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The Potential for the Use of Baculovirus Expression Vector Systems to Produce Recombinant Protein for Clinical Use

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The biologics market, although difficult to estimate, is currently thought to be in excess of $20 billion. In recent years, the growth in the novel therapeutics market has continued to exceed all but the most optimistic of expectations. The number of products in early stage trials may already be over 1,000, with an estimated 40 or so additional products in the process of finally being released to the market. The biologics market is led by relatively few "blockbuster" drugs, but the breadth of novel products continues to expand. This has resulted in exciting times for clinicians but has resulted in concern related to the bottleneck of production capacities for these drugs, as well as the pressure from healthcare agencies to reduce the cost of goods.

Both capacity issues and cost of goods has encouraged manufacturers to investigate alternative strategies for the production of recombinant protein drugs. The cornerstone of all biotherapeutics is based on a cell line. The first recombinant proteins were expressed in microbial cells such as *Escherichia coli* or yeast. Products made in simple systems have the benefit of a many-fold lower cost of goods (CoG) in comparison to eukaryotic production systems. Microbial systems are also able to generate many-fold higher yields than a comparable eukaryotic system, and modification and optimisation of microbial production systems can be completed in a matter of weeks. This is because the production system is not demanding in terms of the growth conditions of the cells, and the growth substrates are significantly less expensive than eukaryotic media. However, the downside to prokaryotic systems is that the proteins produced under these systems often lack the tertiary structure and post-translational modifications seen in mammalian cells. For many proteins, this is not an issue. However, it may affect the bioactivity of the protein or the associated pharmacokinetic properties. Therefore, production in prokaryotic systems has proven unsuitable for many proteins which depend on such modification for their bioactivity. Due to this and other factors, the current trend is that many biologic drugs are being developed and produced in mammalian cell lines.

Historically, murine cells were widely used. These lines produced some of the first monoclonal antibodies tested in humans. Since these early murine-derived products, Chinese hamster ovary (CHO) cells have proven to be the workhorse when considering recombinant protein production. As has been mentioned, the CoG for such products are high and there can be an extended period of up to two years while the production system is optimised. There are potentially a number of other systems that can and have been successfully applied to recombinant protein production, including the human cell line PerC6, marketed by Crucell. Balancing the ability of cells to provide adequate protein folding and tertiary structure, as well as glycosylation patterns with reduction in production costs, still remains a challenge to be resolved. Exciting alternatives such as transgenic sheep, cows, or even rabbits that are able to secrete recombinant proteins in their milk provide headlines in the scientific press, but still require more development to become viable production platforms.

The Baculovirus Expression Vector System (BEVS) is a system based on a virus of insects using insect cells as hosts to produce recombinant proteins. As a technology, this has been available for research purposes for many years. Now, however, this technology could be used to bring inexpensive biotherapeutics to the clinical market. The most frequently used baculovirus in recombinant pro-

Table 1. Advantages of using the baculovirus expression vector system.

High recombinant protein expression levels achieved typically range from 1 to 30 mg/L with some reports of = 500 mg/L.
Mammalian-like post-translational modification — including disulfide bond formation, phosphorylation, glycosylation, oligomerization and proper folding functional glycosylation of glycoproteins.
Protein localisation within the insect cells can be directed to specific cell compartments relevant cellular compartmentalisation of proteins. Secreted, membrane-bound, cytoplasmic or nuclear.
Ability to express multiple genes simultaneously. Capacity of large cDNA. Inserts can accommodate genes up to 15 kb.

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tein expression has been the *Autographa californica* nuclear polyhedrosis virus (AcNPV). This is in a subgroup of the family of viruses termed *Baculoviridae*. These rod-shaped viruses have a large, double-stranded DNA, which can reach 200 kb, packaged into nucleocapsids. During infection of the host insect cell, the virus is released from the cell by budding through the cell membrane during early stages of infection (lasting from ten hours to three days). After this stage, viruses are encased in large protein structures called occlusion bodies, composed predominately of the polyhedrin protein. These are released from the nucleus when the infected cells burst.

