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Process Development and cGMP Manufacturing of a Recombinant Ricin Vaccine: an Effective and Stable Recombinant Ricin A-Chain Vaccine—RVEcTM

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Ricin is a potent toxin and a potential bioterrorism weapon with no specific countermeasures or vaccines available. The holotoxin is composed of two polypeptide chains linked by a single disulfide bond: the A-chain (RTA), which is an N-glycosidase enzyme, and the B-chain (RTB), a lectin polypeptide that binds galactosyl moieties on the surface of the mammalian target cells. Previously (McHugh et al.), a recombinant truncated form of RTA (rRTA1-33/44-198 protein, herein denoted RVEaTM) expressed in *Escherichia coli* using a codon-optimized gene was shown to be non-toxic, stable, and protective against a ricin challenge in mice. Here, we describe the process development and scale-up at the 12 L fermentation scale, and the current Good Manufacturing Practice (cGMP)-compliant production of RVEcTM at the 40 L scale. The average yield of the final purified bulk RVEcTM is approximately 16 g/kg of wet cell weight or 1.2 g/L of fermentation broth. The RVEcTM was >99% pure by three HPLC methods and SDS-PAGE. The intact mass and peptide mapping analysis of RVEcTM confirmed the identity of the product and is consistent with the absence of posttranslational modifications. Potency assays demonstrated that RVEcTM was immunoprotective against lethal ricin challenge and elicited neutralizing anti-ricin antibodies in 95–100% of the vaccinated mice. Published 2011 American Institute of Chemical Engineers *Biotechnol. Prog.*, 27: 1036–1047, 2011

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Introduction

The ricin toxin is a 64 kilodalton (kDa) protein produced by the seeds of the castor oil plant (*Ricinus communis*). The mechanism of action of this opportunistic ribotoxin has been thoroughly studied.^{1,2} The holotoxin is a glycosylated heterodimeric protein that is composed of two chains, A and B, joined by a single disulfide bond.^{3–5} The A-chain (RTA) is an enzyme that inactivates the 28S ribosomal ribonucleic acid (rRNA) by hydrolytic cleavage of the N-glycosidic bond of the essential adenine nucleotide (A4324) of the ribosome.^{6,7} The depurination site in the rRNA is part of a conserved sequence present in nearly all eukaryotic cells and it is known as the sarcin-ricin loop.⁸ The B-chain (RTB) is a

lectin that binds to galactose moieties presented by cell-surface glycopeptides or glycolipids, inducing endocytosis of the holotoxin. The combination of the internalization by the toxin and the cytosolic activity of the A-chain (kcat/Km $\sim 10^7 \text{ M}^{-1} \text{ s}^{-1}$)⁶ allows a single ricin molecule to kill a eukaryotic cell by damaging protein synthesis.⁹ The holotoxin is taken up by cells through endocytosis, incorporated into vesicles, and transported through the Golgi complex until it reaches the endoplasmic reticulum.¹ Therein the disulfide bond is reduced, causing the RTA chain to undergo partial unfolding, and allows it to cross the endoplasmic reticulum into the cytosol. The active depurinase form of RTA is then reconstituted. Outside the cytosol, RTA tends to undergo aggregation, a factor which has significantly slowed down efforts toward development of a vaccine.¹⁰

Interest in the development of a vaccine against ricin has increased in recent years due to threats of bioterrorism and

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the wide availability of castor beans. The Centers for Disease Control and Prevention (CDC) has classified ricin as a level B biothreat agent. One well-known case was the death of a Bulgarian dissident reported in the 1970s, caused by a single ricin pellet.¹¹ On the basis of animal studies, the lethal ricin dose in humans is believed to be 1–25 $\mu\text{g/kg}$ ^{11–13} although such toxicity level occurs only when the toxin is administered either by inhalation or injection.

Previously, protective immunity against ricin was demonstrated in animal models by vaccinating with formalin-treated ricin toxoid¹⁴ and by passive immunity using anti-ricin IgG.^{15,16} Because of the potential for reversion of the formalin-inactivated toxoid to its toxic form and the shortcomings of antibody therapy (cannot neutralize toxin once it has entered cells), other approaches to vaccine development have been investigated. One approach has been to identify essential amino acid residues for enzymatic activity and utilize mutants as potential vaccine candidates. Among those that have been identified include tyrosine (Tyr) 80, Tyr 123, glutamate (Glu) 177, arginine (Arg) 180, asparagine (Asn) 209, and tryptophan (Trp) 211.^{17,18} Although several mutants of RTA have been generated that confer immunoprotection against ricin in animal models, they retain the biophysical tendency to aggregate and precipitate in aqueous environments due to the instability of RTA in the absence of RTB, rendering them ineffective for vaccine process development. A successful approach to stabilizing an RTA derivative using protein engineering and recombinant DNA technology has been reported.^{19,20}

In this approach, a comparative computational analysis of RTA and the similar ribosome-inactivating protein from pokeweed, which is composed of a single monomeric chain, was undertaken. Removal of the highly hydrophobic C-terminus amino acid region (199–267) resulted in a protein with a significant decrease in its behavior for aggregating and precipitating in aqueous solutions. Because residues Asn 209, Trp 211, and Arg 213 are required for rRNA binding, the truncated protein is devoid of N-glycosidase activity and it is therefore not toxic. To further increase stability, a disordered non-immunogenic exposed loop comprised of residues 34–43 was also deleted. This construct behaves as a monodisperse monomer with a secondary protein structure almost identical to that of native RTA; it effectively protected mice against an aerosolized or injected ricin toxin challenge. Furthermore, reformulation of this protein from phosphate-buffered saline into succinate buffer increased the adherence of the protein to aluminum hydroxide adjuvant up to 91%, resulting in a three-fold increase in immunogenicity in a mouse model.²¹ The recombinant form of RTA 1-33/44-198 expressed in *Escherichia coli* (herein called RVEcTM) constitutes a promising candidate for a vaccine against the ricin toxin. This manuscript describes the process development of RVEcTM starting with strain construction through cGMP production of clinical grade vaccine for evaluation in a Phase I clinical study.

Materials and Methods

Analytical methods

Analytical methods were developed for in-process, release, and characterization of RVEc. Table 1 provides a list of routine analytical methods that were developed and their critical attributes. The text below describes methods that required significant development, that is, peptide mapping, intact

mass analysis, potency assay, and an assay for residual testing of kanamycin.

Peptide Mapping of RVEcTM. To perform the trypsin digest, a solution of 20 μg of RVEcTM was adjusted to 50 mM ammonium carbonate by adding 500 mM ammonium carbonate, pH 7.8 stock solution. Samples were reduced with dithiothreitol (5 mM final concentration) for 60 min at 25°C, alkylated with iodoacetamide (10 mM final concentration) in the dark for 30 min at ambient temperature, digested with trypsin (1:20 E:S w/w ratio) for 18 h at 25°C, and then quenched with by adjusting to a final formic acid concentration of 0.1% (v/v).

