.
described [20]. Peptides were purified by reversed-phase HPLC, using Dionex HPLC UltiMate 3000 system (Thermo Fisher Scientific Inc., Vienna, Austria). The identity and mass of each synthetic peptide was confirmed by MALDI-TOF analysis (Microflex, MALDI-TOF, Bruker Daltonics, Billerica, MA) using a CA matrix (α-Cyan4-hydroxycinnamic acid dissolved in 70% acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA)). For the sample preparation a 1:1 mixture of peptide sample and matrix solution was used which was deposited onto a target and air dried. Acquired spectra were analysed with the Bruker DaltonicsFlexAnalysis software. Pure peptides were pooled and lyophilized using CHRiST-Alpha 2–4 LSC Lyophilizer (SciQuip, Newtown, Wem, Shropshire, UK). Purified and lyophilized peptides samples were dissolved in sterile ddH2O before use for ELISA experiments.

2.4. Enzyme-linked immunosorbent assay (ELISA) for measuring antibody isotype and IgG subclass responses

A preS1 nucleotide sequence dataset was downloaded from HBVdb database [21]. The identification of consensus sequences for selected region (aa 13–51) of genotypes A-H was performed using AliView 1.22 software [22] as described in Fig. 3. Information regarding consensus sequences is provided in Fig. 3 and Table S2. A comparison of the consensus sequences established in Fig. 3 with published ancient sequences [23–25] was performed using AliView 1.22 software (Fig. S1).

2.4. Enzyme-linked immunosorbent assay (ELISA) for measuring antibody isotype and IgG subclass responses

The preS-specific antibody responses were assessed by ELISA. For determination of IgG, IgM, IgE, IgG antibody isotypes, as well as IgG1 and IgG4 subclasses, ELISA plates (Nunc MaxiSorp 96-well flat bottom, Thermo Fisher Scientific, Cat. 442404) were coated overnight at 4 °C with preS (2 μg/ml in 100 mM sodium bicarbonate buffer, pH 9.6) and washed two times with PBS 0.05% Tween20 (PBST). Residual binding sites were blocked for 5 h at room temperature with 2% BSA/PBST and then the plates were incubated overnight at 4 °C with sera diluted in 0.5% BSA/PBST (for total IgG: 1:100 for preS and Phil p 5 and 1:50 for peptides; 1:10 for IgG1 and 1:20 for the other isotypes and subclasses). After five times washing with PBST, mouse anti-human monoclonal antibodies were diluted in 0.5% BSA/PBST and applied: IgA1/IgA2 (BD, Franklin Lakes, NJ, Cat. 555886), IgM (BD, Cat. 555856), IgG1 (BD, Cat. 555868), IgG2 (BD, Cat. 555873), IgG4 (BD, Cat. 555878) 1:1000 and IgG2 (Sigma-Aldrich, St. Louis, Missouri, Cat. B3523) 1:5000; after 2 h incubation at room temperature and five times washing plates were incubated for 1 h at room temperature with horseradish-peroxidase (HRP)-linked sheep anti-mouse IgG (GE Healthcare, Chicago, Illinois, Cat. NA931V) at a dilution of 1:2500. For total IgG and IgG detection directly HRP-labeled anti-human IgG (BD, Cat. 555788) diluted 1:5000 and goat anti-human HRP-conjugated IgE (KPL, Gaithersburg, MD, Cat. 5220-0329) diluted 1:2500 were added for 1 h at room temperature. After five times washing, the reaction was developed by incubation with substrate solution: 1 mg/ml 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) di-ammomium salt (ABTS) (Sigma-Aldrich, Cat. A1888) in 70 mM citrate-phosphate buffer containing 0.003% H2O2 (Sigma-Aldrich, Cat.H1009). Absorbance values were measured at wavelength 405 nm (reference wavelength 492 nm) on Tecan Infinite F50 spectrophotometer (Tecan Trading AG, Männedorf, Switzerland).