Over the years, a small number of stable cell lines have been derived from *Lepidoptera* species (butterflies and moths) that are suitable for sustaining the replication of baculovirus. Sf9 and Sf21 are by far the most commonly used cell lines for AcNPV propagation. These cells were derived at the United States Department of Agriculture Insect Pathology Laboratory with a cell line originating from pupal ovarian tissue of the fall armyworm, *Spodoptera frugiperda*. The differences between these cell lines are minimal: Sf21 cells have a wider range in size and a shorter doubling time of 24 hours as compared to 48-72 hours for Sf9 cells. Scientists at the Boyce Thompson Institute developed a third cell line, High Five™, from ovarian cells of the cabbage looper, *Trichoplusia ni* for plant research. High Five cells are reported to be less successful at amplification of baculovirus, but can increase the level of secreted protein, five- to ten-fold, when compared with Sf9 or Sf21. It should be noted, however, that the commercial use of High Five cells is dependent on a licensing arrangement with Invitrogen. AcNPV is the most thoroughly studied baculovirus; its DNA has been fully sequenced and numerous transfer vectors and modified AcNPV DNAs are available, each with special advantages for selection or transfection efficiencies.

The use of insect cells as an alternative production method presents many potentially useful features (Table 2). One very simple feature of insect cells, in contrast to mammalian cells,

Table 2. Companies involved in BEVS.

AB Vector. ProFold™ Technology allows simultaneous expression of a protein of interest and large amounts of molecular chaperones using the same baculovirus vector. The molecular chaperone improves protein folding and thus, bioactivity.

Abgent. Baculoviral expression vector construction, expression, and purification of large-scale production.

ATG Laboratories. Baculoviral expression vector construction, expression, and purification of large-scale production.

BD Biosciences. BD BaculoGold™ expression system.

Biologics Process Development. Cell bank preparation, transfection, plaque purification and screening. Protein production in spinner culture in 5 and 10 L.

Biosciences Research Associates, Inc. Expression and purification of large-scale production.

BlueSkyBiotech. Construction of expression vectors, expression of recombinant proteins in fermentors up to 36 L.

Cambrex. Insect-Express media systems.

Cell Culture Service. Construction of expression vectors, expression of recombinant proteins.

Chesapeake PERL. Baculovirus mediated recombinant expression, using insect larvae as "mini bioreactors." Producing recombinant proteins from milligram to kilogram scale.

Cytostore. TripleXpress™ is a non-lytic, baculovirus-free, plasmid-based expression system for insect cells that allows for continuous production and secretion of recombinant protein.

DIARECT. Generation of a recombinant expression construct, expression in 0.5 L to 40 L, and purification using the 6xHis technology.

Entopath. Larval Express® *in vivo* insect system and production of recombinant protein(s) at research scales.

Expression Systems. Contract production of recombinant protein, titre of virus, media formulation.

Hyclone. Insect cell growth media HyQ SFX-insect medium.

Invitrogen. Insect cell growth media (Sf900, Express Five, Drosophila SFM), BEVS expression systems (EvoQuest, BaculoDirect), insect cells (Sf9, S2).

Kinakeet Biotechnology. Recombinant protein expression and purification using BEVS.

LabFrontier. Construction of expression vectors, expression of recombinant proteins in fermentors >1 L.

Merck Biosciences. Insect cell expression system (Bacvector, Insect Direct, pTriEx™ system, a useful novel expression vector that allows protein expression in bacterial, insect, and mammalian cells from a single plasmid).

Orpigen. Baculovirus expression construction and cloning, seed stock production and characterisation, expression optimisation, and recombinant protein production.

Oxford Expression System. Production of recombinant viruses using flashBAC, optimisation of recombinant gene expression and protein production. Commercial recombinant protein production in suspension and shake cultures.

Paragon Bioservices. Vector construction and expression in up to 200 L fermentors.

Protein Sciences. GeneXpress® recombinant protein production service. Cloning and expression, purification, cGMP Phase I, II, and III materials. (Figure 1.)

Q-Biogene. MERLIN® custom service division uses the BacTen™ baculovirus system for the production of recombinant proteins. Service levels include sub-cloning the gene of interest, construction of the vector, large-scale production of the recombinant protein.

The Wistar Institute. Construction of vector, production of the recombinant protein in 50 ml to L scales.

University of Cambridge. Baculovirus vector construction, transfection, viral amplification, expression optimisation, and expression using Wave bioreactors, 10 L capacity.

University of Minnesota. Baculovirus vector construction, transfection, plaque purification, protein expression analysis, viral amplification, expression optimization, and large-scale expression up to 36 L.

