

BIOMANUFACTURING OF A RECOMBINANT PARVOVIRUS B19 VACCINE USING A DUAL BACULOVIRUS/VECTOR SYSTEM.

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Abstract:

A clinical grade recombinant human Parvovirus B19 (HPVB19) vaccine has been developed to treat transient aplastic crisis (TAC) in patients with sickle cell anemia. The vaccine consists of two viral proteins (VP1 and VP2) in separate baculovirus vectors that are co-infected at the correct MOIs into *Spodoptera frugiperda* (Sf9) cells and that, upon expression, self assemble into immunogenic virus-like particles. Pilot co-infections studies in 1L Wave[®] bioreactors revealed that MOI's of 0.5 for bac1 and 0.2 for bac2 generated self-assembling virus-like particles consisting of 27% VP1 and 73% VP2. The process was scaled to a 20L Wave culture and a purification procedure using fluidized bed chromatography was developed. Dilution of the API to a target concentration of 10µg/ml resulted in loss of the drug due to protein adherence to the vial. Formulation studies utilizing Schott Plus-1 vials combined with sucrose and Tween 80 minimized loss. Formulation and purification studies required a sensitive and specific method to track the vaccine. A sandwich ELISA utilizing both polyclonal and monoclonal antibodies specific for HPVB19 was developed that demonstrated a sensitivity of ≤ 50ng/ml and variability of under 20% within the range of quantitation (100ng/ml to 750ng/ml).

Introduction:

The parvoviruses are small deoxyribonucleic acid (DNA) viruses that infect rapidly dividing cells (Figure 1A) and cause a diversity of diseases in animal species. HPVB19 is the only member of the Parvoviridae family known to cause disease in humans. In healthy individuals, HPVB19 causes fifth disease (Figure 1B) and an arthralgia syndrome in adults. In individuals with underlying hemolysis (Table 1), infections result in TAC, a temporary cessation of red blood cell production with severe and occasionally fatal anemia (Figure 2B). Immunosuppressed individuals may experience persistent and severe anemia and pure red cell aplasia. Infection during mid-trimester pregnancy can result in hydrops fetalis and fetal loss. Immunization is a promising strategy to prevent serious parvovirus infection in high-risk groups, as well as in the general population. Studies in the Hematology Branch of the National Heart, Lung, and Blood Institute (NHLBI) have resulted in the production of recombinant parvovirus capsids, which lack infectious DNA, but retain the immunogenicity of native virions. These empty viral capsids have been utilized in human volunteer studies to elicit neutralizing antibody responses.

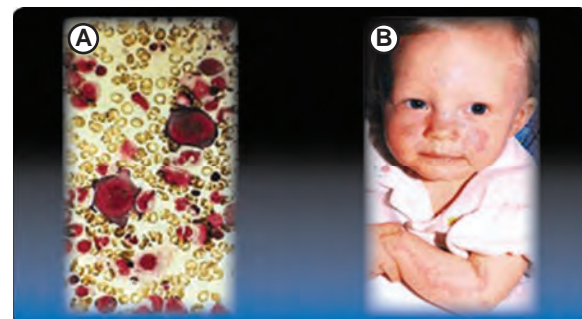


Figure 1: Panel A shows a bone marrow aspirate from an infected patient lacking mature erythroid precursors and with giant pronormoblasts that result from the cytopathic effect of the virus. Panel B shows cutaneous eruptions typical of Fifth Disease, including "slapped" cheeks in children and a more generalized lacy pattern or erythema. (Photos courtesy of Dr. O. Caul.)
Source: Neal S. Young, M.D., and Kevin E. Brown, M.D., Parvovirus B19. N Engl J Med 2004;350:586-97.

