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Vaccination with coxsackievirus B3 virus-like particles elicits humoral immune response and protects mice against myocarditis

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ABSTRACT

Coxsackievirus B3 (CVB3), along with other enteroviruses, is involved in about 50% of myocarditis cases and in the pathogenesis of dilated cardiomyopathy. Prevention of CVB3 infection is therefore highly desirable. Virus-like particles (VLPs) are structurally similar to native virus particles and therefore are far better immunogens than any other subunit vaccines. Recombinant baculoviruses carrying either the intact, entire coding region of CVB3 or the four individual coding regions for virus proteins 1–4 (VP1–4) were constructed. Expression of CVB3 capsid proteins in insect cells infected with recombinant baculovirus was detected by immunofluorescence and Western blot analysis. Sucrose gradient ultracentrifugation fractions of the infected cell lysates contained peaks of CVB3 antigen with an approximate density of 1.14 g/ml. Electron microscopy demonstrated the presence of VLP in these sucrose fractions. The CVB3 VLP was non-infectious in tissue culture. SWR (H-2^q) mice vaccinated with CVB3 VLP developed antibodies to CVB3 capsid proteins after the first boost. Antibody titre was comparable to the level induced by an attenuated CVB3 vaccine. Vaccinated animals were protected from myocarditis when subsequently challenged with cardiovirulent CVB3 (chimera-2). Vaccination with VLP produced from the complete CVB3 coding region gave a greater immune response and afforded better protection than with VLP from the quadruple expression vector. These results demonstrate that CVB3 capsid proteins expressed in insect cells have the intrinsic capacity to assemble into non-infectious VLP, which afforded protection from CVB3 infection to mice when used as a vaccine.

1. Introduction

Coxsackievirus B3 (CVB3) is an important human pathogen that induces acute or chronic viral myocarditis [1]. Between 5–50% of myocarditis and its end-stage, dilated cardiomyopathy (DCM), are attributable to CVB3 infection [2]. However, there are no vaccines or therapeutic reagents for clinical use, although some reports have demonstrated effective candidates in a murine model [3–7].

CVB3 is a non-enveloped, positive-sense, single-stranded RNA virus within the *Picornaviridae* family. This enterovirus genome consists of a single open reading frame that encodes proteins P1, P2 and P3. The P2 and P3 regions encode non-structural proteins (for polyprotein cleavage and RNA replication), while the P1 region encodes the P1 precursor that is cleaved to form VP0, VP1 and VP3. These three proteins spontaneously assemble into icosahedral procapsids and pack the RNA genome into the provirion. Cleavage of VP0 to VP4 and VP2 then occurs by autocatalysis during viral maturation [8–10]. VP1, VP2 and VP3 are surface proteins while VP4 is located at the capsid's inner surface. VP1 contains at least three antigenic regions [11], while VP3 and VP2 also contribute to capsid antigenicity.

Vaccines against CVB could significantly reduce the incidence of serious or fatal viral myocarditis as well as the multitude of diseases associated with CVB infection [12]. Many investigators have used different approaches and demonstrated the potential usefulness of vaccination in the prevention of acute infection or myocarditis in animal models. These include subunit, inactivated virus, attenuated

Abbreviations: CVB3, coxsackievirus B3; VLP, virus-like particle.

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live virus, or DNA vaccines [5–7,13–18]. These approaches are not without risk and may not stimulate an ideal immune response. For example, attenuated and inactivated virus vaccines carry the risks of reversion to a virulent form or incomplete inactivation, which could lead to disease in the recipient. Moreover, the use of live-attenuated vaccines is not recommended during pregnancy due to a theoretical risk to the foetus [19]. Subunit vaccines do not stimulate as great an immune response as inactivated or attenuated virus vaccines [20]. Thus, multiple doses containing high concentration must be used. This increases vaccination costs and reduces applicability and acceptability in some scenarios. Peptide and subunit vaccines often fail, due to antigen presentation to the immune system being ineffective. Moreover, antigenic epitopes are conformation dependent and some are derived from regions of adjacent proteins. As such, conservation of the natural structure of viral proteins is vital for immune stimulation. Virus-like particles (VLPs) are similar in both size and shape to natural virus particles but lack nucleic acid and so are non-infectious and therefore do not carry the risk of reverting to virulent form by reversion, recombination or re-assortment. VLPs are highly immunogenic as they present viral antigens in an authentic conformation; their size also allows uptake by dendritic cells. As such, VLP vaccines stimulate both humoral and cellular immune responses [21–23]. VLP vaccine candidates have been developed for many different types of virus, including picornaviruses. For example, poliovirus empty capsids have been produced that are as immunogenic as virions [24]. Further, immunization of BALB/c mice with VLP of enterovirus 71 has been shown to induce potent and long-lasting humoral immune responses as well as induction of Th1 and Th2 immune responses [25]. Vaccination of pregnant mice with these EV71 VLP conferred protection (survival rate up to 89%) to neonatal mice against subsequent lethal (1000 LD_{50}) viral challenge. The successful development and application of genetically engineered VLP as a vaccine is exemplified by the recently licensed bivalent or quadrivalent products against human papillomavirus [26–28]. As far as we are aware, no one has previously used VLP as a candidate CVB3 subunit vaccine and no subunit vaccines have been licensed for picornaviruses.