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Angra dos Reis, Brazil • September 16-20, 2007

Highlights and Objectives of the Conference

- Latest information on non-viral and viral vectors and expression systems
- Highlights on recent development in cell engineering, cell culture technologies and protein production
- Latest development in systems biology, RNA interference and stem cell/tissue engineering
- Informal discussion with invited speakers and other delegates from both industry and academia
- Latest information on reagents and equipment through vendors' exhibition and workshops

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is their lack of requirement for CO_2 during growth. Insect cells are able to grow on much simpler and more easily defined substrates than mammalian cells. Of particular benefit is their ability to grow on substrates that do not require animal-derived supplements or animal serum for growth. In addition to the cost savings that this presents, there is the additional benefit of not increasing the risk of introducing adventitious viruses from the animal (usually bovine) sourced material. Insect cells are also able to produce a very high yield of proteins—many-fold higher than their mammalian counterparts. Post-translational modifications in insect cells, while in some cases is not identical to mammalian cells, can be sufficiently similar to enable the production of biologically active biopharmaceuticals.

Due to the benefits of the BEVS expression system — one particular section of the biopharmaceutical industry, the market for vaccines — has exploited these features and has brought a number of clinical products through trials. Only one manufacturer, Intervet (a veterinary vaccines company), has taken the lead with a number of products on the market for the prevention of disease. A number of factors have resulted in the vaccine market being more buoyant today than in past decades, and this has been a major target for insect cell-derived biologics. BEVS offers the ability to synthesize large quantities of viral proteins relatively rapidly and at low cost. This is an important feature as many of the agents representing a risk to the population, and for which vaccines are required, emerge incredibly fast. The rapid response to vaccine production that can be allowed by BEVS may help product to be available before any other. This might be of particular importance with the danger of the influenza strain H5N1, which may reach pandemic proportions in less than a year. Cost of goods is an important issue, as well as the ability to manufacture product quickly. Vaccines, in general, are used to treat a large number of individuals, and as such, the cost per dose must be low.

Protein Sciences is one of the leading exponents of the use of the BEVS to produce clinical material. Their product, FluBlØk™, derived from recombinant hemagglutinin (rHA) consists of three rHA proteins corresponding to the flu strains of the annual influenza virus vaccine. The proteins are produced in insect cells and formulated in PBS without preservatives or adjuvants. Phase I and II clinical trials conducted by the National Institute of Allergy and Infectious Diseases (NIAID) demonstrated significant interest. Protein Sciences also has a pipeline of development in other products derived from BEVS. The company was awarded a $2.7M grant by NIAID to produce 2,000 doses of a Severe Acute Respiratory Syndrome virus (SARS) vaccine. The vaccine is based on the manufacture of a recombinant S-protein sub-unit vaccine in insect cells. The vaccine had been shown to elicit an immune response in mice, and early human trials have begun.

The ability to respond rapidly with an effective product ready for manufacture and use in the clinic highlights the effectiveness of this technology. Although today, influenza and SARS vaccines are of international importance, BEVS technology does have some history of use in clinical trials. MedImmune, Inc. (Gaithersburg, MD), announced the clinical trial of a recombinant baculovirus expressed parvovirus B19 virus vaccine as long ago as 1995. The vaccine was based on the ability of the baculovirus to produce empty, non-infectious recombinant parvovirus capsid proteins that are self-assembling. This product was made from the co-expression of viral proteins VP1 and VP2 in *Spodoptera* cells. Insect cells have been uniquely useful in the production of this protein. As the expression of the vaccine was made possible *in vitro*, insect cells are not sensitive to the recombinant viral proteins, in contrast to mammalian cells. The vaccine dem-

