Environmental Impact of Baculoviruses

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Table of Contents

Introduction

This review examines the environmental impact of baculovirus use but does not take a predictive approach to risk assessment. It is primarily intended as a compilation of information to aid decision-makers in developing countries that are considering use of baculoviruses for insect pest control. Emphasis is placed on the impact on non-target organisms of wild-type viruses, currently the only form of baculovirus used in practice for pest control. Wild-type baculoviruses are those which have been taken directly from the field without undergoing selection or improvement prior to mass production and application. However, consideration is also given to the environmental implications of cloned or genetically engineered baculoviruses, now being developed for increased uniformity and speed of action, although they are unlikely to be used in the near future in developing country situations.

Many insect pathogenic viruses show similarities to viruses found in vertebrates or plants: Entomopoxviruses, cytoplasmic polyhedrosis viruses (CPV resemble the reoviruses such as the bluetongue virus and rice dwarf virus), densonucleus virus, small RNA viruses, and iridoviruses (similar to the icosahedral cytoplasmic deoxyriboviruses of fish, frog and green algae). Kurstack and Tijssen (1982) believe that only viruses that differ from those of non-target organisms should be considered for field-use pesticides. And even then only after very stringent investigations of possible deleterious effects on non-target organisms, although some viruses from this group (the CPV for example) have been used in biological control.

A factor that will most favour the potential success of viruses is their relative lack of adverse effects on the environment compared to chemical insecticides. Overall, the environmental effects after releases of insect viruses have not been comprehensively studied, but the observed effects have been minimal. Their major environmental effects are simply the ones for which release is intended, namely, reduction in insect populations and subsequent reduction of damage to crop plants. They can also indirectly reduce populations of beneficial, entomophagous or entomogenous organisms by reducing the population of the common host insect, though this effect is seldom as severe as with chemical insecticides (Fuxa, 1990).

Some thirty years ago Tinsley and Melnick (1973) reminded us that while insect viruses could be useful, the release of living organisms into the environment should not be taken lightly and that at the time the direct evidence of safety came from absence of obvious disastrous effects. They highlighted three potential hazards from the deliberate release of baculoviruses: (1) the infection of non-target insects; (2) the infection of non-insect invertebrates and perhaps even vertebrates; (3) the potential for mutation, recombination or host DNA acquisition leading to changes in pathogenicity or host range.

Some of their concerns have since been largely allayed. There is now, for example, a great deal of evidence to demonstrate the specificity of baculoviruses to arthropods and hence support the notion of safety of baculoviruses to vertebrates. Their concern over impact on non-target insects is, however, still valid but to date no deleterious side effects of virus application on non-targets have been recorded from the field. As to point three above, there is as yet insufficient data to confidently dispute this hypothesis except that as yet these fears have not, as far as we can tell, been substantiated in the field.

However, to date, even with an intervening 15 years or so, the conclusions of the review by Gröner (1986) still stand: it is impossible to avoid contact with baculoviruses as they are in any case naturally occurring pest-control agents. Commercial baculovirus preparations tested for pest control so far have proved safe for beneficial insects and the environment. Extensive safety studies on people, vertebrate and invertebrate non-target species have not found any deleterious effects and baculoviruses, unlike chemical pesticides, are not toxic, teratogenic or mutagenic.

Background Information

In order to appreciate the significance of the implications for the environment and non-target species arising from using baculoviruses in pest control, a general account of their taxonomic and biological characteristics (OECD 2002) is appended below.

1.1 Taxonomic considerations

Baculoviuses are a family of arthropod-specific, rod-shaped (baculum = rod), enveloped viruses with a circular double-stranded DNA genome. Until recently, the family Baculoviridae was divided into two subfamilies, the Eubaculovirinae and the Nudibaculovirinae (Francki *et al*., 1991). Based on the type of virion occlusion (see below) the Eubaculovirinae comprised the genera (i) nuclear polyhedrosis virus (NPV) and (ii) granulovirus (GV). The subfamily Nudibaculovirinae contained the only genus non-occluded baculovirus (NOB) which differed from the Eubaculovirinae in the lack of occlusion body formation and virion morphology (for review see Burand, 1991).

Recently, the International Committee on Taxonomy of Viruses (ICTV) revised the classification of baculoviruses (Murphy *et al*., 1995). The family Baculoviridae is now divided into two genera (i) Nucleopolyhedrovirus (formerly nuclear polyhedrosis virus) and (ii) Granulovirus (formerly granulosis virus). The NOB including the *Oryctes rhinoceros* virus (OrV) and the *Heliothis (=Helicoverpa) zea* virus 1 (HzV-1) has been removed from this family and are not assigned to any virus family.

Some properties used for the taxonomy and classification criteria for baculoviruses are summarised as follows (Murphy et al., 1995) (Fig. 1 in A 2.1).

Baculoviruses exclusively have been isolated from arthropods, primarily from 4 insect orders as Lepidoptera, Hymenoptera, Diptera and Coleoptera (Martignoni and Iwai, 1986; Adams and Bonami, 1991).

During the replicative cycle of baculovirus, two virion phenotypes are produced. One virion phenotype, called occlusion derived virus (ODV), is embeded into a crystalline protein matrix, the occlusion body. Occlusion bodies are polyhedral and contain numerous virions (genus *Nucleopolyhedrovirus*) or ovicylindrical and contain only one (rarely two) virions (genus *Granulovirus*). The ODVs of granuloviruses contain only one nucleocapsid within the viral envelope, whereas NPV ODVs can harbour a single nucleocapsid (SNPV) or multiple nucleocapsids (MNPV) per virion. A second virus phenotype, called budded virus (BV), is generated during early stages of infection. BV consist of single nucleocapsids which bud through the plasma membrane of infected cells into the extracellular fluid. Their membrane envelopes are loose-fitting and contain peplomers of a viral encoded glycoprotein.

The rod-shaped nucleocapsids are 30-55 nm in diameter and 250-300 nm in length and contain a single supercoiled, closed circular doublestranded DNA of 90-160 kb.

1.2 Species included

Among the 633 potential baculovirus species compiled by the ICTV, 15 NPV were categorised as assigned species whereas 483 NPV are tentative species. The GV contains 5

assigned and 131 tentative species (Table 1). In general, the name of a given baculovirus consists of two parts, the name of the host insect where the baculovirus was isolated from and the type of occlusion body formed, e.g. the multiple nucleocapsid nucleopolyhedrovirus of the alfalfa looper *Autographa californica* is termed *Autographa californica* MNPV or AcMNPV.

