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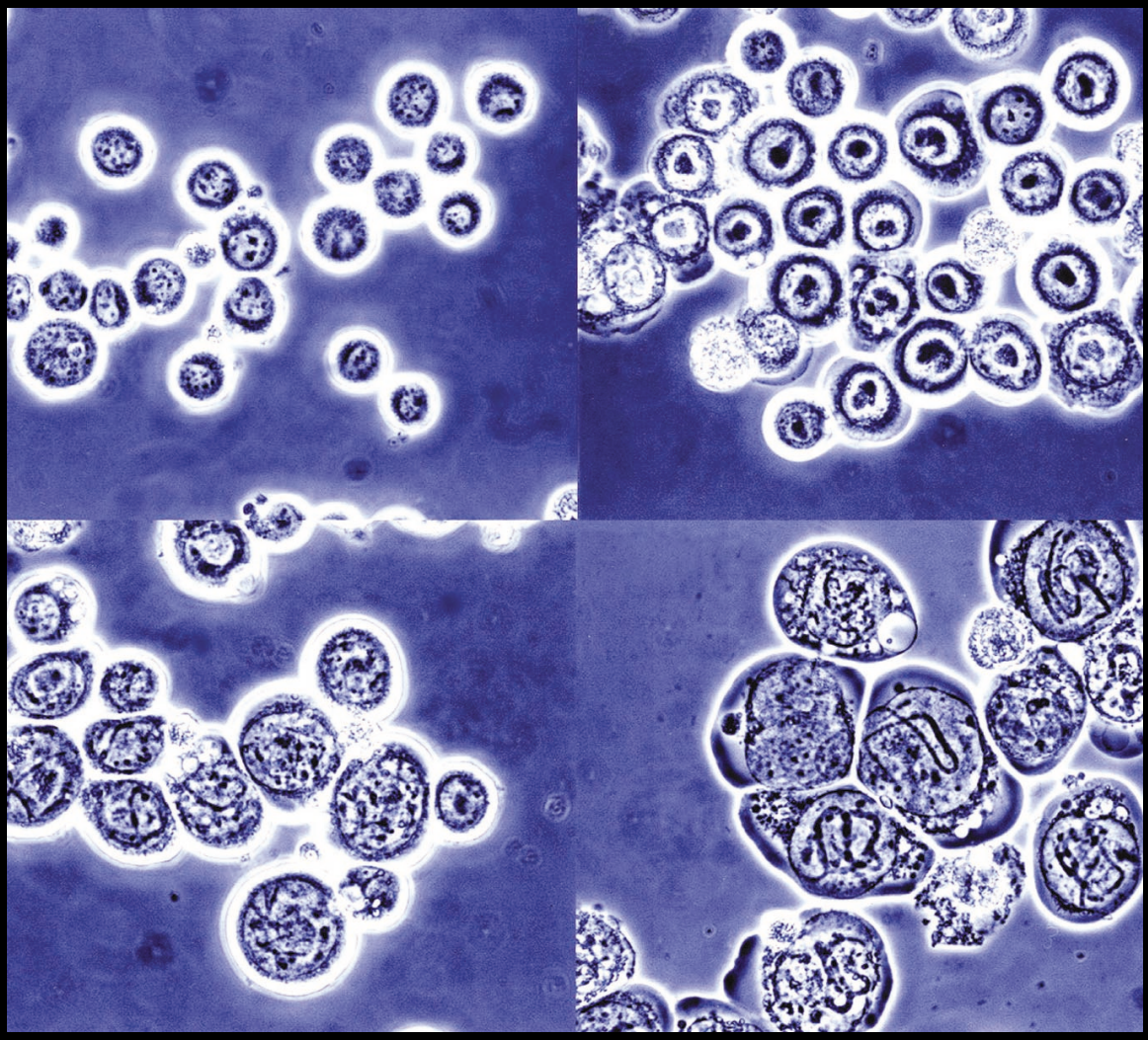

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Regulatory Aspects of Recombinant Protein Products by Baculovirus Expression Systems

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The baculovirus expression system promises to revolutionize the production of recombinant proteins for use as clinical products. The technology is robust, efficient, and low- cost when compared to other cell based systems. The technique may also present an advantage in producing safer products versus the equivalent materials made with mammalian cells. Proteins can be produced in insect cells without animal supplements such as fetal calf serum. In the current climate of concerns over Bovine Spongiform Encephalopathies, and bovine viral risks, this method offers a significant safety, as well as, cost advantage over other production methods.