The trypsin digest was analyzed with a Dionex Acclaim PepMap C18 column (1 mm $\times$ 150 mm, 3 μm particles, 100 Å pores). Solvent A was 0.1% (v/v) formic acid in water and Solvent B was 0.1% formic acid in 50/50 acetonitrile/water. The tryptic peptides were separated by a two step linear gradient (2–10% B in 10 min then 10–100% B in 90 min) at a flow rate of 53 $\mu\text{L/min}$ and monitored at 214 nm.

The cyanogen bromide digest started with a solution containing 50 μg of RVEcTM frozen at -80°C for 2 h and then evaporated to dryness in a centrifugal evaporator without additional heating. The sample was reconstituted in 50 μL of 50 mM ammonium carbonate, pH 7.8. DTT was added to 5 mM, a layer of nitrogen gas was placed over the solution, and the sample was incubated for 18 h at 25°C. The sample was then frozen as before and evaporated to dryness in a centrifugal evaporator without additional heating. The sample was reconstituted in 70% formic acid to a protein concentration of 0.5 mg/mL. Cyanogen bromide was added from a 5 M stock to a final concentration of 250 mM cyanogen bromide. The samples were incubated for 18–24 h in the dark. After digestion, samples were quenched with 2.5 M methionine and incubated for 18 h at 25°C.

The cyanogen bromide digests were analyzed immediately using RP-HPLC with a Grace Vydac C18 column, 4.6 mm $\times$ 100 mm, 3 μm particles, 300 Å pores. Samples were diluted with an equal volume of Solvent A [5% acetonitrile, 0.1% (v/v) TFA in water] and centrifuged at 14,000g for 10 min before being placed in autosampler vials. Solvent B contained 0.1% TFA in 95% acetonitrile and 5% water. The digested peptides were separated by a linear gradient (0–75% B in 10 min) at a flow rate of 2.5 mL/min and monitored at 214 nm.

LC-MS/MS using a nanoflow LC system (Dionex Ultimate 3000 with UV detector) and an Applied Biosystems 4000 QTrap mass spectrometer (ABI-Sciex) was used to identify peptides in the tryptic map. Solvent A was 0.1% (v/v) formic acid (Fluka) in water; Solvent B was 0.1% (v/v) formic acid in acetonitrile (Burdick and Jackson). The column was a Dionex Acclaim C18 PepMap (75 μm $\times$ 15 cm), with a flow rate of 300 nL/min, in a column oven set at 45°C. A gradient of 5–50% B over 90 min eluted the peptides. Information-dependent-acquisition (IDA) methods were used to collect MS and MS/MS data. All peptide identities were confirmed by manual inspection of MS/MS data.

ESI/TOF Intact Mass Analysis and N-Terminus Methionine Determination. Mass spectra of intact proteins were obtained using an Agilent 6210 TOF mass spectrometer equipped with an Agilent 1200 HPLC and orthogonal electrospray ionization (ESI) source. Protein samples were loaded onto an Agilent Zorbax Poroshell 300SB-C8 column (0.5 mm diameter $\times$ 75 mm length, containing 5 μm particles with 300 Å pores) and mass spectra of the intact

Table 1. Standard Analytical Methods for RVEc

HPLC Method	Column Type Manufacture	Solvent (In H ₂ O-HPLC Grade)	Conditions
Reverse Phase	4.6 × 100 mm C ₄ PN:214TP3410 Grace Vydac, Hesperia, CA	A: 5% CH ₃ CN:0.1% TFA B: 95% CH ₃ CN:0.085% TFA	100% A → 70% B over 10 min Col. Temp.: 25°C at 2.5 mL/min Detection: 215 nm
Cation Exchange	4.6 × 100 mm Polysulfoethyl A PN: 104SE0503 PolyLC, Columbia, MD	A: 50 mM MES, pH 6.4 B: Solvent A + 1 M NaCl	5-min hold of 100% A after inj. 100% A → 25% B over 8.5 min Col. Temp.: 25°C at 1 mL/min Detection: 280 nm
Size Exclusion	7.8 × 300 mm TSK-GEL G2000SW _{XL} PN: 08543 Tosoh Bioscience, Montgomeryville, PA	25 mM sodium acetate and 300 mM NaCl, pH 5.5	Isocratic over 30 min Col. Temp.: 25°C at 0.6 mL/min Detection: 214 nm
Tween-20	4.6 × 150 mm Acclaim [®] Surfactant column PN: 063201 Dionex, Sunnyvale, CA	A: 5% CH ₃ CN B: 95% CH ₃ CN	100 A% → 35% B over 11 min 35% B → 100% B over 12 min Col. Temp.: 25°C at 1 mL/min Detection: Evaporative Light Scattering (Millipore 2420) PSI = 45, drift tube = 50, Nebulizer = 45, and detector gain = 100

Analytical Methods	Description
Endotoxin (LAL)	Turbidimetric Pyrotell-T type kinetic assay (Associates of Cape Cod, East Falmouth, MA). Results were considered valid when spike recovery was 50% to 200% of the spiked endotoxin
SDS-PAGE	NuPAGE 10% Bis-Tris gels (cat # NP0301) with the MES buffer system (Invitrogen, Carlsbad, CA) at constant 200 volts. Gels stained with Brilliant Blue R (Sigma PN:B6529 for 1 h and destained with 10% v/v acetic acid and 35% v/v MeOH in H ₂ O)
Western Blot	Gel transferred to a PVDF membrane using 1x NuPAGE transfer buffer containing 10% methanol and a semi-dry blotting apparatus (BioRad) at 20 V for 20 min Blocking: 5% w/v NFD in TBST (20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.4)-1 h 1st Ab: 1:2000 dil of mouse polyclonal anti dg-RTA in TBST – 1 h 2nd Ab: 1:2000 dil of goat anti mouse horseradish peroxidase (KLP PN:074-1806) in TBST-1 h Developed: Amersham ECL Plus [™] , GE Healthcare PN: RPN2132 and x-ray film (Kodak BioMax Light-1, PN: 8689358)
<i>E. coli</i> Protein	ELISA kit from Cygnus Technologies (Southport, NC)
Host DNA	qPCR by Charles River Laboratories-Malvern (Malvern, PA)
Capillary IEF	Capillary isoelectric focusing kit (Beckman Coulter PN: 477490) and a Beckman Coulter PACE MDQ system with detection at 280 nm

RVEc[™] proteins was acquired in positive mode using a fragmentor voltage of 225 V, a capillary voltage of 4000 V, and an *m/z* range of 100–3527. The instrument was calibrated using the Agilent ES-TOF tuning mix to give an accuracy of 0.3 parts per million (ppm) and mass correction was maintained during the acquisition with the Agilent API-TOF Reference mass solution. The acquired spectra were analyzed using the Agilent Mass Hunter Qualitative analysis software. The background was subtracted before de-convolution of the multiple charge state spectrum to remove the signals from internal reference masses and some of the baseline noise. The de-convolution parameters were as follows: mass range of 10000.00–35000.00 atomic mass units (amu) and spectral window of 600.00–2500.0 *m/z*.