Table 1: List of assigned baculovirus species

Family: Baculoviridae	
1. Genus Nucleopolyhedroviruses	***NPV***
Autographa californica MNPV (type species)	AcMNPV
Anticarsia gemmatalis MNPV	AgMNPV
Bombyx mori NPV	BmNPV
Choristoneura fumiferana MNPV	CfMNPV
Galleria mellonella MNPV	GmMNPV
Helicoverpa zea SNPV	HzSNPV
Lymantria dispar MNPV	LdMNPV
Mamestra brassicae MNPV	MbMNPV
Orgyia pseudotsugata MNPV	OpMNPV
Orgyia pseudotsugata SNPV	OpSNPV
Rachiplusia ou MNPV	RoMNPV
Spodoptera exigua MNPV	SeMNPV
Spodoptera frugiperda MNPV	SfMNPV
Trichoplusia ni MNPV	TnMNPV
Trichoplusia ni SNPV	TnSNPV
2. Genus Granulovirus	***GV***
Plodia interpunctella GV (type species)	PiGV
Artogeia rapae GV	ArGV
Cydia pomonella GV	CpGV
Pieris brassicae GV	PbGV
Trichoplusia ni GV	TnGV

Source: (Murphy et al., 1995)

The subject of this document includes the nucleopolyhedroviruses and granuloviruses with emphasis on those that have been used for insect control. Investigations on potential

improvements of application strategies and biological properties predominantly concentrate on species/strains that are infective for lepidopteran hosts.

2.1 Morphological and physicochemical characteristics

Baculoviruses form a distinct and well characterised group of arthropod-specific viruses which can be distinguished from other viruses by a number of unique properties described in the following.

The most prominent characteristic of baculoviruses is the formation of occlusion bodies (OB). The OB are formed in the nuclei of infected cells and can be easily detected by light microscopy (phase-contrast or dark-field) as highly refractile particles.

Nucleopolyhedroviruses form polyhedra-like occlusion bodies of 0.15 to 15µm in size and contain many enveloped virions. The major component of the occlusion body is a single, viral encoded protein of Mr 25-33 x 10^3, called polyhedrin (Hooft van Iddekinge et al., 1983). Polyhedral occlusion bodies normally band at 54-56% sucrose on 40-65% w/w sucrose gradients at 100,000 g. The buoyant density of ODVs in CsCl is 1.18-1.25 g/cm^3, that of BV in sucrose is 1.17-1.18 g/ cm^3.

Electron microscopic observation of polyhedral inclusion bodies reveal two morphotypes: (i) single nucleocapsid nucleopolyhedroviruses (SNPV) contain only a single nucleocapsid within a virion, whereas the virions of (ii) multiple nucleocapsid nucleopolyhedroviruses (MNPV) harbour few to many nucleocapsids. Factors determining and regulating the formation of SNPV or MNPV have not been elucidated.

Granuloviruses generally form ovicylindrical (granule-like) occlusion bodies of 120-300 nm in width and 300-500 nm in length (Crook, 1991). The matrix protein, called granulin, is genetically and serologically closely related to the NPV polyhedrin.

SDS-polyacrylamide gel electrophoresis and serological techniques such as immuno-diffusion, immunoelectrophoresis, radioimmunassay (RIA), enzyme-linked immunosorbent assay (ELISA) and Western-blotting have been used to identify particular NPV and GV and to study their relationship (Summers and Smith, 1975; Reinganum, 1984). ELISA was demonstrated to be a rapid, specific and sensitive method for detecting and quantifying baculoviruses (reviewed by Harrap and Payne, 1979). Polyclonal and monoclonal antisera specific against occlusion body and capsid proteins revealed a high degree of cross-reactivity among NPV and GV (Smith and Summers, 1981). These traits allowed the identification of a single baculovirus species and were used for the first phylogenetic studies of different baculoviruses.

By using monoclonal antibodies in ELISA, it was possible to detect virus antigens in NPV-infected *Helicoverpa armigera* and *Choristoneura fumiferana* larvae at about 6-9 hours after virus exposure, whereas disease symptoms of the larvae could only be observed after 5-6 days (Zhang and Kaupp, 1988; Lu et al., 1995). With an antiserum against the polyhedrin component of the NPV of *Mamestra brassicae*, it was possible to detect polyhedra at a concentration of 3.13 x 10^4 polyhedra/ml by means of immunoelectrophoresis, and as low as 2.44 x 10^2 polyhedra/ml by means of ELISA. Even though this antiserum was specific to MbMNPV, it also cross-reacted with the polyhedrin of *Agrotis segetum* NPV, *Lymantria monocha* NPV and *Neodiprion sertifer* NPV (Riechenbacher and Schliephake, 1988). Similarly, polyhedrin specific antisera were developed for detection of LdMNPV and *Borrelina bombycis* NPV by ELISA in infected host larvae or cultured insect cells (Ma et al., 1984; Shamim et al., 1994). Alternatively,

a monoclonal antibody against the 42K protein of AcMNPV was used for virus detection in dead larvae and for safety investigations (Naser and Miltenburger, 1983).

