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Deletion modification enhances anthrax specific immunity and protective efficacy of a hepatitis B core particle-based anthrax epitope vaccine

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ABSTRACT

Protective antigen (PA) is one of the major virulence factors of anthrax and is also the major constituent of the current anthrax vaccine. Previously, we found that the 2β2–2β3 loop of PA contains a dominant neutralizing epitope, the SFFD. We successfully inserted the 2β2–2β3 loop of PA into the major immunodominant region (MIR) of hepatitis B virus core (HBc) protein. The resulting fusion protein, termed HBc-N144-PA-loop2 (HBcL2), can effectively produce anthrax specific protective antibodies in an animal model. However, the protective immunity caused by HBcL2 could still be improved. In this research, we removed amino acids 79–81 from the HBc MIR of the HBcL2. This region was previously reported to be the major B cell epitope of HBc, and in keeping with this finding, we observed that the short deletion in the MIR not only diminished the intrinsic immunogenicity of HBc but also stimulated a higher titer of anthrax specific immunity. Most importantly, this deletion led to the full protection of the immunized mice against a lethal dose anthrax toxin challenge. We supposed that the conformational changes which occurred after the short deletion and foreign insertion in the MIR of HBc were the most likely reasons for the improvement in the immunogenicity of the HBc-based anthrax epitope vaccine.

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Introduction

The hepatitis B virus core (HBc) protein is synthesized from the second AUG of the hepatitis B virus (HBV) C gene and constitutes a structural component of the hepatitis B virus core antigen (HBcAg) or HBV nucleocapsid (Pumpens and Grens 2001). The natural HBc protein is multifunctional, and due to its unusual flexibility, it can self-assemble into particles and is amenable to genetic modification. Besides, this protein has been found to have high expression levels and efficient particle formation in homologous (Braun et al. 2008) and heterologous expression systems (Pumpens and Grens 2001; Ravin et al. 2012; Freivalds et al. 2011), and it is easy to prepare. Perhaps these factors are the major reasons that the HBc protein occupies a unique position among other virus-like particle (VLP) scaffolds. The crystal structure of HBc was resolved in 1999, and the results showed that the HBc monomer is dominated by a long α-helical hairpin consisting of residues 50–73(α3) and 79–110(α4) (Wynne et al. 1999), which serves as the dimerization interface; the resulting four-helix bundles protrude from the capsid surface as prominent spikes (Walker et al. 2011). An exposed α-helical hairpin loop connects α3 and α4 and is part of the immundominant c/e1 B cell epitope between amino acids (aa) 74 and 84 (Walker et al. 2011). This region is reported to be responsible for the predominant anti-HBc response during HBV infection (Walker et al. 2011), and it is called the major immunodominant region (MIR) (Pumpens and Grens 2001). The primary structures of the HBc molecules are diverse, and they induce the strongest B-cell, T-cell and cytotoxic T lymphocyte response. This property is likely due to the repetitive surface presentation of the B cell epitopes, the presence of potent T cell epitopes, and their ability to act as a T cell independent antigen (Pumpens and Grens 2001; Whitacre et al. 2009). The most valuable factor is that the HBc particles are able to transfer these attributes to the fused foreign sequences (Ulrich

Abbreviations: HBc, hepatitis B virus core protein; HBcAg, hepatitis B virus core antigen; HBV, hepatitis B virus; HBc-N144, N-terminal 144 amino acids of HBc; HBcL2, HBc-N144-PA-loop2; LeTx, Lethal toxin; MIR, major immunodominant region; PA, protective antigen; rPA, recombinant protective antigen; rLF, recombinant lethal factor; VLP, virus like particle; aa, amino acid(s).

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et al. 1998), especially if inserted into the most surface-exposed MIR, thereby enhancing their immunogenicity (Pumpens and Grens 2001; Walker et al. 2011). The HBc protein has a basic C terminal tail for nucleic acid binding that is dispensable for shell formation, as long as it is not truncated before amino acid 140 (Gallina et al. 1989).

The HBc particles have been used as promising virus-like display scaffolds since the 1980s (Zeltins 2013). In the last thirty years, the most successful application of this scaffold has been the influenza vaccine ACAM-FLU-A, produced by Sanofi Pasteur, and the malaria (*Plasmodium falciparum*) vaccine MalariVax (ICC-1132), produced by Apovia (Kushnir et al. 2012). These particles have been expressed in *Escherichia coli* with the N terminal or the MIR of HBc as the foreign insertion site and have already been subjected to Phase I clinical trials (Schotsaert et al. 2009; Neirynck et al. 1999; Fiers et al. 2004; Gregson et al. 2008; Nardin et al. 2004; Oliveira et al. 2005).

Anthrax is a lethal disease caused by the gram-negative, spore-forming bacterium *Bacillus anthracis* (Mock and Fouet 2001). This disease is still endemic in certain parts of the world. Anthrax is found primarily in the herbivores of developing countries, but it can also affect a wide range of species, including humans (Collier and Young 2003). The protective antigen (PA) is one of the most important components of the anthrax toxin and is the predominant target for therapeutic and prophylactic anthrax medicines (Friedlander and Little 2009).

