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Characterization of an Sf-rhabdovirus-negative *Spodoptera frugiperda* cell line as an alternative host for recombinant protein production in the baculovirus-insect cell system

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ABSTRACT

Cell lines derived from the fall armyworm, *Spodoptera frugiperda* (Sf), are widely used as hosts for recombinant protein production in the baculovirus-insect cell system (BICS). However, it was recently discovered that these cell lines are contaminated with a virus, now known as Sf-rhabdovirus [1]. The detection of this adventitious agent raised a potential safety issue that could adversely impact the BICS as a commercial recombinant protein production platform. Thus, we examined the properties of Sf-RVN, an Sf-rhabdovirus-negative *Sf* cell line, as a potential alternative host. Nested RT-PCR assays showed Sf-RVN cells had no detectable Sf-rhabdovirus over the course of 60 passages in continuous culture. The general properties of Sf-RVN cells, including their average growth rates, diameters, morphologies, and viabilities after baculovirus infection, were virtually identical to those of Sf9 cells. Baculovirus-infected Sf-RVN and Sf9 cells produced equivalent levels of three recombinant proteins, including an intracellular prokaryotic protein and two secreted eukaryotic glycoproteins, and provided similar *N*-glycosylation patterns. In fact, except for the absence of Sf-rhabdovirus, the only difference between Sf-RVN and Sf9 cells was SF-RVN produced higher levels of infectious baculovirus progeny. These results show Sf-RVN cells can be used as improved, alternative hosts to circumvent the potential safety hazard associated with the use of Sf-rhabdovirus-contaminated Sf cells for recombinant protein manufacturing with the BICS.

1. Introduction

Since it was first described in the peer-reviewed literature in the early 1980's [2,3], the baculovirus-insect cell system (BICS) has become a widely recognized and heavily utilized recombinant protein production platform [4,5]. The advantages of the BICS include its flexibility, speed, simplicity, eukaryotic protein processing capacity, and ability to produce multi-subunit protein complexes. For nearly 30 years, the BICS was used mainly to produce recombinant proteins for basic research in academic and industrial labs. More recently, however, the BICS has emerged as a *bona fide* commercial manufacturing platform, which is now being used to produce several biologics licensed for use in human (Cervarix®, Provenge®, Glybera® and Flublok®) or veterinary (Porcilis® Pesti, BAYOVAC CSF E2®, Circumvent® PCV, Ingelvac CircoFLEX® and Porcilis® PCV) medicine [6]. In addition, the BICS is being used to produce several other biologic candidates, including potential vaccines against norovirus, parvovirus, Ebola virus, respiratory syncytial virus, and hepatitis E virus, which are in various stages of human clinical trials [6].

The insect cell lines most commonly used as hosts in the BICS are derived from the cabbage looper, *Trichoplusia ni*, or fall armyworm, *Spodoptera frugiperda* (Sf), and most biologics manufactured with the BICS are produced using the latter. The original Sf cell line, designated IPLB-SF-21, also known as Sf-21, was derived from pupal ovaries in 1977 [7]. Other commonly used Sf cell lines include Sf9, a subclone of IPLB-SF-21 [8], and its daughter subclones, Super 9 [9] and Sf900+, also known as *expres*SF+ [10].

Recently, Ma and coworkers [1] discovered that every Sf cell line tested, including Sf21 and Sf9 cells obtained from two reputable commercial sources, was contaminated with a novel rhabdovirus.

Abbreviations: MALDI-TOF MS, matrix assisted laser desorption ionization time of flight mass spectrometry; PCR, polymerase chain reaction; PNGase, peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase; Sf, *Spodoptera frugiperda*.

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This adventitious agent, which is now known as Sf-rhabdovirus, was discovered in a general effort to evaluate the biosafety of the BICS platform. A research group at Takeda Vaccines, Inc. independently confirmed the presence of Sf-rhabdovirus in the Sf9 cells used to produce their norovirus vaccine candidate [11]. In addition, we found that all our lab Sf-21, Sf9, and *expres*SF+ cell stocks, obtained from a variety of sources, are contaminated with this virus (data not shown). Thus, Sf-rhabdovirus appears to be a very common, if not universal, contaminant of the Sf cell lines commonly used as hosts for recombinant protein production in the BICS.

It is important to note that Sf-rhabdovirus is unlikely to be harmful for humans, as it cannot replicate in human or monkey cell lines [1]. Moreover, the only detectable phylogenetic relationship between Sf-rhabdovirus and previously recognized viruses was found in a short sequence, which was related to the L genes of insect and plant rhabdoviruses [1]. Therefore, the potential impact of Sf-rhabdovirus on biosafety of the BICS platform, if any, remains speculative at this time. Nevertheless, whenever adventitious agents are discovered in human biologics, the usual response is to take steps to remove them from the product. Ideally, this involves eradicating the adventitious agent from all stages of the manufacturing process. One notable precedent was the elimination of porcine circovirus from an attenuated live rotavirus vaccine and the Vero cells used to produce that vaccine [12,13].

In this study, we characterized the relevant properties of an Sf-rhabdovirus-negative Sf cell line, Sf-RVN, as a potential alternative host for the BICS. We detected no Sf-rhabdovirus in these cells for 60 continuous passages in culture. Compared to Sf9 cells, Sf-RVN cells had virtually identical growth rates, sizes, morphologies, viabilities after baculovirus infection, and provided equivalent recombinant protein production levels and *N*-glycosylation profiles. Interestingly, Sf-RVN cells produced ~5–10 fold higher levels of infectious baculovirus progeny. These results validate Sf-RVN cells as an alternative host that can be used to circumvent the potential safety hazard associated with the use of Sf-rhabdovirus-contaminated Sf cells for recombinant protein manufacturing in the BICS.