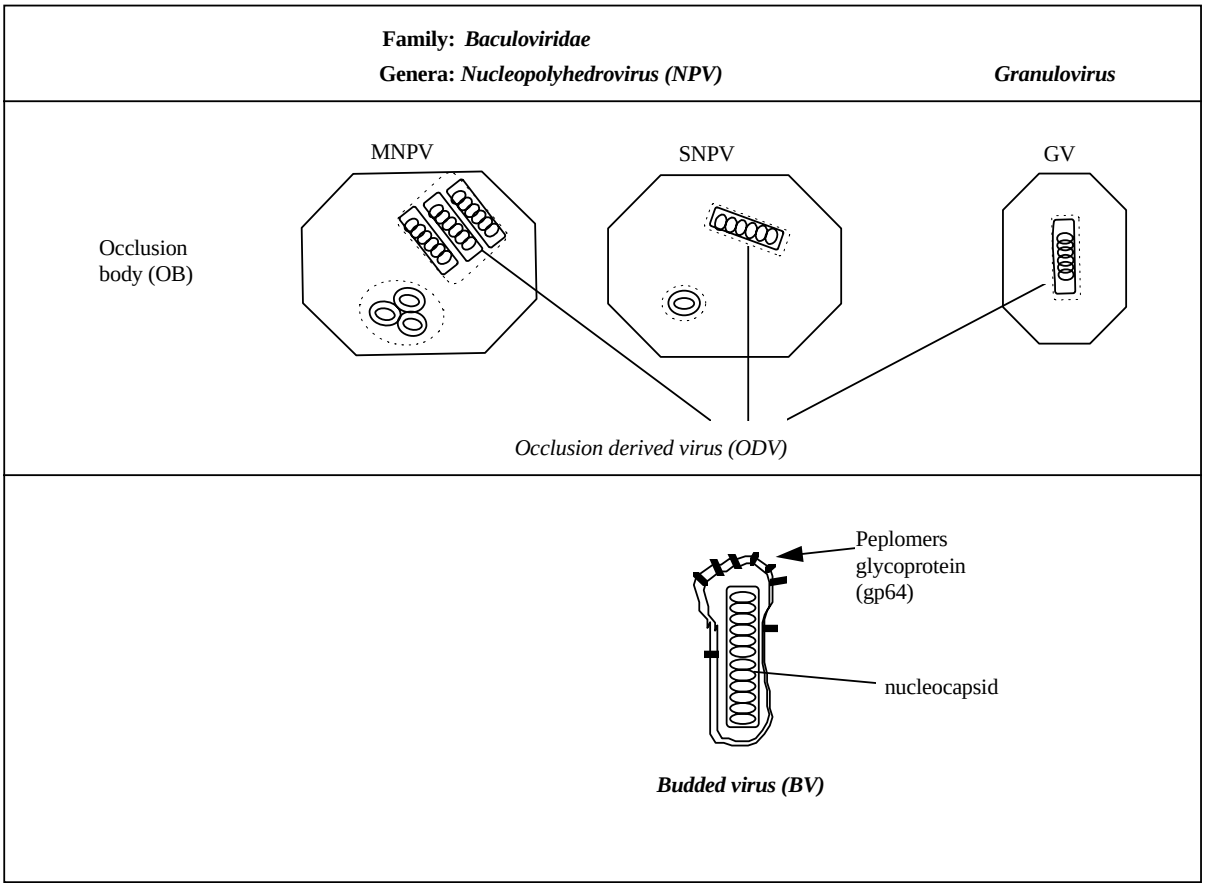

Fig. 1: Morphological characteristics of nucleopolyhedroviruses and granuloviruses

2.2 Biological characteristics

2.2.1 Host range

Host range and cross infectivity of many baculoviruses have been reviewed by Gröner (1986). The infectivity of NPV and GV to alternate hosts was typically evaluated on basis of virus infection and mortality of test larvae after oral virus application. However, these examinations are biased toward Lepidopteran species and economically important insects. So far, a standardisation of bioassays to determine host range and specificity is lacking (Cory *et al*., 1997).

Nucleopolyhedroviruses (NPV)

NPV are widely distributed among more than 400 arthropod species belonging to seven insect orders, which are Lepidoptera, Hymenoptera, Diptera, Coleoptera, Thysanura, and Trichoptera, as well as from Decapoda (class Crustacea) (Murphy et al., 1995). In general, the host range of most NPV is restricted to one or a few species of the genus or family of the host where they were originally isolated. Some of the few exceptions having a broader host range are (i) AcMNPV infecting more than 30 species from about 10 insect families, all within the order Lepidoptera, (ii) *Anagrapha falcifera* NPV infecting more than 31 species of Lepidoptera from 10 families and (iii) MbMNPV which was found to infect 32 out of 66 tested Lepidopteran species from 4 different families (Gröner, 1986; Doyle et al., 1990, Hostetter and Puttler, 1991).

Granuloviruses (GV)

GV infections have been reported for more than 100 insect species, however they appear to infect only members of the order Lepidoptera (Murphy et al., 1995). In contrast to NPV, the host range of GV appears to be even more narrow and mostly restricted to a single species.

2.2.2. Gross pathology

Nucleopolyhedroviruses

The gross pathology post infection (p.i.) of NPV infecting Lepidopteran larvae can be summarised as follows:

- Day 1 - 3 p. i.: Infected larvae normally do not show obvious signs of disease.
- Day 4 - 6 p. i.: Diseased larvae only react slowly to tactile stimuli. The larvae start to appear swollen, glossy and moribund.
- Day 6 - 7 p. i.: Diseased larvae stop feeding and begin to die. Diseased larvae of some species, e.g. *Lymantria spec.*, crawl to the top of the twigs (negative geotropism) on which they were feeding.

– Day 7-10 p. i.: Diseased larvae die and may liquefy, the cuticle ruptures and polyhedra are released.

Granuloviruses

In general three different types of gross pathology with GV can be distinguished (Federici, 1997).

(i) Many GV such as CpGV show a similar gross pathology in infected larvae as described for NPV.

(ii) Some GV, esp. many Noctuid-infecting GV also pass the midgut epithelium but then infect only fat body tissue. Infected larvae do not stop feeding and grow even bigger than healthy larvae. Larval death occurs at 10-15 days.

(iii) The third type of gross pathology has to date only been observed with *Harrisina brillians* GV. Infection is restricted to the midgut epithelium which causes heavy diarrhetic disorder and death within 4 to 7 days.

2.3 Pathological characteristics

Baculovirus pathogenesis has been most extensively studied for its type species AcMNPV, but appears to be similar in all other known baculoviruses (see below).

Most typically, virus replication of NPV occurs in the nuclei of infected host cells, whereas in GV-infected cells the nuclear membrane disrupts during the replication process and loses its integrity. Upon infection, the nuclei appear to become hypertrophic. The occlusion bodies produced by infected cells can be detected by light microscopy.

Divergent tissue tropism is observed with different viruses in their respective hosts. Most NPV specific for lepidopteran species as well as most GV establish a transient infection of the midgut epithelium and then invade other tissues such as fat body, epidermis, tracheal matrix, muscle, nerve, malphigian tubules, and reproductive and glandular tissues. In contrast, NPV specific for Hymenoptera, most Diptera, Trichoptera, Thysanura, and Crustacea as well as the *Harrisina brillians* GV were only found to infect midgut epithelium cells but not any other larval tissue (Federici, 1997).

3.1 In vivo and in vitro ***replication of baculoviruses in permissive hosts***

3.1.1 Initial stages of infection

The replication of AcMNPV has been most extensively studied in larvae of *Trichoplusia ni* and in cultured cells of *Spodoptera frugiperda* and serves as a model for NPV and GV replication in Lepidoptera (reviewed by Granados and Williams, 1986; Federici, 1997; Williams and Faulkner, 1997).

The natural route of infection is the peroral ingestion of viral occlusion bodies by larvae. In the alkaline environment of the midgut (pH > 9.5), the occlusion bodies dissolve rapidly and occlusion-derived virions (ODVs) are released. There is evidence that the dissolution of the occlusion body matrix might be facilitated by an insect derived alkaline protease which is associated with the occlusion body matrix. The ODVs pass through the peritrophic membrane (PM), a proteinaceous-chitinaceous layer which is secreted by the midgut cells to protect the midgut epithelium from direct contact with ingested material. After attachment to the microvilli of the midgut epithelium, the nucleocapsids enter the cell lumen either via fusion of the virion envelope with the epithelial membrane or by viropexis. The nucleocapsids are transported, most likely under involvement of the cellular microtubular structures, to the nucleus and become uncoated at the nuclear pore or within the nucleus where the viral DNA is released and DNA expression and replication is initiated.