Given the benefits of producing recombinant proteins with baculovirus in insect cells, the question arises as to why there are no licensed clinical products that use this technology. One reason could be the uncertainty about how these products would be accepted by worldwide regulatory authorities. As the investment costs of developing novel clinical products continue to escalate, pharmaceutical companies may be wary of investing significant amounts in this technology, while the regulatory and safety aspects remain unknown.

In principle, producing recombinant proteins from insect cells should be no different than using Chinese hamster ovary, or other cells. On deciding to move to preclinical and clinical investigation, a rigorous study of the material and process safety issues will be needed to assure compliance with safety milestones and regulatory requirements. Transfer of a product from the research bench into production requires the use of a cGMP (current Good Manufacturing Practice) environment. Production will normally require master and working cell banks, a master and working seed stock of the baculovirus, and a safety testing and validation plan for the downstream production of the protein.

Specific regulatory guidance for the characterization and testing of insect cells, as well as the products derived from them, is not available. Therefore, manufacturers are encouraged to use the generic guidelines already in place. The principal guidance notes are the International Committee on Harmonisation Q5A: "Quality of biotechnological products: Viral safety evaluation of biotechnology products derived from cell lines of human and animal origin" and Q5D: "Derivation and characterization of cell substrates used for production of biotechnological/biological products." The stated objectives of these guidelines are to provide guidance and appropriate standards for cell lines and other materials used to prepare biological products, and to provide a general framework for the viral testing and clearance studies required for products with intended clinical use.

The guidelines are designed for a cell/reagent bank system, as this is the most practical, safe, and cost effective method for generating clinical product. Banks of materials, such as cells, have a two-tiered format with the creation and complete characterization of a master bank, and the production of a working bank from this master. The product is then generated sequentially from vials of the working banks. In the case of insect cells, the manufacturer would establish master and working cell banks of the production cell line, such as Sf9 or Hi5, and master and working banks of the Baculovirus vector from which the product is made.

Safety Testing Requirements

Cell Line / Vector History

When assessing the suitability of cell lines for clinical production, the history of the cells must be documented. As insect cells and Baculovirus are infrequently used for such production, these materials will require a clearly documented culture history. Frequently, cell lines have been passed between a number of laboratories before they are used for the production of a clinical product.

For example, Sf9 cells were cloned in 1983 from parental line (IPLB-SF 21 AE), which was originally derived from

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Table 1. Safety testing schedule for insect cell products

Test	Master Bank	Master Virus Bank	Working Bank	Bulk Harvest	Final Product
Sterility	+	+	+	+	+
Mycoplasma	+	+	+	+	+
Electron microscopy examination	+	+		+	
Species specific tests	+	+			
Specific retrovirus tests	+	+		+	
In vivo assay	+	+			
In vitro assay	+	+		+	
Residual DNA					+
Residual protein					+
Pyrogenicity					+
Abnormal toxicity					+

the ovarian tissue of *Spodoptera frugiperda* (described by Vaughn et al., in 1977). Finally in the late 1980's, the cells were transferred to the American Type Culture Collection where they were expanded, laid down as a bank, and made available for release to any laboratory.[1]

It can be assumed that between 1977 and today, there has been a considerable amount of culturing and sub-culturing done with this cell line by different operators, and with a number of different media types. Therefore, there is the potential that the cell line has been exposed to any number of materials that could affect its safety profile. Complete documentation may not be available on the physical, chemical, or biological procedures used with the cells, and thus present difficulties.

The guidelines note that, on some occasions, it is impossible to document all of the manipulations carried out before the final point of use, and this will be taken into consideration. However, the significant manipulations that affect the production characteristics of the cells should be recorded. Potential exposure to infectious agents should also be noted, as regulators require knowledge of any bovine serum, enzymes, or other cell growth promoters of animal origin that were used during the culture or cell manipulation.

The accumulation of such data allows a thorough risk analysis of the possibility that the cells contain adventitious agents, and directs the testing strategy to establish product safety. A number of companies now offer characterized cell banks for use in the production of therapeutic products. These cells are based on clones from Sf9 or Hi5 cells, but care should be taken to determine that the characterization satisfies applicable and current guidelines.

