Development and Field Testing of Genetically Modified Baculovirus Insecticides

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Abstract

Baculovirus insecticides are attractive alternatives to synthetic chemical pesticides. Naturally occurring baculoviruses are recognized as playing important roles in regulating insect populations in a variety of ecosystems and as possessing none of the negative health and environmental attributes associated with many chemical pesticides. However, baculovirus insecticides have a relatively slow speed of action, as compared to chemicals, and this has been a deterrent to commercial development. Through genetic engineering, numerous recombinant baculoviruses have been constructed which kill insects faster. In order to address the potential environment/health issues of recombinant insecticides, several recombinant virus strategies have been developed that allow for enhanced pesticidal activity and reduced probabilities of negative ecological consequences. These recombinant strategies have been field tested in the United States and the United Kingdom.

Introduction

Baculoviruses belong to a large group of double-stranded DNA viruses which have been isolated from arthropods. Several hundred baculoviruses have been described and most infect lepidopterous insects, many of which are important agricultural and forestry pests (Bilimoria, 1986). They are the largest and best studied insect-pathogenic viruses.

It has been recognized for more than a century that natural baculovirus epizootics can play an important role in the regulation of insect pest populations. Unfortunately, these natural epizootics, which reduce pest populations to minor levels, usually occur after the insect pests have caused significant economic damage. A goal of biological control has been to create artificial viral epizootics prior to the development of unacceptably high pest populations (Huber, 1986). This approach has been successfully used to control an insect pest in Brazil. For the past decade, the *Anticarsia gemmatalis* nuclear polyhedrosis virus (AgMNPV) has being applied to more than two million acres of soybean fields to control the velvetbean caterpillar, *A. gemmatalis* (Moscardi and Sosa-Gomez, 1993).

Accordingly, baculoviruses, along with many other biological control agents, are attractive alternatives to chemical insecticides - they are natural insecticides. Unlike some synthetic chemical pesticides, naturally occurring baculovirus pesticides have no known health safety problems and have never elicited any known environmental perturbations. The problems associated with the commercial use of biological control

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agents have generally been restricted to cost/benefit ratios which have favored the use of synthetic chemical pesticides. The problems associated with baculovirus pesticides include their slow speed of action, high production costs and formulation/application technologies which have produced only limited field persistence and infection rates. Recent advances in biotechnology have resulted in significant progress towards solving these problems (Wood, 1995).

Of particular note has been the use of biotechnology to enhance the pesticidal properties of baculoviruses. Typically, larval death may occur 5-15 days after infection, resulting in significant pest damage even after viral infection. Through genetic engineering it has been possible to insert foreign genes into baculoviruses, resulting in viruses with shorter times to death and/or, more importantly, reduced times to cessation of feeding (Wood, 1995 ; Wood and Hughes, 1995).

Using the baculovirus expression vector technology (Pennock *et al.,* 1984; Smith *et al.,* 1983), foreign pesticidal genes can easily be inserted into baculoviruses. To date, several pesticidal genes have been inserted into the *Autographa californica (Ac)* MNPV and *Bombyx mori (Bm)* NPV and evaluated under laboratory conditions for their improved pesticidal properties. A few of these recombinant viruses have been tested in the field. Typically, the foreign genes have been placed under the transcriptional control of the very late baculovirus polyhedrin or *p10* gene promoters.

The expression of genes isolated from insects has been used in an attempt to disrupt normal physiological processes. The expression of an insect-derived juvenile hormone esterase (Bonning *et al.,* 1992 ; Hammock *et al.,*1990) a prothoracicotropic hormone (O'Reilly *et al.*, 1995) a diuretic hormone (Maeda, 1989) and an insect chitinase (Gopalakrishnan *et al.,* 1995) gene to improve baculovirus pathogenicity has met with marginal success. However, significant enhancements in baculovirus pathogenicity have been achieved through the insertion and expression of insect-specific neurotoxin genes from a scorpion (McCutchen *et al.,* 1991; Stewart *et al.,* 1991), mite (Tomalski and Miller, 1991; 1992), hornet (Tomalksi *et al.,* 1993)and spider (Hughes *et al.,* 1997.). Currently there is considerable interest in the commercialization of these recombinant viruses.

