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Patents and Literature

Use of Viral Insecticides for Pest Control and Production in Cell Culture

DAVID W. MURHAMMER

Department of Chemical and Biochemical Engineering, The University of Iowa, Iowa City, IA 52242

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ABSTRACT

This review discusses a number of issues relevant to the production and use of baculoviruses for pest control. Following this review, patents pertaining to baculovirus use in pest control are summarized.

Index Entries: Viral insecticides; insect cell culture.

INTRODUCTION

Although a significant reduction in agricultural losses has resulted from the use of chemical pesticides, their future effectiveness is threatened by increasing insect resistance (*1*). In addition, it is commonly believed that chemical pesticides pose real or at least potential health and environmental hazards (*2*). Integrated pest management (IPM), which utilizes biological control agents and other control methods to minimize chemical insecticide use, is an important tactic in addressing the problems associated with chemical pesticide use (*1*). The use of biological control agents, e.g., toxins produced by *Bacillus thuringiensis* (Bt toxins), insect predators, and insect viruses, is an important component of IPM. Insect viruses, specifically baculoviruses, are ideally suited for use in IPM since they are compatible and often synergistic with other control agents. It has been estimated that baculoviruses are capable of replacing 80% of the chemical insecticides currently used in agricultural applications (*3*). For example, baculoviruses have been demonstrated to be synergistic with chemical insecticides in both forest (*4*) and agricultural (*5*) applications, thereby reducing the concentration of chemical insecticides required for pest control.

This review begins with a brief overview of IPM and then discusses the potential for use of baculoviruses in insect pest control, and methods that are being developed to improve the pest control effectiveness of baculoviruses. Issues relevant to the large-scale production of viral insecticides, particularly in insect cell culture, are then discussed. Finally, representative patents related to the use of baculoviruses for pest control are summarized.

INTEGRATED PEST MANAGEMENT (IPM)

IPM was conceptually introduced by Smith and Allen in 1954 (*6*). Most of the issues addressed are still applicable today. For example, they noted that resistance to chemical insecticides indicated that insect pest control cannot be assured simply by inventing new and more potent chemical insecticides. In addition, they noted that chemical insecticides kill nontarget pests, including those that are natural predators of the target pest. To combat this problem, they recommended that chemical control be used in conjunction with the natural factors influencing insect populations, i.e., an integrated control program should be used in which all the resources of ecology are utilized to give the most permanent and economical insect control. As a specific example, they recommended that an insect virus and *Bacillus thuringiensis* (Bt) toxin be applied to alfalfa to control the alfalfa caterpillar population.

The key components of IPM are prevention, monitoring, and control (*7*). The goal of such an approach is to maintain crop damage within economically acceptable levels, i.e., killing the insects *per se* is not the primary goal. The preventive aspect of IPM includes rotating crops, destroying plant residues that can harbor pests, conserving natural enemies of pests by minimizing chemical insecticide use, and planting resistant crop varieties. Monitoring involves periodically checking plants, animals, and soil for pests. This practice can be supplemented by improved pest forecasting with knowledge of weather, crop history and growth, and pest behavior and development. Control involves the use of chemical treatment only when necessary to prevent economically important damage to crops. In addition, methods should be used that reduce pest populations later in the year, on the next year's crop, and in adjoining fields.

The major principles involved in IPM (*7*) include:

1. Potentially harmful species will continue to exist at finite, but tolerable levels (this may be advantageous as these pests will provide food for natural predators);
2. Manipulating ecosystems to hold pests at tolerable levels while avoiding disruptions of the systems;
3. The use of natural control agents is maximized by emphasizing factors in the ecosystem that limit insect pest growth, e.g., the use of fungal, bacterial, and viral pathogens;

4. Any control procedure may produce unexpected and undesirable results; and
5. An interdisciplinary approach is essential, including expertise in agronomy, economics, meteorology, engineering, sociology, mathematics, and various pest control sciences.

Baculoviruses are well suited for use in IPM as they:

1. Are compatible with other control agents;
2. Have minimal effect on nontarget insects, e.g., honey bees or predators of the target pest;
3. Do not present the safety and health problems associated with chemical insecticides; and
4. Have continuous effectiveness, i.e., insects do not develop resistance to them (*8*).

The benefits of using baculoviruses in IPM to control pests in both forest (*4,7*) and agricultural (*5,9*) applications have been demonstrated.

THE USE OF BACULOVIRUSES TO CONTROL INSECT PEST POPULATIONS

Baculoviruses are a family of invertebrate viruses with a double-stranded DNA genome of 88 to 153 kilobase pairs (*10*) that are divided into two subgroups: nuclear polyhedrosis viruses (NPVs) and granulosis viruses (GVs) (*11*). NPVs are present in two morphologically distinct forms, specifically the budded virus (BV) (extracellular form) and the occluded virus (OV) (intracellular form). The BV acquires its envelope (one virus encased within a membrane) by budding through the cell's plasma membrane. It is the form that is infective in cell culture and is responsible for the spread of the viral infection within an individual infected insect. The OV is enveloped within the cell's nucleus via an unknown mechanism. Depending on the virus, there are either multiple nucleocapsids (MNPV) or a single nucleocapsid (SNPV) within an envelope. Polyhedra result when the enveloped OVs are encased within a polyhedrin protein matrix in the cell nucleus. The polyhedra, which are deposited on foliage upon insect death, are responsible for the spread of the viral infection between insect pests. The polyhedrin protein matrix is dissolved in the alkaline conditions of the insect gut following ingestion and leads to a repeat of the viral infection process. GVs also exist in extracellular and intracellular forms. The intracellular (i.e., occluded) form of the GV is singly enveloped and these enveloped viruses are individually encapsulated in a protein matrix, i.e., granulin protein. Most of the baculoviruses with potential for use as viral insecticides are NPVs, although a few GVs have also shown potential.