Determination of Residual Kanamycin. This method detects the presence of kanamycin to the limit of 2 μ M or approximately 1 μ g/mL. Samples were analyzed both as spiked with kanamycin (2 μ M final concentration) and neat with 5 mL of sample spiked whereas another 5 mL remained unspiked. Hereafter, both spiked and unspiked sample will be generically referred to as “the samples.” The samples were then processed independently in quintuplicate. Controls were run spiked and unspiked using the buffer without RVEc[™].

Sample isolation and clean-up: Solid-phase extraction (SPE) cartridges were processed in parallel using a vacuum manifold at a flow rate of 2 mL/min or less. Carboxylic acid SPE cartridges (J. T. Baker speed disk # 8172-06, 10 μ m, 100 mg, 3 mL capacity) were preconditioned by applying 2 mL of methanol, 1 mL of purified water, and then equili-

brated with 2 mL of 50 mM sodium phosphate buffer, pH 7.4. An aliquot, 925 μ L, of the sample was applied to a SPE cartridge, washed with 2 mL of 50 mM sodium phosphate buffer, pH 7.4, 4 mL of 12.5 mM sodium tetraborate, pH 9.0, and finally with 3 mL of purified water and allowed to dry under vacuum. The samples were eluted by adding 2 mL of 100 mM sodium tetraborate, pH 10.5, drying the cartridges under vacuum, and applying an additional 1 mL of the same buffer to the cartridge. The pH of the eluate was verified to be between 10 and 10.5 to ensure elution. The eluate was used in the subsequent steps.

Fluorescent Labeling: The fluorescent label stock solution was prepared by dissolving 1 mg of Alexa-Fluor[®] 488 (Invitrogen, catalog #30005) in 100 μ L dimethyl sulfoxide (DMSO). The stock was stored as 10 μ L single-use aliquots at –20°C. The stock was diluted 80-fold with purified water on the day of use. For each sample, 50 μ L of sodium carbonate, pH 8.2 was added to 240 μ L of sample. The labeling reaction was started by addition of 40 μ L of label working solution. The reactions were either incubated in the dark at ambient temperature for 1 h or at 60°C for 10 min. After the incubation period, the reaction was quenched by the addition of 20 mM Tris base. Water (160 μ L) was added to the sample and the sample was mixed before analysis by capillary electrophoresis.

Analysis by Capillary Electrophoresis: Capillary electrophoresis was performed on a Beckman Coulter PACE MDQ system with laser-induced fluorescence (LIF) detection that used an argon laser with an excitation line at 448 nm and monitoring emission at 520 nm. Uncoated, bare capillaries

75 μm (ID) $\times$ 60 cm total length with 50 cm to window (MicroSolv Technologies Corp.) and windows burned in-house. The separation was performed using a commercially available dynamic capillary coating system, CELixerTM (MicroSolv Technologies Corp.). This two-component system (initiator solution part number 06025-CE and accelerator solution part number 06125-CE) supplies the capillary coating and the separation buffer (pH 2.5). The sample compartment and the capillary cartridge temperature were controlled at 25°C. The separation program contains multiple pressure rinses at 20 psi as follows: (1) 1 N hydrochloric acid for 2.00 min; (2) 0.1 N sodium hydroxide for 5.00 min; (3) water for 2.00 min; (4) initiator for 1.00 min; and (5) accelerator for 1.00 min. The sample is injected by pressure injection at 0.5 psi for 5 s and followed with a water plug injection at 0.1 psi for 10 s. The electrophoretic separation starts with a 1 min ramp to 25 kilovolts (kV) under normal polarity (negative terminal on window side of capillary) with total voltage separation time of 12 min.

Results were interpreted based on a set peak area threshold, 20,000 RFU*min. Samples that were unspiked with kanamycin peak areas below threshold were reported as below the detectable limit. Unspiked samples with areas less than the spiked control but greater than the threshold were reported as less than 2 μM of kanamycin. Unspiked samples with areas greater than the spiked control and the threshold were reported as greater than 2 μM of kanamycin.

Potency of the Vaccine by Ricin Challenge. The potency of RVEcTM was determined using a modified in vivo assay previously described.²¹ Briefly, three groups of 10 female BALB/c mice were intramuscularly vaccinated at study days 0 and 21 with 20 μg of RVEcTM absorbed to 0.2% Alhydrogel[®]. One group of 10 female mice (control mice) was sham vaccinated with vaccine diluent. One week after the last vaccination, blood was collected for serological analysis and the mice were challenged intraperitoneally (i.p.) the following day with 5, 20, or 50 LD₅₀ of ricin toxin. Control mice were challenged i.p. with 5 LD₅₀ of ricin toxin. Mice were observed for mortality or morbidity for 7 days with percent survival in each group being calculated along with neutralizing antibody titers and concentration.

Determination of Serum Neutralization Antibody Concentration and Titer. The neutralizing anti-ricin antibody concentration and endpoint titer in the mouse serum was determined by a colorimetric cell proliferation toxin neutralization assay (TNA) as previously described.²²

Molecular biology and strain development

Construction of RVEcTM Plasmid for Production in *E. coli*. The gene for the over-expression of rRTA 1-33/44-198 was previously obtained by PCR and was ligated into a plasmid vector containing a T7 promoter;¹⁹ this work was based on the original sequence by Lamb.²³ For this study, the pET24a plasmid was used for the T7-promoter dependent expression of the RVEcTM gene. The gene for RVEcTM (rRTA 1-33/44-198) was optimized for expression in *E. coli* according to the codon-bias.