As with synthetic chemical pesticides, naturally occurring and recombinant viral pesticides will be required to satisfy the requirements of regulatory agencies prior to registration. The potential hazards associated with chemical and viral pesticides differ mainly because viral pesticides replicate and, therefore, environmental contamination can increase in concentration and area. Accordingly, health safety and environmental considerations made prior to the small scale field testing of recombinant viral pesticides are far more substantive than those required for the small scale testing of chemical pesticides.

The risk factors associated with recombinant baculoviruses include potential interactions with non-target invertebrate species and the health safety of the foreign-pesticidal protein to vertebrate species. Since little is known about the ecology of baculoviruses, particular attention must be paid to potential ecological interactions.

Laboratory data cannot always predict environmental consequences of the release of a recombinant organism into the environment. Therefore it has been appropriate to use physical and/or biological containment strategies while evaluating the potential risk factors associated with the field testing of recombinant organisms (Wood, 1995; Wood and Granados, 1991; Wood and Hughes, 1995). Clearly, the approach to evaluating the environmental impact of pesticides has changed since the 1950s from a reactive to a proactive approach

Field testing of recombinant baculoviruses

Prior to field testing of recombinant baculoviruses in the U.S., approval must be obtained from the Environmental Protection Agency's Office of Pesticide Programs as well as appropriate state agencies. If the project has federal funding, the release conditions must also satisfy the requirements of the National Environmental Policy Act. Similar review processes are required in the United Kingdom.

Three types of recombinant field testing programs have been conducted to date with baculoviruses. The first program was designed to test the commercial potential of recombinant viruses under conditions of physical containment. The second type has been the evaluation of genetic engineering strategies designed to mitigate potential environmental interactions. The third type of program has been designed to evaluate if the high expression of an insect-specific neurotoxin gene can be used to improve the commercial potential of a baculovirus and also limit its environmental impact.

Marked virus release

The field testing of genetically engineered isolates of baculoviruses was pioneered by scientists at the Natural Environment Research Council's (NERC) Institute of Virology in Oxford, UK2. In 1986 they field-tested a recombinant isolate of AcMNPV which contained a synthetic oligonucleotide, 80 bp in length. The insert was non-coding and was inserted downstream of the polyhedrin gene coding region. In laboratory tests the marked virus was shown to have biological properties identical with those of the wild-type virus. The test was conducted to evaluate the genetic stability and persistence of recombinant viruses under field conditions. Late instar *Spodoptera exigua* larvae were infected with the marked virus in the laboratory and placed on sugar beet plants in the field.

Since this was the first release of this type, considerable efforts were made to ensure a high level of physical containment. The release site was surrounded with a 2-meter-high wire fence, covered with a fine mesh net and surrounded with formalin traps and buried wire netting. At completion of the testing, all residual virus in the test site was inactivated by formalin treatment of the soil. The studies showed that the marked virus was genetically stable in the environment and had the same level of stability in the soil as the wild type virus.

Polyhedrin-minus virus release

In 1987 the NERC Institute of Virology conducted a second field test with a recombinant AcMNPV isolate which lacked a polyhedrin gene (Bishop *et al.,* 1988). The polyhedrin gene and promoter were replaced with a 100 bp oligonucleotide insert. Laboratory-infected insects were placed on plants in the field enclosure identical with that used in the 1986 marked virus release.

Crystallization of the polyhedrin protein around baculovirus particles (forming polyhedra) affords protection of the virus at death of the host. Deletion of the polyhedrin gene resulted in the production of only non-occluded progeny virus which, under laboratory conditions, was shown to be completely inactivated soon after larval death. Following field release and death of the larvae, as predicted from laboratory testing, no virus infectivity could be detected on foliar or soil samples at seven days after death. The marked polyhedrin-minus virus was therefore referred to as "self-destructive".