2. Materials and methods

2.1. Insect cell culture

The isolation of Sf9 cells has been described [8] and the isolation of Sf-RVN cells will be described (Geisler, C., Maghodia, A.B., and Jarvis, D.L., submitted) elsewhere. Both cell lines were routinely maintained as shake-flask cultures at 28 °C in ESF 921 medium (Expression Systems, Woodland, CA).

2.2. Sf-rhabdovirus and mycoplasma detection

Samples of Sf9 and Sf-RVN cultures containing 1×10^6 cells were harvested and the cells were pelleted by low speed centrifugation. The cell-free supernatants were filtered through a 0.22 μm filter (CELLTREAT Scientific, Shirley, MA) and then ultracentrifuged at 131 000 × g for 22 h at 4 °C. Total RNA was extracted from both the low speed cell and high speed cell-free pellets using the RNA*Solv* reagent (Omega Bio-Tek, Inc., Norcross, GA), according to the manufacturer's protocol. The RNAs were then quantified and used as templates for cDNA synthesis with the ProtoScript II First Strand cDNA synthesis kit (New England Biolabs, Ipswich, MA) and either an Sf-rhabdovirus-specific (320-SP1) primer or the oligo(dT) 23-VN primer included in the kit, according to the manufacturer's protocol. Equivalent amounts of each cDNA preparation were then used for PCR's with *Taq* DNA polymerase, ThermoPol reaction buffer (New England Biolabs), and either Sf-rhabdovirus- (Mono-1 and Mono-2; [1]) or Sf ribosomal protein L3- (SfRPL3-SP and SfRPL3-ASP) specific primers, respectively. The reaction mixtures were incubated at 94 °C for 3 min, cycled 35 times at 94 °C for 30s, 55 °C for 1 min, and 72 °C for 1 min, and finally incubated at 72 °C for 10 min. For Sf-rhabdovirus-specific RNA detection, one μL of each primary PCR was then used as the template for secondary PCR's under the same conditions, except the primers were nested Sf-rhabdovirus-specific primers (Mono-1i and Mono-2i; [1]). The sequence of each primer used for these assays is given in Table 1, which is an extended version of Table 1 in the publication by Ma and coworkers [1]. Primary and secondary PCR products were analyzed by agarose gel electrophoresis with ethidium bromide staining. In addition, the gel-purified primary PCR product obtained with primers Mono-1 and -2 was further purified on a HiBind DNA Mini Column (Omega-Biotek, Norcross, GA) according to the manufacturer's protocol, and then directly sequenced using the same primers (Genewiz, South Plainfield, NJ).

We also tested samples containing about 10^5 Sf-RVN and Sf9 cells for mycoplasma using the Universal Mycoplasma Detection kit from American Type Culture Collection (Manassas, VA), according to the manufacturer's protocol.

2.3. Cell growth properties, morphologies, and diameters

Sf-RVN and Sf9 cells were seeded at a starting density of 1.0×10^6 cells/mL in 50-mL shake flask cultures, triplicate samples were removed every 24 h for 4 days, and viable cell densities and sizes were measured using a Countess® automated cell counter (ThermoFisher Scientific, Inc.). Doubling times were calculated using the formula: $Td = T \times Log_2/Log(Q2/Q1)$ where Td = doubling time, T = time (h) elapsed since the last passage, Q1 = cell seeding density, and Q2 = viable cell count. Cell morphologies were documented by collecting phase contrast images at a magnification of 10× using an Olympus FSX-100 microscope and FSX-BSW imaging software.

2.4. Baculovirus expression vectors

A baculovirus expression vector designated BacPAK6-ΔChi/Cath encoding full-length, untagged *Escherichia coli* β-galactosidase (β-gal) was produced in two sequential steps. In the first step, BacPAK6 viral DNA was recombined with a plasmid encoding *E. coli* β-glucuronidase under the control of the baculovirus *p6.9* promoter. In this plasmid, the *p6.9*-ß-glucuronidase gene was inserted in place of the AcMNPV *chiA* and *v-cath* genes and embedded within wild type AcMNPV flanking sequences. The desired recombinant was tentatively identified by its blue plaque phenotype in the presence of X-GlcA (RPI Corp., Mount Prospect, IL). The recombination site was confirmed by PCR with primers specific for the β-glucuronidase gene and 5′ UTR of the AcMNPV *gp64* gene, which were internal and external to the transfer plasmid, respectively. This virus was amplified and viral DNA was isolated and digested with I-*Sce*I to delete the entire β-glucuronidase expression cassette. In the second step, Sf9 cells were transfected with the I-*Sce*I-digested viral DNA. The resulting progeny were resolved by plaque assay in the presence of X-GlcA and the final recombinant baculovirus, BacPAK6-ΔChi/Cath, was identified by its white plaque phenotype.

The recombinant baculovirus expression vectors designated AcP(−)p6.9hSEAP and AcP(−)p6.9hEPO encoded 8X HIS-tagged forms of human secreted alkaline phosphatase (hSEAP) and human erythropoietin (hEPO), respectively, under the control of AcMNPV *p6.9* promoters and honeybee prepromellitin signal peptides. Synthetic genes encoding mature SEAP and EPO (Genbank NP_001623.3 amino acids 23-511 and Genbank NP_000790.2