Baculovirus expression vectors undergo a greater level of manipulation than the production cell lines. The Baculovirus genome is modified to include the foreign transgene that is capable of producing the desired recombinant protein. The various methodologies used to insert these genes and produce the vectors are reviewed in Kitts (1996).[2]

As with the history of the cell line, the vector history must be documented and include all of the significant manipulations. Baculovirus vectors, that are suitable for clinical production, can be obtained from commercial sources. Again, care should be exercised to assure that the supplier's safety data satisfies current guidelines.

After the cell and virus banks have been prepared, they must be characterized and tested according to the current guidelines. The guidelines also encourage manufacturers to apply "state-of-the-art" methods and technological improvements to the safety testing protocols, provided the novel protocols are at least as good as those already in place.

Identity Testing

The initial test required for both the production cell line and the Baculovirus vector is usually one for identity. A phenotypic or genotypic characteristic may be used for this purpose, and must include a description of the cell or virus morphology, along with an assay that can identify the species of cell or type of virus. Karyology is sometimes part of the characterization of a cell bank, but may not be required in the case of insect cells, due to the polyploid nature of many insect cells.

Tests for cell identity normally use molecular assays, such as isoenzyme analysis, which take advantage of the inherent amino acid composition variability in the ubiquitous enzyme systems found in different species. These protein differences are visualized using electrophoretic techniques. More recently, DNA fingerprinting has been approved by regulatory authorities for use in identifying insect cells.

The tests for Baculovirus vector identity may require specific immunoassays, or examination by molecular analysis of the viral genome. To ensure vector stability, the transgene must be sequenced, both in the master virus seed and in the virus at the time of product harvest. In addition, characterization of the promoters, or other such elements, may be required.

Sterility and Mycoplasma Testing

The majority of any product testing lies in the assays for contaminating microorganisms (Table 1). Two assays are required to ensure that banks are free of bacteria, fungi and protozoa. First, a sterility test is performed on one percent of the total containers in a cell bank, and on the containers used at other stages of production (Table 1). To ascertain sterility, the test material is inoculated on agar, and in broth culture systems, to allow the growth of any contaminating microorganisms. To test for viable forms of bacteria and fungi, thioglycollate medium and tryptone soya broth (TSB) are used. Thioglycollate medium is used for the detection of aerobic and anaerobic bacteria, while TSB is used for the detection of aerobic bacteria and

fungi. After inoculation, the samples are incubated at two temperatures and observed for a period of at least fourteen days.

Mycoplasma can frequently be a contaminant from the environment or operators. Detecting mycoplasma, and similar organisms, requires a cultivation period in media (agar and broth), plus inoculation into cultures containing specified cells. The cells are subsequently stained for mycoplasma using published techniques.

For compliance, these protocols require that a qualification experiment be used to show that the test material (insect cells or Baculovirus) will not inhibit the detection of contaminating microorganisms. It is known that the pH, salt concentration, and detergents present in insect cell media can frequently inhibit the growth of some microorganisms. This qualification demonstrates that microbes can grow in the presence of the test material under the specified culture conditions.

Assay qualification can be cumbersome. For example, in the case of mycoplasma, the controls include *Spiroplasma citri*, *Mycoplasma pneumoniae*, *Entomoplasma lucivorax*, *Mesoplasm entomophillum* and *Mycoplasma synoviae*. Since the specifics of the sterility and mycoplasma protocols can differ amongst the various regulatory bodies, manufacturers should seek the tests that apply for the geographical location of the product's intended use.

Virus Safety Testing

Virus contamination may pose the most serious risk in biologics production. Indeed, the reason for much of the safety testing used in the bioprocessing industry is due to past viral contamination. Virus safety testing is divided into three sections: detection of species-specific viruses, detection of adventitious viruses, and detection of retrovirus.

Detection of species-specific viruses denotes the testing for viruses that may have been present during the preparation of the production cell line or Baculovirus. These are viruses that originated from animal material, and in the case of insect cell lines, would have been present in the insect tissue from which the cells were originally harvested. In the case of Sf9 cells, this would be *S. frugiperda* ovarian tissue.

Determining which viruses to test for can present a problem. Table 2 lists the known families and genera of viruses that infect invertebrates. However, given the diversity of insect species, it is likely that the number of viruses is underestimated. Aside from Baculoviridae, five families of viruses have been described that can infect normal production cells. Ascovirus, Nodavirus, Metavirus, Reovirus, and Tetraviridae can infect Sf9 cells, and more frequently, Hi5 cells.

