VIROLOGICA SINICA, April. 2007, 22 (2):83-93
CLC number: Q2 Document code: A Article ID: 1003-5125(2007)02-0083-11

Insect Cell Culture and Biotechnology

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Abstract: The continued development of new cell culture technology is essential for the future growth and application of insect cell and baculovirus biotechnology. The use of cell lines for academic research and for commercial applications is currently dominated by two cell lines; the *Spodoptera frugiperda* line, SF21 (and its clonal isolate, SF9), and the *Trichoplusia ni* line, BTI 5B1-4, commercially known as High Five cells. The long perceived prediction that the immense potential application of the baculovirus-insect cell system, as a tool in cell and molecular biology, agriculture, and animal health, has been achieved. The versatility and recent applications of this popular expression system has been demonstrated by both academia and industry and it is clear that this cell-based system has been widely accepted for biotechnological applications. Numerous small to midsize startup biotechnology companies in North America and the Europe are currently using the baculovirus-insect cell technology to produce custom recombinant proteins for research and commercial applications. The recent breakthroughs using the baculovirus-insect cell-based system for the development of several commercial products that will impact animal and human health will further enhance interest in this technology by pharma. Clearly, future progress in novel cell and engineering advances will lead to fundamental scientific discoveries and serve to enhance the utility and applications of this baculovirus-insect cell system.

Key Words: Human vaccines; Immuno-therapeutics; Recombinant proteins

Following the first attempt to culture insect cells *in vitro* in 1915, almost 50 years passed before the methods for the establishment of continuous cell lines were successfully established. By 1980, the science of invertebrate cell culture had matured and many cell lines from different species were available for the study of insect and plant viruses. With the advent of the baculovirus-insect cell expression system a new exciting application for insect cells in biotechnology was realized. The need for new and superior insect cell lines for expression of recombinant proteins became important and research in insect cell culture gained renewed interest. In addition to the expression of recombinant proteins for basic research in cell and molecular biology, baculovirus biotechnology has been accepted by industry for the commercial production of reagents, therapeutics, and vaccines for use in agriculture and human health (32). This mini- review will examine; the early attempts to grow baculoviruses in cell culture, the development of widely used cell lines for protein expression, current directions for insect cell culture research, and commercial products

Received: 2006-12-18, Accepted:2007-02-12

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that are in development or in the marketplace.

INSECT CELL CULTURES AND BACULOVIRUS STUDIES

Early history

The first attempt to grow insect tissues *in vitro* was attributed to Goldschmidt who in 1915 attempted to culture follicle cells of male gonads from pupae of the Cercropia moth (*Samia cercropia*) in the hemolymph of this species (12). Cells remained alive for a few days but he observed no mitosis in the culture. From this early and seminal observation, the science of insect cell culture was born. The implications and value of insect tissue cultures in the study of insect viruses were realized very early. In 1917, just two years after Goldschmidt's pioneering attempts to culture insect cells, Glaser (11) attempted to grow a polyhedral virus in cultures of insect hemocyte cells maintained *in vitro* without success.

No further attempts were documented until the classical paper published in 1935 by William Trager (49) who reported the first successful cultivation of a baculovirus in primary ovarian insect cell cultures from silkworm, *Bombyx mori*, larvae. Two media compositions were used, A and B. The composition of each was surprisingly very simple and only contained a sugar, maltose, 5 inorganic salts, and distilled water. Medium B differed from A in that it contained an egg albumin digest. Both media were supplemented with 10% non-heat inactivated silkworm hemolymph. Medium B provided the best growth of cells. Late instar larvae were used as the source of ovarian tissue and collection of the silkworm hemolymph. Using a hanging drop culture technique he was able to achieve very good ovarian cell growth for up to 3 weeks. He found that the monolayer cells could not be kept growing indefinitely but he was able to subculture them at least once. This was a remarkable achievement considering the state of the art and the non-complex medium that he used. When he inoculated actively growing cell cultures with hemolymph taken from *B. mori* nucleopolyhedrovirus (BmNPV)-infected larvae, he was able to observe polyhedra formation within 24 hrs and in most of the cells within 48 hrs. Other interesting observations that Trager made included: 1) cell-free insect hemolymph was needed for optimum growth of the cells, 2) more and larger polyhedra are formed in active healthy cells than in less active, less healthy cells; 3) cell growth was inhibited by the virus, 4) the virus had multiplied (as measured by virus titration in larvae) through 8 passages of virus in cultured ovarian cells, and 5) the virus does not, with successive tissue culture passages, lose any of its characteristics properties. These remarkable early observations are similar to those recorded today by many researchers using optimum baculovirus and cell culture methods.