3.1.2 Production of budded viruses (BVs) and occlusion derived viruses (ODVs)

The replicative cycle of baculoviruses is biphasic and generates two distinct viral phenotypes, the budded virus (BV) and the occlusion derived virus (ODV). The two phenotypes are structurally distinct and destined for two different functions, both of which are essential for virus survival in nature. ODVs are released from the inclusion bodies and infect midgut epithelium cells, where a first round of virus replication takes place. The newly produced nucleocapsids traverse the nuclear membrane, the cytosol and bud through the basal lamina of the midgut cells into the hemolymph. These budded virions (BV) acquire a new envelope which consists of plasma membrane containing peplomers of a viral encoded glycoprotein, termed gp64. Gp64 appears to be pivotal for the interaction between the BV envelope and susceptible host cells through a possible interaction with a cell membrane receptor molecule and then a final fusion with the endosomal membrane (for review see Blissard, 1996). In cultured cells the production of BV peaks during the late phase of gene expression, between 10 - 20 hr p.i., whereas the very late occlusion phase can be observed between 16 - 72 hr p.i.

For most NPV and GV infecting lepidopteran host larvae, virus occlusion is not observed in midgut epithelial cells. These cells release BV into the hemolymph which then systemically spreads the virus infection among susceptible cells and tissues. In contrast, NPV of Hymenoptera, Diptera and Crustacea and the *H. brillians* GV infect only midgut cells where occluded viruses are produced (for review see Federici, 1997). However, there is also evidence that some nucleocapsids might traverse the midgut epithelial cells without replication and bud directly into the hemolymph (Granados and Lawler, 1981).

Engelhard *et al*. (1994) used a recombinant AcMNPV mutant expressing *lacZ* reporter gene (beta-galactosidase) to study the infection pathway in fourth instars of *Trichoplusia ni*. Based on the observation of early infection of midgut tracheoblast and the tracheal matrix, they postulated that the tracheol system might directly contribute to the systemic spread of BV. This finding, however, is not supported by others who used a similar approach (Flipsen *et al*., 1993; 1995).

Altogether, the spread of BV and systemic infection starts from the midgut and continues to hemocytes, tracheal cells, fat body, muscle and nerve cells as well as reproductive and glandular tissues. In the final step of infection, occlusion bodies are formed and the nuclei are packed with occlusion bodies which causes the cellular hypertrophy and swollen appearance of the infected larvae.

The different role of BVs and ODVs in the reproduction of baculoviruses can be summarised as follows: ODVs transmit infection from one larvae to another within an insect population, whereas the budded virus (BV) spreads the infection within susceptible larval tissues.

3.2 Genes involved in host range determination

So far, a number of baculovirus genes involved in differential host cell and host larval specificity have been identified. There are several examples where the expression or deletion of these host range determinants by recombinant NPV allowed the specific extension or restriction of host specificity. These results, mainly obtained with AcMNPV, provide evidence that a baculovirus which is infective to different host species relies on specific genes to establish infection and virus replication and that these sets of genes might differ slightly from host species to host species.

Non-target effects

The specificity of the interactions of baculoviruses with arthropod and the corresponding narrow range of species which are susceptible to productive infection by a particular virus, is the basis of their innocuousness for a large spectrum of non-target organisms (Gröner, 1986). In the course of safety assessments toxicity and pathology studies have been performed on mammals, birds and other wildlife animals including beneficial insects such as the honeybee and silkworm. The studies with animals other than arthropods up to 1986 were extensively reviewed by Gröner (1986), demonstrating an absence of any adverse effects. To summarise:

(1) Toxicity studies on mammals with a variety of NPVs, using the spectrum of application routes as conventionally tested for chemical pesticides, never resulted in any indications of toxicity or pathogenicity using doses 10 to 100 times the per-acre field rate equated to a 70-kg man. Also, no indications of teratogenic or carcinogenic effects in mammals were found with challenges of NPVs .

(2) No side effects on birds after oral application and on aquatic vertebrate and invertebrate animals could be observed. Such laboratory studies were supplemented by some extensive monitoring for pathological effects of wildlife birds and mammals after (aerial) applications of different NPVs.

(3) Consistent with their restriction of infectivity to the family or at least order of their original host, no infectivity or adverse effects on beneficial insects like pollinators (bees) have been observed. Baculovirus infection interferes with the multiplication of parasitoids within the same host. This interaction seems to be described most adequately as a competition for the same resource. No productive infection of the parasitoids is observed.

(4) No geno-toxic effect was observed by cytological studies after challenging mammals or cell cultures.

Similar studies with granuloviruses are smaller in number but gave the same results.

Small mammals or birds and also parasitoids feeding on insects infected by a baculovirus may take up and transport intact baculoviruses (e.g. in their digestive tract). Excretion of infective viruses may contribute to virus dispersal.

A. Safety to vertebrates

It has long been a widely held view that baculoviruses are harmless to mammals including man (Krieg *et al.* 1980, Entwistle 1983, Cunningham 1988, Black *et al*. 1997). This seems to be true for vertebrates in general as, to date, no single instance of baculovirus pathogenicity to a vertebrate has been recorded. There is much circumstantial evidence to support this view.

In a draft statement submitted to, and endorsed by, the Working Group on the Safety of Microbial Control Agents of the Society for Invertebrate Pathology, at the International Colloquium on Invertebrate Pathology and the XIth Annual Meeting of the Society for

Invertebrate Pathology, held in Prague, Czechoslovakia, in September 1978, Krieg *et al.* (1980) reviews the safety tests that have been carried out on baculoviruses that are of actual or potential interest for the control of pest species of arthropods. It is stated that absolutely no health or environmental hazard has yet been demonstrated which would prevent the replacement of toxic chemical pesticides by baculoviruses in the control of certain pests. It is considered desirable to investigate genetic aspects of the safety of baculoviruses; such projects have already been initiated.