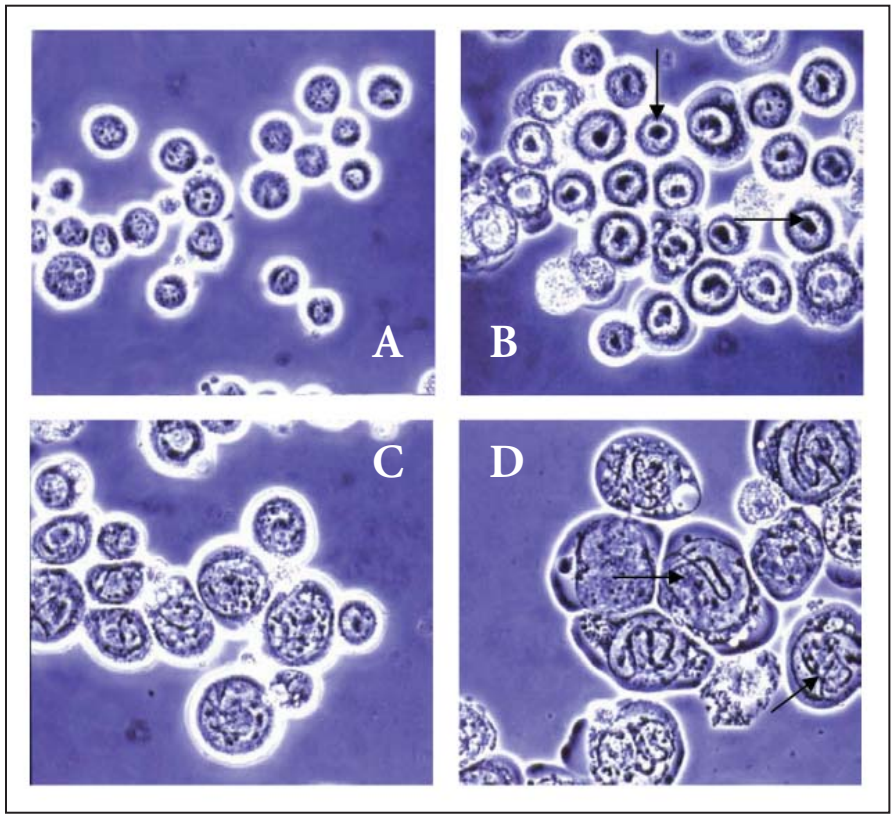

Figure 1. Visualization of the progression of baculovirus infection in expresSF+® insect cells. A) Uninfected cell culture, cell size is approximately 17 – 18 microns; B) Early stage infection (18 – 24 hours post-infection). The cells enlarge with pronounced stroma formation (arrow); C) Mid-stage infection (30 – 40 hours post-infection). Cells continue to enlarge and stroma structures begin transformation into fibrillar structures; D) Late stage infection (48 – 72 hours post-infection). Cell size reaches 22 – 25 microns and well formed snake-like fibrillar structures are present (arrows). (Courtesy of Protein Sciences Corp.)

onstrated promise in pre-clinical and early clinical phase development but has yet to be released as a licensed product.

MedImmune has, however, licensed the technology to GlaxoSmithKline (GSK) who has developed a vaccine against *Human papilloma* virus (HPV); a cause of cervical cancer, the second highest incidence of reported cancer worldwide. The GSK product, Cervarix, is competing against a similar product, Gardasil, manufactured by Merck & Co., that has already received FDA approval (June 2006). Both products contain virus-like particles (VLP), protein structures which resemble the form of viruses but contain no viral genetic elements. The Merck product is manufactured using yeast cells in contrast to the insect cell expression system used by GSK.

GSK is presently carrying out late stage clinical trials with the HPV vaccine. Both products elicit an effective immune response and are, therefore, comparable in respect to efficacy. Cervarix contains two types of HPV, whereas Gardasil contains four, and therefore, potentially may represent an ability to prevent infection by a wider range of HPV sub-types. The glycosylation ability of the host cell production system appears to have an effect on the clinical efficacy of these products. From this example, it is clear that baculovirus-generated products can reach marketing authorisation in a similar timeframe to those produced in the microbial systems, traditionally thought to be the fastest to market, and have the potential to achieve similar cost of product.

There are reports in the literature of a number of other viral vaccines that are produced using insect cells. The highly pathogenic subtypes of influenza virus (avian influenza or "bird-flu") have received a significant degree of interest recently. The immunogenic proteins, haemagglutinin and nuraminidase, of the influenza virus have been expressed in insect cells and have been successfully used as a vaccine to elicit protective immune responses in lab animals. The interest in the use of insect cells has been heightened recently as these cells have the ability to express high levels of recombinant protein which may allow large quantities of vaccine to be produced in a relatively short time. This ability to react rapidly to a new viral threat such as influenza H5N1 may be essential in preventing a pandemic. Protein Sciences was asked to produce a vaccine against avian (H5N1) influenza following an emergency request from the Centers for Disease Control. The vaccine was a BEVS-produced recombinant influenza haemagglutinin (HA) gene product forming a trimer protein, and 1,700 doses were rapidly produced. This illustrates the speed at which these new threats can be responded to.

Clearly, production issues can be satisfied by the use of insect cells. However, the biosafety aspects of insect cell-derived products and the characterisation of the product are a potential cause for concern. The ICH Q5A and Q5D guidelines which have been enacted as guidelines in the United States of America, the European Union, as well as Japan, clearly set out the safety testing and characterisation strategy for cell lines used in the production of biologics for humans. These guidelines were written based on the expectation that mammalian cells would be the method of choice for the eukaryotic production system of choice. To this extent, the use of insect cells does raise some issues. One problem is estimating the virus risks in insect cells.

