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Synthesis of empty african horse sickness virus particles

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ABSTRACT

As a means to develop African horse sickness (AHS) vaccines that are safe and DIVA compliant, we investigated the synthesis of empty African horse sickness virus (AHSV) particles. The emphasis of this study was on the assembly of the major viral core (VP3 and VP7) and outer capsid proteins (VP2 and VP5) into architecturally complex, heteromultimeric nanosized particles. The production of fully assembled core-like particles (CLPs) was accomplished *in vivo* by baculovirus-mediated co-synthesis of VP3 and VP7. The two different outer capsid proteins were capable of associating independently of each other with preformed cores to yield partial virus-like particles (VLPs). Complete VLPs were synthesized, albeit with a low yield. Crystalline formation of AHSV VP7 trimers is thought to impede high-level CLP production. Consequently, we engineered and co-synthesized VP3 with a more hydrophilic mutant VP7, resulting in an increase in the turnover of CLPs.

1. Introduction

African horse sickness virus (AHSV), an arbovirus (genus *Orbivirus*) of the family *Reoviridae*, is the causative agent of African horse sickness (AHS), a highly infectious disease of equids of which the mortality rate in susceptible horse populations may exceed 95% (Coetzer and Guthrie, 2004). The AHSV genome is composed of 10 segments of linear double-stranded (ds) RNA, which encode four nonstructural proteins (NS1 to NS4) and seven structural proteins (Bremer, 1976; Grubman and Lewis, 1992; Zwart et al., 2015). The virion is a nonenveloped icosahedral particle (Oellermann et al., 1970; Burroughs et al., 1994) composed of three concentric protein layers (Manole et al., 2012). The core particle comprises of a VP7 surface layer and an underlying VP3 layer, which encloses the genomic dsRNA segments and three enzymatic minor proteins (VP1, VP3 and VP6). The core is surrounded by an outer capsid composed of VP2 and VP5, with VP2 being the major serotype-specific determinant and neutralizing antigen (Martínez-Torrecuadrada et al., 1994, 1996; Roy et al., 1996; Scanlen et al., 1998).

African horse sickness is endemic in sub-Saharan Africa, but sporadically escapes from its geographical area and extends to North Africa, the Middle East, the Arabian Peninsula and Mediterranean countries (Carpano, 1931; Alexander, 1948; Lubroth, 1988; Howell, 1963). Since no effective treatment exists for AHS, control of the disease relies on preventative vaccination programmes. To date, nine different serotypes of AHSV (AHSV-1 to AHSV-9) have been identified in Africa (Howell, 1962). In endemic areas, vaccination is performed by administration of combinations of representative live, attenuated strains of the different AHSV serotypes. Although serotypes 5 and 9 are excluded from vaccine formulations, these serotypes partially cross-reacts with serotype 8 and serotype 6, respectively (von Teichman et al., 2010). Despite their efficacy, AHS attenuated live virus vaccines are associated with several disadvantages. These include, amongst other, the risk of gene segment reassortment between field and vaccine strains, the risk of

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introducing foreign topotypes into new geographical regions since vaccines are based on South African strains, and the lack of DIVA (differentiating infected from vaccinated animals) capability (Van Dijk, 1998; Mellor and Hamblin, 2004; Maclachlan and Guthrie, 2010).

The shortcomings of currently available AHS attenuated live virus vaccines have motivated efforts to develop alternative safe and efficacious vaccines. Over the years, vaccines based on baculovirus-expressed AHSV capsid proteins (Martínez-Torrecuadrada et al., 1994; Roy et al., 1996; Scanlen et al., 2002), DNA vaccines (Romito et al., 1999) and vaccines based on the use of poxvirus expression vectors (Chiam et al., 2009; Guthrie et al., 2009) have been pursued with varying degrees of success. A particularly promising approach for the development of an inherently safe AHS vaccine may be the production of virus-like particles (VLPs). These are self-assembling, non-replicating, non-pathogenic synthetic particles that mimic the native virus, as well as contain a diverse, repetitive high-density display of viral epitopes in the native conformation. VLP-based vaccines not only elicit humoral immune responses, but are also effective in stimulating $CD4^+$ proliferative and cytotoxic T lymphocyte (CTL) responses (Grgacic and Anderson, 2006; Kang et al., 2009). These attributes render VLPs safer than the conventional vaccines and superior to recombinant single-protein subunit vaccines in eliciting strong, long-lived protective immune responses, even in the absence of adjuvants (Noad and Roy, 2003; Grgaciac and Anderson, 2006). Notably, VLPs of the prototype orbivirus, bluetongue virus (BTV), produced by baculovirus-mediated co-expression of the four major structural proteins (VP2, VP5, VP7 and VP3) in insect cells, were shown to stimulate a long-lasting protective immune response in vaccinated sheep (Roy et al., 1992, 1994; Stewart et al., 2010).