Baculoviruses are naturally occurring pathogens of arthropods. Their host range is exclusively restricted to arthropods. No member of this virus family is infective to plants or vertebrates. The long and close relationship between baculoviruses and arthropods has led to specific evolutionary adaptations by this family of viruses. These adaptations, which allow efficient exploitation of the arthropods by baculoviruses, have also resulted in a restriction of their host range. The best illustration of this coevolution is the existence of the occlusion body, a proteinaceous crystal that provides environmental stability to the fragile virions but is extremely sensitive to the high pH found in the insect midgut. This allows the baculovirus occlusion body to survive on a leaf surface long enough to infect a host insect yet readily release the encased infectious virions during its short transit through the insect digestive tact**. In the neutral or acidic environment of vertebrate digestive tracts, the occlusion bodies pass through the digestive tract and are either excreted intact or become inactivated during digestion.**

Baculoviruses are ubiquitously present naturally in the environment and have been used for biological insect control for more than 100 years. Circumstantial evidence for the safety of baculoviruses emerges from the history of contact between baculoviruses and humans without any detrimental effect. Baculoviruses often occur at high levels (Miltenburger, 1980) such that man, domestic animals and wildlife are generally exposed by ingestion, inhalation and contact. During natural epizootics vast quantities of virus are liberated into the environment. The occurrence of epizootics, for example in *B. mori* cultures, pass with no reports of harmful effect to man or other vertebrate. An outbreak of NPV in pine sawfly (*Neodiprion sertifer*), a common forest pest in the UK, could easily result in production of $3x10^{15}$ virus particles per hectare per year (Entwistle, 1983). Epizootics have been regularly observed in many insect species. In the Douglas-fir tussock moth, *Orgyia pseudotsugata,* NPV appears to be the main cause of the cyclic nature of outbreaks of the pest. Major outbreaks are followed by dramatic population declines caused by NPV. Large amounts of NPV are washed down into the soil where they can remain for periods of several years; Smirnoff (1976) reports persistence of 40 years. Crook and Jarrett (1991) list several other lepidopteran species for which epizootics are documented and these include *Spodoptera exigua, S. exempta, Pieris rapae, Lymantria dispar and Trichoplusia ni.*

During the 1970s, the US Environmental Protection Agency established the Guidance for Safety Testing of Baculoviruses, which also became a guideline of baculovirus safety tests in many other countries (Anonymous, 1975; Summers *et al*., 1975). This guidance included *in vivo* and *in vitro* safety studies and was applied for commercial baculovirus insecticides, such as *Helicoverpa zea S*NPV (Elcar®), *Orgyia pseudotsugata* NPV (TM Biocontrol-1) and many others. *Helicoverpa zea* SNPV was the first commercial baculovirus insecticide and is one of the most extensively tested entomopathogenic viruses (Ignoffo, 1975). During the past 40 years extensive testing of the safety of more than 30 baculoviruses resulted in a long and complete safety record (extensively reviewed by Ignoffo, 1973; Burges *et al*. 1980a, 1980b; Gröner, 1986). No adverse effect on human health has been observed in any of these investigations, indicating that the use of baculovirus is safe and does not cause any health hazards.

These tests were performed on 10 different mammalian species including rats, mice, dogs, guinea pigs, monkeys, and humans, The baculoviruses in these tests were administered by a variety of routes including orally, intravenous injection, intra-cerebral injection, intra-muscular injection, and by topical application. In all cases, there were no indications of toxicity, allergic response, or evidence of pathogenicity due to the baculovirus (Ignoffo and Heimpel, 1965). When adjusted to the weight of a 70-kg (160 lb) man, the doses in many of the tests were 10-100 times the per-acre field rate. In no case did exposure to the baculovirus result in deleterious effects to test animals. The results of these tests have been summarised in reviews (Doller, 1985; Burges *et al., 1980a;* Ignoffo, 1973, 1975).

In one study, 230 white mice and guinea pigs were exposed to *Helicoverpa zea* NPV polyhedra, occlusion-derived virus, or polyhedrin protein. Routes of administration included inhalation, feeding, and intra-dermal, intra-peritoneal, or intra-cerebral injection. A single death occurred due to acute pneumonia. All other animals remained healthy (Ignoffo and Heimpel, 1965). Long-term studies on *Helicoverpa zea* baculovirus administered orally and parentally to rats were also conducted (Barnes *et al., 1970).* These tests included a 2-year feeding study with Sprague-Dawley rats. No baculovirus-related deaths were observed, and the incidence of neoplasia between the baculovirus-fed and control groups was not significantly different. Rats that received the highest dose of baculovirus received an amount (on a weight basis) that would equate to feeding a man sufficient virus to treat 100 acres.

The safety of baculoviruses in humans has been demonstrated both directly and by indirect evidence. *Helicoverpa zea* NPV polyhedra ingested by ten men and women at a dose of almost 6 billion polyhedra over a 5-day period (Heimpel and Buchanan, 1967) caused no ill effects. Six persons exposed to the *Helicoverpa zea* NPV during 26 months of production also showed no adverse effects (Huang *et al.,* 1977). Analysis and bioassay of blood samples did not detect infectious baculovirus, baculoviral antigens, or baculoviral antibodies.

Virus production workers might be considered as individuals at risk of high level exposure to baculoviruses. Rogoff (1975) considered the case of *Heliothis zea* NPV production staff. He reported the work of Ignoffo and Shapiro whose observations indicate that in individuals in intimate contact with *Heliothis* materials over extended periods, neither clinical symptomology nor immune response indicating possible sub-clinical infection was detected, nor has there been an indication that the virus material is a general allergen.

Miltenburger (1980) considered hazard evaluation for non-target organisms and safety testing. He includes mention of several groups of workers such as farmers, forestry staff and production personnel who have received prolonged occupational exposure to baculoviruses but who have shown no adverse reactions. What the evidence of these cases point to is a high level of baculoviruses in certain environments to which individuals are exposed. Despite this, there are no documented cases of individuals suffering ill effects through natural exposure to baculoviruses.

In addition to the circumstantial or anecdotal evidence, there is a large body of more scientific evidence which also points to the non-pathogenicity of baculoviruses to mammals. Burges *et al* (1980a) provide an extensive review of safety tests on NPV and GV up to 1980.

As noted, the indigenous baculovirus load in the environment is quite high. Heimpel *et al.* (1973) have shown that polyhedron counts made on cabbage taken from store shelves or collected from the field vary between 2×10^6 polyhedra/in^2 to 7×10^7 polyhedra/in^2 on epizootic plots. Using these numbers, it has been estimated that a typical cole-slaw serving (16 in^2 of

cabbage) contains an average 1.12 x 10^8 polyhedra. Therefore, not only are large amounts of baculoviruses present in the environment, but people who eat raw cabbage in any form consume very large numbers of baculovirus particles. Thus, Krieg *et al*, 1980 have also shown that marketed autumn cabbage may contain about 10^6 polyhedra per cm^2 of leaf surface resulting from natural epizootics in cabbage loopers (*Trichoplusia ni*). This ubiquitous and harmless association between baculoviruses and humans underscores their safety.

It is clear from the extensive testing summarized above that wild-type baculoviruses pose no safety hazard to man, fish, birds, or other vertebrates. The safety of baculoviruses to vertebrates should not be changed by the addition of a gene for an insect-selective toxin, enzyme, or hormone. However, prior to registration of a recombinant baculovirus as a biopesticide, the US EPA requires data to confirm vertebrate safety. Tests designed to supply these data are specified in a set of guidelines published by the US EPA (United States Environmental Protection Agency, 1996).