Baculoviruses currently registered for use as pest control agents in the United states (*12*) include:

1. *Heliothis zea* NPV (1975) for cotton bollworm and budworm control;
2. *Orgyia pseudotsugata* NPV (1976) for Douglas fir tussock moth control;
3. *Lymantria dispar* NPV (1978) for gypsy moth control;
4. *Spodoptera exigua* NPV (1993) for beet armyworm control;
5. *Autographa californica* NPV (1994) for alfalfa looper control;
6. *Anagrapha falcifera* NPV (1995) for control of lepidopteran species; and
7. *Cydia pomonella* GV (1995) for codling moth control.

No baculovirus for use as insecticides are presently sold in the United States; Sandoz, Inc. discontinued selling *H. zea* NPV (Elcar) in the early 1980s owing to low sales. *L. dispar* NPV (Gypchek) has been utilized by the USDA in control programs that proved to be effective under a wide variety of conditions (*13,14*). The USDA Forest Service currently produces 10,000 to 25,000 hectare equivalents of Gypchek in insect larvae annually for use in experimental studies (*15*). Some baculoviruses are currently used elsewhere in the world for agricultural crop protection, including Multigen (*Anticarsia gemmatalis* NPV) to control velvetbean catepillar populations, a major soybean pest, in Brazil; *Oryctes rhinoceros* NPV to control the coconut rhinoceros beetle, a pest of palm and coconut trees; in the South Pacific (*8,16,17*); and *Cydia pomenella* GV to control the codling moth, a pest of apples, pears, and walnuts, in Europe (*18*).

In addition to these baculoviruses, many others have demonstrated the potential to control pests in agricultural applications (*19*). A partial list includes *Trichoplusia ni* NPV (cabbage looper) for use on cole crops, and *Mamestra brassicae* NPV (cabbage moth) for use on cabbage. Whereas most baculoviruses have a relatively narrow range of host species, there are two baculoviruses—*Autographa californica* NPV (AcNPV, alfalfa looper) and *Anagrapha falcifera* NPV (AfNPV, celery looper)—that have significantly broader host ranges (*20*). AcNPV and AfNPV are applicable to the control of a wide range of insect pests in agricultural applications as they are orally infective to the most economically important groups of lepidopteran pests, including genera such as *Heliothis, Trichoplusia,* and *Spodoptera.*

Although there are many advantages of using baculoviruses in insest pest control programs, cost and field performance has hindered their use (*8,21*). Specifically, problems include the following:

1. Baculovirus are slow at killing their host, and so, the insect pest continues to feed for many days following initial exposure to the baculovirus;
2. A given baculovirus has a restricted host range—insecticidal agents that control all potential insect pests are generally preferred (this characteristic of baculoviruses is also commonly cited as being an advantage since beneficial insects are not adversely affected);

3. Baculoviruses are sensitive to environmental factors, e.g., UV light; and
4. Baculoviruses are significantly more expensive than chemical insecticides, e.g., treatment of forests for the control of gypsy moth populations with Dimilin 4L (the chemical pesticide of choice) costs \$2.25–4.50/acre (*22*) compared to a total cost of \$20–30/acre for Gypchek (two applications at 2×10^{11} polyhedra/acre each) (*23*).

This cost includes approx \$10 for producing the virus, \$10–20 for formulating and applying the virus, and overhead. This, however, is probably not representative of the current cost of producing baculoviruses in insect larvae. For example, Bell and Hardee (*24*) utilized mechanized rearing procedures to produce *Heliothis zea* NPV at a cost of \$2.16/acre (a single application to cotton at 2.4×10^{11} polyhedra/acre). This cost included both production and application costs, but did not include overhead costs. Regarding the control of gypsy moth, an alternative biocontrol agent (Bt toxin) costs approx \$8–15/acre (including overhead) (*25*). A factor that may reduce the effective cost of using baculoviruses in pest control is that application in 1 yr can control insect populations in succeeding years, which is in contrast with chemical insecticides and Bt toxins. A specific example is that the application of *Orgyia pseudotsugata* NPV to Douglas fir trees in British Columbia, Canada in yr 1 resulted in the absence of insect larvae for the following 2 yr of the study, which was in stark contrast to the untreated plants (*26*). The cost estimates given here also ignore the socio-economic and environmental benefits of using baculoviruses in pest control (which are not easily quantifiable) (*10*).

APPROACHES TO REDUCING THE KILL TIME OF BACULOVIRUSES

Genetic engineering of baculoviruses has been investigated as a method of reducing the time to kill insect pests. The methodology involves adding genes to the baculovirus genome that code for hormones, toxins, or other compounds that inhibit insect feeding and/or decrease the time needed to kill insect pests. Any gene can be considered effective for pest insect control if its expression can safely block feeding, disrupt metamorphosis, or otherwise change normal behavior such that there is a reduction in crop damage (*16*). The effectiveness of these genetically engineered viruses are generally determined by two types of bioassays: (1) the time to kill 50% of the larvae at a given dose (LT_{50}) (alternatively, survival time, ST_{50}, is sometimes used) and (2) the dose of virus to kill 50% of the larvae (LD_{50}). Time and dose are both important variables as it is desired to stop insect feeding as rapidly as possible in order to minimize crop damage and reduce the required dosage in order to minimize costs. Another use-

ful basis on which the effectiveness of pest control agents can be compared is larval weight gain, which is related to the level of crop damage (e.g., foliage damage).