A Normal Subjects B Patients with Transient Aplastic Crisis

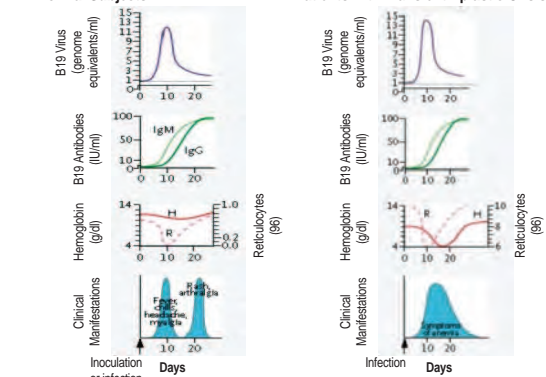


Figure 2: Pathophysiology of HPVB19 infection is compared between normal subjects and patients with transient aplastic crisis. Detection of virus genomes and development of IgG and IgM follow nearly identical time courses and durations. In normal subjects, reticulocytopenia (R) occurs, but hemoglobin (H) levels do not fall below normal. In subjects with TAC, reticulocytopenia causes cessation of erythropoiesis and severe anemia. Normal subjects experience a two phase infection whereas subjects with TAC experience prolonged anemia.
Source: Neal S. Young, M.D., and Kevin E. Brown, M.D., Parvovirus B19. N Engl J Med 2004;350:586-97.

Indication	Target Population	Population Size
Transient Aplastic Crisis: Life Threatening Anemia.	Sickle Cell and Hemolytic Anemias, Immune compromised.	In 2005, the Sickle Cell population is estimated at ~85,000 U.S. and ≥ 2.8 million globally with ~1,800 new cases per year in the U. S. and up to 400,000 globally. 40% of existing population is seronegative and eligible for the vaccine. All new cases would be vaccinated between 2 to 6 years.
Erythema Infectiosum: Fifth disease, Slapped Cheek Syndrome.	Children 2 to 6 years old.	Vaccinate all children 2 to 6 years due to the fact that Fifth disease is so prevalent among children it could be classified as an occupational hazard for pregnant daycare workers, teachers, and mothers with infected children.
Hydrops Fetalis: B19 Fetal infection transmitted by the mother.	Women of child bearing age, not yet pregnant, who could be exposed to children or intend to have children.	B19 infection rates for pregnant woman is 20-30% for occupational related contact and 30-50% for mothers with infected children. Seroprevalence data indicates that 50% of pregnant women are susceptible to B19 infection. For infected women, transplacental transmission rate is 30% with 5 to 9% chance of fetal loss. - If 1,000 pregnant teachers were exposed, 500 could develop B19 infection. - 150 would transmit B19 to their fetus. - 7.5 to 13.5 could develop hydrops fetalis and fetal loss.

Table 1: Incidence rates of parvovirus B19 infection.

Vaccine Design:

HPVB19 has a small linear, single-stranded DNA genome, which encodes 2 capsid proteins. The major capsid protein, VP-2, constitutes about 95% of the capsid structure, the minor capsid protein, VP-1, is identical except for an additional 226 amino acids at the amino terminus. Studies conducted in a CRADA between a previous industry partner and the Hematology Branch of the National Heart Lung and Blood Institute (NHLBI) demonstrated that the individual capsid proteins can be expressed in a baculovirus system (Figure 3) and, when recombinant vectors are cotransfected (Figure 4), VP-1 and VP-2 spontaneously assemble into empty parvovirus capsids or virus like particles (VLP). Neutralizing linear epitopes cluster in the VP1-unique and VP1-VP2 junction regions. Enrichment of VLPs by manipulating multiplicity of infection (MOI) for the baculoviruses produce capsids containing 20% to 40% VP-1, that are particularly effective in promoting neutralizing antibody response. Expressed VLPs lack infectious DNA but retain the immunogenicity of native virions. Animal and human studies in have shown that HPVB19 VLPs elicit neutralizing antibody responses as assayed in vitro.

Recombinant VLP's formulated with an aluminum hydroxide adjuvant elicited low neutralizing antibody titers. Investigation of other adjuvants determined that using a squalene-based emulsion adjuvant with the VLP's yielded excellent neutralizing antibody responses in rhesus monkeys. The adjuvant, designated MF59, is produced by Chiron Corporation. The proposed candidate vaccine consists of recombinant HPVB19 VLP's containing 20% or greater VP1 formulated with MF59 adjuvant and excipients to prevent adherence to the glass vial.