The self-destructive nature of the polyhedrin-minus virus was very attractive from an environmental standpoint. After killing the insect pest, there would be no residual virus. However, the instability of the non-occluded virus particles also meant that there would be problems with commercial production and application.

Co-occluded virus release

It was discovered that the instability of polyhedrin-minus baculoviruses could be overcome through a process called co-occlusion (Hamblin *et al.,* 1990; Miller, 1988; Shelton and Wood, 1989; Wood *et al.,* 1990). By co-infecting cells with both the wild type and polyhedrin-minus viruses,the polyhedrin protein produced by the wild type virus will crystallize around both the wild type and polyhedrin-minus virus particles, thereby forming polyhedra containing both virus types. By co-occlusion, polyhedrin-minus virus particles can be stabilized in a form which can be used to infect larvae in the field.

Laboratory studies were used to develop a predictive model concerning the persistence of a co-occluded, polyhedrin-minus AcMNPV in a virus population during successive passages of virus from larva to larva. The model predicted that under field conditions the polyhedrin-minus virus would be eliminated quickly from the virus population (Hamblin *et al.,* 1990). The model was field tested in 1989 by scientists at the Boyce Thompson Institute and Cornell Agricultural Experiment Station (Wood *et al.,* 1993). This was the first field release of recombinant virus in the USA. Since the removal of the polyhedrin gene provided an appropriate level of biological containment, no physical containment or decontamination procedures were required.

A 2-acre planting of cabbage infested with *Trichoplusia ni* larvae was originally sprayed with polyhedra containing 49% polyhedrin-minus and 51% wild-type AcMNPV particles. In the second and third years, the progeny polyhedra contained only 9% and 6% polyhedrin-minus virus, respectively (Wood *et al.,* 1993). Based on the predictive laboratory model, there was a low probability of co-occlusion of the polyhedrin-minus virus passed from one insect to another under field conditions.

A second co-occluded virus release was initiated in 1993 by scientists at the Boyce Thompson Institute, the University of Massachusetts and the U.S. Forest Service. The recombinant virus was a polyhedrin-minus isolate of the *Lymantria dispar* (gypsy moth) MNPV in which the polyhedrin gene was replaced with a bacterial lacZ gene. The production of beta-galactosidase was used as a reporter to monitor movement and persistence of the recombinant virus in time and space. The test site was an oak forest infested with gypsy moth larvae. The test analyses will be completed in 1996, but the preliminary data indicate that since 1993 the recombinant virus has moved less than 100 meters outside of the release site and was quickly lost from the resident virus population (D'Amico *et al.*, in preparation).

Pre-occluded virus release

During the AcMNPV co-occlusion field studies(Wood *et al.*, 1993), it was observed that the soil served as a long-term reservoir for biologically active polyhedra. This observation raised questions of potential environmental consequences with increasing polyhedra concentrations under agronomic conditions.

In order to preclude any potential environmental problems associated with high levels of recombinant virus contamination, the pre-occluded strategy was developed. It is based on previously unrecognized properties of the Oxford "self-destructive" system which results in zero environmental contamination.

Late in the replication cycle of occluded baculoviruses, the viral nucleocapsids become membrane-bound within the nucleus. The polyhedrin protein crystallizes around these membrane-bound particles to form polyhedra. In the absence of a polyhedrin gene and polyhedra formation, it was discovered that the membrane-bound particles accumulate in the nucleus (Wood *et al.*, 1993). These particles which were destined to become occluded are called pre-occluded virions (POV). The pre-occluded virions are highly infectious *per os* with susceptible host larvae (Wood *et al.*, 1993). The POV can be produced *in vitro* and are stable at 4℃. They can also be produced *in vivo* if the larvae are freeze-dried prior to death (Hughes and Wood, 1995). The rehydrated tissues contain high concentrations of POV that are infectious *per os*.