The specific foreign genes that have been expressed in baculoviruses to enhance their ability to kill insect pests include those coding for diuretic hormone, juvenile hormone esterase, Bt toxins, scorpion toxins, and mite toxins (*16*). Use of hormones and enzymes, particularly when taken from the target pest species, offers the advantage that the virus will be producing products, at an increased level, that are normally synthesized by larvae (*17*). Chances of an insect becoming resistant to its own product are remote. The expression of foreign toxins is also a useful alternative, to which the risk of the host developing resistance is also considered minimal as the insect would also have to become resistant to the viral infection. The timing and strength of the viral promoter, in addition to the potency of the foreign gene product, are important considerations for selecting the optimal promoter for foreign gene expression. For example, Tomalski and Miller (*27,28*) have investigated the effect of using different promoters on the ability of a genetically modified AcNPV expressing a mite toxin to minimize the weight gain and the time required for paralysis of *T. ni* larvae. They found that a novel promoter containing both late and very late promoter elements performed best.

Overexpression of insect hormones by baculoviruses aims to disrupt metabolic processes within the insect that are normally carefully regulated, e.g., water balance and molting (*29*). Maeda (*30*) found that *Bombyx mori* larvae fed a recombinant BmNPV expressing the diuretic hormone of the tobacco hornworm (*Manduca sexta*), which plays an important role in the regulation of water balance, died 20% faster than when fed wild-type BmNPV. Bonning et al. (*31*) inserted the juvenile hormone esterase (JHE) gene of *Heliothis virescens* into the viral genome of AcNPV behind a duplicated p10 promoter. JHE breaks down the juvenile hormone that would otherwise extend the life of virally infected insects. Therefore, infection of an insect pest with a recombinant AcNPV expressing JHE should result in more rapid death than infection with the wild-type virus. However, a genetically modified AcNPV propagated in *Spodoptera frugiperda* Sf-21 cells had a minimal effect on larval feeding. Similar results were obtained by Eldridge et al. (*32*). It was hypothesized that the JHE was unstable owing to the presence of amino acid sequences to which ubiquitin attached and degraded the JHE (*33*). Consistent with this hypothesis, removal of ubiquitin binding sites stabilized the JHE and thereby reduced the kill time of larvae infected with the recombinant AcNPV. Another potential hormone for enhancing the insecticidal activity of baculoviruses is the eclosion hormone (EH) from *Manduca sexta*. EH triggers the shedding of old cuticle at the end of each molt by regulating many events associated with ecdysis. Eldridge et al. (*32*) have demonstrated that a recombinant AcNPV can efficiently express biologically active EH in infected Sf-21 cells. Injection of recombinant EH into *S. frugiperda* or *M. sexta* larvae resulted in the init-

iation of eclosion behavior. When the recombinant AcNPV was used directly as an insecticide, however, no enhancement in terms of larval death or reduced feeding were observed. This lack of enhanced insecticidal activity is believed to be related to the timing and complexities associated with the action of EH (*8*).

Recombinant baculoviruses expressing toxin genes isolated from *Bacillus thuringiensis* (Bt toxins) is another approach that has been investigated to improve the effectiveness of the wild-type baculoviruses. Bt toxins are a variety of crystalline proteins dissolved in the insect gut to release one or more biologically active protein(s) of 27–140 kDa (*34*). Each Bt toxin is toxic to a relatively narrow range of insect pests. Bt toxins have been used commercially in various forms for many years and are by themselves an important component of IPM. The use of Bt toxins as pesticides, however, requires periodic application to crops (more than once per growing season in some cases). Therefore, a potential advantage of using recombinant baculoviruses expressing Bt toxins over using Bt toxins alone is that the baculovirus pesticide will be replicated and persist longer to control insect pest populations. Merryweather et al. (*35*) found that *T. ni* larvae treated with extracts of insect cells infected with a modified AcNPV expressing a Bt toxin gene were killed more rapidly than larvae infected by extracts of cells infected with wild-type virus. It has also been demonstrated that active Bt toxin is produced within insect larvae infected with a recombinant AcNPV (*35,36*).

Insect-specific toxin genes isolated from the scorpions *Androctonus australis* and *Buthus eupeus*, expressing AaIT and BeIT, respectively, have been investigated for their ability to improve the insecticidal activity of AcNPV. AaIT causes a modification of the Na^+ conductance of neurons, producing a presynaptic excitatory effect leading to paralysis and death of insects. BeIT is also a paralytic neurotoxin. Stewart et al. (*37*) found that *T. ni* larvae injected with a recombinant AcNPV in which the AaIT gene was inserted behind the p10 promoter resulted in more rapid death than when infected with wild-type virus. Specifically, they found that the LD_{50} and ST_{50} were reduced from 44 to 31 polyhedra/larvae and 113.1 to 85.8 h (17 polyhedra/larva), respectively. Similarly, McCutchen et al. (*38*) found that this recombinant AcNPV decreased the LD_{50} and LT_{50} in *Heliothis virescens* larvae from 21.9 to 13.3 polyhedra/larvae and 125 to 88.0 h (250 polyhedra/plug of diet), respectively. Maeda et al. (*39*) found that feeding of *Bombyx mori* larvae infected with recombinant BmNPV expressing AaIT stopped at 40 h postinfection followed by paralysis and death by 60 h postinfection. The wild-type virus took 96 h to kill the larvae. The effectiveness of a recombinant AcNPV expressing AaIT has also been demonstrated in field trials (*40*). Carbonell et al. (*41*) did not detect paralytic activity when recombinant AcNPV containing the BeIT gene was used to infect *T. ni* and other larval species.

A mite neurotoxin gene expressing an insect-specific product has also been inserted into the baculovirus genome to increase the effectiveness of

a viral insecticide. Specifically, Tomalski and Miller (*27,28*) demonstrated that a recombinant AcNPV expressing TxP-I paralyzed 50% of the infected *T. ni* larvae within 24 h and that all of the larvae were paralyzed within 48 h, thereby terminating pest feeding.

