Vaccine 31 (2013) 1197–1203

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

# A novel therapeutic hepatitis B vaccine induces cellular and humoral immune responses and breaks tolerance in hepatitis B virus (HBV) transgenic mice☆

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ARTICLE INFO

*Article history:*
Received 10 August 2012
Received in revised form
13 December 2012
Accepted 23 December 2012
Available online 7 January 2013

*Keywords:*
HBV
ISCOMATRIX™ adjuvant
Protein vaccine
T cells

ABSTRACT

Therapeutic vaccines are currently being developed for chronic hepatitis B and C. As an alternative to long-term antiviral treatment or to support only partially effective therapy, they should activate the patient's immune system effectively to fight and finally control the virus. A paradigm of therapeutic vaccination is the potent induction of T-cell responses against key viral antigens – besides activation of a humoral immune response. We have evaluated the potential of a novel vaccine formulation comprising particulate hepatitis B surface (HBsAg) and core antigen (HBcAg), and the saponin-based ISCOMATRIX™ adjuvant for its ability to stimulate T and B cell responses in C57BL/6 mice and its ability to break tolerance in syngeneic HBV transgenic (HBVtg) mice. In C57BL/6 mice, the vaccine induced multifunctional HBsAg- and HBcAg-specific CD8+ T cells detected by staining for IFNγ, TNFα and IL-2, as well as high antibody titers against both antigens. Vaccination of HBVtg animals induced potent HBsAg- and HBcAg-specific CD8+ T-cell responses in spleens and HBcAg-specific CD8+ T-cell responses in livers as well as anti-HBs seroconversion two weeks post injection. Vaccination further reduced HBcAg expression in livers of HBVtg mice without causing liver damage.

In summary, this study demonstrates therapeutic efficacy of a novel vaccine formulation in a mouse model of immunotolerant, chronic HBV infection.

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## 1. Introduction

HBV infects the livers, where it establishes either transient or persistent infection and may cause necroinflammatory liver disease – hepatitis B. Vertical transmission from mothers to their neonates, or infection during the first year of life, results in persistent, often life-long infection in >90%. In contrast, infection during adulthood is cleared in most cases, and results in life-long protective immunity [1]. A polyclonal and multispecific T-cell response is characteristic for cleared acute infection [2,3], while a weak and oligoclonal response is associated with chronic infection [4,5].

Despite the availability of an effective prophylactic vaccine, worldwide more than 350 million humans are chronically infected with HBV being at risk to develop liver cirrhosis or hepatocellular carcinoma. Current treatment options for chronic hepatitis B depend on interferon α or nucleos(t)ide analogs, which efficiently control virus replication but rarely eliminate the virus. HBV covalently closed circular DNA (cccDNA) persists in the host cell nucleus and drives a viral rebound and recurrent disease once therapy is discontinued. Therefore, cost-intensive long-term treatment is required.

*Abbreviations:* HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; IFNγ, interferon γ; anti-HBs, antibodies against HBsAg; anti-HBc, antibodies against HBcAg.

☆ *Financial support*: This work was in part supported by the Technologie-Programm Wirtschaft (PTJ-001-9910-0v08) to Rhein Biotech, the German Center for Infection Research (DZIF) and by the Helmholtz Alliance "Immunotherapy of Cancer" HA-202 to U. P.

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0264-410X/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.vaccine.2012.12.074

Immunotherapies designed to activate either nonspecific or HBV-specific immune responses could achieve sustained viral control after timely limited treatment [6]. Support for the efficacy of T-cells has been gained from the clinical observation that chronic HBV infection may resolve in bone marrow transplant patients receiving bone marrow from an HBV immune donor [7,8]. Therapeutic vaccination can induce T cell responses [9], and preclinical studies in chimpanzees had a promising outcome [10].

First clinic attempts based on available prophylactic vaccines, alone or in combination with interferon-α and/or antiviral compounds [11–13] were not able to induce an immune response in chronic hepatitis B [14].

Failure was mainly attributed to the fact that the aluminum adjuvanted vaccines induce a pronounced Th2 type immune response and do not stimulate cytotoxic T lymphocytes (CTL). Hence, novel therapeutic vaccine formulations were developed to induce a CTL response. The lipopeptide Theradigm®, containing an HBcAg 18–27 HLA-A2 peptide epitope and an universal tetanus toxoid helper T cell epitope induced a CTL response in healthy volunteers [15], but had no therapeutic effect in chronic hepatitis B patients [16]. HBsAg adjuvanted with MPL, QS21 and an oil-in-water emulsion induced HBsAg-specific T cells and antibodies in healthy volunteers [17], but in patients with HBeAg positive chronic hepatitis B under lamivudine treatment failed to increase HBeAg seroconversion rates [18]. HBsAg complexed with anti-HBs also showed only minor effects in clinical studies [19].

All these approaches had in common that a single HBV antigen was included in the vaccine. In addition, particulate antigens (*e.g.* virus-like particles) combined with a potent adjuvant seem better suited to induce robust CD4+ and CD8+ T-cell responses. To induce multispecific CTL responses, which are key to resolution of HBV infection [20,21], we tested a combination of particulate HBcAg and HBsAg adjuvanted with saponin-based ISCOMATRIX™ adjuvant for its immunogenicity in naïve as well as in HBVtg mice.