Other insect cell lines, such as Drosophila, have a completely different profile of viruses to which they are susceptible. The guidelines indicate that if a potential virus contaminant is recognized in a cell line, steps should be taken to ensure that the line is free of the agent. Therefore, specific assays should be designed to detect each of these virus types in the cell and virus master banks, though the methodology of detection may vary.

Traditionally, cell culture or animal assays are used to detect many viruses. The sample to be tested is inoculated into cultures of specific detector cells, or directly into animal species. Then the cells are observed for specific morphological changes, or the virus is measured with immunoprobes. In the case of animal experiments, the animals are monitored for clinical signs. These assays are particularly useful as they will only identify replicating virus and are designed to detect a single infectious unit.

Currently, molecular amplification techniques, such as polymerase chain reaction (PCR), can be used in a simple assay to identify specific viruses. These tests have the advantage of being faster to complete than cell culture assays. PCR assays are available to detect a number of common insect cell contaminants, such as Nodaviruses, Tetraviruses, and Picornaviruses. These assays, whether cell culture or PCR based, must be qualified and validated to demonstrate their specificity and sensitivity.[3]

It remains a question as to the extent production cells can be infected by the remaining invertebrate virus families. Virus families, such as Iridovirus and Parvovirus, can infect a wide range of invertebrate species, and are highly likely to initiate an infection in Sf9 or Hi5 cells. A great deal of scientific investigation is required to discover the full extent that viruses can be harbored by insect cells.

Adventitious Viruses

Tests for adventitious viruses are in place to monitor the breakdown of cGMP, or the introduction of virus via the cell growth media. In the latter instance, the assays are typically used to identify non-insect viruses. In the published literature, there are relatively few articles on the susceptibility of production cell lines to virus infection. Zhang described infection of Sf9 cells by St. Louis encephalitis virus.[4]

Fourteen other viruses, including a number of human pathogens, were investigated, but few demonstrated survival. However, Yellow Fever, Dengue Fever, and Equine Encephalomyelitis virus demonstrated some level of survival in Sf9 cell culture media, plus some evidence of replication. Although these particular viruses are unlikely contaminants, they serve to illustrate the importance of safety testing for adventitious viruses.

Adventitious agent tests are based on three assays that have traditionally been used to detect the presence of viruses: observation of the cells by transmission electron microscopy, the in-vitro cell culture assay, and the in-vivo animal assay.

The electron microscope affords a non-specific means of monitoring for the presence of any virus type. A snapshot is taken of representative cells from the master cell bank or any other appropriate sample type. The samples are fixed, stained, and finally observed by transmission EM for characteristic morphology. Again, guidelines can be specific regarding the number of cells that must be reported for examination. The electron microscope is seen as the "gold standard" of virus identification, as this is the only method

Table 2. Families and genera of viruses infecting invertebrates			
Poxviridae (Entomopoxvirinae)	Circoviridae	Reoviridae (Cypovirus)	Rhabdoviridae
Iridoviridae (Iridovirus, Chloridovirus)	Parvoviridae (Densoviridae)	Birnaviridae (Entomobirnavirus)	Bunyaviridae
Baculoviridae		Tetraviridae	Togaviridae
Polydnaviridae (Ichnovirus , Bracovirus)		Picornaviriade	Flaviviridae
Ascoviridae		Metaviridae (Errantivirus)	Nodaviridae

where viruses can be visualized. However, the disadvantage is that any virus must be at a concentration of at least 10^5-10^6 particles per ml before it would be observed (Q-One Biotech internal data).

Used in combination, cell based and animal assays allow the detection of the most likely contaminants. The in-vitro assay utilizes the ability of viruses to cause a cytopathic effect on living cells in culture. The guidelines are quite specific on the number and types of cell lines used. At least three cell lines must be used, including a human diploid line, a primate line, and a cell line of the same species as the production line (in this case insect). The guidelines also specify the culture period and the observations that should be made at the end of the assay.