Another approach that has been taken to enhance the ability of baculoviruses to kill insect larvae involves the deletion of the viral gene that codes for the enzyme ecdysteriod UDP-glucosyltransferase (*egt*). *Egt* has been shown to be capable of catalyzing the conjugation of either glucose or galactose to ecdysteroids (*42*). Evidence indicates that ecdysteroids in vivo are actually conjugated with galactose. Egt expression interferes with normal insect development by blocking molting in infected larvae, thereby extending the larval feeding time and the time during which viral synthesis can occur (*43*). O'Reilly and Miller (*44*) found that deletion of the *egt* gene significantly improved the insecticidal properties of AcNPV by reducing ST_{50} and LC_{50} from 127.2 to 99.7 h (1.25×10^7 polyhedra/cm^3) and 2.39 to 1.42×10^6 polyhedra/cm^3 of infecting diet fed to *S. frugiperda* larvae.

A major concern with using genetically modified baculoviruses for pest control is the potential persistence of the virus in the environment that could lead to its uncontrolled spread (*45*). An approach that addresses this issue involves the use of a mixed formulation consisting of wild-type virus and recombinant virus lacking the polyhedrin gene, i.e., the so-called co-occlusion strategy (*45,46*). Field studies were conducted by Wood et. al. (*45*) in which an AcNPV formulation consisting of 48% genetically altered virus (i.e., deficient in the polyhedrin gene) and 52% wild-type virus was applied to cabbage plants infested with *T. ni* larvae. Consistent with the goal of containment, the percent of polyhedrin deficient AcNPV declined over the course of their studies.

APPROACHES TO EXPANDING THE HOST RANGE OF BACULOVIRUSES

Expanding the host range of baculoviruses to additional insect pests in an environmentally sound manner would be commercially attractive. Many approaches have been developed that offer hope that this goal can be achieved. First, Carbonell and Miller (*41*) demonstrated that the host range of AcNPV can be extended by utilizing the Rous sarcoma virus long terminal repeat (RSV-LTR) promoter to express toxic proteins. It was demonstrated that the virus was taken up and the toxic protein synthesized by nonpermissive dipteran cells. Second, replication of *B. mori* NPV in the usually nonpermissive *S. frugiperda* Sf-21 cell line was induced with coinfection with AcNPV. Progeny virus resulting from this coinfection were plaque purified in *B. mori* BmN cells. These viruses were then capable of replicating in cell lines nonpermissive to BmNPV, thereby indicating the appearance of new viruses with expanded host ranges (*47*). These

viruses were demonstrated to be recombinants consisting of portions of the genomes of each of the parent viruses. Third, a cocktail of engineered viruses with different host ranges has been utilized (*33*). Fourth, Shapiro and Dougherty (*48*) have demonstrated that viruses (AcNPV and the *Amsacta* entomopox virus) not usually infective to gypsy moth larvae caused infection when fed with optical brightener Phorwite AR.

APPROACHES TO INCREASING BACULOVIRUS RESISTANCE TO ULTRAVIOLET (UV) LIGHT

It is well known that exposure to UV radiation rapidly inactivates baculoviruses. Therefore, baculoviruses used in agricultural and forest applications must be formulated to increase their resistance to UV exposure. A variety of compounds have been demonstrated to protect baculoviruses from UV inactivation, including molasses (*49*), adjuvants and sunscreens (*50*), congo red (*51*), and optical brighteners (*48,52–54*). Of the substances tested, optical brighteners, which are commonly used in detergents and fabric softeners, appear to be the most promising. Not only are many optical brighteners able to completely protect virus from UV exposure that totally inactivate unprotected virus (*52*), they also can significantly reduce the LC_{50} and LT_{50}. Shapiro and Robertson (*53*) tested *Lymantria dispar* NPV (LdNPV) formulations containing 1% solutions of five different optical brighteners and found a reduction in the LC_{50} from over 18,000 polyhedra/cm^3 to 10–44 polyhedra/cm^3 and the LT_{50} from 11.3–14.4 to 6.9–7.4 d (10^5 polyhedra/cm^3) and from 11.7–12.7 to 5.9–6.4 d (10^6 polyhedra/cm^3). Shapiro and Dougherty (*48*) used Phorwite AR optical brightener in a formulation containing *L. dispar* cytoplasmic polyhedrosis virus and found that the LC_{50} was reduced from 1.2×10^5 to 1.4×10^2 polyhedra/cm^3 and that the LT_{50} was decreased from 13.2 to 8.4 d at 10^6 polyhedra/cm^3. Webb et al. (*54*) compared the effectiveness of LdNPV formulations containing optical brighteners to those using the widely used sunscreen Orzan. Compared to Orzan, the optical brighteners Phorwite and Blankophor resulted in a significantly higher viral infectivity and a shorter kill time. It has been hypothesized (*53*) that optical brighteners inhibit or alter the chitinous peritrophic membrane to create gaps through which the virus can pass.

METHODS OF BACULOVIRUS PRODUCTION

Baculoviruses can be produced in either insect larvae or in cell culture bioreactors. Commercial production to date has been in insect larvae owing primarily to lower costs for small-scale production. As the scale increases, however, the relative cost of production in bioreactors becomes more competitive. It has been estimated that cell culture production

becomes more economical than production in insect larvae when the bioreactor volume exceeds 7500 L (*55*). There are also other advantages to production in cell culture (*56*), including (1) absence of contaminating microorganisms, insect proteins and cuticles found in larvae-derived product, and (2) superior control over the process. A number of strategies can be used to reduce baculovirus production cost in cell culture, including:

1. Development of prolific, high yield per volume cell lines;
2. Simplification of culture media;
3. Propagation of high pathogen titers in selected cell lines; and
4. Design and development of plant-scale equipment and routine production procedures.

It should be noted that no susceptible cell cultures exist for many baculoviruses; e.g., few stable cell lines exist that are susceptible to granulosis viruses (*18*). The remainder of this review article addresses issues relevant to AcNPV, AfNPV, and LdNPV production in cell culture.

