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Antibiotic-dependent expression of early transcription factor subunits leads to stringent control of vaccinia virus replication

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ABSTRACT

The use of vaccinia virus (VACV) as the vaccine against variola virus resulted in the eradication of smallpox. VACV has since been used in the development of recombinant vaccine and therapeutic vectors, but complications associated with uncontrolled viral replication have constrained its use as a live viral vector. We propose to improve the safety of VACV as a live-replicating vector by using elements of the *tet* operon to control the transcription of genes that are essential for viral growth. Poxviruses encode all enzymes and factors necessary for their replication within the host cell cytoplasm. One essential VACV factor is the vaccinia early transcription factor (VETF) packaged into the viral core. This heterodimeric protein is required for expression of early VACV genes. VETF is composed of a large subunit encoded by the A7L gene and a small subunit encoded by the D6R gene. Two recombinant VACVs were generated in which either the A7L or D6R gene was placed under the control of *tet* operon elements to allow their transcription, and therefore viral replication, to be dependent on tetracycline antibiotics such as doxycycline. In the absence of inducers, no plaques were produced but abortively infected cells could be identified by expression of a reporter gene. In the presence of doxycycline, both recombinant viruses replicated indistinguishably from the wild-type strain. This stringent control of VACV replication can be used for the development of safer, next-generation VACV vaccines and therapeutic vectors. Such replication-inducible VACVs would only replicate when administered with tetracycline antibiotics, and if adverse events were to occur, treatment would be as simple as antibiotic cessation.

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1. Introduction

Vaccinia virus (VACV) is the prototype member of the genus *Orthopoxvirus* within the family *Poxviridae*. This genus also includes cowpox virus, monkeypox virus, and most notably variola virus, the causative agent of smallpox. Immunization of humans with VACV was used to successfully eradicate naturally occurring smallpox worldwide (Wehrle, 1980). Since then, VACV has been utilized as a viral vector for the development of recombinant vaccines for humans and animals, as well as cancer immunotherapies and oncolytic therapies. In addition, there is still significant interest in the development of next-generation smallpox vaccines to be used in case of a bioterrorist event or the emergence of other orthopoxvirus threats. However, the safety of live VACV vectors is always a concern, as uncontrolled replication can result in complications from vaccination or therapeutic use. Although VACV has not been associated with any disease, it can cause mild to severe complications that include accidental infection, generalized vaccinia, eczema vaccinatum, progressive vaccinia, and post-vaccinial encephalitis (Casey et al., 2005; Fenner et al., 1988; Fulginiti et al., 2003; Henderson et al., 1999; Lane and Goldstein, 2003a). The occurrence of complications has been correlated with pre-existing conditions such as atopic dermatitis, cardiac disease, and immunosuppression due to infection (e.g., HIV/AIDS) or drug therapy. Consequently, individuals with such conditions or with contacts that have these conditions are contraindicated for vaccination or treatment with replication-competent VACVs (Kemper et al., 2002; Lane and Goldstein, 2003b).

A number of strategies have been attempted to enhance the safety of VACV vectors, including the selection of natural strains

Abbreviations: VACV, vaccinia virus; VETF, vaccinia early transcription factor; TET, tetracycline; DOX, doxycycline; ATC, anhydrotetracycline; DPI, days post-infection.

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of lower virulence such as those derived from the New York City Board of Health (NYCBH) strain, the development of highly attenuated strains such as modified vaccinia Ankara (MVA), NYVAC, and LC16m8 (Drexler et al., 1998; Kenner et al., 2006; Tartaglia et al., 1992), inactivation of virulence factors such as the thymidine kinase gene (Buller et al., 1985), deletion of immunomodulatory and other non-essential viral genes (Legrand et al., 2004; Verardi et al., 2001), and expression of attenuating genes such as cytokines (Flexner et al., 1987; Giavedoni et al., 1992; Grigg et al., 2013; Legrand et al., 2005; Ramshaw et al., 1987). However, these safety improvements can lead to a reduction in the effectiveness of the vectors. For example, vaccine efficacy is typically compromised with attenuation, next-generation smallpox vaccines must provide immunogenicity in clinical trials equivalent to current licensed vaccines such as ACAM2000 (a clone derived from NYCBH) (Greenberg and Kennedy, 2008), the effectiveness of oncolytic VACV vectors is contingent upon the replication-competence of the vector, and expression of cytokines can lead to unforeseen immune activation and complications (Bakacs et al., 2012; Tisoncik et al., 2012).