Some testing on an AaIT-expressing AcMNPV has already been reported (Possee *et al.*, 1993b). These tests included a subcutaneous injection of 1 x 10^6 polyhedra into rats, oral feeding of 1 x 10^6 polyhedra also to rats, and acute dermal exposure of the recombinant virus to guinea pigs. No adverse effects were recorded for any of the test animals.

Safety tests of baculoviruses included

In *vitro and* in vivo *replication of baculoviruses in vertebrates and mammals*

The possibility of replication of baculoviruses in vertebrates and mammals was investigated by challenging many vertebrate and human cell lines with OB and BV of many baculoviruses. Although virus uptake of these cells was frequently reported, no evidences of virus replication or cytopathological effects were observed. The few early reports, which stated baculovirus replication in vertebrate cell lines (Himeno *et al*., 1967; McIntosh and Shamy, 1980) could never be demonstrated or confirmed in other laboratories. After oral uptake of baculoviruses by man, mice, chickens, rabbit, pigs, and other mammals, no specific antibody production, which would indicate replication of the virus used to challenge the host, was observed (reviewed by Gröner, 1986). In contrast, a specific immunological response against CpGV was observed in woodmice (*Apodemus sylvaticus*) which were trapped in an apple orchard sprayed with CpGV. It is conceivable that an antigenic challenge may have occurred via the nasal mucous membrane, virus replication or a negative effect to the animals was not observed (Bailey and Hunter Fujita, 1987).

Using a recombinant AcMNPV containing the *cat* gene under the control of the Rous sarcoma virus terminal repeat promoter and the β-galactosidase gene under the control of the very late polyhedrin promoter reporter gene, expression was analysed in different invertebrate and vertebrate cell lines (Carbonell *et al*., 1985; Carbonell and Miller, 1987). No *cat* or β-galactosidase activity was detected in transfected mouse or human carcinoma cells. On the other hand, recent reports showed that recombinant AcMNPV virus is efficiently taken up by human hepatocytes via an endosomal pathway. Recombinant AcMNPV carrying the *Escherichia coli lacZ* reporter gene under control of the Rous sarcoma virus promoter and mammalian RNA processing signals showed considerable expression levels in the human liver cell line HepG2, but at very low levels, or not at all, in cell lines from other tissues (Hofmann *et al*., 1995; Boyce and Bucher, 1996). Based on these findings it was suggested that baculovirus might be exploited for

liver-directed gene therapy. From the view of baculovirus safety this results also show that careful attention has to be paid to the promoters used to control heterologous gene expression in recombinant baculoviruses.

Acute oral and intra-peritoneal toxicity of mammal

Acute toxicity of *Helicoverpa zea* NPV has been tested in many mammals, e.g., rat, mouse, rabbit, guinea pig and man, at doses from 6×10^9 to 3×10^{12} OB/kg, which is up to 1000 times the average field rate per acre. Similar tests were conducted with TnSNPV, SeMNPV, AcMNPV, LdMNPV, CpGV and others (Ignoffo, 1975).

Sub-acute dietary-administration to mammals

In order to test potential subacute toxicity or pathogenicity *Helicoverpa zea* NPV was fed or subcutaneously injected to mice (about 5×10^{10} OB/kg of animal), rats (4×10^9 to 4×10^{11} OB/kg), beagle dogs (7×10^9 OB/kg), rhesus monkeys (10^8 to $1.6\ 10^{10}$ OB/kg) and man (10^9 OB/day for 5 days). Similar tests were performed with rats for OpMNPV, LdMNPV and others (Ignoffo, 1975). Furthermore, health monitoring of workers who were involved in production of HzSNPV (for the viral pesticide) for extended time periods did not show any clinical symptomatology, nor any serological response or any indications that the virus is allergenic (Rogoff, 1975).

Eye- or skin-irritation to mammals

Eye irritation tests were negative, when 1×10^5 to 20×10^5 OB/eye were applied to rabbit eyes. Skin irritation sensitivity tests were conducted with *Helicoverpa zea* NPV in rabbits, guinea pigs and man at doses of 10^3 to 10^6 OB/mm^2 of skin. Dermal and eye applications have been also conducted with *Neodiprion sertifer* NPV, AcMNPV, SeMNPV and others without any adverse reactions (Ignoffo, 1975).

Cytogenetical implications

In studies on the activation of endogenous C-type retrovirus by baculoviruses in three mammalian cell lines (mouse, rat, and man) no activation of C-type retrovirus could be detected (Schmidt, 1981). When cultured frog cells (ICT-2A) were challenged with TnSNPV no virus multiplication and no chromosome aberrations were observed over a 4-week period of time (McIntosh, 1975). No chromosome aberrations in Chinese hamster cells, mouse cells after oral uptake of BV or OB of AcMNPV and MbMNPV was observed (Miltenburger, 1978).

Carcinogenicity, teratogenicity, mutagenicity to mammals.

Potential carcinogenicity of *Helicoverpa* NPV were conducted in mice 10×10^9 to 4×10^{11} OB/kg) or rats (3.5×10^{12} OB/kg), teratogenicity tests were performed in rats at a dose of 10^9 OB/kg. No evidence of carcinogenic or teratogenic effects was found (Ignoffo, 1975).

In contrast to the NPVs, the body of safety testing has been considerably smaller for the GVs (Saik *et al*, 1990). Bailey *et al* (1982) performed tests in which they examined small mammals exposed to field spraying of *Cydia pomonella* GV. Detectable levels of antibody were found in these animals (Döller, 1985). Döller and Huber (1983) collected faecal samples daily for three weeks after exposing mice to virus. Biologically active virus was detected along with

antibody production, however, within 80 days post feeding no virus-specific antibodies were detected by radio immunoassay. Tests for vertical transmission of the virus were negative. Sera from young born of inoculated mothers were found free of antibodies. Several other studies with GV are reported by Saik *et al* (1990) all of which gave similar results

Thus today we have considerable experimental evidence for the non-infectivity of vertebrates or their cells by baculoviruses, which are not known to be toxic or pathogenic to vertebrate animals. Therefore the classical dose-response relationship cannot be established and only the non-effect of doses can be demonstrated (Krieg *et al,* 1980).

The development and widespread adoption of mammalian tissue culture techniques has allowed a proliferation of baculovirus testing in mammalian cells. However, the World Health Organisation (1973) points out that insect viruses tend to be less specific *in vitro* than *in vivo*. The fact that a virus is capable of infecting cultured cells from a particular host does not necessarily imply that it is a pathogen for that host. In the macroscopic world of course, the gut provides an additional barrier or ecological separation between baculoviruses and mammalian cells.