## 2. Materials and methods

### *2.1. Antigens, adjuvant, vaccine formulation*

HBcAg particles (lacking the nucleic acid binding region) of genotype D subtype ayw (ay) were produced in *Escherichia coli* and purified as described [22]. Endotoxin concentration was 65 endotoxin units/mg corresponding to 0.65 units per dose. Recombinant HBsAg particles of genotype A/subtype adw, and genotype C/subtype adr were produced in the yeast H. polymorpha [23]. Saponin-based ISCOMATRIX™ adjuvant capable of inducing both humoral and cellular immune responses was obtained from CSL Limited (Parkville, Victoria, Australia). The vaccine formulation contained 10 μg HBsAg, 10 μg HBcAg, and 4 μg ISCO™ Units ISCOMATRIX™ adjuvant, respectively, in a volume of 100 μl per mouse dose, if not otherwise stated.

### *2.2. Mice and immunizations*

All animals received human care and study protocols were in compliance with institutional guidelines. Mice were immunized s.c. on day 1 and day 22 unless otherwise indicated. Spleens, livers and sera were collected day 14 after the last immunization for subsequent analysis. Liver associated lymphocytes (LAL) were prepared as described [24,25]. C57BL/6 HBV1.3.32 transgenic mice (line 1.3.32 [26]), kindly provided by F. Chisari (The SCRIPPS Institute, La Jolla, CA, USA), carry a 1.3-fold overlength HBV genome. HBsAg(adw) and HBsAg(adr), both heterologous to HBV genotype D/subtype ay expressed in HBVtg mice, proved to be equally immunogenic (data not shown). Therefore, HBsAg(adr) was used in most experiments.

### *2.3. Stimulation and intracellular cytokine staining of HBV-specific T cells*

Freshly isolated splenocytes ($2–6 \times 10^6$ cells per well) or LAL were incubated with 1 μg/ml or 0.25 μM HBcAg 93–100 MGLKFRQL, HBsAg (ay) 208–215 ILSPFLPL, HBsAg (adw) 208–215 IVSPFIPL or control peptide SIINFEKL (jpt Peptide Technologies, Berlin, Germany) and 5 μg/ml brefeldin A (Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany) for 4 h. After stimulation, cells were washed and incubated with Ethidium monoazide to assess viability. After stained for CD8 and CD4 cells were fixed and permeabilized using the BD Cytofix/Cytoperm™ Kit (BD Bioscience, Heidelberg, Germany). Fluorochrome conjugated antibodies were added for intra cellular cytokine staining: IFNγ-FITC, TNFα-PECy7 (BD Bioscience) and IL-2-AF647 (eBioscience, Eching, Germany). Cells were analyzed by flow cytometry to determine the number of responding cells per $10^5$ CD8+ T cells. Background levels of cytokine production from control peptide stimulated samples were subtracted from the corresponding responses.

### *2.4. Enzyme linked ImmunoSpot*

$1.5 \times 10^6$ splenocytes per well were plated on 96-well plates for ELISpot assays for IL-4 or IL-5 release using ELISpot plus for Mouse Kits (MABTECH, Nacka Strand, Sweden). Cells were unstimulated or stimulated with 2 μg/ml PMA/Ionomycin (Sigma–Aldrich, Taufkirchen, Germany) or with 5 μg/ml recombinant HBsAg or HBcAg for 48 h at 37 °C. After washing and counterstaining, spot forming cells/well (SFC) were counted on a CTL ImmunoSpot S5 UV Analyser. Positive results were defined as ≥5 SFC. In unstimulated samples, SFC background was <2. Negative results were defined as <5 SFC when positive controls were ≥50 SFC.

### *2.5. Detection of serological and biochemical parameters*

Serum alaninaminotransferase (ALT) activity was determined using bioreaction strips on a Reflovet® Plus reader (Roche Diagnostics, Mannheim, Germany). For C57BL/6 mice, anti-HBsAg antibodies were quantified using IMx AUSAB reagents (Abbott Laboratories, Abbott Park, IL, USA). For HBVtg mice, HBsAg and hepatitis B e antigen (HBeAg) as well as anti-HBs and anti-HBc were measured in 1:20 diluted sera using standardized assays (AXSYM™, Abbott). IgG1 and IgG2b antibody isotypes were determined by ELISA. HBV DNA was quantified in the serum by quantitative real time PCR as described [19].

### *2.6. Liver histology*

Liver tissue specimens were fixed in 4% buffered formalin for ≥24 h and embedded in paraffin. Four μm sections were stained with hematoxylin and eosin (HE). Ten vision fields per mouse were randomly chosen and inflammatory foci per visual field were counted in a 100-fold magnification. Desmet scores: 0 = no fibrosis (0–2 inflammatory foci), 1 = mild fibrosis (periportal fibrous expansion or 2–5 foci); 2 = moderate fibrosis (porto-portal septa or 6–10 foci); 3 = severe fibrosis (portcentral septa or >10 foci); 4 = cirrhosis were applied [27]. Immunohistochemistry was performed using polyclonal rabbit anti-hepatitis B core antigen (HBcAg) (Dako Hamburg, Germany, dilution 1:500). Positive HBcAg staining was evaluated separately for nuclei and cytoplasm of hepatocytes.

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