The measurement of preS- and peptide-specific IgG1 and IgG4 levels was performed by ELISA and quantified by using a standard curve established with purified human monoclonal antibodies specific for Phil p 2 (IgG1) [26] and Phil p 5 (IgG4) [27] and the corresponding purified recombinant grass pollen allergens (Biomay AG, Vienna, Austria). The plates were coated with preS, peptides A, B, C, Phil p 2, Phil p 5 for 5 h at room temperature, washed and blocked overnight at 4 °C as described above. To establish the standard curve, the purified human monoclonal antibodies were serially diluted with 2% BSA/PBST to the concentrations 50, 150, 450, 900 and 1350 ng/ml and incubated on the plates, as well as sera, for 2 h at 37 °C and 1 h at room temperature. The sera were applied in serial dilutions from 1:40 to 1:1280 depending on the intensity of colorimetric reaction. The detection was performed using either biotin-labeled mouse anti-human IgG1 (BD, Cat. 555869) or biotin-labeled mouse anti-human IgG4 (BD, Cat. 555882) in dilution 1:1000 mixed with HRP-conjugated streptavidin (BD, Cat. 554066) in dilution 1:2500 and incubated overnight at 4 °C. The reaction was developed and detected as described above. The standard curve and serum concentrations were calculated using SoftMaxPro 4.8 software.

ELISAs were controlled by performing omission of antigen, serum or detection antibodies and the buffer control (i.e., omission of serum) was used to establish the cut-off. Plate-to-plate normalization was done by including reference sera and a reference antigen (i.e., rPhil p 5) (Biomay AG, Austria) on each of the plates. All measurements were performed in duplicates with deviations <5%. The results are shown as means of plate-plate harmonized OD values after subtraction of the buffer control.

2.5. Data evaluation and statistical analysis

Plots and pie charts were created using Prism 6.0 software (GraphPad, San Diego, CA). Heat maps were generated using Heatmapper web server [28]. Statistically significant intergroup differences were defined by Wilcoxon rank-sum test. Spearman’s r coefficient was used to determine the correlation. P < 0.05 was considered as significant. Statistics were calculated using Prism 6.0 software (GraphPad, San Diego, CA).

3. Results

3.1. Design of the clinical study investigating different forms of dosing of BM32

In this study we investigated the effects of different pre-seasonal dosage regimens of BM32 on the induction of an antibody response specific for HBV-derived preS. The clinical trial (ClinicalTrials.gov Identifier: NCT02643641) was performed to study the effects of three, four or five injections of the recombinant grass pollen allergy vaccine, BM32 on the induction of grass pollen allergen-specific IgG levels. The grass pollen allergy vaccine BM32 consists of four recombinant fusion proteins (20 μg each of BM321, BM322, BM325, BM326 adsorbed to aluminum hydroxide) containing preS as a carrier protein with attached hypoallergenic peptides derived from the major timothy grass pollen allergens Phil p 1 (BM321), Phil p 2 (BM322), Phil p 5 (BM325) and Phil p 6 (BM326), which had been administered by subcutaneous injection before the grass pollen season of the year 2016 as indicated in Fig. 1. Thirty four grass pollen allergic patients received five injections of placebo containing aluminum hydroxide alone in approximately monthly intervals (visit 3–visit 7) before the grass pollen season, a second group of thirty three subjects received two placebo injections followed by three active vaccines, a third group (n = 30) got one placebo followed by four active injections and the fourth group (n = 31) received five monthly active injections before the grass pollen season (Fig. 1). The demographic characteristics of the study subjects summarized in Table S1 show that they were balanced regarding age and gender (Table S1).

The general architecture of the BM fusion proteins used for vaccination is illustrated in Fig. 2a. They consist of a central preS core with the fourth group (n = 31) received five monthly active injections before the grass pollen season (Fig. 1). The demographic characteristics of the study subjects summarized in Table S1 show that they were balanced regarding age and gender (Table S1).

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peptide C) comprising the NTCP binding site and the accessory domain important for docking on liver cells, two peptides overlapping with and flanking peptide C (i.e., peptides A and B) as well as a set of eight HBV peptides representing the NTCP binding site and the accessory domain important for the inhibition from the consensus sequences of the HBV genotypes A, B, C, D, E, F, G, and H (Fig. 3). The consensus sequences selected to represent genotype A, B, C and D have been conserved for more than several hundred years (genotype C) up to 4400 years (genotype D) with the exception of a few amino acid changes (Fig. S1). Furthermore, despite existing geographical distribution, the sequences representing the different genotypes have been reported from all over the world (Table S2) [29].