CHOICE OF HOST INSECT CELL LINES

The most general requirement for a host insect cell line used to produce a baculovirus is that it is susceptible to the corresponding viral infection. For wide host range baculoviruses, e.g., AcNPV and AfNPV, a range of cell lines are potential candidates, whereas only a few cell lines are potential candidates for narrow host range baculoviruses, e.g., LdNPV. After the potential host insect cells are identified, the goal is to find one that is able to (1) produce high volumetric polyhedra yields, (2) produce highly pathogenic polyhedra, (3) grow and produce virus in inexpensive serum-free medium, (4) grow and produce virus in suspension cultures, and (5) be able to maintain these characteristics in large-scale bioreactors. In reality, it is unlikely that all of these goals can be fully realized but they are useful guides to use in reaching the ultimate goal of minimizing the cost of viral insecticide necessary for effective pest control.

Much information obtained regarding the host cells used with the baculovirus expression vector system (BEVS) is directly applicable to AcNPV production. The most widely used cell lines with the BEVS (using AcNPV) are the *S. frugiperda* Sf-9 and IPLB-Sf-21-AE cell lines. The major advantages of these cell lines is that they are easily adapted to and grown in suspension cultures and they produce relatively high product levels. It has been found that these cell lines require no significant adaptation period when transferred from stationary to suspension culture. For example, Murhammer and Goochee (*57*) found that the Sf-9 insect cell line taken directly from tissue culture flasks could be grown in spinner flasks without adaptation when either serum-supplemented or serum-free media was used. The recombinant protein expression levels in suspension cultures were comparable to those obtained in stationary cultures. In addition, it

has been demonstrated that the Sf-9 cell line has long term stability in suspension cultures (*58*). We have also recently demonstrated that the Sf-21 insect cell line does not require adaptation to suspension growth in spinner flasks (unpublished results). In contrast to our findings in spinner flasks, Wu et al. (*59*) found that about six successive passages were required to adapt Sf-21 cells to suspension growth in shaker flasks. Over the course of these passeges, they noted an increase in the maximum cell density from 5×10^5 to 3×10^6 cells/cm^3. This difference in adaptation characteristics may be a result of more intense shear forces present in the shaker flasks than in the spinner flasks. Methods are available for economic production of serum-free media that support growth and product production as well as or better than serum-supplemented media (*60*). There are also many commercially available serum-free media, e.g., Sf900 II (Gibco-BRL) and Excell 400 and 401 (JRH Biosciences).

Another cell line frequently used with the BEVS is the *T. ni* BTI-Tn-5B1-4 cell line available under the trade name High Five from Invitrogen, Inc. (San Diego, CA). The expression levels of many recombinant proteins in the High Five cell line are significantly higher than those in the Sf-9, Sf-21, and other cell lines in low density attachment cultures (*61,62*). The per-cell expression levels in the High Five cell line, however, decrease rapidly as the cell density increases. In addition, it has been demonstrated that the High Five cell line is capable of producing two and three times more polyhedra per cell than the Sf-21 cell line (*63*). Although originally believed to be an attachment-dependent cell line (*62*), the High Five cell line has subsequently been adapted to suspension growth in many laboratories. One approach to adapting these cells involves adding 50 to 2000 Units/cm^3 heparin to prevent clumping in suspension cultures (*63*). The heparin must be removed prior to expressing products because of heparin's inhibitory effect on viral uptake. This and other approaches used to adapt the High Five cell line to suspension culture involves isolating individual cells, i.e., removing cell clumps. Studies are currently being conducted in our laboratory and others regarding the long-term stability of these cells in suspension culture and whether they retain their desirable characteristics under these conditions. Commercial serum-free media have been developed for the High Five cell line, e.g., Express Five (Gibco-BRL) and Excell 405 (JRH Biosciences). Media development is also ongoing at companies interested in the commercial production of viral insecticides with the goal of reducing cost and maximizing obtainable cell densities and volumetric productivity.

Many of the cell lines that are susceptible to infection by AcNPV can also serve as hosts for AfNPV production (*20*), e.g., the High Five and Sf-9 cell lines. There is essentially no information available in the literature specifically regarding the production of AfNPV.

A limited number of cell lines are susceptible to LdNPV infection owing to its narrow host range. Some cell lines isolated from *L. dispar*, including IPLB-Ld-652Y, IPLB-LdFB, IPLB-LdFB-c1, IPLB-LdEG, IPLB-

LdEI, IPLB-LdEIt, IPLB-LdEIta, and IPLB-LdEP, are susceptible to LdNPV infection (*64–66*). The IPLB-LdEIta cell line grown in suspension formed aggregates of 50–200 cells, which may present mass transfer limitations upon scale-up (*67*). It is possible, however, that utilizing an approach similar to that used to adapt the High Five cells to suspension will eliminate this cell-clumping problem. The LdFB cell line grown in suspension was found to be especially sensitive to hydrodynamic stress, probably owing to its relatively large size (*68*). Finally, the LdFB-c1 cell line, a clone isolated from the LdFB cell line, has been used in a 40-L airlift bioreactor to produce LdNPV (*66*).

PRODUCTION OF BACULOVIRUSES IN LARGE-SCALE BIOREACTORS

There are three major modes of operation that can be used to produce baculoviruses in insect cell cultures: batch, continuous, and fed-batch. Batch bioreactors have been the most widely used owing mainly to their simplicity. Continuous bioreactors, consisting of a bioreactor devoted to cell growth followed by one or more bioreactors devoted to virus production, have been attempted but suffer from the accumulation of defective interfering particles as discussed later in this paper. Finally, the development of fed-batch bioreactors for baculovirus production, in which nutrients (e.g., glucose and glutamine) are fed as needed by the cells, is in its infancy. In all three of these operational modes, the cells are most efficiently grown and infected in suspension cultures oxygenated through direct sparging. Sparging, however, can result in severe cell damage. Murhammer and Goochee (*57,69,70*) demonstrated that Sf-9 cells can usually be protected from the adverse effects of sparging by supplementing the medium with the surfactant Pluronic F-68 (or a related compound), which is now a widely used additive in both insect and mammalian cell media.

