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SCIENCE DIRECT
Vaccine 24 (2006) 2506–2513
Vaccine
www.elsevier.com/locate/vaccine

Rapid, high-level production of hepatitis B core antigen in plant leaf and its immunogenicity in mice

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Received 3 October 2005; received in revised form 29 November 2005; accepted 13 December 2005
Available online 28 December 2005

Abstract

Hepatitis B core antigen (HBc or HBcAg) self-assembles into capsid particles and is extremely immunogenic. HBc has been extensively studied for its production in various expression systems and for the use of HBc particles for high-density, immunogenic presentation of foreign epitopes. Here we reported the high-level transient expression of HBc in plant leaf and its immunogenicity in mice. By using a novel plant viral expression system, HBc was produced in *Nicotiana benthamiana* leaves at levels up to 7.14% of total soluble protein (TSP) or 2.38 milligrams HBc per gram of fresh weight at 7 days post-infection (dpi). Plant-derived HBc (p-HBc) assembled into virus-like particles (VLPs) as revealed by sucrose gradients and electron microscopy. Partially purified p-HBc stimulated strong serum antibody responses in mice as *Escherichia coli*-derived HBc upon intraperitoneal (i.p.) injection. Furthermore, mice immunized mucosally (orally and intranasally) with p-HBc in the absence of adjuvants also developed HBc-specific serum IgG as well as intestinal IgA. Taken together, our results indicate the potential usefulness of p-HBc-VLP as a carrier for immunogenic presentation and mucosal delivery of foreign epitopes.

Keywords: Transient expression; Hepatitis B core antigen; Virus-like particle; Plant-made vaccine

1. Introduction

Hepatitis B virus (HBV) core antigen (HBc or HBcAg) is a 21 kDa protein that self-assembles into subviral nucleocapsid particles packaging the viral polymerase and pregenomic RNA during HBV infection. HBV-infected individuals develop early, long-lasting and high-titered anti-HBc responses. The extremely high immunogenicity of HBc is probably due to its polymeric nature [1], the presence in its primary sequence of a number of potent T helper epitopes [2], its ability to directly activate B cells [3,4], and the presence of encapsidated nucleic acids [5,6]. Interestingly, it was found that HBc-specific helper T cells can also help B cells produce antibodies against a separate antigen, hepatitis B surface antigen (HBsAg) [7].

HBc monomers assemble into virus-like particles (VLPs) of two different sizes, composed of 180 or 240 subunits arranged with $T = 3$ or $T = 4$ icosahedral symmetry [1], making it an attractive carrier for high-density presentation of foreign epitopes. HBc fusion proteins with insertion of various foreign B cell and T cell epitopes retained the ability to form chimeric VLPs and stimulated a high level of B cell and T cell immunogenicity to foreign epitopes, especially when they are inserted into the tips of spikes of HBc particles (reviewed in [8–10]). Besides being a carrier molecule, HBc also has an immuno-enhancing effect on co-delivered HBsAg [11,12], and was hence suggested to be included in the design of more potent therapeutic and preventive vaccines against HBV infection.

Recombinant HBc or HBc fusions can be produced in various expression systems, including yeast [13], mammalian cell cultures [14,15], vaccinia virus [16,17], and bacteria such as *Escherichia coli* [18]. Transgenic plants have also been used for HBc production [19]. Transgenic plant-derived HBcAg

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doi:10.1016/j.vaccine.2005.12.024

assembled into VLPs and showed serologic properties comparable to that from *E. coli*; however, the expression levels were very low and the immunogenicity was not tested [19].

We report here that the rapid, high-level (up to 7% of total soluble protein (TSP)) production of HBc can be achieved in plant leaf by using a novel plant viral expression system. The plant-derived HBc (p-HBc) self-assembles into VLPs that are morphologically and physically similar to *E. coli*-derived HBc (*e*-HBc)-VLPs. We also show that partially purified p-HBc induced strong antibody responses in mice upon intraperitoneal (i.p.) injection as did *e*-HBc. Furthermore, mice given p-HBc mucosally (orally and intranasally) without any adjuvant developed HBc-specific serum IgG and intestinal IgA. Theses results indicate the potential usefulness of plant-derived HBc-VLPs as a carrier for immunogenic presentation and mucosal delivery of foreign epitopes.