3.2. Five monthly injections of BM32 induce higher preS-specific IgG, IgG1, and IgG4 levels than three or four injections

Fig. 4 shows the development of preS-specific isotype and IgG subclass responses in the groups having received three, four or five injections of BM32 one month and four months after the last injection. We found no relevant development of preS-specific IgA, IgM and IgE responses in the groups with three, four or five BM32 injections as compared to placebo (Fig. 4). However, a strong and significant induction of preS-specific IgG responses was detected in the three groups treated with BM32 one month after the last injection (visit 8) which was maintained but was lower after four months (visit 11) (Fig. 4). The group treated with five injections had significantly higher preS-specific IgG levels at visit 8 and visit 11 as compared to patients treated with four and three injections, respectively (Fig. 4).

The preS-specific IgG response consisted mainly of an IgG1 and IgG4 subclass induction. preS-specific IgG1 responses were highest at visit 8 and lower at visit 11 whereas IgG4 levels appeared more sustainable when comparing visit 8 and visit 11 (Fig. 4). Significantly higher levels of IgG1 were observed in the group treated with five injections versus the group with four injections at visit 11. Five injections built up significantly higher levels of preS-specific IgG4 at visit 8 as compared to the groups treated with three or four injections (Fig. 4). Only a modest induction of preS-specific IgG2 levels was observed in the actively treated groups and no relevant induction of preS-specific IgG3 could be detected upon BM32 treatment (Fig. 4).

3.3. BM32-induced IgG responses are mainly directed against the N-terminal portion of preS and target the NTCP binding site and the accessory domain involved in the inhibition of infection

Using a set of overlapping peptides spanning the preS sequence (Fig. 2b), we mapped the binding sites of preS-specific IgG responses (Fig. S2). Three, four and five injections of BM32 induced significant increases of IgG antibodies specific for peptides 1, 2 and 3 as compared to placebo treatment (Fig. S2). The highest levels of IgG were directed against peptide 1 which contains the NTCP binding site of HBV (Fig. S2, Fig. 2b). We therefore investigated and quantified preS-specific IgG1 and IgG4 responses using three additional peptides A, B, and C of which peptide C contained the NTCP binding site and the accessory domain involved in the inhibition of infection (Fig. 2c).

The quantification of preS-specific IgG1 and IgG4 levels performed at visits 8 and 11 (Fig. 5) confirmed the results obtained with the semi-quantitative ELISA (Fig. 4). Five injections of BM32 induced significantly higher preS-specific IgG concentrations at visit 11 than four injections and, at visit 8, IgG4 levels were significantly higher in the group having received five injections than in groups treated with three or four injections (Fig. 5). preS-specific IgG1 concentrations in the group treated with five injections of BM32 ranged from 23.8–984.8 μg/ml with a median of 106.8 μg/ml and IgG4 concentrations ranged from 3.2–929 μg/ml with a median of 29.7 μg/ml (Table S3). We also found a significant induction of IgG1 and IgG4 concentrations against the preS-derived peptides A, B, C containing the NTCP binding site and the accessory domain involved in the inhibition of infection for the groups treated with three, four and five injections of BM32 at visits 8 and 11 (Fig. 5). Of note, a considerable amount of preS-specific IgG1 and IgG4 antibodies was directed against the sequential peptide C epitope containing both the NTCP binding site and the accessory domain involved in the inhibition. The median peptide C-specific concentrations of IgG1 at visit 8 were 24.8 μg/ml whereas the median IgG4 concentrations were 5.8 μg/ml (Table S3).

The kinetics of preS-specific IgG1 and IgG4 antibody developments were different from each other. In each of the three active groups (three, four, five injections of BM32) preS-specific IgG1 antibodies rose quickly and strongly at visit 8 but also declined quickly until visit 11. The decline was less in the group having received five injections compared to patients treated with three or four injections (Table S3). preS-specific IgG4 did rise less strongly than IgG1 at visit 8 but showed a much lower decline until visit 11 (Fig. S3, Table S3).