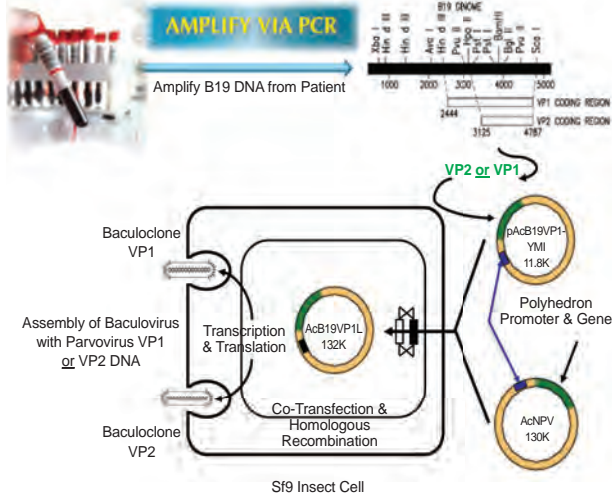


Figure 3: An almost full length copy of the HPVB-19 genome strain Au encoding the VP1 and VP2 proteins, was obtained from a child with sickle cell disease and TAC and subcloned into pUC19 and pUC7 vectors and propagated in *E. coli* JM101. HPVB19 DNA for VP1 and VP2 was cloned into baculovirus vector pAcB19VP1-YM1 or pAcB19VP2-YM1 with wildtype baculovirus in Sf9 insect cells produced baculoviruses possessing DNA for either the VP1 or VP2 capsid proteins. Expression of recombinant VP2 protein alone can assemble VLPs but is only mildly immunogenic. Co-expressed VP2 and VP1 proteins self-assemble into VLPs that elicit neutralizing antibody response in volunteers.

Methods and Results:

Pilot production of the candidate vaccine has been performed using suspension cultures of Sf-9 cells in Wave Bioreactors. A pilot production lot was made using the ACB and the AVB's as a 5 liter culture in the Wave Bioreactor at an MOI of 1:0.5 for bacVP1:bacVP2 to confirm expression levels of each viral protein in the VLP's.

Production of VLP's:

Production size lots of B19 VLP's have been prepared in 20 liter suspension cultures in the Wave Bioreactor. One vial from the Sf-9 WCB was expanded through a series of shake flask cultures until 4 x 1 liter cultures were obtained. The cultures then were transferred to a 50 liter Wave bag at 1.5 x 10⁶ cells/ml and allowed to expand until 20 liters of culture at a cell density of 1.5 to 2 x 10⁶ were obtained, after which, the culture was infected simultaneously with bacVP1 and bacVP2 at an MOI of 1 and 0.5 respectively (Figure 4). The bioreactor was incubated at 26° to 28°C for 4 days and harvested when the cell viability was less than 50%. The temperature, pH and rocking speed and angle were monitored. Cell density and viability were monitored daily. At harvest, a sample of the culture was removed for End of Passage (EOP) cell testing. The culture was harvested by centrifugation at 800 x g for 30 minutes after which the fluid was decanted and the cell paste stored at -60° to -80°C. A sample of the cell paste was removed to test for protein expression by SDS-PAGE and silver staining.

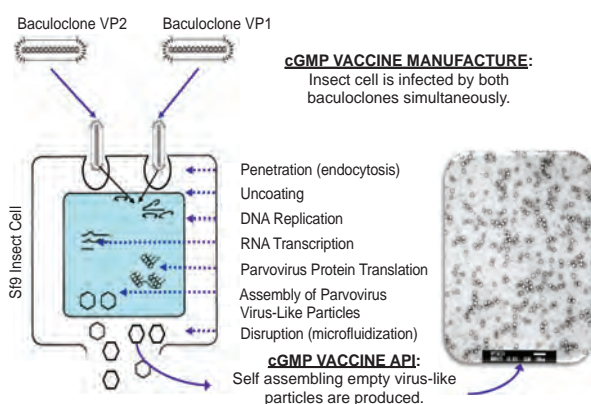


Figure 4: Schematic of VLP production. Two baculoviruses possessing DNA encoding for the VP1 or VP2 capsid proteins are co-infected into Sf9 cells (left). Baculoviruses penetration, uncoating and protein transcription/translation occurs. Expressed proteins self assemble into VLPs. The panel on the right shows an EM of empty VLPs.