Transformation of Plasmids and Screening of RVEcTM Producing Clones. The RVEcTM pET24a plasmid (RVEcTM, pET24a-CBI-RVEc) was transformed into the *E. coli* BLR(DE3) competent cells (Lot # N70786, Cat. # 69053 Novagen, Madison, WI). The cells were thawed on ice and 50 ng of plasmid was added to each vial, mixed well

and kept on ice for 10 min. Heat shock was applied for 50 s at 42°C, after which the cells were placed back on ice. SOC medium (150 μL ; Lot # N65145, Cat. # 69319, Novagen) was added and the transformed cells were allowed to grow at 37°C in an incubator shaker for 1 h. A volume of 50 or 100 μL of each of the transformed cells were plated on LB-lennox/kanamycin plates (Lot # 10200, MBL). Kanamycin-resistant colonies were obtained after overnight incubation at 37°C. Four colonies were picked and inoculated into 15 mL LB-Lennox medium (Lot # 10177, MBL) with 50 $\mu\text{g}/\text{mL}$ of kanamycin (Lot # 10135, MBL) and grown overnight in an incubator shaker at 37°C. The overnight cultures from different colonies were used as inocula for two flasks per colony with each flask containing 15 mL TB medium (Lot # 10168, MBL) with 50 $\mu\text{g}/\text{mL}$ of kanamycin. These flasks were incubated at 37°C with shaking (250 rpm) and grown to a final OD measured at 600 nm (OD₆₀₀) of 0.1. The expression of RVEcTM was induced with a final concentration of 1 mM β -isopropylgalactoside (IPTG) (EMD Biosciences, San Diego, CA). After induction, the cultivation temperature was reduced to 30°C and 500 μL samples were taken at 2 and 6 h post-induction and centrifuged at 13,200 rpm for 5 min at room temperature. To evaluate expression of the RVEcTM, 1 mL of 1 OD₆₀₀ (or 5 OD₆₀₀ for small scale analysis) of each cell pellet was dissolved into 200 μL of 1X working solution of SDS-PAGE, heated in boiling water for 10 min, and centrifuged at 16,000g for 10 min. A volume of 10 μL of supernatant was loaded per well into NuPAGE 12.5% SDS-PAGE gels (Invitrogen, Cat. # NP0302BOX). Western blots were performed on the samples as described above.

Fermentation of *E. coli* BLR(DE3) + pET24a-CBI-RVEcTM

A semi-defined medium was used for both shake flask and fermentation studies and is a modification of Kilikian et al.²⁴ The medium consisted of 13.0 g/L potassium phosphate, monobasic (KH₂PO₄), 10.0 g/L potassium phosphate, dibasic (K₂HPO₄), 3.0 g/L ammonium phosphate, dibasic [(NH₄)₂HPO₄], 4.6 g/L sodium phosphate, monobasic, monohydrate (NaH₂PO₄·H₂O), 30.5 g/L yeast extract, and 0.5 mL/L of antifoam KFO 673.^{25,26} After sterilization, 1.2 g/L of 2 M magnesium sulfate, heptahydrate (MgSO₄·7 H₂O), 8 g/L glucose, 50 $\mu\text{g}/\text{mL}$ of kanamycin, and 3 mL/L of a modified *E. coli* trace elements solution were added by filter sterilization.²⁷ The modified *E. coli* trace elements solution had the following composition: 27 g/L of ferric chloride, hexahydrate (FeCl₃·6H₂O), 2 g/L of zinc chloride (ZnCl₂), 2 g/L of cobalt chloride, hexahydrate (CoCl₂·6H₂O), 2 g/L of sodium molybdate, dehydrate (Na₂MoO₄·2H₂O), 1 g/L of calcium chloride, dihydrate (CaCl₂·2H₂O), 1.27 g/L of cupric chloride, dihydrate (CuCl₂·2H₂O), 0.5 g/L of boric acid (H₃BO₃), 8.4 g/L of ethylenediamine tetraacetic acid (EDTA), and 100 mL/L of concentrated hydrochloric acid (HCl). The feed solution for the fed-batch phase consisted of 30 g/L of yeast extract, 50% w/w of glucose, and 22.7 g/L of MgSO₄·7H₂O. The medium was supplemented with 30 g/L of yeast extract to overcome the isoleucine auxotrophy requirement of the BLR strain.²⁸

A 250 μL aliquot of a glycerol stock was the inoculum for 300 mL of the semi-defined medium in a 1 L conical flask. After 12–14 h of growth at 37°C and 250 rpm in a Innova 4200 incubator shaker (New Brunswick Scientific, Edison, NJ), the 300 mL shake-flask culture with an OD₆₀₀ of 2–4 was used as the inoculum for 6 L of fermentation

medium in a 19 L fermentor (NLF Bioengineering AG, Switzerland). The fermentation was monitored for OD, pH, and off-gas using a Thermo Fisher VG Prima δ B Process mass spectrometer (Thermo Fisher Scientific), WCW, and glucose concentration. The temperature was maintained at 37°C during the batch and during the fed-batch phase until the OD reached 40–50. The pH set point was maintained at 7.0 throughout the entire fermentation. The oxygen concentration was kept at >40% saturation using a standard cascade by supplementing the air flow rate of 6 L/min with pure oxygen after the agitation reached 800 RPM. The vessel pressure was maintained at 0.2 bar. Once the glucose was consumed as indicated by dissolved oxygen (D.O.) spiked during the batch phase, a feed rate of 5 g/L/h of feed solution [30 g/L yeast extract, 50% (w/w) glucose, 22.7 g/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 100 $\mu\text{g/mL}$ of kanamycin] was started until the WCW reached 40–60 g/L. At this point, induction was started by first adjusting the fermentor temperature to 22°C and then adding IPTG to a final concentration of 1 mM based on estimated volume of the fermentor contents. A second fed-batch phase was started, consisting of 2 h at 5 g/L/h, 2 h at 4 g/L/h, 2 h at 3 g/L/h, and 1 h at 2 g/L/h to maintain the glucose concentration below 1 g/L. At this point (7 h induction time), the cells were harvested by centrifugation using an Evolution RC Sorvall centrifuge at 11,344g for 30 min and the cell paste was stored at –20°C. The average yield was 24 mg of RVEcTM/g WCW or 1.2 g of RVEcTM/L fermentation broth.

Purification of RVEcTM at the benchscale

Frozen cells were suspended at 10% w/v solids (20 g in 200 mL of 25 mM Bicine, pH 9.0) and disrupted by two passes through a Model EH-110 microfluidizer (Microfluidics Corp., Newton MA) at a pressure of 17,000–20,000 psig at 10–11°C. The homogenate suspension was centrifuged at 11,344g for 70 min at 4°C, the supernatant was diluted five-fold with 50 mM MES, 70 mM NaCl, pH 5.5 to adjust the pH to ~5.70 and the conductivity below 3 mS/cm. The diluted lysate was filtered through a 0.8 μm /0.45 μm Fluorodyne II membrane (Pall Corp., East Hills, NY). RVEcTM content was monitored by the SCX-HPLC method.