A landmark paper by Silver Wyatt in 1956 (54) was an important step in the evolution of improved methods for growing insect cells *in vitro*. Wyatt began her work in an attempt to corroborate Trager's work and to adapt her new techniques for routine use in the study of virus replication and titration. Instead, her study turned into an investigation of improved methods for the growth of insect tissue cultures. Wyatt attempted to culture *B. mori* ovarian cells in Trager's medium with 10% hemolymph and obtained poor cell growth. She also inoculated these cells with *B. mori* NPV and the infection was uneven with few infected cells. She attributed the poor infection levels to poor growth conditions for the cells and turned her attention to the growth of *B. mori* ovarian cells in a

new medium formulation based in part on an analysis of the amino acids and sugar composition of insect hemolymph (53). Unlike Trager's medium that contained only 6 ingredients, Wyatt's new growth medium was composed of 34 ingredients including 5 inorganic salts, 3 sugars, 4 organic acids, and 22 amino acids. Ten percent heat-treated silkworm hemolymph was added to the final complete medium. With this formulation, Wyatt was able to obtained cells in culture "superior to any previously obtained" and was able to maintain the cells alive for up to 3 weeks. No subculturing nor baculovirus infection of these cultures was attempted.

Following Wyatt's work, it was generally accepted by most researchers that new media containing all the factors necessary for growth still needed to be developed. However, before this was accomplished, a major breakthrough-the establishment of the first insect cell line-was reported by Shangyin Gaw and associates in 1959 (10). They reported the continuous culture of a *B. mori* cell line using the simple medium composition developed by Trager in 1935. They used Trager's solution B (see above) supplemented with 10% non-heat inactivated hemolymph and were able to grow ovarian cells and maintain them for at least 22 passages. They also reported that the cells were susceptible to BmNPV and polyhedra formation occurred after 48 postinfection. After submission of their paper for publication, they subcultured the cells through passage 24 before losing the cultures to a bacterial contamination (T.U. Zia, personal communication).

A major step in the evolution of an improved insect cell culture medium was reported by Grace (16). Grace composed a medium that was essentially the same as Wyatt's medium with the following changes: 1) ten members of the vitamin B complex were added; 2) The ionic ratios of the Na/ K and Ca/Mg were altered to values found in the blood of Saturnid moths; 3) The osmotic pressure was changed from an equivalent of 0.72%NaCl to an equivalent of 0.99% NaCl; and the pH was changed from 6.35 to 6.5. Five percent of heat-treated hemolymph was added to the medium. With these changes, Grace was able to establish lines from ovarian tissue of *Antheraea eucalypti* (16), larval tissues of the mosquito *Aedes aegypti* (15) and ovarian tissue of *B. mori* (14). In subsequent studies, Grace also reported that BmNPV was able to infect *A. eucalypti* cell clones (17) to varying degrees.

The research by Grace which resulted in numerous publications reporting new established cell lines from moths and mosquitoes coupled with his description of Graces insect cell culture growth medium, a medium that is still widely used today, opened the floodgates for the development of many other insect cell lines in laboratories around the world. For his many early contributions Tom Grace is considered by many as the pioneer who was most responsible for providing the leadership and scientific breakthroughs that led to a new era in insect cell culture. It is estimated that 350 to 400 cell lines were established between 1960 and 1990 and for years many of these established lines were important research tools to study the pathology of insect viruses and insect transmitted viruses of animals and man.

Another significant scientific contribution that greatly synergized the interest in insect cell culture research was the development of the baculovirus-insect cell expression system (43, 47). The baculovirus-insect cell expression system is now considered a highly effective, proven, and robust technology for the expression of thousands of recombinant proteins for use in cell and molecular biology research with

both insect and mammalian cells (32). Commercial production of recombinant proteins is a growing industry and several companies are using this expression system for the commercial development of immuno-therapeutics, animal vaccines and human vaccines.

