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Research Paper

Immunotherapy With the PreS-based Grass Pollen Allergy Vaccine BM32 Induces Antibody Responses Protecting Against Hepatitis B Infection

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ABSTRACT

Background: We have constructed and clinically evaluated a hypoallergenic vaccine for grass pollen allergy, BM32, which is based on fusion proteins consisting of peptides from the IgE binding sites of the major grass pollen allergens fused to preS (preS1 + preS2), a domain of the hepatitis B virus (HBV) large envelope protein which mediates the viral attachment and entry. Aim of this study was the characterization of the HBV-specific immune response induced by vaccination of allergic patients with BM32 and the investigation of the vaccines' potential to protect against infection with HBV.

Methods: Hepatitis B-specific antibody and T cell responses of patients vaccinated with BM32 were studied using recombinant preS and synthetic overlapping peptides spanning the preS sequence. The specificities of the antibody responses were compared with those of patients with chronic HBV infection. Furthermore, the capacity of BM32-induced antibodies, to inhibit HBV infection was investigated using HepG2-hNTCP cell-based *in vitro* virus neutralization assays.

Findings: IgG antibodies from BM32-vaccinated but not of HBV-infected individuals recognized the sequence motif implicated in NTCP (sodium-taurocholate co-transporting polypeptide)-receptor interaction of the hepatitis B virus and inhibited HBV infection.

Interpretation: Our study demonstrates that the recombinant hypoallergenic grass pollen allergy vaccine BM32 induces hepatitis B-specific immune responses which protect against hepatitis B virus infection *in vitro*.

1. Introduction

Allergy affects >25% of the population. There are several forms of intervention to manage allergic disease. They include allergen avoidance, symptomatic pharmacotherapy, biologics and allergen-specific immunotherapy (AIT) but only AIT has disease-modifying and long-lasting effects (Durham et al., 1999; Larché et al., 2006; Jacobsen et al., 2007). In order to improve safety, efficacy, convenience and patients' compliance, hypoallergenic allergy vaccines based on recombinant allergen derivatives or synthetic allergen-derived peptides have been developed and advanced into clinical trials (Valenta, 2002; Jutel and Akdis, 2014; Marth et al., 2014; Sandrini et al., 2015).

The concept of B cell epitope-based allergy vaccines is based on recombinant fusion proteins which consist of *per se* non-allergenic peptides from the IgE binding sites of major allergens and an allergen unrelated carrier protein which provides T cell help without activating pro-inflammatory allergen-specific T cell responses (Focke et al., 2010; Focke-Tejkl and Valenta, 2012). As candidates for the allergen-unrelated carrier proteins, viral proteins from rhinovirus and hepatitis B virus have been considered (Edlmayr et al., 2011).

We have recently developed such a hypoallergenic vaccine for grass pollen allergy, BM32, which is based on fusion proteins consisting of non-allergenic peptides from the IgE binding sites of the four major grass pollen allergens, Phl p 1, Phl p 2, Phl p 5 and Phl p 6, fused to the hepatitis B virus-derived surface protein preS (preS1 + preS2),

Abbreviations: HBV, hepatitis B virus; HBcAg, hepatitis B virus core antigen; HBeAg, hepatitis B virus e antigen; HBsAg, hepatitis B virus surface antigen; NTCP, sodium-taurocholate co-transporting polypeptide; AIT, allergen-specific immunotherapy.

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(Focke-Tejkl et al., 2015) (Fig. 1). The protein preS was selected as a carrier in BM32 because it has been used in HBV vaccines and was found to be safe (Rendi-Wagner et al., 2006) and preS-fusion proteins could be produced under Good Manufacturing Practice (GMP) conditions in a quality suitable for clinical trials. Due to lack of conformation the allergen-derived peptides are non-allergenic and the recombinant fusion proteins accordingly lacked IgE reactivity when tested with sera from grass pollen allergic patients and showed almost no induction of basophil activation. Furthermore, upon immunization of animals they induced IgG antibodies which recognized the natural grass pollen allergens and inhibited allergic patients' IgE binding and allergen-induced basophil activation (Focke-Tejkl et al., 2015). Skin testing of grass pollen allergic patients with the recombinant preS fusion proteins showed that they did not induce any relevant immediate or late phase skin reactions (Niederberger et al., 2015). Vaccination of grass pollen allergic patients with aluminium hydroxide-adsorbed preS-based fusion proteins revealed that the BM32 vaccine induced allergen-specific IgG responses and protected patients from grass pollen-induced rhinitis as determined in a pollen challenge chamber (ClinicalTrials.gov number: NCT01445002). Since it has been shown that preS1-specific antibodies neutralized HBV infectivity (Neurath et al., 1986; Glebe et al., 2003) we aimed to evaluate whether the preS-based grass pollen allergy vaccine BM32 could also induce HBV-specific immune responses. Using synthetic peptides spanning the preS-sequence, we mapped preS-specific antibody and T cell responses and compared the antibody responses of BM32-treated subjects with that of individuals, suffering from chronic HBV infection to explore the possible use of BM32 for therapeutic vaccination in the latter group. Furthermore we investigated the potential of BM32-induced antibodies to protect against HBV infection using an *in vitro* assay based on HBV receptor (sodium-taurocholate co-transporting polypeptide, NTCP) expressing HepG2 cell lines.