Column chromatography was performed using a BioCad Vision Chromatography system (Applied Biosystems, Foster City, CA). Dynamic resin-binding studies determined that maximum recovery of RVEcTM was achieved when the RVEcTM load did not exceed 2.5 mg of RVEcTM per mL of resin for all columns. The clarified lysate was loaded onto a 5.0 $\times$ 18.5 cm SP-Sepharose FF column (Waters AP-5, Millipore, Milford, MA) at a linear velocity of 150 cm/h. The column was washed with five column volumes (CVs) of 50 mM MES, 70 mM NaCl, pH 5.5, followed by a 10 CV gradient from 70 mM to 250 mM NaCl in the same MES buffer, with detection at 280 nm (UV₂₈₀). The column was cleaned with two CVs of 1 M NaCl in the same MES buffer. The RVEcTM peak was collected and diluted three-fold with 50 mM MES, pH 5.5, to bring the conductivity below 7 mS/cm and passed through a 370 mL Q-Sepharose FF (5 $\times$ 19 cm) column at a linear flow rate of 150 cm/h. Under these conditions the RVEcTM did not bind to the column resulting in a negative purification step. The flow-through was directly loaded onto a 360 mL SP-Sepharose HP column (GE Healthcare BioSciences, Piscataway, NJ) (5.0 $\times$ 18 cm, Waters AP-5) at a linear flow rate of 120 cm/h pre-equilibrated with

50 mM MES, pH 5.5. The elution protocol is as follows using 50 mM MES, pH 5.5: (1) wash with one CV of 70 mM NaCl; (2) a gradient of 10 CVs from 119 mM to 166 mM NaCl; (3) an isocratic elution step at 166 mM NaCl; and (4) an isocratic final elution step at 1 M NaCl. During the 10 CV gradient, fractions were collected across the asymmetric elution peak, first at 0.25 CV per fraction, then 1.0 CV per fraction and then at 3 CV per fraction. Fractions were analyzed by SDS-PAGE, Western blot, and SCX-HPLC; those that contained RVEcTM at purities of 99% or greater based on SCX-HPLC were pooled. Tween-20 was added to the pooled product at a final concentration of 0.12% w/v before the final diafiltration. The pooled fraction was first concentrated to 1 mg of RVEcTM/mL using a PES 5,000 molecular mass cutoff (MWCO) membrane (2 ft², 0.5 mm channel height) mounted in an OPTISEP 3000 membrane holder attached to a Model 5 cross-flow membrane filtration unit (SmartFlow Technologies, Apex, NC) and processed at ambient temperature. Once the RVEcTM was concentrated to 1 mg/mL, a five-fold constant volume diafiltration step using 20 mM succinate, 0.1 M NaCl, pH 6.5 was performed. The concentration of RVEcTM was calculated from the absorbance of the solution at 280 nm with the spectrophotometer blanked against pure water using an extinction coefficient of 0.91 for a 1 mg/mL solution. Before bulking, the Tween-20 concentration was measured (Table 1) and the concentration of Tween 20 was readjusted to ensure a concentration of 0.12% w/v. The final product was analyzed by SDS-PAGE, Western blot, HPLC (SCX, RP, and SEC), intact mass, peptide mapping, Tween-20, residual kanamycin, residual endotoxin, residual host DNA, residual host protein, and potency.

Process scale-up

The bench-scale process was scaled up five-fold and run in a manner identical to how the process would be performed in the cGMP suite. The fermentation was identical to that described above and cell mass was frozen for at least 24 h before use. All columns, equipment, tubing, and storage containers were depyrogenated by overnight treatment with 0.5 N sodium hydroxide (NaOH). Pyrogen-free water was generated by filtration of reverse-osmosis purified water through a Kleenpak Posidyne filter from Pall. Buffers were prepared with this water, but only the buffers for the final two steps were tested for endotoxin before use. Purification was performed with a process chromatography skid (SmartFlow Technologies, Inc S/N: 5037) equipped with inline UV-visible (Model 660), pH (Model 600), and conductivity (Model 620) detectors from Wedgewood Technologies (San Carlos, CA).

For the engineering runs, 100 g of frozen cell paste was thawed and the cell paste was suspended in 1 L of 25 mM Bicine, pH 9.0. The cells were lysed by microfluidization as described above. After centrifugation for 70 min at 4°C and 11,344g, the supernatants were combined (1.2 L total volume) and transferred into a 20 L HyClone[®] 2D BioProcess Containers[®] bag (BPC) (Thermo Fisher, Logan, UT) using a peristaltic pump. From this point forward, unless otherwise noted, processing was performed in a closed system using appropriately sized Hyclone bags. The column size for each of the three chromatography steps were 10 cm $\times$ 21 cm 1.65 L SP-Sepharose FF column, 10 cm/21 cm 1.65 L Q-Sepharose FF, and a 10 cm $\times$ 22 cm 1.73 L SP-Sepharose HP column. The elution conditions for all columns are as described above. The RVEcTM pool was concentrated to ~1.5 L and diafiltered

Figure 1. Effect of induction temperature on the production of RVEcTM per g WCW as determined by SCX-HPLC.

as described above using a SmartFlow Technologies, Inc Model 10 cross-flow membrane filtration system with an OPTISEP 3000 equipped with a 5,000 MWCO Regenerated Cellulose (RC) membrane, 0.5 mm channel height and area of 0.196 m². The final product was filtered through a Fluorodyne II (0.2 µm) filter, after which 1,015 mL of a 1.15 mg/mL solution of RVEcTM was obtained (1.17 g). RVEcTM is routinely stored at -80°C.

cGMP production of RVEcTM

The cGMP production of RVEcTM was performed in the UN-L BPDF cGMP suite equipped with a New Brunswick Scientific (NBS) MPP80 fermentor and a NBS gas blending system for addition of oxygen. Fermentation followed the same process as previously described above except for an increase in volumes. Cells were harvested by centrifugation for 30 min at 4°C 11,344g using an Evolution RC Sorvall Centrifuge. The exact same downstream processing equipment described above was used for the cGMP run. The Purification Development Laboratory and the cGMP suite have the exact same downstream processing equipment, minimizing technology transfer into the cGMP suite. The shakedown runs performed in the cGMP suite and the cGMP run processed 400 g of cell paste which is a 20-fold scale up from the bench-scale. The column sizes for the cGMP runs were increased fourfold over what was previously described above.

Results

Optimization of the RVEcTM fermentation

A T7-promoter dependent expression system was used to express the *E. coli* codon-optimized RVEcTM gene using the pET24a expression vector. We opted to use the *recA*⁻, *lon*⁻, and *ompT* deficient *E. coli* strain BLR(DE3) to minimize the potential for plasmid recombination.²⁹ This *E. coli* strain has a mutation that prevents the biosynthesis of isoleucine, resulting in a significant decrease in the rate of cell growth and product yield during fermentation using a defined medium.²⁸ The defined medium used in these studies was supplemented with 30 g/L of yeast extract (now a semi-defined medium), which was a source of additional nutrients and served as a source of isoleucine. The glucose feed solution was also supplemented with 30 g/L of yeast extract.

Fermentation parameters were optimized for RVEcTM quality, that is, purity and stability, so as to meet the requirements for production of Phase I clinical material. The yield

Figure 2. Production of RVEcTM during fermentation with semi-defined medium after induction.