Insect cell lines used with the baculovirus-insect cell expression system

The *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is the most widely used virus vector for protein expression (32). T86 virus AcMNPV has a broad *in vitro* host range and numerous established cell lines have been evaluated for their growth characteristics, baculovirus susceptibility, and ability to express high levels of recombinant proteins (4, 24, 46). Although the versatile cell line Tn368, developed by Fred Hink in 1970 (24), was initially widely used for basic studies with AcMNPV, three other cell lines became the dominant lines that are currently used for protein expression around the world by academia and industry. These are the *Spodoptera frugiperda* line, SF21 (and its clonal isolate, SF9), and the *Trichoplusia ni line*, BTI TN5B1-4, commercially known as the High Five cells.

The SF21 cell line was established by James E. Vaughn at the USDA Insect Pathology Laboratory in Beltsville, Maryland, in 1970. SF21 cells were developed from ovarian tissue of *S. frugiperda* pupae (13) and at passage 22 it was reported to be susceptible to *S. frugiperda* NPV. The cell line designated IPLB-SF-21 was established in hemolymph-supplemented medium (13) and was shown to be highly susceptible to AcMNPV. A detailed description of the establishment of this line was published in 1977 (51) and a strain of the IPLB-SF-21 line was adapted to a hemolymph-free medium by Gardiner and Stockdale in England (9). This strain which was designated IPLB-SF-21AE was later used by the Vaughn lab for virus studies and is the origin of the highly popular SF21 cells that are currently used today (J. L. Vaughn, personal communication). In 1983 the SF9 cells were cloned from the parent line IPLB-SF21AE (ATCC No. CRL-1711). The SF9 clone and SF21 cells are similar in their characteristics with the following properties: They are 1) robust cells and easy to culture in monolayer or suspension, 2) highly susceptible to AcMNPV, 3) able to grow rapidly to high cell densities and produce high budded virus (BV) titers, and 4) readily adaptable to serum-free medium and scaleup culture. Recently, a new cell line was established from the SF9 cells by selection in serum-free medium supplemented with human insulin. This new line, which is reported to be genetically and morphologically distinct from the parent SF9 cells, is known commercially as expressSF+ (Protein Sciences Corp., Meriden, Ct).

A significant contribution in the development of new cell lines for the production of high levels of recombinant proteins, was the establishment of the *T. ni* cell line, BTI-Tn5B1-4 (19). The Tn5B1-4 cells are a clonal isolate of a parental line Tn5B1 which was established from *T. ni* eggs in 1986 for studies of the replication of a *T. ni* granulovirus and NPV (18). The clonal isolate, Tn5B1-4 normally grows in monolayer culture and has the following characteristics. They are: 1) highly susceptible to AcMNPV, 2) adaptable to grow in suspension, 3) able to grow to moderate cell densities and produce moderate BV titers, 4) readily adaptable to serum-free media and scaleup procedures, and 5) able to produce very high levels of many recombinant proteins. The ability of these cells to outperform SF21 and SF9 cells in their expression of most but not all recombinant proteins (4) has led to

the acceptance of Tn5B1-4 cells as a widely used line for gene expression. This cell line is available for research purposes under the trade name “High Five” cells (Invitrogen Inc, Carlsbad, Ca). Recently, two novel, clonal cell lines (Tn-H5CL-B and Tn-H5CL-F) were isolated from the High Five cells at passage 90 (34). Both H5CL-B and H5CL-F cells showed higher levels of B-galactosidase production and expressed two-fold more secretory alkaline phosphatase when compared to High Five cells. The clonal lines also exhibited higher resistance to nutrient stress. Both the parental cells and the novel clones produced 2.5-fold more wild type AcMNPV occlusion bodies than SF9 cells. The clonal lines have a population doubling time similar to the High Five cells, grow as attached mono-layers and can be adapted to serum-free medium.