2. Material & Methods

2.1. Expression and Purification of Recombinant PreS, Synthesis of PreS Overlapping Peptides, Sequence Alignments

The procedure of the expression and purification of a hexahistidine-tagged recombinant preS protein (preS1 + preS2; genotype A; subtype adw2, GenBank: AAT28735.1) in *Escherichia coli BL21* (DE3, Stratagene, La Jolla, CA) is described elsewhere (Niespodziana et al., 2011).

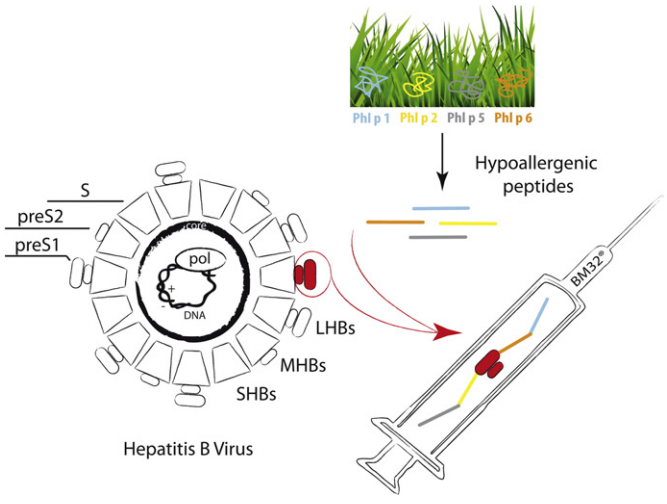

Fig. 1. Scheme for the construction of the BM32 vaccine. BM32 contains fusion proteins consisting of the preS domain (i.e., preS1 and preS2) of the large hepatitis B virus envelope protein (LHB) fused with allergen-derived peptides. LHB: Large hepatitis B virus envelope protein; MHB: Middle hepatitis B virus envelope protein; SHB: Small hepatitis B virus envelope protein.

Eight peptides at a length of approximately 30 amino acids and an overlap of 10 amino acids spanning the complete sequence of preS (genotype A, subtype adw2; Supplemental Fig. 1) were synthesized by a Fmoc (9-fluorenylmethoxycarbonyl) - strategy with HBTU [2-(1H-Benzotriazol-1-yl)1,1,3,3 tetramethyluronium hexafluorophosphat] activation (Liberty Microwave Peptide Synthesis, CEM Corporation, Matthews, NC) as described previously (Focke et al., 2001). Peptides were purified by preparative HPLC and their identity was confirmed by mass spectrometry (Microflex MALDI-TOF, Bruker, Billerica, MA).

An alignment of the preS genotype A, serotype adw2 sequence and peptide sequences thereof with HBV genotypes B–H was performed with CLUSTAL Ω using reference sequences from the HBV data base (HBVdb: https://hbvdb.ibcp.fr/HBVdb/HBVdbIndex),(Hayer et al., 2012).

2.2. Immunization of Rabbits and Mice

Specific rabbit antibodies against recombinant preS were raised by immunization of a New Zealand white rabbit with purified preS (200 μg per injection) using Freund's complete adjuvant (CFA) for the first and incomplete Freund's adjuvant (IFA) for the second and third injection (Charles River, Kisslegg, Germany). In addition, New Zealand white rabbits were immunized three times with a mix containing 20 μg (n = 2) or 40 μg (n = 2) of each of the four BM32 components (BM32–20/BM32–40) using $Al(OH)_3$ as adjuvant (Focke-Tejkl et al., 2015).

Furthermore, rabbit antibodies specific for the registered HBV vaccine ENGERIX-B were obtained by immunizing New Zealand white rabbits (n = 2) three-times with commercially available ready-to-use prefilled syringes (20 μg HBsAg/ml) at an interval of one month.

With approval of the animal ethics committee, six-week-old BALB/c mice (Charles River) (six animals/group) were immunized three times in a four weeks interval subcutaneously with $Al(OH)_3$-adsorbed mixes containing 10, 20 and 30 μg of each of the BM32 components. Animals were kept in the animal care unit of the Department of Pathophysiology and Allergy Research, Medical University of Vienna according to the local guideline for animal care. Serum samples were obtained before immunization and approximately four weeks after the third immunization and stored at −20 °C until analysis for preS-specific antibodies (Fig. 2).

2.3. Human Subjects

Serum samples from BM32 immunized subjects were obtained in the course of two clinical studies. One set of serum samples was obtained in the course of a safety and dose-finding phase IIa study (ClinicalTrials.gov number: NCT01445002) during which patients received three injections of $Al(OH)_3$-adsorbed BM32 (i.e., mixes of 10, 20 or 40 μg of each BM32 component or placebo, i.e., $Al(OH)_3$). Sera were collected before and four weeks after the third immunization and stored at −20 °C until usage. This study was approved by the Clinical Pharmacology Ethics Committee of the city of Vienna. A second set of serum samples was obtained in the course of a phase IIb study (ClinicalTrials.gov number: NCT01538979) during which patients were treated over a period of two years with seven subcutaneous injections of $Al(OH)_3$-adsorbed BM32 (mixes of 20 or 40 μg of each BM32 component) or $Al(OH)_3$ as placebo. An overview of the latter study is provided in Fig. 3. Supplemental Table 1 summarizes the characteristics of the patients (n = 30). This study was approved by the Ethics committee of the Medical University of Vienna, Austria (EK2092/2012) and all procedures were performed in accordance with the Declaration of Helsinki. Informed consent was obtained from all subjects included in the study.

In addition, serum samples were obtained from individuals suffering from chronic HBV infection which was diagnosed based on clinical data,