The SCX-HPLC chromatograms correspond to 0, 3.7, 7.2, 12.3, 15.0, 17.9, 21.0, and 23.9 h induction time.

of RVEcTM per gram of wet cell mass was more than sufficient (24–37 mg/g WCW), allowing optimization studies to focus on the quality of the RVEcTM. Shake flask and fermentation studies evaluated a glucose-supplemented animal-free complex medium. Shake flask inocula, which was the same complex medium used for fermentation, grew slowly reaching an OD of 1–2 over a 12 h compared to an OD of 3–4 using a semi-defined medium. The batch and fed-batch phases of fermentation using a complex medium only reach ODs of 15–20 before induction. Evaluation of the whole cell lysate by Western blot from shake flask and fermentation growth before induction showed low levels of RVEcTM (data not shown). This unintended “auto-induction” was detrimental to cell growth and productivity. It was postulated (though not confirmed) that trace amounts of lactose in the complex media were causing unintended induction as seen by Studier.²⁹ Given that BLR(DE3) is an isoleucine auxotroph, a defined medium supplemented with either isoleucine or yeast extract was investigated. The best growth and RVEcTM production was observed with the medium supplemented with yeast extract.

The effect of temperature on the production of RVEcTM was investigated. The batch and fed-batch temperature was 37°C and once 100 g/L of WCW was reached the temperature was reduced to either 22°C, 26°C, and 37°C and 1 mM IPTG was added once the temperature reached the new set point. Induction occurred for 24 h and samples were analyzed by SCX-HPLC (Figure 1). The results indicated that 22°C or 26°C were preferred over 37°C with 22°C selected because of the slightly slower growth rate and the cells continued to produce RVEcTM.

The effect of residual glucose concentration during the induction was evaluated. Studies showed that glucose concentration in excess of 1–2 g/L during induction reduced the production of RVEcTM by 50% as compared to glucose levels maintained between 0.1 and 0.4 g/L (data not shown). Glucose feed rates were optimized to maintain residual glucose concentrations to <0.5 g/L. The intracellular production of RVEcTM increases up through 24 h of induction and produced up to 80 mg RVEcTM/g WCW (data not shown). Analysis of the cell lysates during induction by SCX-HPLC (Figure 2) showed the accumulation of a less basic

Figure 3. Profile of temperature, wet cell weight, RVEcTM, and glucose concentration during the cGMP run.

contaminant (9.6 min elution time) starting after 7 h of induction. Attempts to remove the contaminant during purification were unsuccessful and the decision was made to reduce the induction time to 7 h to avoid the basic contaminant. The time course of a fermentation is presented in Figure 3 and shows the production of RVEcTM, the WCW, glucose feed profile, and the residual glucose level, which was maintained below 0.4 g/L.

Purification of RVEcTM at the benchscale and engineering runs

Cell Disruption. The theoretical isoelectric point for RVEcTM is 7.40 and cell disruption studies were optimized from pH 6.5 to 10.5 with NaCl concentration from 0 to 2 M. Small-scale (1.5 mL) cell disruption studies were performed with a Bead Beater (BioSpec Products, Bartlesville, OK) and samples analyzed by SDS-PAGE and Western blot. Results indicated that the best condition for release of RVEcTM was 25 mM Bicine, pH 9.0, 2 mM EDTA, and no NaCl. As the NaCl concentration increased so did the amount of RVEcTM that precipitated. The yield of RVEcTM using a microfluidizer at 17,000 psig after two passes averaged 20% higher as compared to the bead beater. Analysis of the supernatant after frozen cells were thawed before cell disruption showed that approximately 60% of the RVEcTM was released due to the thawing process alone. A study showed that processing frozen cells (−20°C) yielded about 25% more RVEcTM than fresh cells using the standard disruption procedure of two passes at 17,000 psig. Based on this study freezing at −20°C was instituted as an overnight hold step after fermentation.

Capture Column—SP Sepharose. The optimal pH for release of RVEcTM from cells by disruption is basic and thus several anion exchange resins were screened for capture of RVEcTM. It was found that even at pH 10.5, the binding of RVEcTM to Q-Sepharose was not satisfactory and resulted in losses of RVEcTM in the flow-through. This is consistent with previous purifications results.^{19–21} Interestingly, a number of impurities bound to the Q-Sepharose and eluted at 1 M NaCl with no RVEcTM detected in the 1 M fraction, indicating that Q-Sepharose would be a suitable negative purification step, which is discussed later.

RVEcTM binds tightly to cation exchange resins¹⁹ at pH < 7.0. Before initiating these studies, the lysate was exchanged

from pH 9 to pH 6 using a five volume diafiltration with 50 mM MES at pH 6 using a 5,000 MWCO PES ultrafiltration membrane. Unfortunately, this resulted in RVEcTM losses as high as 73%. We determined that RVEcTM was susceptible to precipitation below pH 9 and exposure to a liquid-air interface. Every effort was made to avoid a air-liquid interface, which proved difficult with a cross-flow membrane filtration system. The alternative was to dilute the homogenate fivefold with 50 mM MES pH 5.5, 2 mM EDTA, and 10 mM NaCl (note: NaCl was added to increase the conductivity as much as possible and still bind RVEcTM to the SP-Sepharose). The addition of 10 mM NaCl was helpful in reducing the amount of contaminants binding to the SP-Sepharose FF column. The reduction in pH from 6.0 to 5.5 improved the binding of RVEcTM and it was critical to reduce the conductivity by dilution to at least 4 mS/cm to maximize binding to the SP-Sepharose FF column. Using this approach RVEcTM losses were less than 4% before loading onto the capture column.

The optimum dynamic binding capacity of four different cation exchange resins, that is, SP-Sepharose FF, Source 30S, SP-Sepharose HP, and Toso Haas SP-650M, was <3 mg/mL as RVEcTM broke through all of the resins with loads above 3 mg/mL. To ensure maximum recovery from the capture step, the RVEcTM loading density was maintained at 2.5 mg/mL of resin or less. There was no obvious difference in performance between all four resins, so SP-Sepharose FF was selected as it was the least expensive resin. Purity of the RVEcTM pool (fraction 2—Figure 4), as judged by SCX-HPLC was >95% and the average yield for 10 runs from bench-scale to cGMP was 87% (Table 2).

Second Purification Column—Q Sepharose. The second step was a negative purification step using a Q-Sepharose column which was very effective at removing a majority of host cell protein contaminants (Figure 5). It was important to reduce the conductivity to at least 6 mS/cm using a threefold dilution using 50 mM MES, pH 5.5. This conductivity was necessary for RVEcTM to bind to the final cation exchange polishing column.

Third Purification Column—SP-Sepharose HP. The Q-Sepharose flow-through was loaded directly onto a high performance SP-Sepharose HP column at 2.5 mg RVEcTM/mL of resin. A shallow 15 CV NaCl gradient from 119 mM to 190 mM NaCl eluted the RVEcTM in an asymmetric peak

Figure 4. Chromatogram for SP-Sepharose FF capture column and SDS-PAGE of the fractions.
L = load, FT = flow-through, W = Wash, F1 – F4 = fraction 1 through fraction 4.