As for the in-vivo assay, this typically involves the inoculation of one or more animal species with a preparation made from the master banks. As before, the guidelines specify the species, age, and number of animals to use in the assays, as well as the observations to be made and the assay duration.[5,6] Experience has shown that insect cells and Baculovirus are not readily tested with these procedures, since the samples frequently contain chemicals that are toxic to both the cells and animals used in the experiments. Therefore, pre-treatment steps are usually required to reduce this toxicity.

Retrovirus

The final virus type specified in the guidelines for investigation is retrovirus. Retroviruses are given special consideration due to their many unique attributes. Retroviruses can exist as two types: endogenous and exogenous. Endogenous retroviruses may exist as a "provirus" in the chromosomes of many, if not all, living animal cells. The virus is passed in the germ line and is thereby present in all members of the same species. Exogenous retroviruses are acquired by infection, in a manner similar to most other viruses. Retroviruses have been identified in insect species, and the risks posed by the presence of these viruses should be investigated.

Traditionally, the electron microscope examination was the method of choice for detecting retroviruses in production cells and the virus seed stock. However, this method has been hampered by its lack of sensitivity. The polymerase based reverse transcriptase (PBRT) assay is now most frequently applied to detect retrovirus. The PBRT assay would normally be applied to all mammalian and avian cell banks, or virus seed materials, to test for retrovirus. The FDA, Division of Vaccines and Related Products, released a letter to advise viral vaccine manufacturers that vaccines produced with insect cells would not require the same tests as vaccines produced with mammalian or other cell types.[7] The justification was that insect cells "would not be expected to be contaminated by adventitious retroviruses."

When compared to the intensive work that has been done with mammalian retroviruses, the status of insect retrovirology is in its infancy. As more information becomes available, the opinions of the FDA may well change. Should endogenous or exogenous retrovirus be seen as a potential contaminant in insect cell production, further validation studies would be required to show that this risk could be contained. [For an overview of validation studies, see: Notes on virus validation studies: The design, contribution, and interpretation of studies validating the inactivation and removal of viruses, CPMP/BWP/268/95.]

A final consideration for the safety testing of insect cells is the possibility that these cells will be capable of inducing tumors. Novel cell lines may have to be investigated for their tumorigenic effects in animal studies.[8] This is also dependent on the nature of the downstream processing, and levels of residual cellular DNA. Discussions with the appropriate regulatory authority are advised before performing any of these studies.

After the safety of the master and working virus seeds and cell banks has been established, the testing requirements continue throughout the downstream purification process, and are completed only when the product is finally filled. The first sample to be examined is the crude bulk harvest from the production run. This sample will require a repeat of all the assays performed on the master banks (omitting the species-specific tests). After the downstream processing, the bulk clinical lot, and the final vialed product, require testing for sterility and mycoplasma, but not virus testing.

The final product does require some additional tests for residual DNA and the proteins generated by the production cells. Specific guidelines are in place for the amount of DNA that can be in a product (no more than 100pg DNA per dose - FDA guidelines).[9] The usefulness of this type of analysis is currently under active discussion with many regulators, and the question of insect cell protein contamination in the final product is one that will be raised. This type of analysis cannot be easily resolved, given the large number of proteins that may be present in the final product. Again, specific guidance should be sought from the regulatory authorities on the types of assays that should be used for each product.

On the clinical lot of product, two other tests are required for clinical release. These tests may vary, but normally an assessment of bacterial endotoxin is required. Usually, the rabbit pyrogen test, or the Limulus Amoebocyte Lysate (LAL) assay,[10] is also required. The last test to be performed is normally an assessment for general toxicity, which is required by both the European and US Pharmacopoeia.[11] It is conducted on "a representative sample, in the final container, from every final fill of each product lot." This is an animal based assay where a prescribed dose of test material is inoculated into mice and guinea pigs. Each animal is examined daily, and any signs of ill health are noted. The preparation passes the test if none of the animals show signs of ill health.

Conclusion

Utilizing the Baculovirus expression system is a relatively new method for the production of recombinant proteins. However, there are no fundamental components of this system that create a greater safety issue than those of any other similar production system. The primary disadvantage of the insect cell system appears to be the lack of products in trials or on the market, which would otherwise give manufacturers the confidence that this is a safe method to use. Therefore, insect cell-based products are in a difficult situation.

Until someone moves a product through preclinical and clinical trials, and has marketing authorization, a more general adoption of this technology will be delayed. Exactly when this will happen is unknown, but a number of groups are successfully moving through the process, and several products could emerge within the next few years.

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