Commercial development of products produced in insect cells

Numerous small to midsize startup biotechnology-companies in North America and the Europe are currently using the baculovirus-insect cell technology to produce custom recombinant proteins for research and commercial applications. Furthermore, since this system is an accepted technology for the production of viral antigens with vaccine potential, several compa-nies are now in the developmental testing stage with therapeutic or vaccine products for animal or human purposes. An excellent review by van Oers (50) summarizes the potential use of the baculovirus-cell system for animal and human vaccines. Currently, there are two animal vaccines that are on the market and several immuno-therapeutic and human vaccines are being developed and produced in SF21, SF9, expressSF+, or High Five cells (Table 1 and 2). In Europe, two commercial subunit vaccines for classical swine fever are produced in *S. frugiperda* cells by Intervet, Leiden, The Nether lands (50). Several human vaccines that are being produced in *S. frugiperda* cells and are in mid to late phase clinical trials are:1) Provenge, an immuno-therapeutic vaccine for prostate cancer de- veloped by Dendreon Inc, Seattle, WA., 2) Flublok, a vaccine for human influenza virus developed by Protein Sciences Inc., Meriden, CT., and 3) Chimigen vaccines for chronic hepatitis B and C developed by Virex Medical Corp., Calgary, Canada.

Commercial products in development using *T. ni* High Five cells include: 1) FavId (idiotype/KLH) an immuno-therapeutic vaccine for B-cell non-Hodg kin’s lymphoma developed by Favrille Inc, San Diego, CA. and 2) Cervarix, a vaccine for cervical cancer developed jointly by Medimmune,Gaithersburg, Md and GlaxoSmithKline, Rixensart, Belgium. Cervarix may be the first insect cell produced vaccine to be commercialized for human use. GlaxoSmithKline filed for regulatory approval in Europe in March 2006 and expect approval by early 2007. They intend to filefor regulatory approval in the United States by April, 2007. A new vaccine produced in insect cells would be a major advancement for women’s health since it is estimated that approximately 250 000 women world- wide die each year of cervical cancer. The commercial availability and probable success of vaccines for animal or human use in the near future will provide even greater impetus for the application of the baculovirus-insect cell technology in research and medicine.

CELL CULTURE TECHNOLOGY

Future directions

While existing insect cell lines provide excellent results in baculovirus expression vector systems, there

Table 1. Commercial products in development using *Spodoptera frugiperda* cells

Product	Company	Status
Provenge Vaccine • Prostate Cancer	Dendreon	Phase 3 Clinical
Flublok Vaccine • Human Influenza	Protein Sciences	Phase 3 Clinical
Chimigen Vaccines • Chronic Hepatitis B • Chronic Hepatitis C	Virexx Medical Corp.	Phase 1&2 Clinical
Swine Fever Vaccine • Classical Swine Fever	Intervet	Commercial

Table 2. Commercial products in development using *Trichoplusia ni* "High five cells"

Product	Company	Status
Cervarix HPV Vaccine • Cervical Cancer	Medimmune / GlaxoSmithKline	Market 2007
Idiotype/KLH Vaccine • B-Cell Non-Hodgkin's • Lymphoma	Favrille	Phase 3 Clinical

is enormous potential for future development and improvement of insect cell lines. One promising direction is the engineering of cell lines for improved growth, or for high quantity or quality protein expression. Limitations of current insect cell lines maybe overcome by constitutively expressing either single proteins or entire metabolic pathways in a cell line which can then be used for infection by a baculovirus expression vector. In addition, insect cell lines can be engineered and used directly for protein production. Indeed, the expression of proteins from an uninfected engineered cell line can provide certain advantages in protein quality, when compared with protein expression from a baculovirus expression vector.

Perhaps the first demonstration of a stably transformed lepidopteran cell line was the engineering of Sf9 cells by the laboratories of Donald Jarvis, Linda Guarino, and Max Summers (27). Beta galactosidase (β -gal) and human tissue plasminogen activator (t-PA) were constitutively expressed in Sf9 cells, by placing each under the control of a "promiscuous" baculovirus early promoter, the *ie-1* promoter. Other promoters that have been used to generate stably transformed lepidopteran cell lines include the *ie-2* and *gp64* promoters from OpMNPV, and several forms of the *Bombyx mori A3 cytoplasmic actin* gene promoter (6, 23, 39). In contrast to the very high levels of protein production typically generated from the lytic infection by a baculovirus expression vector, the constitutive protein expression from stably transformed cells may be at considerably lower levels. However, this may be advantageous for proteins requiring extensive or complex post-translational processing such as the human t-PA protein. For example, in comparison with t-PA produced from a baculovirus expression vector, t-PA produced in stably transformed Sf9 cells is more efficiently processed (27). The use of certain parental cell lines, promoter constructs, and examples of results from protein expression in stable lepidopteran cell lines is detailed in a recent review (5).