The concentrations of preS-specific IgG1 and IgG4 antibodies which had developed in the groups treated with three, four or five injections of BM32 at visits 8 and 11 were comparable to IgG1 and IgG4 concentrations against the major timothy grass pollen allergen Phl p 5 (Fig. S4).

3.4. Correlations of preS-specific IgG subclass responses

Fig. 6 shows the development of IgG1 and IgG4 antibody responses to preS and preS-derived peptides A, B and C for each of the patients in each group from visit 3 (baseline) to visits 8 and 11 in the form of a heat map displaying antibody concentrations. The heat map shows that the development of preS-specific IgG1 and IgG4 responses is not uniform and not always completely synchronized. For example, patient 68 develops strong preS-specific IgG1 at visit 8 but does not develop a strong IgG4 response (Fig. 6a, b). Likewise, patient 40 does not develop a strong preS-specific IgG1 response at visit 8 but responds strongly with IgG4 antibodies at the same time (Fig. 6a, b). Approximately one third of BM32-treated patients showed high levels of preS-specific IgG1 and lower levels of preS-specific IgG4 (e.g. patients 44, 45, 55, 57, 60, 65, 67, 74, 80, 81, 82, 89, 91, 92, 94, 97, 98, 103, 104, 106, 110, 112, 114, 115, 119, 122, 128) whereas for several subjects the opposite was observed (e.g., patients 38, 40, 58, 66, 108, 120, 127). The scatter plots displaying the correlations between preS-specific IgG1 and IgG4 concentrations determined at visits 8 and 11 for the groups treated with three, four and five injections of BM32 also indicate that preS-specific IgG1 and IgG4 responses are not fully correlated. A significant correlation was observed for the groups treated with three and four injections at visits 8 and 11 but preS-specific IgG1 and IgG4 concentrations in the group treated with five injections were not correlated.

Next, we analyzed the epitope specificities for peptides A, B and C within each of the two IgG subclasses (Figs. S6–S8). We found significant correlations of IgG1 responses to peptides A and B for each of the three BM32 groups at visits 8 and 11 (Fig. S6a). Likewise, we found significant correlations of peptide A- and B-specific IgG4 responses for each of the three BM32 treatment groups at visits 8 and 11 (Fig. S6b). Furthermore, IgG1 and IgG4 responses to peptides A and C as well as to peptides B and C were significantly correlated at visits 8 and visit 11 (Figs. S7 and S8).

3.5. The NTCP binding site and the accessory domain involved in the inhibition of infection are highly conserved evolutionarily and in different HBV genotypes

In order to select the consensus sequences representing the NTCP-binding site and the accessory domain involved in the inhibition of infection (i.e., amino acid 13–51 of preS) of different HBV genotypes A–H, we evaluated the complete preS1 dataset taken from the HBV
Fig. 4. Comparison of preS-specific isotype and IgG subclasses responses in the four groups of patients. Shown are optical density (OD) values (y-axes) corresponding to preS-specific IgG, IgA, IgM, IgE and IgG subclass (IgG1–IgG4) levels in patients immunized with 3, 4 or 5 injections of BM32 or with placebo (see inlay) at baseline (V3), after one (V8) or four (V11) months treatment (x-axes). Medians (horizontal bars) and significant differences are indicated: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Fig. 5. Quantification of IgG1 and IgG4 subclass responses in patients treated with placebo or different numbers of BM32 injections. Shown are absolute increases of serum concentrations of IgG1 and IgG4 (y-axes: µg/ml) specific (a) for preS, (b) for peptide A, (c) for peptide B and (d) for peptide C from baseline (V3) to visits 8 and 11 (x-axes) in patients immunized with placebo, 3, 4 or 5 injections of BM32. Medians (horizontal bars) and significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Fig. 6. Kinetics and fine specificities of anti-preS antibody responses in the individual subjects of the placebo group, and the groups having received 3, 4 or 5 injections. The heat maps depict the log_{10} of the serum concentrations of preS- or peptide-specific (a) IgG1 and (b) IgG4. Missing values are indicated in gray.
BM32-induced IgG antibodies at visits 8 and 11 reacted with peptides the group having received 5 injections of BM32, we found that 3.7. preS- and peptide C-specific ability, we quantified IgG responses were directed against a sequential epitope containing the N-terminal portion of preS which contains the critical NTCP binding of the virus and accordingly the use of the region showed that there were only few differences as compared to sequences determined in the more recent times (Fig. S1).