2. Materials and methods

2.1. Plasmid construction

Binary vectors, pICH11599, pICH15879, pICH14011 and pICH-GFP (pICH7410) (Fig. 1), were described previously [20] and in Fig. 1. Plasmid pHBV33 (obtained from Axis Genetics) contains a plant codon-optimized HBc gene (GenBank accession in progress) under the control of CaMV 35S promoter. The HBc gene was released by NcoI/SacI digestion of pHBV33 and inserted into pICH11599 from the same sites, resulting in pICH-HBc (Fig. 1).

2.2. Agroinfiltration procedure

ICON binary vectors were separately introduced into *Agrobacterium tumefaciens* GV3101 by electroporation. The resulting strains were grown in YENB medium (7.5 g/l yeast extract, 8 g/l nutrient broth) supplemented with rifampicin (60 mg/l) and carbenicillin (50 mg/l) at 28 °C. The cells of an overnight culture (OD_{600} = 0.5–1.0) were sedimented by centrifugation (10 min, 4500 g) and resuspended in infiltration buffer (10 mM 2-(*N*-morpholino)ethanesulphonic acid (MES), pH 5.5, 10 mM Mg_2SO_4). The bacterial suspensions were adjusted to a final OD_{600} of 0.05 and equal volumes (5′ module:3′ module:integrase module = 1:1:1) were mixed before infiltration. Using a 1 ml syringe without a needle, mixed *Agrobacterium* suspensions were injected through a small puncture into fully expanded leaves of 6–8 weeks old *N. benthamiana* plants grown in greenhouse as previously described [21].

2.3. HBc extraction and quantification

Total protein from infiltrated *N. benthamiana* leaf was extracted by using a Fastprep FP120 machine (Bio101, Vista, CA) in ice-cold extraction buffer [1 × PBS (50 mM sodium phosphate, 100 mM NaCl, pH 7.4), 10 mM EDTA, 0.1% Triton X-100, 5 mg/ml sodium ascorbate, and 10 μg/ml leupeptin]. Total soluble protein was measured using a Bradford protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as reference standard. HBc was quantified by a sandwich ELISA. Briefly, microtiter plates were coated with 50 μl per well of a rabbit polyclonal anti-HBc (Cat# D44410130, BiosPacific, Emeryville, CA) diluted 1:5000 in 1 × PBS buffer and incubated at 4 °C overnight; then and after each of the following steps, the plates were washed three times with PBST buffer (1 × PBS with 0.05% tween-20). Consecutively, 200 μl/well of 5% skim milk in PBST was added for blocking and incubated at 37 °C for 1 h; plant extracts or *E. coli*-derived recombinant HBc standard (ViroGen, Watertown, MA) diluted in PBST plus 1% skim milk were added

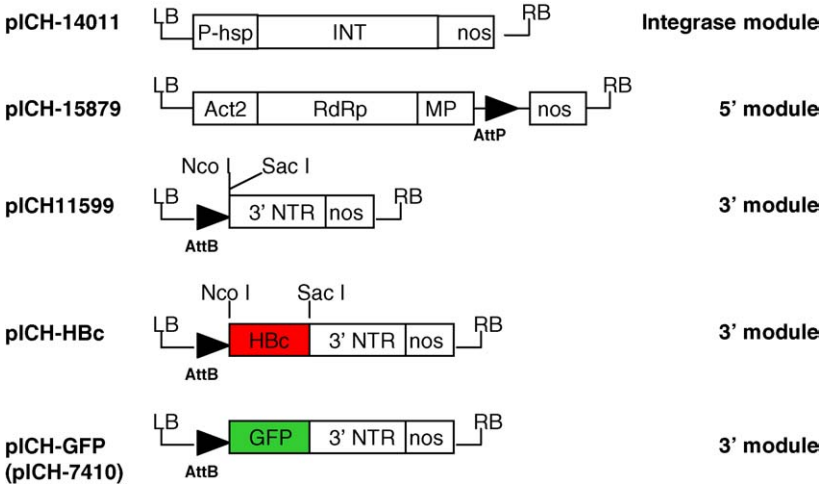

Fig. 1. Schematic representation of the T-DNA regions of the ICON constructs used in this study. P-hsp, Arabidopsis heat shock protein 81.1 promoter; INT, the *Streptomyces* phage PhiC31 integrase; AttP and AttB, PhiC31 integrase recombination sites; Act2, *Arabidopsis* actin 2 promoter; RdRp, RNA-dependent RNA polymerase; MP, movement protein; 3′ NTR, 3′ nontranslated region; nos, nopaline synthase terminator; LB and RB, the left and right borders of the T-DNA region.