Previously, we identified a dominant neutralizing epitope in the 2β2–2β3 loop of PA domain 2 and successfully inserted the loop into the MIR of the HBc (Zhang et al. 2006). The resulting fusion structure HBc-N144-PA-loop2 (HBcL2) effectively produced anthrax-specific protective antibodies in an animal model (Yin et al. 2008). However, the protective immunity caused by HBcL2 could still be improved. In this research, we modified the HBc particles used as a scaffold in HBcL2 to abrogate the intrinsic HBc antigenicity/immunogenicity while still retaining or increasing the PA sequence-specific immunogenicity. We also analyzed the conceivable reasons for the change of protective immunity induced by this modification.

Materials and methods

Plasmid construction

The C-terminal His tag and extra pET21a sequences were excised from plasmid pET21a-HBcL2 (Yin et al. 2008) using the restriction sites *Nde/Hind* and the primers pETNu (5-cccatatgatggacattgacccgtat-3)/pETNd (5-cgcaagcttttacggaagtgttgataagatag-3) to generate the plasmid pET21a-H144L2. Using pET21a-H144L2 and the primers H144DL2f (5-actaaatcctgcagatacactccca-3) and H144DL2z (5-agggaatta gtagtcagctatgtc-3), we deleted the 79–81 amino acids of HBc through inverse PCR, producing plasmid pET21a-H144DL2. A brief description of all constructs used is shown in Fig. 1. The content of the plasmids was verified by DNA sequencing.

Protein expression and purification

H144L2 and H144DL2

The constructs were transformed into *E. coli* BL21 (DE3). When the OD_{600} reached 0.6–1.0, the cell cultures were induced by adding IPTG to a final concentration of 1 mM for 5 h at 28 °C. The bacteria were concentrated by centrifugation and lysed by sonication, and the clear supernatants were loaded onto a DEAE sepharose column (GE Healthcare, USA) and equilibrated with a solution of 20 mM Tris–HCl and 50 mM NaCl, at pH 8.0. The fusion proteins

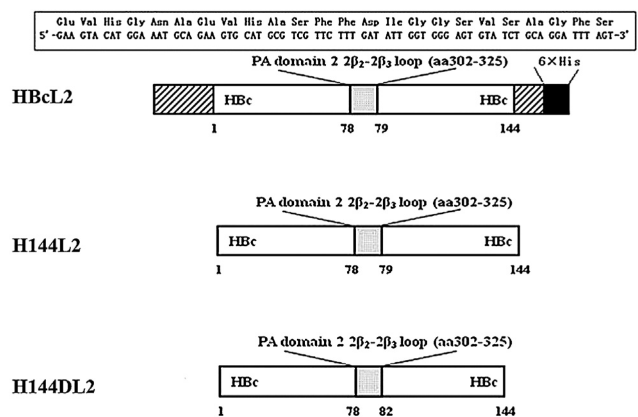

Fig. 1. Modification of the HBcL2 based structures. The general organization of HBcL2. The scaffold portion, HBc-N144, encompassed the assembly domain (aa 1–144) of the HBc protein, with the C-terminal basic domain replaced by a 6× His tag. The shadow represents the extra sequences from pET21a. The sequences from PA loop2 were inserted between Asp78 and Pro79 of HBc-N144. The H144L2 were generated without the 6× His tag and the extra sequences from pET21a. The H144DL2 were generated without the 6× His tag and the extra sequences from pET21a and the aa 79–81 of HBc was deleted.

were not retained but appeared in the flow-through while some bacterial proteins were kept on the column. A solution of 20 mM Tris–HCl and 1.0 M $(NH_4)_2SO_4$ at pH 8.0 was then added to the flow-through to a final concentration of 0.2 M $(NH_4)_2SO_4$. The precipitate was collected and dissolved in PBS at pH 7.2 (20 mM NaH_2PO_4, 150 mM NaCl) at 4 °C for 1–2 h. The solutions were centrifuged at 4 °C and 12,000 rpm for 20 min, and the resulting supernatants were harvested and filtered through a 0.2 μm filter to remove any undissolved proteins. The solution containing the fusion protein was then loaded onto a Sephacryl S-200 Hiload 26/60 column (GE Healthcare, USA), which was equilibrated in PBS, at pH 7.2. The eluate was assessed at OD_{280}, and the peak fractions were collected and analyzed by SDS-PAGE. LPS contamination was identified by the tachyplens amebocyte lysate (TAL) assay (Chinese Horseshoe Crab Reagent Manufactory, China).

Recombinant anthrax protective antigen and lethal factor

Recombinant anthrax protective antigen (rPA) and lethal factor (rLF) was prepared as described previously (Yin et al. 2008; Liu et al. 2013). Briefly, a plasmid carrying the gene of PA or LF with an OmpA signal sequence was transformed into *E. coli* DH5α and the bacteria were induced to express rPA or rLF by IPTG. The recombinant proteins which were secreted to the periplasmic space of the bacterial cell was purified with chromatography. The final proteins were about 95% pure and the contamination of LPS was <20 EU/mg determined by TAL assay.

SDS-PAGE and western blot analysis of the HBc particles

SDS-PAGE electrophoresis was performed on 12% SDS polyacrylamide gels. For immunological detection, the SDS-PAGE gels were blotted to the NC membranes, which were then probed with specific primary antibodies and peroxidase-conjugated secondary antibodies with chemiluminescent substrates. The monoclonal antibody (mAb) 10E11 (mouse mAb, Santa Cruz) was used against the HBc; a mAb 5E12 was used against 2β2–2β3 loop of PA domain 2. The working concentration of both mAb was 0.1 μg/ml.

Electron microscopy

The purified proteins HBc-N144 (Yin et al. 2008), H144L2, H144DL2 were analyzed by negative staining electron microscopy

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