Table 2. Summary of the Production Yield of RVEc™ for All Runs

	Fermentation g RVEc/ kg WCW	% Yields Across Each Step based on SCX-HPLC							
		Cell Rupture + 5X Dilution	Capture SP-FF	3X Dilution	Negative QS-FF	Polishing SP-HP	UFDF	Overall Yield	g RVEc/ kg WCM
RVEc Benchscale Run 1	37.5	96.1%	95.9%	87.4%	93.7%	87.0%	91.3%	59.9%	22.5
RVEc Benchscale Run 2	37.5	94.4%	102.4%	90.0%	104.1%	84.1%	82.0%	62.5%	23.4
RVEc Benchscale Run 3	37.5	100.0%	102.0%	100.3%	96.9%	82.8%	90.0%	73.9%	27.7
RVEc Benchscale Run 4	26.1	100.0%	91.5%	100.0%	97.8%	70.7%	90.0%	56.9%	14.9
RVEc Benchscale Run 5	30.0	87.0%	100.0%	97.5%	98.0%	71.2%	90.0%	53.3%	16.0
RVEc Engineering Run 1	27.1	78.5%	95.2%	81.1%	97.0%	83.0%	76.2%	37.2%	10.1
RVEc Engineering Run 2	29.2	93.8%	84.8%	119.0%	81.6%	74.9%	74.1%	42.9%	12.5
RVEc Shake Down Run 1	24.8	44.4%	67.5%	100.0%	91.0%	90.1%	105.7%	26.0%	6.4
RVEc Shake Down Run 2	25.5	79.1%	77.2%	100.0%	93.7%	107.1%	79.2%	48.5%	12.4
RVEc cGMP Run	23.8	99.9%	73.1%	100.0%	63.7%	90.4%	99.9%	42.0%	10.0

(Figure 6) and separated contaminants that eluted before and after the RVEc™ peak as determined by SCX-HPLC (Figure 7). The criterion for pooling RVEc™ was that fractions (5–8) must be greater than 99% pure (Figure 6).

Final Diafiltration and Bulking Step. The RVEc™ was susceptible to precipitation and was particularly sensitive to precipitation at an air–liquid interface. Precipitation started as white “stringy” filaments that were visible upon close inspection and was independent of protein concentration (0.2–1 mg/mL). It was critical during cross-flow membrane filtration that bubbles were removed from the recirculation path and that the liquid returning back to the tank was completely submerged to eliminate any liquid–air interface. Even with these precautions there was still some precipitation. Three different detergents, Tween-20, Tween-80, and Triton X-100, were evaluated at concentrations from 0.05% to 0.2% w/v in an effort to minimize precipitation. Just before the concentration/diafiltration step, the detergent was added at the respective concentration. The diafiltration buffer, 20 mM sodium succinate pH 6.50 and 0.1 M NaCl, did not contain detergent. The selection criterion was based on visual obser-

vation of precipitate both during and after diafiltration. Both Tween-80 and Triton X-100 showed noticeable precipitation at all concentrations, while Tween-20 showed very little to no precipitation at the 0.1–0.2% (w/v). At the completion of the diafiltration, the Tween-20 concentration was measured and it was found that the concentration was reduced by 50 to 70% after five diafiltration volumes. Tween-20 was added back to the final retentate at a concentration of 0.15% (w/v).

Analysis of the RVEc™ Bulk Drug Substance. The summary of analysis of the RVEc™ bulk drug substance (BDS) for all runs is presented in Table 3. RVEc™ is 100% pure based on SCX-HPLC and RP-HPLC, 99.5% pure based on SEC-HPLC (Figure 8), and is a single band by SDS-PAGE and Western blot for all runs and capillary isoelectric focusing showed a variance in the pI ranging from 7.14 to 7.22 (Table 3). Residual host cell proteins for all runs range from 1.42 to 7.2 ng/mg RVEc™. The residual host cell DNA for cGMP Shakedown Run 1 was reported below the detection limit of <80 pg/mg RVEc™, while cGMP Shakedown Run 2 and the cGMP Production Run were <10 pg/mg RVEc™. Charles Rivers Laboratory-Malvern, who was contracted to

perform the assay, was able to improve the sensitivity of the assay. Kanamycin was undetected in all final product samples and is not reported in Table 3.

RVEcTM was characterized at the molecular level using intact mass analysis. The ESI-TOF determined an intact mass range of 21447.1 to 21447.5 amu for all runs listed in Table 3, which compares favorably to the theoretical mass of 21447. On the basis of the charge envelope, it was determined by intact mass analysis that the percentage of RVEcTM missing an N-terminal methionine ranged from 6.5% to 12.1%. LC-MS/MS was able to identify the N- and C-termini peptides and determined that a majority of the RVEcTM N- and C- termini were intact. LC-MS/MS also determined that methionines 165 and 179 were partially oxidized.

The stability of the RVEcTM BDS was evaluated at 4°C and -80°C over 3 weeks. RVEcTM did not show any signs of proteolytic degradation at 4°C or -80°C after 3 weeks based on SDS-PAGE (Figure 9).

Potency Results. The data for the potency assay on various lots of RVEcTM are summarized in Table 3. For all the

lots that were tested, 90–100% of the mice survived for 7 days after an i.p. challenge with 5 LD₅₀s of ricin toxin. Survival rate at various ricin LD₅₀s were used to calculate the midpoint of the dose-response curve (ED₅₀) by probit analysis (Table 3). In all 10 lots tested, the ED₅₀ ranged from 300 to 700 µg/kg for mice vaccinated with two doses of RVEcTM whereas the ED₅₀ for diluent control mice was 10–20 µg/kg

The anti-ricin neutralization antibody concentrations as determined by the TNA are summarized in Table 3. With the exception of lot shakedown run 2 (67%), 90–100% of the mice vaccinated with the other nine lots of RVEcTM developed anti-ricin neutralization antibodies. For all 10 lots, the anti-ricin neutralizing antibody concentration was 54–163 µg/mL. None of the buffer controls developed anti-ricin antibodies.

Discussion

The biopharmaceutical industry, in concert with the FDA, has implemented a Quality by Design (QbD) approach to process development and manufacturing. QbD builds quality

Figure 5. Chromatogram for the Q-Sepharose FF. SDS-PAGE of the fractions.
L = load, FT = flow-through (RVEcTM), F = 1 M NaCl wash.

Figure 7. SCX-HPLC chromatograms of the fractions from the SP Sepharose HP column.
Numbers to the left are the fraction numbers from the chromatogram shown in Figure 6.

Figure 6. Chromatogram of the SP-Sepharose HP column.
The fractions are indicated by the vertical marks (MRK) on the chromatogram.