Previously, co-infection of insect cells with single-gene recombinant baculoviruses expressing the VP3 or VP7 proteins of AHSV was shown to permit the assembly of core-like particles (CLPs), albeit that this approach resulted in a low yield of mostly partially assembled CLPs (Maree et al., 1998). Consequently, the production of multilayered, heteromultimeric protein complexes, such as VLPs, has remained an elusive goal in the case of AHSV. Here, we report a recombinant baculovirus expression system that allowed the expression of various combinations of the four major AHSV-9 structural proteins and the assembly of CLPs, partial VLPs or complete VLPs. The efficient assembly of CLPs composed of VP3 and more soluble, but structurally similar mutant VP7 trimers, is also described.

2. Materials and methods

2.1. Insect cells and viruses

Spodoptera frugiperda clone 9 cells (Sf9; ATCC CRL-1711) were grown in suspension or in monolayer cultures at 27 °C in Grace's medium (Lonza) supplemented with 10% (v/v) fetal bovine serum (FBS) and an antibiotics–antimycotic solution (streptomycin, penicillin and fungizone). Recombinant single-gene baculoviruses, 9.2, 9.3 and 9.7, which expresses the VP2, VP3 and VP7 proteins of AHSV-9, respectively, have been described previously (Maree et al., 1998; Venter et al., 2000).

2.2. Construction of recombinant baculovirus transfer plasmid DNA

Baculovirus pFastBAC-dual transfer plasmids (Invitrogen Life Technologies), containing different combinations of the coding regions of the four major structural proteins (VP2, VP5, VP3 and VP7) of AHSV-9, were constructed, as described below, according to standard procedures (Sambrook and Russell, 2001). The integrity and orientation of cloned inserts relative to the *polyhedrin* and *p10* promoters were verified by nucleotide sequencing.

To construct pFBd9.3–9.7, the VP7 coding sequence was isolated from plasmid pBR-9.7 (Maree et al., 1998) as a *Bgl*II fragment and cloned into the *Bam*HI site of pFastBAC-dual to generate pFBd9.7. The VP3 coding sequence was recovered from plasmid pBR-9.3 (Maree et al., 1998) by digestion with *Bgl*II and then cloned into the *Bbs*I site of pFBd9.7 to complete construction of pFBd9.3–9.7. In this construct, the VP7 and VP3 coding sequences are under the transcriptional control of the *polyhedrin* and *p10* promoters, respectively.

The wild-type VP7 coding sequence of pFBd9.3–9.7 was also replaced with a mutant version of VP7, which contains an insertion of six amino acids (KLSRVD) between position 177 and 178. (Rutkowska et al., 2011). For this purpose, the wild-type VP7 coding sequence was excised from pFBd9.3–9.7 by digestion with *Pst*I and partial digestion with *Bam*HI (5′ internal site) and replaced with the mutant VP7 coding sequence, which was recovered as a *Bam*HI/*Pst*I DNA fragment from pFB9.7mt. The resulting plasmid was designated pFBd9.3–9.7mut.

For construction of pFBd9.6–9.7, the VP5 coding sequence was isolated as a *Bam*HI fragment from plasmid pBS-9.6 (Du Plessis and Nel, 1997) and then cloned into the corresponding site of pFastBAC-dual to yield pFBd9.6. The VP7 coding sequence was recovered from pBR-9.7 as a *Sma*I/*Sal*I fragment and cloned into the *Sma*I and *Xho*I sites of pFBd9.6. The derived construct, pFBd9.6–9.7, contains the VP5 and VP7 coding sequences under the transcriptional control of the *polyhedrin* and *p10* promoters, respectively.

To construct pFBd9.2–9.3, the VP3 coding sequence was recovered from plasmid pBR-9.3 by digestion with *Bgl*II, treated with Klenow polymerase and then blunt-end cloned into the *Sma*I site of pFastBAC-dual to generate pFBd9.3. The VP2 coding sequence was isolated from plasmid pBS-9.2 (Venter et al., 2000) as a *Sal*I/*Xba*I fragment and cloned into the corresponding sites of pFBd9.3. The resulting plasmid, pFBd9.2–9.3, contains the VP2 and VP3 coding sequences under the transcriptional control of the *polyhedrin* and *p10* promoters, respectively.

2.3. Generation of recombinant baculoviruses

A recombinant baculovirus series, containing the expression cassettes as described above, was constructed by means of the BAC-to-BAC™ baculovirus expression system (Invitrogen Life Technologies), following the manufacturer's instructions. This series included 9.3–9.7 (VP3.VP7), 9.3–9.7mut (VP3.VP7mut), 9.6 (VP5), 9.6–9.7 (VP5.VP7) and 9.2–9.3 (VP2.VP3) baculoviruses. The progeny baculoviruses were plaque-purified and high-titre virus stocks were prepared according to the methods described by O'Reilly et al. (1994).

2.4. Analysis of AHSV-9 capsid proteins expressed in insect cells

Sf9 cells were infected with the recombinant baculoviruses at a multiplicity of infection (MOI) of 5 pfu/cell. At 48–72 h post-infection, whole-cell lysates were analyzed by SDS-PAGE. Alternatively, radiolabelled proteins were prepared by starving infected cells of methionine for 1 h in methionine-free Eagle's medium (BioWhittaker) at 26 h post-infection, followed by addition of 15 μCi [^{35}S]-methionine/ml and incubation for 3 h. At 30 h post-infection, radiolabeled proteins in whole-cell lysates were separated by SDS-PAGE and detected by autoradiography.