We also compared the NTCP-binding site and the accessory domain involved in the inhibition of infection (i.e., amino acid 13–51 of preS) in recently discovered ancient HBV sequences from Bronze Age to the Medieval ages [23–25]. This evolutionary analysis of the aa 13–51 preS region showed that there were only few differences as compared to sequences determined in the more recent times (Fig. S1).

3.6. IgG antibodies induced by vaccination with BM32 react with the NTCP binding sites and the accessory domain involved in the inhibition of infection of HBV genotypes A–H

In order to investigate if BM32 induces IgG antibodies recognizing the region comprising the NTCP binding site and the accessory domain involved in the inhibition of infection of different HBV genotypes we synthesized peptides comprising aa 13–51 of preS from the consensus sequences of HBV genotypes A–H (Fig. 3b) and tested them for reactivity with sera from vaccinated patients. As exemplified for the group having received 5 injections of BM32, we found that BM32-induced IgG antibodies at visits 8 and 11 reacted with peptides from all eight HBV genotypes (Fig. 7).

3.7. preS- and peptide C-specific IgG1 and IgG4 concentrations in sera capable of neutralizing HBV in vitro

In order to relate preS-specific antibodies with in vitro virus neutralizing ability, we quantified preS and peptide C-specific IgG1 and IgG4 levels in eight serum samples which had been assessed for HBV neutralization in vitro in an earlier study [17]. Our results showed that peptide C-specific antibodies in BM32-treated subjects (Table S4: B3, B6, B8, B17, B30, A1, A9) were always associated with neutralization whereas serum from a placebo-treated subject (Table S4: B15) lacking peptide C-specific IgG1 and IgG4 like observed in the placebo group of our current study did not induce neutralization. A more than 80% neutralization was observed with serum from subject A9 which had peptide C-specific IgG1 and IgG4 levels in the range of the median levels observed for the group having received 5 injections at visit 8 (Table S3). However, the levels of peptide C-specific IgG1 and IgG4 were not associated with the extent of neutralization. For example, serum from subject B17 containing very high levels of peptides C-specific IgG1 and IgG4 had a lower neutralization capacity (i.e., 50.25%) than serum from subject A9 (84.81%) which contained much lower peptide-specific antibodies (Table S4).

4. Discussion

We have previously reported that the recombinant grass pollen allergy vaccine BM32, which contains preS domain of LHBs, as immunogenic carrier molecule for fostering antibody responses to attached grass pollen allergen peptides also induced a preS-specific antibody response [17]. Moreover, we found that the preS-specific antibodies induced by immunization with BM32 protected against HBV infection in vitro using an infection model based on a cultured hepatocellular line (i.e., HepG2-NTCP cells) [17]. Our study adds some important new results, which encourage further studies to evaluate if BM32 may be used for therapeutic vaccination in patients with HBV-induced chronic hepatitis or as an alternative preventive approach for those who fail to build protective response with conventional vaccines (e.g., immunocompromised or elderly subjects). We found that a course of vaccination consisting of five monthly injections of BM32 was most effective in inducing preS-specific IgG responses as compared to three or four injections. Although preS-specific IgG levels varied, we did not find any non-responder in the group having received 5 injections of BM32. Since we were able to analyze sera from 128 subjects with more than 30 subjects in each of the four treatment groups these results seem to be quite representative. Importantly, we could confirm the results from our initial study, demonstrating that preS-specific IgG responses were directed against the N-terminal portion of preS which contains the NTCP binding site and the accessory domain involved in the inhibition of infection which are critical for the infection of liver cells by HBV. Using the synthetic peptide C which comprises these sites we could show that IgG antibodies were directed exactly against a sequential epitope containing the NTCP binding site and the accessory domain. This finding has relevance for the potential use of BM32 for therapeutic vaccination because antibodies blocking the NTCP binding site of HBV should inhibit the possibly ongoing recurrent infection of liver cells in patients with chronic hepatitis [30]. In fact, therapeutic vaccination has been attempted earlier with a vaccine containing preS2/S (Gene-Hevac B®, Pasteur-Merieux) [31]. Although this study offered evidence that the vaccine may decrease HBV replication [31], the results might have been improved, perhaps by including preS1, which contains the critical NTCP binding of the virus and accordingly the use of
preS1-containing vaccines has been proposed [3,11]. BM32 would meet this requirement because it potently induced IgG antibodies not only against preS2 but especially against preS1 and the NTCP binding site. A recent study by Wang et al. shows significant preventative and therapeutic effects achieved by vaccination with preS1 carried by ferritin nanoparticles, including the induction of anti-preS IgG up to 60 μg/ml and subsequent reduction of viral covalently closed circular DNA (cccDNA) in the liver [32]. However, this study has yet been done only in a mouse model and the relative importance of re-infection for persistence is unclear [30].