Here we propose an approach to generate replication-inducible VACV vectors that are significantly safer, yet replicate to the same levels as their parental strains, and therefore maintain their full immunogenic and oncolytic potential. These replication-inducible VACV vectors, based on elements of the transposon Tn*10* operon that confers tetracycline (TET) resistance in bacteria, would replicate only in the presence of tetracyclines. Tetracyclines such as TET and doxycycline (DOX) are commonly used broad-spectrum antibiotics that inhibit translation in gram-positive, gram-negative, and atypical bacteria by binding to the 30S bacterial ribosome subunit (Chopra and Roberts, 2001). In the *tet* operon, the Tet repressor (TetR) is unable to bind to *tet* operators in the presence of tetracyclines, allowing transcription of the efflux gene that confers resistance to tetracyclines (Hillen and Berens, 1994). The *tet* operon has been adapted to a variety of organisms for inducible gene expression, including prokaryotes, yeast, insect, plant, and mammalian cells, and transgenic organisms (Bertram and Hillen, 2008; Faryar and Gatz, 1992; Gatz and Quail, 1988; Stebbins et al., 2001; Stieger et al., 2009; Yao et al., 1998; Zhu et al., 2002). The *tet* system has also been adapted to control gene expression in a number of viruses (Cunningham et al., 2010; Gall et al., 2007; Hedengren-Olcott and Hruby, 2004; Yamaguchi et al., 2012), and in some instances for conditional replication of herpesviruses, adenoviruses, and retroviruses (Legrand et al., 2012; Manoussaka et al., 2013; Yao and Eriksson, 1999; Yao et al., 2010; Zhang et al., 2009). In particular, inducible VACVs have been generated by expressing the TetR gene (*tetR*) constitutively and inserting a *tet* operator element (O_2) immediately after the transcriptional start sites of VACV genes, enabling their expression to be regulated by tetracyclines (Traktman et al., 2000; Unger and Traktman, 2004). Using this system, inducible genes are expressed at minimal levels in the absence of inducer and at high levels in the presence of TET or DOX, without any apparent effect on VACV replication (Grigg et al., 2013; Traktman et al., 2000). Therefore, we propose to improve the safety of VACV as a live-replicating vector by using elements of the *tet* operon to control the transcription of genes that are essential for viral growth. These replication-inducible VACVs would replicate to wild-type levels in the presence of low concentrations of tetracyclines and would be unable to replicate in the absence of these antibiotics, thus allowing treatment of adverse events resulting from uncontrolled replication of the virus to be as simple as antibiotic cessation.

The vaccinia early transcription factor (VETF) is a heterodimeric protein required for the expression of early VACV genes (Broyles et al., 1988). VETF is composed of an 82 kDa large subunit encoded by the A7L gene and a 70 kDa small subunit encoded by the D6R gene (Broyles and Fesler, 1990; Gershon and Moss, 1990). The VACV A7L gene is referred to as A8L in some literature; currently the A8 open reading frame is considered to be A8R and to encode a subunit of an intermediate transcription factor (Sanz and Moss, 1999). The VETF subunits are produced late in infection and packaged into virions to be used in the next round of replication. VETF provides early promoter specificity by binding to early promoters and recruiting the RNA polymerase (Baldick et al., 1994; Li and Broyles, 1993b). A7 interacts with the core region of the early promoter while D6 interacts with the region downstream from the transcriptional start site (Cassetti and Moss, 1996). D6 also contains the DNA-dependent ATPase activity of the transcription factor (Li and Broyles, 1993a). Both D6R and A7L genes have been shown to be essential for VACV replication (Hu et al., 1996, 1998; Li et al., 1994; Yang and Moss, 2009). Thus, we used elements of the *tet* operon to design and construct VACVs that inducibly express the D6R or A7L genes. We evaluated and characterized the growth properties of these VACVs and showed that these viruses fail to replicate in the absence of tetracyclines, but replicate indistinguishably from wild-type VACV in the presence of tetracycline antibiotics.

2. Materials and methods

2.1. Cells and viruses

Cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). African green monkey BS-C-1 (CCL-26) and human HeLa-S3 (CCL-2.2) cells were grown in Dulbecco's modified Eagle medium (D-MEM; Life Technologies, Gaithersburg, MD) supplemented with 10% tetracycline-tested fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA). All cells were grown at 37 °C in 5% CO_2. The L-variant of VACV strain Western Reserve (WR) was obtained from ATCC (VR-2035) and a clone (9.2.4.8) derived by sequential plaque purification was used to generate the recombinant viruses in this study. High-titer stocks of VACV were obtained in HeLa-S3 cells and titered in BS-C-1 cells.

2.2. Construction of the VACV transfer vectors

The schematic representation of the VACV transfer vector backbone used for the generation of the recombinant VACVs is shown in Fig. 1C and D. The two transfer vectors were generated in multiple steps by a combination of DNA synthesis (DNA2.0, Menlo Park, CA), PCR cloning, and standard subcloning, using engineered restriction endonuclease sites (not shown) to facilitate construction. The *gpt*-EGFP fusion gene for combined xanthine–guanine phosphoribosyl transferase (*gpt*) selection and enhanced green fluorescent protein (EGFP) screening was developed by DNA synthesis of the *Escherichia coli gpt* gene (based on the sequence in plasmid pMSG, GenBank: U13860) and the EGFP gene (based on the sequence in plasmid pEGFP-1, GenBank: U55761), using a previously developed strategy (Cao and Upton, 1997). The *tetR* gene (based on GenBank: X00694) was synthesized with an internal VACV early transcriptional termination sequence (TTTTTNT) removed from the middle of the gene (Leu codon at position 358 changed from TTA to CTT) to ensure early gene expression. The tetR and *gpt*-EGFP genes were placed under back-to-back $P_{E/L}$ synthetic promoters (sequence TATTTATATTCCAAAAAAAAAAAATAAAATTTCAATTTTTAACT-GCAGTTAAAAATTGAAATTTTATTTTTTTTTTTTGGAATATAAATA) (Chakrabarti et al., 1997). The transfer vectors also contained the putative D6R promoter region or a modified P_{11} late VACV promoter with a *tet* operator (O_2) (Hillen and Berens, 1994) placed immediately after the late transcriptional initiator element sequences, as shown in Table 1. Each cassette was surrounded by 600 bp of VACV genomic sequences to the left and to the right of the insertion points shown in Fig. 1B (based on GenBank: NC_006998) to direct homologous recombination and insertion

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