Both airlift and agitated sparged bioreactors have been utilized to grow insect cells in the batch mode. The growth and viral infection of many insect cell lines in both of these types of bioreactors has been widely reported with AcNPV infection (*see* ref. *71* for a review). Only one such study, however, has been reported for LdNPV production (*66*), and no such studies have been reported regarding AfNPV production.

In addition to production of AcNPV in batch cultures, a number of studies have been conducted in which the continuous production of AcNPV has been attempted. An economic analysis (*3*) concluded that the utilization of a continuous system would reduce total production costs by approx 50% when compared to a batch system. The first reported attempt at actually running a continuous system was by Kompier et al. (*72*), who utilized a two stage bioreactor system for the continuous production of AcNPV in Sf-21 cells. The first stage was utilized for cell growth, while

the second stage was used for virus production. A 60-h mean residence time was maintained in each vessel and a decreased in productivity was noted after approx 30 d, which was probably owing to the passage effect, i.e., defective interfering particle accumulation as described later in this paper. Up to this dropoff in productivity, the system produced approx 25 polyhedra and 250 BV/cell. They also found that 65% of the cells in stage 2 were infected, i.e., contained polyhedra, which compared well with the 68% theoretical value.

van Lier et al. (*73*) produced AcNPV in Sf-21 cells with two different systems. The first system consisted of a bioreactor devoted to cell growth followed by a bioreactor devoted to virus production. The second system consisted of one bioreactor for cell growth followed by two bioreactors for virus production. In the second system, each of the two bioreactors used for virus production was one-half the volume of the growth bioreactor. Productivity dropped off more rapidly in the second system, in which the productivity declined after 10 d and eventually leveled off at a new steady state. Prior to the dropoff in productivity, the fraction of cells in the first and second viral production bioreactors containing polyhedra were 30–35% and 50–70%, respectively, as compared to the theoretical values of 45 and 81%. After the dropoff in productivity, only 55% and 10% of the cells in the first and second viral production bioreactors, respectively, contained polyhedra. A mathematical model demonstrated that higher passages occur sooner when the number of vessels in the series of infection reactors is increased, thereby leading to a more rapid mutant accumulation.

In fed-batch bioreactors, critical nutrients are provided as needed. This can lead to a more efficient use of nutrients and a significant extension in the productive life of the culture. Recently a number of investigators have demonstrated that the maximum cell density and productivity of the Sf-9 cell line can be significantly enhanced by using a fed-batch bioreactor, although there is some discrepancy regarding the specific nutrient supplementation that is necessary. Wang et al. (*74*) achieved a twofold increase in recombinant epoxide hydrolase production by adding glucose and glutamine immediately after infection and then adding glucose daily thereafter. In contrast, Nguyen et al. (*75*) found that the addition of glucose and glutamine failed to increase the maximum cell density or the level of recombinant human nerve growth factor (rhNGF) synthesis. The addition of glucose, glutamine, and yeastolate, however, did result in a significant increase in the maximum cell density and rhNGF production. The rhNGF productivity was further enhanced by the addition of a lipid emulsion. Similarly, Reuveny et al. (*76*) found that the level of recombinant β-galactosidase production could be significantly enhanced by adding glucose, glutamine, and yeastolate to the culture. A greater enhancement in productivity was obtained by a combination of medium replacement and supplementation with glucose, glutamine, and yeastolate. In addition to enhanced recombinant protein productivity, Nguyen et al. (*75*) extended the maximum cell density to 1.2×10^7 cells/cm^3 (vs $\sim 5 \times 10^6$

cells/cm^3 in batch cultures) by feeding glucose, glutamine, and yeastolate. It is likely that utilizing fed-batch bioreactors would also enhance polyhedra productivity in insect cell culture. The optimal operation of fed-batch bioreactors is currently limited by the lack of on-line real-time monitoring systems that could be used to maintain constant critical nutrient concentrations.

MUTANT FORMATION UPON PASSAGE IN CELL CULTURE

Serial passage of wild-type baculoviruses in insect cell culture usually results in the high frequency generation of few polyhedra (FP) mutants. The FP phenotype results in a considerable reduction in the number of polyhedra produced per infected cell when compared to the wild-type virus. For example, AcNPV FP mutants produced less than 10 polyhedra per infected *T. ni* Tn-368 cell as compared to over 50 polyhedra for the wild type many polyhedra (MP) phenotype (*77*). In addition, polyhedra produced by FP mutants contain few, if any, virus particles. FP mutants have a selective advantage in cell culture by producing more budded virus, perhaps at the expense of polyhedra. In general, the FP phenotype results from reduced levels of the 25K protein. The reduced levels of this protein frequently result from the disruption of the gene by the insertion of host insect cell DNA. The 25K protein is either directly or indirectly associated with an enhancement of polyhedrin synthesis and nuclear localization at the beginning of the occlusion phase of infection (*78*). This enhancement effect appears to be necessary to ensure the normal assembly of viral occlusions. Beames and Summers (*79*) demonstrated that inserting the *Escherichia coli* β-galactosidase gene within the 25K gene in AcNPV led to FP mutations. In addition, Fraser et al. (*80*) demonstrated that co-transfecting cells with purified DNA from FP mutants and a restriction fragment from MP AcNPV containing the 25K gene lead to a significant increase in the number of MP plaques, i.e., expressing the 25K gene was able to overcome the FP phenotype.