In this study we were for the first time able to quantify the preS-specific IgG response, in particular the IgG1 and IgG4 responses which we found to dominate over the other IgG subclasses and isoelectrofocusing earlier [17]. This finding was also confirmed in the current study. By using a quantitative ELISA for IgG1 and IgG4, based on purified standard curves established with purified human subclass-specific standards and the corresponding antigens, we were able to quantify the preS- and peptide C-specific IgG1 and IgG4 concentrations in serum. We found that immunization with five injections of BM32 induced a fast and strong initial IgG1 response accompanied by a lower but more sustained IgG4 response. The kinetics of preS-specific IgG1 and IgG4 responses were similar to the kinetics of allergen-specific IgG1 and IgG4 responses measured in a recent study [14]. Of note, at visit 8, 4 weeks after the fifth injection, the preS-specific cumulative IgG1 and IgG4 responses reached up to 1.8 mg/ml with median preS-specific IgG1 and IgG4 level of 106.8 μg/ml and 30 μg/ml, respectively, and thus should eventually be able to neutralize HBV even when soluble preS antigen is present in the blood of chronic HBV patients. While preS-specific IgG1, declined quickly, preS-specific IgG4 was maintained and from allergen-specific immunotherapy studies conducted with BM32 we know that IgG1 titers can be restored and IgG4 continues to grow after only one booster injection [14]. Our current study provides thus support for the latter speculation and encourages performing clinical trials with BM32 to investigate if it can be indeed used for therapeutic vaccination. Another major question, which we cannot answer at this time is if BM32 can overcome immune tolerance against HBV reported for patients suffering from chronic HBV infections [1]. Not yet published data (Tulaeva and Valenta, unpublished data) would indeed suggest that it is possible to build up robust preS-specific IgG responses in murine models for chronic HBV infections [33], but this needs to be verified in clinical trials in patients.

It has been suggested that it may be of advantage for achieving cure in chronic HBV that a therapeutic vaccine would not only neutralize HBV and prevent continuous infection of liver cells but also lead to a gentle elimination of infected cells [2,34]. Only a clinical trial studying vaccination of chronic HBV patients with BM32 can answer the question if BM32 vaccination can bring benefit and a form of cure, be it complete or functional. However, the available data would point towards this possibility, because BM32 was found to induce preS-specific CD4+ and mild CD8+ responses [17]. The mixed IgG1 and IgG4 response observed in our study would support this hope further because preS-specific IgG antibodies may mediate complement- or ADCC-mediated elimination of infected cells, whereas sustained IgG4 responses will mainly achieve neutralization of the virus without mediating inflammation because they do not activate complement or ADCC. In this context, it may be considered to modulate the short-lived IgG1 response by different treatment schedules because it can be easily boosted by additional injections and quickly declines after injection. However, the role of antibody-dependent cytotoxicity is not clear because HBV-infected cells may eventually not express preS on the surface.