The purpose of this study was to explore the VLP approach for the development of enterovirus vaccines, using a sensitive murine model of CVB3-induced myocarditis. This paper describes the construction and characterization of a potential CVB3 VLP vaccine and its efficacy in protecting mice from CVB3-induced myocarditis. It is envisaged that this proof of principle work will stimulate further research into enterovirus vaccine development.

2. Materials and methods

2.1. Construction of transfer vector pBluBac4.5/cb3

Plasmid pG4Z-2 [29], containing a full-length cDNA copy of a cardiovirulent CVB3 genome, was used as a PCR template. To obtain the entire coding region, encompassing residues 741–7299 of the CVB3 genome, three fragments were prepared. Fragment 1 (residues 741–2079) was amplified using LiF1 sense (5′-AACTGCAGA**ATG**GGAGCTCAAGTATC-3′) and LiR1 antisense (5′-GGCCGAACCAACAGAACATAAA-3′) primers. The LiF1 primer had a built-in *Pst* I site (underlined) at its 5′ end to facilitate cloning. A translation initiation codon (ATG; bold) was also incorporated in the sense primer LiF1. Fragment 2 (residues 2079–6673) was obtained after digestion of pG4Z-2 with *Hind* III. Fragment 3 (residues 6316–7299) was amplified from pG4Z-2 using the LiF2 sense (5′-GGGACATCCTCTCTAAGAAGAC-3′) and LiR3 antisense (5′-TTCCCCCGGG**TAA**AAGGAGTCCAACCACTTC-3′) primers. The LiR3 primer had a built-in *Sma* I site (underlined) at its 5′ end. A termination codon (bold) was also incorporated in the anti-sense primer LiR3. Pfu DNA polymerase (Stratagene Ltd., UK) with a proof reading activity was used in order to reduce the risk of introducing errors; the sequences of amplified products were verified by DNA sequencing.

The three fragments were cloned into the vector pBlueBacHis2A (Invitrogen BV, Bleiswijk, The Netherlands) using standard procedures. The resulting 6.6-kb fragment, constituting the entire coding region of CVB3, was excised from the pBlueBacHis2A vector with *Pst* I and *Sal* I and ligated into similarly digested pBlueBac4.5 (Invitrogen) to produce pBlueBac4.5/cb3. The procedures employed to prepare this vector are described and illustrated in Supplementary methods and Fig. S1, respectively. The ligation reaction was transformed into competent *Escherichia coli* strain XL1-blue (Stratagene, Stockport, UK) and the transformed cells were selected by blue/white screening on Ampicillin and Tetracycline containing LB-Agar plates. White recombinant bacteria were inoculated into LB medium containing Ampicillin and the recombinant transfer vector pBlueBac4.5/cb3 DNA was extracted. Presence, orientation and sequence identity of the entire 6.6-kb insert in the pBlueBac4.5/cb3 vector were confirmed by restriction enzyme analysis and DNA sequencing.

2.2. Construction and identification of recombinant baculovirus rB2

To obtain recombinant virus rB2, pBlueBac4.5/cb3 plasmid DNA was co-transfected in cultured Sf9 cells with linearised Bac-N-Blue™ AcMNPV DNA (Invitrogen) according to the manufacturer's instructions. The medium of the co-transfected cells was then used to perform plaque purification to obtain pure recombinant virus. Recombinant rB2 virus was identified by white-blue screening and its identity confirmed by PCR.

2.3. Construction of a quadruple expression vector and recombinant baculovirus rB4

In an attempt to increase protein expression, as demonstrated by Bräutigam et al. [30], the VP1, VP2, VP3 and VP4 coding regions were cloned into the multiple promoter transfer vector pAcAB4 (Pharmingen, San Diego, USA). Expression of each coding region was under the control of independent promoters. To generate recombinant virus rB4, the transfer vector pAcAB4/VP1423 was co-transfected in cultured Sf9 cells with linearized BaculoGold™ AcMNPV DNA (see Supplementary methods).

2.4. Expression of CVB3 proteins

To analyse the expression (see Supplementary methods) of CVB3 capsid proteins in recombinant virus (rB2 or rB4)-infected insect cells or attenuated virus-infected mammalian cells, 20 μg of total protein extract from recombinant virus-infected or wild-type baculovirus-infected Sf9 cells (ovarian cells isolated from *Spodoptera frugiperda* – Fall Armyworm), cardiovirulent CVB3 (chimera-2)-infected or mock-infected Vero (African Green monkey kidney) cells were run on 12% SDS-PAGE and analysed by Western blotting and immunofluorescence using either the anti-VP1 MAb 5-D8/1 (Dako Ltd., UK) or antibody $VP2_{150–168}$ (provided by Dr Marlen Aasa-Chapman, Dept. of Molecular Pathology, ICSM, London, UK) [31].

To determine if the expressed CVB3 proteins assembled into VLP, CVB3 proteins were purified from infected cells by sucrose density gradient centrifugation. The lysates from recombinant virus-infected Sf9 cells and cardiovirulent CVB3 (chimera-2)-infected Vero cells were subjected to low-speed centrifugation (15,000 × *g* for 15 min at 4 °C), and the supernatants were then centrifuged through a 10% (w/v) sucrose cushion. Western blotting