Glycosylation

The baculovirus expression vector system and insect cells have been used extensively for the production of secreted glycoproteins, and this represents one of their most important biotechnological applications. Many or most secreted animal proteins that have been examined are expressed and secreted in abundance in the baculovirus expression system. An important difference between mammalian glycoproteins that are expressed in insect vs. mammalian cells, is the nature of carbohydrates that are posttranslationally added and modified. The addition and processing of carbohydrates during transit through the human secretory pathway typically results in glycoproteins that contain terminal sialic acid. However, N-glycosylation observed in Sf9 or High Five cells is typically of the

high mannose or paucimannose type and lacks terminal sialic acid. Several reviews (26, 32) describe concerted efforts aimed at engineering insect cell lines for production of terminally sialylated or "humanized" glycoproteins. An engineered cell line derived from the Sf9 line and engineered to express glycosyltransferases (galactosyltransferase and sialyltransferase) is commercially available from Invitrogen under the trade name "Mimic™ Sf9 Cells". A detailed review describing protein N-glycosylation in insect cells infected with recombinant baculoviruses or stably transformed with glycosyltransferases was recently published (22).

Improving cell lines through expression of other proteins

Cell growth and resistance to stress from culture conditions can also be improved by engineering lepidopteran cell lines. The best example to date is the constitutive expression of baculovirus P35, an inhibitor of apoptosis, in lepidopteran Sf9 cells. The P35 protein from AcMNPV is perhaps the most broadly active of all caspase inhibitors identified to date, and P35 has been studied extensively (2, 3, 8). As an inhibitor of downstream caspases, P35 inhibits the induction of apoptosis by a large variety of stimuli, and in a broad range of cell types. By engineering P35 expression in lepidopteran Sf9 cells, and selecting for cells that were resistant to actinomycin-D, Lin and colleagues (36) identified several engineered cell lines with remarkable resistance to nutrient stress. Using P35, IAP proteins, and/or combinations of apoptosis inhibitors, it may be possible to engineer cells for growth and protein expression at extremely high densities, and in less costly media formulations.

Use of stable cell lines for experimental approaches

In addition to the biotechnological applications described above, engineered lepidopteran cell lines have also been used for studies of the *in vivo* roles or functions of important or essential viral proteins. Examples include studies of knockouts or substitutions of the AcMNPV P35 gene (1) and the AcMNPV GP64 gene (39). In the case of proteins like P35 which are very important but not essential for virus replication, cell lines expressing P35 were used as important tools for examining the function of P35 in the context of a viral infection. In the case of AcMNPV GP64, which is essential for productive viral replication in cell culture, cell lines expressing GP64 proteins have been used both for complementing knockouts and for substitutions of modified forms of GP64 in the virus genome (25, 37, 39, 41, 55) and directly for studies of GP64 function (45). The same cell transformation techniques developed for studies of baculovirus biology can also be used for studies of other viruses infecting lepidopteran cells, or for functional studies of insect genes.

Expression of proteins in Sf9 cells as a non-lytic heterologous system

A variety of studies have used engineered lepidopteran insect cell lines as a non-lytic heterologous system for expression and for studies of protein function. Examples include studies using stably transformed lepidopteran cell lines to express proteins such as the human mu opioid receptor (hMOR) (29) and other G-protein-coupled receptors (30, 31), factor X (44), and invertebrate GABBA receptors (48). Additionally, several indirect approaches for using stably transformed lepidopteran cell lines provide exciting new avenues for future research and applications. Stable cell lines expressing proteins that enhance the efficiency of protein transport or processing may be valuable tools. One example is enhanced protein

folding (33). Another potentially powerful approach is the use of double stranded RNA-mediated interference (RNAi) in stably transformed lepidopteran cells (35) to reduce or knockdown the expression of cellular genes. Genes that may limit, interfere with, or modulate protein expression in stably transformed cells could be down regulated by this method. In addition, viral or cellular genes that may interfere with or compete for resources in cells infected with baculovirus expression vectors could be similarly manipulated by RNAi approaches.