The analysis of the IgG1 and IgG4 responses at the individual patient levels suggested that the vaccine has built up rather independent and quite unrelated IgG1 and IgG4 memory responses because they were poorly correlated. However, within each subclass and at the epitope levels responses were highly correlated and focused on the NTCP-binding site of HBV. In this context it is also of note, that at least 20% of the cumulative preS-specific IgG1 and IgG4 antibody responses were directed against the NTCP-binding site of HBV which is important to achieve inhibition of virus entry. The calculated 20% are a relatively conservative estimation because only antibodies reactive with the sequential peptide C epitope were quantified whereas antibodies reacting with conformational epitopes could not be measured with peptide C. In fact, we measured a median cumulative sum of 30 μg/ml of peptide C-specific IgG1 and IgG4 antibodies in subjects one month after having received five injections of BM32. In this context we found earlier that the nonmonoclonal antibody MA18/7 recognizing the peptide C-defined region inhibited in vitro hepatitis B infection when used at a concentration of 14 μg/ml [17]. The concentration of peptide C-specific antibodies was actually also quite comparable with the concentrations of myrcludex B, a 47 amino acids long myristoylated peptide derived from the N-terminus of preS suggested for treatment of chronic HBV infections, which was measured in the plasma of subjects two hours after having received 20 mg of the peptide by intravenous infusion [35] in terms of molarity. However, in contrast to myrcludex B, which disappeared from the circulation already approximately 15–20 h after infusion, we could detect more than 30% of the peptide C-specific IgG1 and IgG4 antibodies four months after visit 8. Due to the kinetics of myrcludex B extended treatment might be required, which may lead to inhibition of NTCP function and partial retention of bile acid transportation. Furthermore it is conceivable that much more myrcludex is needed to saturate the receptor on liver cells than antibodies are needed to prevent the binding of virus to the receptor. Therapeutic vaccination with BM32 would therefore have the advantage that one can build up a quite long-lasting concentration of antibodies which are approximately equimolar as compared to an infusion of 20 mg myrcludex B. In this context it should be mentioned that only one single booster injection of BM32 was sufficient to boost specific antibody levels [14]. Therapeutic vaccination would thus have considerable advantages over other small moleular inhibitors because only few injections with booster injections in long intervals (i.e., several months) would maintain protective antibody levels and the costs for treatment would be also low. However, the determination of preS and peptide C-specific IgG levels in sera from BM32-treated subjects (n=7) and in one placebo-treated subject which had been analyzed for their ability to neutralize HBV infection in vitro showed that the neutralizing capacity depended on the presence of peptide C-specific antibodies but not on their titers (Table S4). Therefore, additional factors such as avidity and ability to recognize the native virus seem to play an important role for neutralization which can only be evaluated in clinical trials.

Finally, we addressed another important question in our study: Will vaccination with BM32 induce antibodies which may cross-protect against the naturally occurring HBV genotypes? To address this question we synthesized a set of peptides comprising the NTCP binding sites and the accessory domains involved in the inhibition of infection from consensus sequences of the eight common HBV genotypes A–H and tested BM32-induced IgG antibodies for cross-reactivity. Our results showed that vaccination with five injections of BM32 containing genotype A-derived preS induced IgG antibodies which reacted with all eight genotypes in an almost comparable manner. Since preS sequences are highly conserved whereas there is evidence for frequently occurring S gene mutations [3], these findings would indicate that vaccination with BM32 may be used for treatment and eventually prevention of infections by the most common HBV genotypes.

In summary, our study provides several new and important results which encourage to further study BM32 for vaccination in chronic HBV infections in clinical trials. In fact, a first phase I/II trial in this direction has already been initiated using BM325, the most immunogenic component of BM32 (ClinicalTrials.gov Identifier: NCT03625934). This trial is currently ongoing and is expected to provide a basis for clinical trials for preventive and therapeutic vaccination of HBV infected patients with BM32.
Declaration of Competing Interest

Rudolf Valenta has received research grants from the Austrian Science Fund (FWF), from Viravaxx AG, Vienna, Austria and from HVD Life-Sciences, Vienna, Austria and serves as a consultant for Viravaxx AG. Rainer Henning is an employee of Viravaxx AG. Rainer Henning, Carolin Cornelius and Rudolf Valenta have patent application (WO2017037280) regarding the vaccine convincing. The other authors have no conflict of interest to declare.

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Supplementary materials

Supplementary material associated with this article can be found in the online version, at doi:10.1016/j.ebiom.2020.102953.