Mammalian viruses are well characterised in many cases and an estimate of potential risks can be made. The literature available on the viruses that are able to infect insect cells used in production is very limited at best. The risk therefore, of infection with endogenous insect viruses or adventitious viruses which enter into the production system and are able to infect the insect cells, can be difficult to quantify and therefore, increased caution should be applied to the viral safety assessment of insect cells. There is already a well known family of viruses termed the *Arboviruses* that contain thousands of species which infect insects and will infect man. Some of the well recognised viruses which are in

this group include *Japanese encephalitis* virus, *Yellow fever* virus, and *Dengue* virus. Although these viruses are spread by mosquitoes or biting flies, they have no recorded growth in *Lepidoptera* cells (used in BEVS production). However, the ability for similar viruses to grow on such different species (mammals and insects) would indicate a potential ability to grow on *Lepidoptera* cells, should the opportunity arise. Aside from the viruses which are known to be able to replicate in both human and insect cells, there are a number of virus species which are known in insects but have never been described in humans.

One of the bigger concerns regarding the viral safety issues of insect cells is the unknown or poorly understood viruses that can infect insect cells. Viruses of the family *Nodaviridae* and *Tetraviridae* have not been described in humans but do infect a number of insect species. The safety guidelines for the production of human therapeutics are designed to ensure that manufacturers show their cell lines and that production systems result in a product which is safe and free from endogenous and adventitious microbes. In a number of cases, the specified assays for microbial safety assessment can be common between mammalian and insect cells.

For example, checks for sample sterility and freedom from mycoplasma can be common between cell types. However, insect cells are also able to be potential hosts for spiroplasma and hence, should also be tested for these organisms. Adventitious viruses can infect insect cells that are in common in the same way that mammalian cells can be infected. However, testing for insect viruses sometimes requires a slightly different approach. Mammalian cells on infection with many viruses will typically show a characteristic cytopathic effect which, on simple visual observation, are obvious. This is the basis for the majority of virus detection assays used. In contrast, insect cells on infection can show little or no visible cytopathology. This presents some difficulty during production. In mammalian cells, if a virus has entered the system, the cells will lyse or show other easily distinguished morphological features. Insect cells, on the other hand, can and will remain ostensibly healthy from a visual observation. Many of the methodologies used to detect viruses can be difficult to apply to the testing of samples derived from insect cells. Testing for viruses using *in vitro* (cell-based assays) or *in vivo* (laboratory animal assays) methods can be difficult as the media used to grow insect cells can be toxic for the cell systems or animals used in testing for viruses. The solution can be to dilute the test material before it is inoculated in the assay. However, this clearly reduces the sensitivity of the assay. It should be noted that some cell culture systems such as the baby hamster kidney cell line, can be particularly useful in detecting some *Arboviruses* which have the potential to be a contaminant during production. Careful consideration should be made of using such appropriate systems to identify contaminants.

The production of biologics using BEVS and insect cells has already been successfully applied in the veterinary field and many products continue to yield promising results in clinical trials. Thus far, the safety profile of insect cells has been excellent. However, the number of patients treated with such products still remains small when compared with the numbers treated with products produced on mammalian cells, or even prokaryotic cells. The potential for BEVS to produce other types of recombinant proteins does have drawbacks, particularly is the glycosylation pattern. Insect cells do not have the ability to put the sialic acid or the penultimate galactose on many sugar complexes. Instead, manose is the terminal sugar of choice. Despite this, insect cells are similar enough to mammalian glycosylation in many cases, so the proteins expressed in BEVS have similar biological activity.

In an attempt to achieve full mammalian glycosylation, a number of modifications have been applied to insect cells. The enzymes involved in post-translational modification have been genetically altered and the cells are grown in specific substrates to provide the sugars necessary to mimic mammalian glycosylation. The potential that these new systems offer make such modifications worthwhile and commercially viable. For example, complex proteins such as nerve growth factors have been expressed in insect cells and have shown clinical potential.

In Conclusion

Insect cells and the BEVS are potentially one of the most useful systems already in place to allow biotechnology to provide drugs at low cost. The near completion of the regulatory hurdles by the GSK product, Cervarix, should herald an improved confidence with manufacturers using this technology. From this, the use of insect cells should expand and develop. Entopath, has presented one of the most interesting possibilities using the BEVS technology system. Their Larval Express product is a self-contained kit that supplies insect larvae in cups that allow the larvae to survive. The larvae can be infected with baculovirus that express a target gene. On infection, the baculovirus will kill the larvae while expressing large amounts of the therapeutic or prophylactic protein. The protein can be harvested and recovered in a similar way to cell culture harvests. This system may provide the ultimate, low-cost solution for the production of recombinant protein.