The issue of allergenicity has not yet been fully answered although Burges (1981) stated that no allergic reactions have been reported to insect pathogenic viruses. In all safety tests on registered insect pathogens, no allergies have been reported. Slight eye irritation from extremely high doses of Gypcheck, which contains gypsy moth NPV, was due to insect remnants (Lewis, 1981).

B Aquatic systems

Baculovirus applications, especially those made aerially inevitably lead to viruses reaching rivers or lakes. Infected insects adjacent to waterways may fall into water, and some lepidopteran species represent during their larval stage a significant dietary component in certain aquatic habitats. Dejoux and Elouard (1990) considered the infectivity of baculoviruses to freshwater invertebrates and fish. The results of many studies which have investigated pathogenicity in invertebrates such as *Notonecta* spp, *Daphnia* spp, the penaeid shrimps *Penaeus* spp of several NPVs including *A. californica* NPV have shown no evidence of infectivity. Several species of Salmonidae have also been tested as have fish cell cultures and these also gave negative results. Rainbow trout, white suckers, bluegills and brown trout are also among those reported by Dejoux and Elouard (1990) as being unaffected by high baculovirus doses. Dejoux and Elouard (1990) conclude that these results point to baculoviruses being non-toxic to aquatic invertebrates as well as fish, although they add a cautionary note that the effects of long term exposure have not been studied.

Relatively few microbials have been tested for safety in selected non-target estuarine and marine species (Couch and Foss, 1990). Lightner *et al* (1973) carried out the first test of an insect baculovirus in non-insect arthropods - the white shrimp, *Penaeus sertiferus* and the brown shrimp *Penaeus aztecus*. Early and late juvenile instars were exposed to *A. californica* NPV but there was no exposure-related mortality.

The grass shrimp, *Palaemonetes vulgaris* (Crustacea, Decapoda) was selected by Couch *et al.* (1984) as a representative aquatic species against which to test the nontarget effects of purified *A. californica* NPV. Serological tests, histological and electron microscope examinations

revealed no evidence of viral infection in test individuals following 30 days dietary exposure to high doses of *A. californica* NPV.

Recently, toxic effects were observed with a larval test that was considered to be useful for assessing adverse effects of microbial pest control agents on non-target bivalves because of its simplicity, precision, and sensitivity. Larvae of the coot clam *Mulinia lateralis* were challenged for 48 h during the straight hinged stage of development with the LdMNPV at a density of 10^6 OB/ml. Mortalities observed were significantly higher than those obtained with a heat killed control (Gormly *et al*., 1996).

C Non-target Invertebrates

Non-Lepidopteran Arthropods

With the safety of baculoviruses to vertebrates firmly established, attention has focused on the safety of baculoviruses to non-target invertebrates. Most of the wild-type baculoviruses being-considered for use as biopesticides and all of the genetically engineered baculoviruses have host ranges limited to the order Lepidoptera. This specificity makes baculoviruses an excellent tool for use in integrated pest management programs. By controlling specific insect pests in the field and leaving the complement of beneficial arthropod predators and parasites unharmed, baculoviruses augment natural control of pests rather than supplant it, as do many chemical pesticides.

Most emphasis has been laid on the effect of baculoviruses on honeybees (*Apis mellifera*) and other such useful insects as the silkworm (*Bombyx mori* and others). Gröner (1990) provides tables summarising tests of baculoviruses against these two groups. No deleterious effect of baculoviruses on honey bees has ever been found and only two cases of cross-infectivity of baculoviruses to non-homologous silkworm hosts are cited. The first is that of *A. californica* NPV in the silkworm *Anisotera senatoria* (Lep.:Saturniidae) and the second is that of *B. mori* NPV in *Samia cynthia* (Lep.:Saturniidae). There are no examples of cross-infectivity to *B. mori*.

Concern has been expressed in India over use of *Heliothis zea* baculovirus and the possibility of adverse effects on the silk moth *B. mori*, but results of cross-infectivity studies showed no harmful effects of *H. zea* NPV on *B. mori* (Padhi and Maramorosch, 1983). Viron/H had significant effect but was found to contain almost pure *B. cereus*.

The tasar silkworm, *Antheraea mylitta* (Dru.), as well as *B. mori* were also tested in India by injection for susceptibility to NPV of the armyworm *Mythimna* (*Pseudaletia*) *separata* (Wlk.) but no symptoms of infection occurred (Dhaduti and Mathad, 1979). The *M. separata* NPV is successfully used in the biological control of this species in India.

One experimental approach to use vectorised transport by honey bees as a non-intrusive means for virus dispersal has been field tested, although concerns on safety and non-target innocuousness of baculoviruses have not been allayed in the public arena. An applicator in a specifically designed substructure of a conventional beehive caused honey bees to take up (by surface contamination) and disseminate a talc formulation of HzSNPV into fields of *Trifolium incarnatum*. Increased HzSNPV induced mortality was observed in the clover fields foraged by the bees. A good persistence of baculovirus infectivity in honey was noted. An increased knowledge about the intersection of bee and target organism behaviour determining the virus transmission was considered to be essential, in order to further investigate the feasibility of the approach (Gross *et al.*, 1994b).

Recombinant DNA Technology and advances in genomic mapping have been combined to improve the insecticidal qualities of naturally occurring baculoviruses (reviewed by Bonning and Hammock 1996). Although the host specificity of wild-type baculoviruses make them ideal components of integrated pest management (IPM) programs, their usage in pest suppression has been limited to plants able to tolerate moderate levels of feeding damage while viral infection slowly kills the pests. Recently, the lengthy infection cycle has been significantly shortened by incorporating insecticidal toxin genes into the viral genomes. Genes coding for diuretic hormone (Maeda 1981), juvenile hormone esterase (Hammock *et al*. 1990), maize mitochondrial protein (Korth and Levings 1993), mite neurotoxin (Popham et al. 1997, Tomalski and Miller 1991), and insect-specific scorpion neurotoxins (Carbonell *et al.* 1988, Maeda *et al*. 1991, McCutchen *et al*. 1991, Stewart *et al*. 1991) have been successfully incorporated to produce recombinant baculoviruses able to cause lethal infections and terminate feeding in their respective host larvae more rapidly than their wild-type progenitor viruses.

The addition of a toxin, enzyme, or hormone gene to the genome of a baculovirus raises the question, "Would beneficial insects such as predatory spiders or parasitic wasps be harmed by the ingestion or parasitism of an insect larvae infected with the recombinant baculovirus?" For example, could the finite amount of toxin expressed in a target insect by such a recombinant virus result in death of a predatory arthropod or larvae of a parasitic wasp? To address this issue, a number of studies have been conducted with various recombinant AcMNPV viruses that express the insect-specific toxin AaIT.