Another type of mutant baculovirus, referred to as a defective interfering particle (DIP), also accumulates upon serial passage in insect cell cultures. DIP mutants, unlike FP mutants, cannot be plaque purified; i.e., they will only replicate in cells that are also infected by a normal virus particle (*81*). Consistent with this, low multiplicity of infection (MOI) favors normal virus synthesis; i.e., a low probability exists for infection of a given cell by both a DIP and a normal virus. The DIPs can apparently replicate faster than standard particles, perhaps owing to a smaller genome. Indeed, AcNPV DIPs lack 43% of the genome present in the standard viruses (*82*). Electron microscopy of DIPs produced in *T. ni* Tn-368 and *S. frugiperda* Sf-9 insect cells demonstrated that the DIPs are smaller (~200 nm) than the standard sized virions (~330 nm). At equilibrium, there

were approx 28% standard sized particles, 60% DIPs, and 12% 400 nm particles.

FUTURE DIRECTIONS

The prospect of commercial production of a viral insecticide (e.g., AcNPV, AfNPV, and/or LdNPV) in the United States in the future is encouraging. For example, American Cyanamid, a subsidiary of American Home Products, recently received approval from the EPA to conduct small-scale field tests of their genetically altered AcNPV (*83*). Specifically, their recombinant AcNPV has the *egt* gene deleted and expresses AaIT (*84*). It should be noted that the present cost of producing baculoviruses in insect larvae is competitive with Bt toxin, the current standard for biopesticides. As mentioned previously, the cost of producing baculoviruses for treating cotton is $2.16/acre/application (excluding overhead) (*24*) compared to $1.50–6.00/acre/application for Bt toxin (including overhead) (*25*). In addition, Bt toxin costs $9–14/acre/application (including overhead) for controlling pests on vegetables. Although industrial production costs are proprietary, it is believed that large-scale baculovirus production costs in insect cell culture will be competitive with the production costs in insect larvae (*55*). It is likely that a number of advances in insect cell cultures and a general improvement in understanding insect cell behavior can further reduce production costs. First, since it is likely that production scale bioreactors will be 10,000s of liters, it is imperative that an understanding of insect cell behavior in these environments is obtained. For example, in a high-density bioreactor of this size it is likely that significant environmental heterogeneities will exist (*85*). Thus, as cells circulate throughout the bioreactor, they are likely to experience an ever-changing environment. Second, optimization of a fed-batch bioreactor system would greatly enhance productivity and thereby reduce production costs. The critical advancement needed to realize this goal is a continuous, real-time system for the on-line monitoring of critical nutrient concentrations. Third, media need to be developed that can maximize productivity at the lowest possible cost. Fourth, cell lines capable of higher productivity are needed, especially for LdNPV production.

PATENTS

This section covers US patents regarding the use of baculoviruses as viral insecticides from January 1987 to June 1995. The abstracts have been edited for clarity and conciseness. Copies of these patents can be obtained from the Commissioner of Patents, US Patent and Trademark Office, Box 9, Washington, D. C. 20231.

Asplirot, J., Biache, G., Delattre, R., and Ferron, P.
Process for the biological control of insects which destroy crops, and insecticidal compositions
US Pat. 4,668,511, May 26, 1987
Assignees: Institut National de la Recherche Agronomique and Recherches du Coton et des Textiles Exotiques (both of Paris, France)

A process in which crops were conjointly treated with a baculovirus (from *Spodoptera littoralis or Mamestra brassicae)* and a photostable pyrethrinoid was described. Using such combinations to control noctuid Lepidoptera resulted in increased effectiveness and significantly reduced doses as compared to either insecticide used individually.

Fraser, M. J., Rosen, E. D., and Ploplis, V.A.
Recombinant baculovirus occlusion bodies in vaccines and biological insecticides
US Pat. 4,870,023, Sept. 26, 1989
Assignee: American Biogenetic Sciences (Copiague, NY)

Recombinant baculoviruses that encode fusion polyhedrin proteins capable of forming occlusion bodies containing foreign peptides were developed. The recombinant baculoviruses are formed by insertion of foreign DNA fragments into regions of the polyhedrin gene that are not essential for occlusion body formation. The resulting recombinant occlusion bodies have uses as biological insecticides and in other applications.

Hostetter, D. L. and Puttler, B.
Multiple embedded nuclear polyhedrosis virus from celery looper with activity against lepidoptera
US Pat. 4,911,913, March 27, 1990
Assignee: The United States of America as reprsented by the Secretary of Agriculture (Washington, D. C.)

A multiple embedded nuclear polyhedra virus isolated from the celery looper, *Syngrapha falcifera,* was found to have broad insecticidal activity against Lepidoptera species.

Granados, R. R.
Baculovirus proteins and viral pesticides containing same
US Pat. 4,973,667, Nov. 27, 1990 and US Pat. 5,011,685, April 30, 1991
Assignee: Boyce Thompson Institute for Plant Research, (Ithaca, NY)

Nuclear polyhedrosis viruses, for example, *Autographa californica* nuclear polyhedrosis virus (AcNPV), useful in the control of lepidopteran larvae, e.g., cabbage looper *Trichoplusia ni,* were found to have enhanced infectivity when mixed with certain proteins obtained from the granulin fraction of *T. ni* granulosis virus (TnGV) or *Heliothis armigera* granulosis virus (HaGV). The enhanced infectivity was found to be correlated to biochemical and structural changes in the *T. ni* peritrophic membrane.

Vail, P. V.
Novel virus composition to protect agricultural commodities from insects
US Pat. 5,023,182, June 11, 1991
Assignee: The United States of America as represented by the Secretary of Agriculture (Washington, D. C.)

A potent, stable virus composition useful for protecting agricultural commodities from insects and a simple, efficient, economical, and labor-saving method to produce and formulate large quantities of the virus composition are described. The method is particularly useful in preparing nuclear polyhedrosis virus or granulosis virus compositions for control of postharvest pests such as the Indianmeal moth.