Table 3. Summary of RVEcTM Characterization and Analytical Results for All Runs

Run	Bench Scale Run 1	Bench Scale Run 2	Bench Scale Run 3	Bench Scale Run 4	Bench Scale Run 5	Scale Up Run 1	Scale Up Run 2	cGMP Shake Down Run 1	cGMP Shake Down Run 2	cGMP Run
Strong Cation Exchange HPLC (%)	100	100	100	100	100	100	100	100	100	100
Reverse Phase HPLC (%)	100	100	100	100	100	100	100	100	100	99
Size Exclusion HPLC (%) (% monomer)	99.6	99.5	99.6	99.6	99.3	99.7	99.4	99.6	99.2	99.9
SDS-PAGE ^A (%)	>95	>95	>95	>95	>95	>95	>95	>95	>95	>95
2100 Bioanalyzer ^B (%)	100	100	100	100	100	100	100	100	N/D	N/D
N-terminal Met Content (%)	93.5	93.6	93.7	92.0	91.0	91.1	92.3	86.3	89.0	87.9
Tween-20, % w/w	0.08	0.11	0.12	0.12	0.08	0.14	0.08	0.18	0.16	0.16
Cap. Isoelectric Focusing (pI)	N/D	N/D	N/D	N/D	N/D	7.10	7.12	7.18	7.14	7.22
Intact Mass Analysis (Da)	21447.1	21447.5	21447.4	21447.4	21447.4	21447.4	21447.3	21447.4	21447.5	21447.1
Endotoxin (EU/mL)	N/D	N/D	N/D	N/D	N/D	1.33	0.91	8.61	<0.098	1.1
Host Cell DNA (pg/mL)	<5000	<5000	<5000	<5000	<5000	<5000	<5000	<80	<10	<10
Host Cell Protein (ng/mL)	4.45	5.20	4.29	1.47	6.71	1.64	1.42	7.55	5.70	7.2
Potency Assay										
% of Mice Surviving 5 LD ₅₀	100	100	100	100	90	100	89	100	95	100
ED ₅₀ (μg/kg)	380	708	380	580	340	360	420	460	300	500
% Immune Response in TNA	100	100	100	100	100	100	100	100	67.3	93.3
Anti-ricin neutralization Ab (μg/ml)	163.1	128.9	120.7	91.0	109.8	58.0	136.0	96.8	54.5	74.1

N/D, not determined.

Figure 8. Strong cation exchange (A), reverse phase (B), and size exclusion (C) HPLC of the RVEcTM BDS.

and robustness into the product starting with product development through commercial launch and is evident through all stages of process development, scale-up and cGMP manufacturing. A bench-scale process was developed that met

Figure 9. SDS-PAGE of RVEcTM BDS from 1 to 5 μg (A).

SDS-PAGE (5 μg) of RVEcTM BDS stored at 4°C and -80°C for 3 weeks (B).

expectations for yield, quality and potency and served as a reference point for process scale-up and manufacturing. The robustness and consistency of the bench-scale process was based on five different bench-scale runs with three purifications performed with the same cell mass (runs 1, 2, and 3) and two purifications from separate fermentations (runs 4 and 5). Runs 1–3 averaged 65% overall recovery from the same fermentation and runs 4 and 5 had recoveries of 57 and 53%, respectively. The average yield for all five bench-scale runs was 61%. The analytical testing of the products from all 5 runs is presented in Table 3. The purity of RVEcTM based SCX, RP, and SEC HPLC is 99.5% or greater, the percentage of RVEcTM containing an N-terminal methionine ranged from 91 to 93.7%, host protein ranged from 1.47 to 6.71 ng/mL and the MW based on intact mass analysis ranged from 21447.1 to 21447.5 for all 5 runs. These results indicate a consistent and robust process at the bench-scale and are a starting point for establishing the technical specification for the release tests for the cGMP product. An approved Standard Operation Procedure (SOP) was generated for all analytical methods listed in Table 3. The

standard process is to qualify all release methods which allows for the SOPs to be finalized and in combination with all of the results from the bench-scale and scale up activities, an approved technical specification document is generated that is used for release of the cGMP material. In this case, the methods were not qualified, but 7 different process runs were performed at 2 different scales with full testing with the approved SOPs to generate the technical specification document.

The average yield of the process scale-up runs was 40% which is 21% less than the bench scale runs. The major difference between the bench-scale and process scale-up runs was going from a very low hold-up volume Biocad system to a process chromatography system with a bubble trap and $\frac{1}{2}$ " sanitary piping, which will reduce the resolution of the process chromatography system. Tighter cuts of the product peak were made to insure the desired quality was maintained (Table 3) at the expense of yield.

cGMP shakedown run 1 showed a significant reduction in the yield across the first step (44%), as compared to cGMP shakedown run 2 and the cGMP run. This was attributed to the method of mixing of the initial cell paste before cell disruption. The bench-scale studies used a high-shear mixer to re-suspend the frozen cell paste for a very short period of time (1–2 min). It was determined that before the first pass through the homogenizer, nearly 60–70% of the cells were lysed due to the freeze-thaw. When the process was transferred into the cGMP suite, the mixing time was arbitrarily set to 30 min in the batch records. It was found that there was a significant loss of RVEcTM during this extended high-shear mixing and it is hypothesized that the RVEcTM released from the freeze-thaw was precipitating during the high-shear mixing. The high-shear mixer was replaced with a marine impeller mixer for re-suspension of the cell mass, which corrected the problem as seen by the yields for the cell disruption and 5X dilution step for cGMP shakedown run 2 and the cGMP run (Table 2). The average overall yield for the process scale-up runs, shakedown run 2, and the cGMP run was 43%.

The release testing and characterization of the cGMP runs are presented in Table 3. The purity by SXC, RP, and SEC HPLC was identical to the bench-scale runs and the process scale-up runs. The host protein increased very slightly 5.70–7.55 ng/mL (or ng/mg), which is well within expected limits and the host DNA was less than 10 pg/mL (pg/mg). With an upper dose of 100 μ g the maximum amount of host protein and DNA would be 0.76 ng and <1 pg, which is well below the FDA's recommendations for host protein and DNA.³⁰

The process as described is able to produce 1.2 g RVEcTM/L of fermentation broth and assuming a dose of 100 μ g, the yield is 12,000 doses per L of fermentation broth. A 60 L fermentation would produce 720,000 doses. The fermentation is the step that could have the highest impact on increasing yield. As mentioned, it was possible to produce 80 mg RVEcTM/g of wet cells by increasing the induction from 7 to 24 h. The problem was that a "basic" impurity based on SCX HPLC started to show up and a decision was made to shorten the induction so as to eliminate the need to reconfigure the purification process to remove this impurity.

Further process optimization and scale-up will be dependent on what the human dose and schedule is determined to be and the total amount of vaccine required for clinical evaluation and stockpiling. A Phase 1 escalating, multiple-dose

study to evaluate the safety and immunogenicity of RVEcTM in healthy adults is underway beginning in April 2011 at USAMRIID. Results from this study will provide initial data upon which future developmental needs are required.

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