Purification of VLP's:

VLPs are purified by resuspending cell paste in 20mM Tris-HCl and 0.4M NaCl at pH 8 with 1.6µM Leupeptin, and microfluidized to prepare a cell lysate. Protocols transferred in the IND from the previous industry partner utilized filtration to remove insoluble particles with a single DEAE anion exchange chromatography step. Figure 5 shows a silver stain and western blot of materials purified using filtration. This filtration step was changed to improve manufacturability and product yields.

Lysate was diluted with 20mM Tris-HCl to reduce salt concentration to 0.08M and subjected to fluidized bed ion-exchange chromatography with Streamline DEAE media in lieu of filtration (Figure 6). Eluate containing VLP's was diluted with 20mM Tris-HCl to reduce salt concentration to 0.08M and subjected to Fractogel DEAE ion exchange column chromatography equilibrated with 20mM Tris-HCl, 0.2M NaCl buffer, pH 8. After washing with Tris-HCl buffer, pH 8, containing 0.24M NaCl, the product was eluted with 50mM phosphate buffer, pH 6, containing 0.2M NaCl.

After adjustment of the pH with 1M Tris to 8.6 the product was subjected to TMAE ion exchange chromatography equilibrated with 20mM Tris-HCl, pH 7, containing 0.2M NaCl. Product was eluted with 20mM Tris-HCl, pH7, containing 0.28M NaCl. Sucrose and Tween 80 were added and final concentrated bulk product was filtered through a 0.22µm filter.

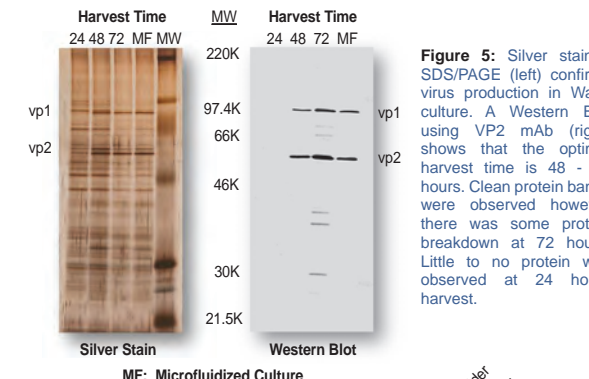


Figure 5: Silver stained SDS/PAGE (left) confirms virus production in Wave culture. A Western Blot using VP2 mAb (right) shows that the optimal harvest time is 48 - 72 hours. Clean protein bands were observed however there was some protein breakdown at 72 hours. Little to no protein was observed at 24 hours harvest.

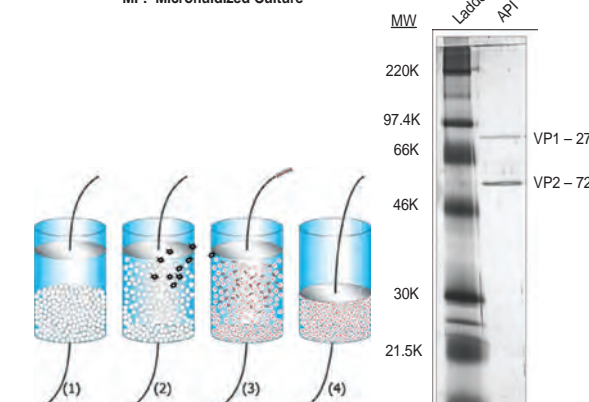


Figure 6: Methodology for Ion-exchange chromatography via fluidized bed technology using Streamline DEAE media is shown diagrammatically in panel A. Steps 1 and 2 involve expansion of the DEAE bed. Microfluidized culture is passed over the bed to remove cellular debris in step 3. The column is then packed, the flow reversed, and VLPs eluted in step 4. harvest. Panel B represents a silver stained SDS/PAGE of API adjusted to 10mg/ml showing clean bands of VP1 at 27.3% and VP2 at 72.7%.