References

[1] Gerlich WH. Medical virology of hepatitis B: how it began and where we are now. Virol J 2013;10:1.doi: 10.1186/1743-422X-10-239.
[2] Revill PA, Chisari FV, Block JM, Dandri M, Gehring AJ, Guo H, et al. A global scientific strategy to cure hepatitis B. Lancet Gastroenterol Hepatol 2015;204:39–67. doi: 10.1016/j.bshy.2015.07.021.
[3] Gerlich WH. Prophylactic vaccination against hepatitis B: achievements, challenges and perspectives. Med Microbiol Immunol 2015;204:39–67. doi: 10.1007/s00430-014-0737-y.
[4] Neurath AR, Kent SBH, Strick N, Parker K. Identification and chemical synthesis of a host cell receptor binding site on hepatitis B virus. Cell 1986;46:429–45. doi: 10.1016/0092-8674(86)90663-X.
[5] Verrier ER, Colpitts CC, Sureau C, Baumert TF. Hepatitis B virus receptors and protective. Vaccine 1989;7:234–43. doi: 10.1016/0264-410X(89)90235-1.
[6] Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, et al. Sodium taurocholate cotransport polypeptide derived from the large viral surface protein. J Virol 2005;79:1613–22. doi: 10.1128/JVI.1613-1622.2005.
[7] Gripon P, Cannie I, Urban S. Efficient inhibition of hepatitis B virus infection by acetylated peptides derived from the preS1 region of the hepatitis B virus (HBV) envelope (env) protein are virus-neutralizing and protective. Vaccine 1989;7:234–43. doi: 10.1016/0264-410X(89)90235-1.
[8] Neurath AR, Seto B, Strick N. Antibodies to synthetic peptides from the preS1 region of the hepatitis B virus (HBV) envelope (env) protein are virus-neutralizing and protective. Vaccine 1989;7:234–43. doi: 10.1016/0264-410X(89)90235-1.
[9] Greiner S, Flicker S, Scheiblhofer S, Zafred D, Pavkov-Keller T, Thalhamer J, et al. Conversion of grass pollen allergens human immunoglobulin E to investigate the allergenic activity of major grass pollen allergen Phl p 5. Clin Exp Allergy 2011;41:270–80. doi: 10.1111/j.1365-2222.2010.03696.x.
[10] Babicki S, Arndt D, Marcu A, Liang Y, Grant JR, Maciejewski A, et al. Heatmapper: web-enabled heat mapping for all. Nucleic Acids Res 2016;44:W147–53. doi: 10.1093/nar/gkw419.
[11] Velkov S, Orr J, Protzer U, Michler T. The global hepatitis B virus genotype distribution assessed from available genotyping data. Genes 2018;9:495. doi: 10.3390/genes9040495.
[12] Herrerscheid C, Roingeard P, Blanchard E. Hepatitis B virus entry into cells. Cells 2020;9:1488. doi: 10.3390/cells9061488.
[13] Pol S, Nalpas B, Driss F, Michel M-L, Tiollais P, Denis J, et al. Efﬁcacy and limitations of a speciﬁc immunotherapy in chronic hepatitis B. J Hepatol 2001;34:97–11. doi: 10.1053/jhep.2000.0029-8.
[14] Wang W, Zhou X, Bian Y, Wang S, Chai Q, Guo Z, et al. Dual-targeting nanoparticle vaccine elicits a therapeutic antibody response against chronic hepatitis B. Nat Nanotechnol 2020;15:5(8):406–16. doi: 10.1038/s41565-020-0648-y.
[15] Bourque M, Dion S, Godon O, Guillen G, Michel M-L, Aguilar JC. Optimization of immune responses induced by therapeutic vaccination with cross-reactive antigens in a humanized hepatitis B surface antigen transgenic mouse model. Virol- ogy 2012;430:10–9. doi: 10.1016/j.virol.2012.04.007.
[16] Li D, He W, Liu X, Zheng Q, Q Y, Li H, et al. A potent human neutralizing antibody is prepared by independently established HBV infections. Elife 2017;6:e26738. doi: 10.7554/elife.26738.
[17] Blank A, Markert C, Hohmann N, Carls A, Mikus G, Lehr T, et al. First-in-human application of the novel hepatitis B and hepatitis D virus entry inhibitor myrcul- bex. J Hepatol 2016;65:483–9. doi: 10.1016/j.jhep.2016.04.013.