In 1993 and 1994, AgriVirion Inc. conducted field releases with the POV form of AcMNPV in cabbage plots infested with *T. ni* larvae. No foreign genes were inserted in the virus. As observed by Bishop *et al.* (1988), following the death of the infected larvae in the laboratory or under field conditions, all the infectivity of the progeny virus was lost within seven days after death. In the absence of stabilizing conditions, the nonoccluded POV are inactivated in the decaying larval tissues, resulting in zero environmental contamination or persistence.

AaIT virus release

The first field study of a genetically enhanced viral pesticide was conducted in 1993 by scientists at the NERC Institute of Virology (Cory *et al.*, 1994). The test involved a recombinant isolate of AcMNPV which expressed the *AaIT* toxin gene from the scorpion

Androctonus australis. The *AaIT* toxin is an insect-specific neurotoxin. Because of the expression of the scorpion toxin and the lack of a biological containment strategy, field testing was performed under conditions of strict physical containment, and the field sites were again treated with formalin at the end of the experiment.

The field data indicated that the expression of *AaIT* toxin reduced the mean time to death of infected *T. ni* larvae by approximately 15%, as compared to wild-type virus infections. More importantly, the *AaIT*-expressing virus infections resulted in a reduction in larval-feeding damage to cabbage plants as compared to the wild-type virus infections. This was the first field study to show that genetically enhanced viral pesticides can exhibit marked improvements over the pesticidal properties of naturally occurring viral pesticides.

EGT-minus virus release

In 1994 the American Cyanamid Company conducted a field release with a recombinant AcMNPV which lacked the viral gene coding for ecdysteroid UDP-glucosyltransferase (*EGT*). Removal of the *EGT* gene results in small reductions in feeding damage and time to death (O'Reilly and Miller, 1991). Since the recombinant virus only had a gene deletion, no physical or biological containment was required for this release.

EGT-minus, AaIT virus release

In 1995 the American Cyanamid Company performed a small scale field release of their EGT-minus isolate of AcMNPV which also expressed the scorpion neurotoxin, previously field-tested in the UK. Unlike the UK experiment, physical containment facilities were not employed. In laboratory studies, the recombinant virus exhibited significant reductions in time to death, reduced feeding damage, and an approximate 90% reduction in polyhedra production when compared to the wild-type virus infections. Accordingly, the American Cyanamid Company considered that the increased virulence of the recombinant virus resulted in reductions in progeny polyhedra which conferred an appropriate degree of biological containment. This release project will be repeated on a larger scale in 1996.

LqhIT virus release

The Dupont Company has made an application to perform a small scale field release of a recombinant in 1996. The recombinant AcMNPV (AcLqhIT) expresses an insect-specific neurotoxin gene from the scorpion *Leiurus quinquestriatus hebraeus*. The AcLqhIT virus has a functional EGT gene but has similar biological control properties to those of the *AaIT* virus being tested in the UK and by the American Cyanamid Company. Larvae infected with the AcLqhIT virus produce fewer progeny polyhedra than the wild-type virus. Accordingly, employing the same reasoning as used by the American Cyanamid Company, Dupont is proposing that this feature will provide an appro-

priate level of biological containment in the field.

Conclusion

During the past decade there have been a significant number of changes in the economic factors which control the pesticide industry. These changes have made biological control agents such as baculoviruses attractive alternatives to synthetic chemical pesticides. One approach to the commercial development of baculovirus insecticides has been to use the techniques of biotechnology to enhance the pesticidal properties of these agents. New biotechnology methods are being used also to reduce both *in vivo* and *in vitro* production costs and to produce pesticides which pose no or little risk to the environment.

The construction and commercialization of genetically enhanced baculovirus pesticides is in an early stage of development. The results to date clearly indicate that future research in this area will lead to the development of recombinant pesticides with cost benefit ratios equivalent to those of many of the synthetic chemical pesticides. However, as this industry progresses, care must be taken that the environmental benefits associated with naturally occurring biological control agents are maintained in the final products.

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