Vialing:

During initial production runs, the API was diluted to a target concentration of 10µg/ml resulting in the loss of the drug due to protein adherence to the vial. An aggressive formulation program was initiated to resolve the problem. Combinations of buffers, excipients, and vials were evaluated to determine which formulation would provide protection from adherence to the glass vial.

Vial, Buffer and Excipient

- Standard @ 400ng per lane
- Schott 280mM NaCl, 20mM Tris, 5% Sucrose, 0.005% Tw 80
- Schott 280mM NaCl, 20mM Tris, 5% Maltose, 0.005% Tw 80
- Schott 280mM NaCl, 20mM Tris, 5% Sucrose, 0.005% Tw 80, 10mM Histidine
- Schott 280mM NaCl, 20mM Tris, 5% Maltose, 0.005% Tw 80, 10mM Histidine
- Schott 280mM NaCl, 5% Sucrose, 0.005% Tw 80, 10mM Histidine
- Schott 280mM NaCl, 5% Maltose, 0.005% Tw 80, 10mM Histidine
- Kimble, same as 2
- Kimble, same as 3
- Kimble, same as 4
- Kimble, same as 5
- Kimble, same as 6
- Kimble, same as 7

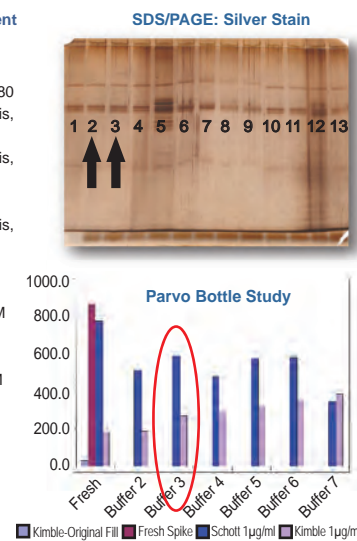


Figure 7: The best formulation contains sucrose or maltose and Tween 80 as the excipients. The Schott Plus 1 vial is clearly superior to the Kimble vial in this study.

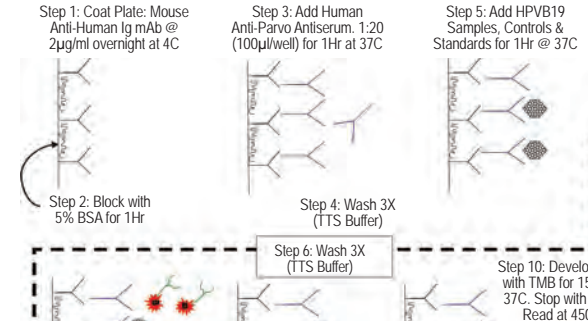


Figure 8: Capture assay used to test for parvovirus vlp's in the presence of various excipients. A direct assay is in development now that the excipients have been finalized.

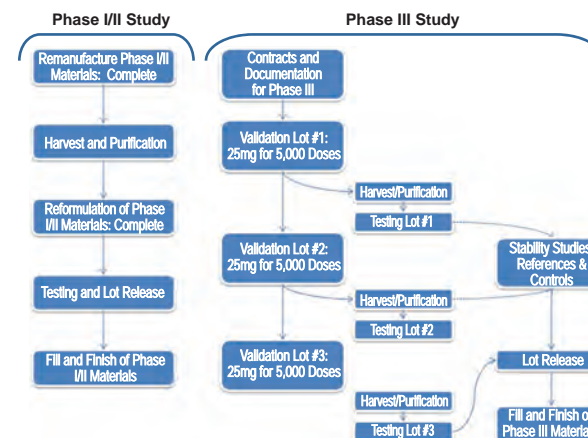


Figure 9: Phase I/II is scheduled for early 2006 with Phase III studies beginning in 2007.

Conclusions:

The combination of Wave technology, expanded bed technology and reformulation of the buffer will enable manufacture of parvovirus vaccine at scales necessary for Phase III and commercialization for Transient Aplastic Crisis.

Collaboration with the NIH, NHLBI and DMID is on target to get this important vaccine through clinical trials.

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