Engineered insect cell lines have proved to be extremely useful although these examples represent a very small portion of the possible uses of this exciting technology. The development of new cell lines and new tools for rapidly engineering and manipulating lepidopteran insect cells will be important for increasing the utility and applications of these cells.

Novel insect cell lines

In the past 4 decades, cell lines have been established from species represented in 8 orders of insects including Lepidoptera. The Order Lepidoptera (moths and butterflies) is the second largest order of insects and has more than 200,000 species in 130 families. Cell lines from Lepidoptera are used primarily for the study of insect pathogenic viruses including baculoviruses and consequently most lines have come from insect species that are serious agricultural pests. Of the 29 baculovirus genomes that have been sequenced, 19 are NPVs (including AcMNPV) which are lepidopteran-specific viruses (28). Of these 19 sequenced genomes, all have been isolated from insects representing only 4 families (3% of total). All 4 families are comprised of moth species, many of which are serious pests of agricultural crops and forests. Furthermore, the vast majority of the cell lines used for the study of these 19 sequenced NPVs also came from the same 4 lepidopteran families and most were from the family Noctuidae. In fact, noctuid species are the origins of the three most popular cell lines used in baculovirus biotechnology; the fall armyworm *S. frugiperda* (SF21 and SF9 cells) and the cabbage looper *T. ni* (High Five cells).

There is a need to develop established cell lines from insect species representing other lepidopteran families. A small number of such cell lines have been established (38) and the few that have been tested suggest that some non-pest species may have useful applications for gene expression. For example, the saltmarsh caterpillar (*Estigmene acrea*, family Arctiidae) is an occasional pest in agriculture and an *E. acrea* hemocyte cell line (BTI EAA) was established for the study of insect viruses (20, 21). The cell line was cloned and the clonal line, Ea4 was found to produce greater amounts of complex N-linked glycolsylation on recombinant proteins that the SF9 cells (40, 52). Similarly, the Monarch butterfly (*Danaus plexippus*, family Danaidae) is not a plant pest and an established cell line (DpN1) was reported to be capable of complex N-glycosylation and sialylation of recombinant proteins (42). These two studies provided further evidence that it was possible for non-engineered insect cells to produce recombinant proteins that underwent complex-type glycosylation and suggests that cells from unusual, non-pest species may be an important source of new lines for use in basic research and biotechnology.

Growth factors and *in vitro* culture

The importance of growth factors in controlling the proliferation of insect cells has been known for many years (7). Many researchers including Wyatt (53, 54) and Grace (14, 17) noted that unidentified growth

factors in insect hemolymph may be needed by cells in culture. Currently, there are many commercial insect cell culture media available and these media may have as many as 30 components in their formulation. It is not clear if all the ingredients are essential for cell growth. Gaw *et al*. (10) succeeded (see above) in establishing an insect cell line in a simple medium containing only 6 ingredients supplemented with non-heat inactivated hemolymph. It is possible that some unknown growth factor(s) in the hemolymph may have been important for the growth and establishment of the cultured cells. Only a few insect growth-promoting substances in insect hemolymph have been identified and tested in cultured insect cells (7). Research is needed in this area to assist in the development of defined insect culture media that can be used to help us better understand cell growth, insect development and gene expression.

CONCLUSION

The continued development of new cell culture technology is essential for the future growth of insect cell and baculovirus biotechnology. The long perceived prediction that the potential application of the baculovirus-insect cell system for protein expression was immense has been achieved. The versatility and recent applications of this popular expression system have been reviewed by Kost *et al*. (32) and it is clear that this system has been widely accepted for biotechnological applications. The use of the baculovirus-insect cell-based system for the development of several commercial products that will impact animal and human health (Tables 1 and 2) will further enhance interest in this technology by pharma. Clearly, future progress in novel cell line production and in cell engineering will serve to enhance the utility and applications of the baculovirus-insect cell system.

Acknowledgements

The authors gratefully acknowledge support from Boyce Thompson Institute project B00103-R06-1252, USDA project 2002-35302-12342, and Chinese Science Foundation project 30370963.

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