Miller, D. W.
Mixed baculovirus compositions and uses thereof
US Pat. 5,071,748, Dec. 10, 1991
Assignee: Genetics Institute (Cambridge, MA)

A mixed composition polyhedra was developed containing a mixture of nucleocapsids of at least two genetically distinct baculoviruses. At least one of the baculoviruses was genetically engineered to contain at least one heterologous gene. Ingestion of the mixed composition polyhedra by an insect host resulted in a mixed viral infection in the insect and the production of additional copies of the mixed composition polyhedra and the heterologous protein(s) encoded by the genetically engineered baculovirus(es).

Biache, G. A. and Guillon, M. R.
Insecticide based on a virus from the baculovirus group, and use thereof for the destruction of *Phthorimaea operculella*
US Pat. 5,091,179, Feb. 25, 1992
Assignee: Calliope S. A. (Beziers, France)

The *Mamestra brassicae* nuclear polyhedrosis virus was found to cause considerable mortality when ingested by *Phthorimaea operculella* caterpillars. This finding is quite unexpected as *P. operculella* caterpillars are not closely related to *Mamestra brassicae*. In fact, *P. operculella* and *M. brassicae* belong to different families, the first belonging to the noctuidae family and the latter to the gelechiidae family.

Hammock, B. D. and Philpott, M. L.
Juvenile hormone esterase for insect control
US Pat. 5,098,706, March 24, 1992
Assignee: The Regents of the University of California (Berkeley, CA)

The use of juvenile hormone esterase to control insect populations was discussed. This enzyme fatally disrupts normal insect larvae development and is preferably administered by infecting insects with a recombinant baculovirus including the gene coding for the enzyme.

Shapiro, M., Dougherty, E., and Hamm, J. J.
Compositions and methods for biocontrol using fluorescent brighteners
US Pat. 5,124,149, June 23, 1992
Assignee: The United States of America as represented by the Secretary of Agriculture (Washington, D. C.)

Formulations consisting of a baculovirus and a fluorescent brightener analog that provide UV protection and enhance biological activity of the baculoviruses are discussed. Specifically, compositions are disclosed that are comprised of a baculovirus such as a nuclear polyhedrosis virus (NPV), cytoplasmic polyhedrosis virus (CPV), entomopox virus (EPV), or granulosis virus (GV), and a fluorescent brightener such as 4,4′-diamino-2,2′-stilbene disulfonic acid that absorbs UV radiation and may have chitin synthetase inhibitory properties.

Shapiro, M., Lynn, D. E. and Dougherty, E. M.
More virulent biotype isolated from wild-type virus
US Pat. 5,132,220, July 21, 1992
Assignee: The United States of America as represented by the Secretary of Agriculture (Washington, D. C.)

A method has been found to select the most virulent biotypes from a wild-type virus population by selection of desirable characteristics after serial passages through in vivo and in vitro systems. Using this method, a strain of *Lymantria dispar* NPV was isolated that has increased biological activity and increased speed of kill.

Summers, M. D. and Oker-Blom, C. E. G.
Baculovirus dual promoter expression vector
US Pat. 5,169,784, Dec. 8, 1992
Assignee: The Texas A & M University System (College Station, TX)

The design and construction of a recombinant baculovirus that utilizes different baculovirus promoters and is therefore capable of temporal regulation of successive protein synthesis are discussed. A potential application is the construction of a recombinant viral insecticide.

Miller, L. K. and O'Reilly, D. R.
Biological insect control agents and methods of use
US Pat. 5,180,581, Jan. 19, 1993
Assignee: University of Georgia Research Foundation, (Athens, GA)

Insect control agents comprising a gene coding for a protein that affects the growth, development, or behavior of an insect are discussed. The gene is either activated to prevent insect molting and pupation or is inactivated to reduce the feeding behavior, inhibit growth, and result in the earlier death of the insect hosts. Methods for producing the insect control agent and methods of controlling insects by exposing them to the insect

control agent are also included. A specific example involves an *Autographa californica* nuclear polyhedrosis virus in which the naturally occurring ecdysteroid UDP-glucosyl transferase gene was inactivated. The virus was demonstrated to have advantages over the wild-type virus as an insect control agent.

Maeda, S.
Insecticide making use of viruses and preparation process thereof
US Pat. 5,266,314, Nov. 30, 1993

A process was developed to form and isolate hybrids of two baculovirus species with expanded host ranges.

Granados, R. R.
Cell line isolated from larval midgut tissue of *Trichoplusia ni*
US Pat. 5,298,418, March 29, 1994
Assignee: Boyce Thompson Institute for Plant Research (Ithaca, NY)

Two new insect cell lines, derived from midgut (BTI-TN-MG1, ATCC CRL 10860) and embryonic tissue (BTI-TN-5B1-4, ATCC CRL 10859) of *T. ni* (cabbage looper), were established and characterized. These cell lines are susceptible to various baculoviruses, including TnNPV and AcNPV, and may be used to replicate such viruses for use as insecticides or otherwise.

Granados, R. R.
Trichoplusia ni cell line which supports replication of baculoviruses
US Pat. 5,300,435, April 5, 1994
Assignee: Boyce Thompson Institute for Plant Reaearch (Ithaca, NY)

An insect cell line derived from embryonic tissue (BTI-TN-5B1-4, ATCC CRL 10859) of *T. ni* (cabbage looper) has been established and characterized. Similar to the US Pat. 5,298,418.

Miller, L. K. and O'Reilly, D. R.
Biological insect control agents and methods of use
US Pat. 5,352,451, Oct. 4, 1994
Assignee: University of Georgia Research Foundation (Athens, GA)

Similar to US Pat. 5,180,581

McIntosh, A. H.
Heliothis subflexa cell line for the production of baculoviruses
US Pat. 5,405,770, April 11,1995
Assignee: The United States of America as represented by the Secretary of Agriculture (Washington, D. C.)

A cell line was isolated from *H. subflexa* (BCIRL-HS-AM1). The cell line is particularly useful in the production of baculoviruses.

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