Recombinant viruses of *Autographo californica* nucteopolyhedrovirus (AcMNPV) and *Helicoverpa zea* NPV (HzSNPV) have been engineered to express the insect-selective toxin genes of the scorpion *Leirus quinquestriatus hebraeus* (Birula) (Scorpiones: Buthidae) (abbreviated as LqhIT2). These viruses naturally infect *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) and *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), key pests of cotton, *Gossypium hirsutum* (L.) (Groner 1986). Non-target species, such as predators and parasitoids of the heliothine pest complex, are not able to support viral replication; thus they should not be directly affected by these toxin-producing viruses (Huang *et al*. 1997), However, if these scorpion toxin recombinants were applied to cotton, natural enemies would be likely to indirectly encounter the expressed toxins by consuming infected heliothines actively producing the toxins.

Predators

Gröner (1990) provides a list of 18 instances of viruses being dispersed by predators in most cases as a consequence of there being viral activity in the predator's faeces. Predators include species in the Orthoptera, Dermaptera, Heteroptera, Coleoptera and Hymenoptera.

Predators seem to be little affected by feeding on infected prey. Part of this resistance maybe due to the more acid digestive systems of predators. Their possible role as disseminators of defecated polyhedra is mentioned by Vinson (1990). Boucias *et al* (1987) have suggested that invertebrate predators feeding on infected *Anticarsia gemetalis* larvae in soybean fields are potential dispersal agents of AgNPV while not being susceptible themselves.

Several studies have been conducted that examine the effect of feeding larvae infected with a recombinant baculovirus to several generalised insect predators. The predatory arthropods include a carabid beetle *(Pterostichus madidus) (Possee et al.*, 1993b), the green lacewing (a predatory Neuroptera; *Chrysopa carnae),* a predatory Hemiptera *(Orius insidiosus) (*Heinz *et al.*,

1995), a funnel web spider *(Ixeuticus* spp.), and the Chinese mantid (Ten*odera aridifolia sinensis)* (American Cyanamid, 1994; Treacy *et al., 1997).* No adverse effects were observed. Furthermore, honeybees *(Apis mellifera)* injected with the budded form of AaIT-expressing recombinant AcMNPV also showed no ill effects (Heinz *et al.,* 1995).

Indirect data on the safety of a recombinant baculovirus to non-target arthropods also comes from surveys of their population densities during two small field trials of an AaIT-expressing AcMNPV conducted by American Cyanamid during 1995. Results showed that population levels of nontarget arthropods (18 different non-lepidopteran insect families in addition to various spiders) were not adversely affected by weekly applications of doses of up to 2×10^{12} polyhedra per hectare (Treacy and All, 1996; Treacy *et al.,* 1997).

Laboratory studies have examined both predator-host-virus and parasitoid-host-virus interactions. Predators apparently do not suffer deleterious effects from consuming wild-type or recombinant virus-infected lepidopteran larvae (Abbas and Boucias 1984, Ruberson *et al*. 1991, Heinz *et al*. 1995, McNitt *et al*. 1995, Li *et al*. 1999). Similarly, in a field study conducted by Smith *et al*. (2000), no differences were detected among recombinant and wild-type viruses in the densities and diversities of the predator populations in cotton up to 6 d after initial applications of the viruses. After feeding on infected larvae, however, results from laboratory studies have shown that predators excrete viable wild-type baculovirus (Boucias *et al*. 1987, Young and Yearian 1987, Fuxa *et al*. 1993, Vasconcelos *et al*. 1996). Smith *et al*. (2000) was able to detect viral DNA in 2% of predators collected from virus-treated cotton fields, confirming they had fed upon infected heliothines. Thus, mobile predators could represent one potential avenue by which a recombinant baculovirus may survive for longer than would occur on treated plants and be dispersed outside of target agroecosystems through predator frass.

Parasitoids

Researchers have suggested that baculoviruses used for biological control might be vectored by hymenopteran parasitoids. However not all infected hosts are accepted as oviposition sites by parasitoids. Kelsey (1962) reported that *C. glomerata* would not oviposit in virus-infected laboratory *Pieris rapae* larva. Host discrimination was reported for *Cotesia melanoscelus*, parasitizing *Lymantria dispar*. The parasitoid-host contact did not differ between infected and uninfected host *L. dispar* larvae but ovipositional attempts were significantly greater in uninfected larvae (Versoi and Yendol, 1982). *Hyposoter exiguae* females on the other hand do not discriminate between virus-infected and non-infected *Trichoplusia ni* host larvae.

The progeny of parasitoids which oviposit in infected hosts are not likely to survive. *H. exiguae* larvae in hosts exposed to virus prior to parasitisation died when their hosts died from virus infection (Beegle and Oatman, 1974). In most cases parasitoid mortality is simply due to premature death of the virus-infected host although viruses may also compete for nutrients. Where parasitism precedes inoculation by a significant margin the chances of successful emergence are improved. Parasitism can influence the level of food consumption, both increasing and decreasing it, and this must influence exposure to viruses. In addition parasitism itself appears to influence susceptibility to virus infection. Examples of both positive and negative correlations exist (Vinson, 1990). Beegle and Oatman (1974) found that non-parasitised *Trichoplusia ni* were twice as susceptible to *T. ni* NPV (at the LD_{50} level) compared with those parasitised by *Hyposoter exiguae* (Hymenoptera: Ichneumonidae).

Recombinant baculoviruses are more likely to negatively affect parasitoids than predators. Larval endoparasitoids developing within infected hosts not only resume diseased tissues, but they may be bathed in the expressed insect toxins. In addition, their host could die more quickly than hosts infected with wildtype virus, resulting in incomplete parasitoid development. A general pattern detected from laboratory studies with wild-type baculovirus is that if infection precedes or occurs 0-2 d after parasitisation of the host, larval parasitoids are not likely to complete development because of host death (Beegle and Oatman 1974, Murray *et al*. 1985, Teakle *et al*. 1985, Eller *et al*. 1988, Brown *et al*. 1989). Larval hosts parasitised at least 3 d before consuming wild-type virus inoculum are less susceptible to becoming infected and are more likely to support parasitoid development to completion.

In a laboratory study, McCutchen *et al*. (1996) showed the percentage of larval *Microplitis croceipes* Cresson endoparasitoids successfully completing development in *H. viriscens* infected with wild-type virus to be similar to parasitoid emergence percentages observed for hosts infected with recombinant viruses. However, adult parasitoids that developed within infected by a scorpion toxin recombinant virus were significantly smaller than adults that emerged from uninfected or wild-type virus-infected larvae (McCutchen *et al*. 1996). In addition, parasitoids could represent another potential avenue for recombinant virus movement. Parasitoid adult females that either developed successfully in virus-infected hosts or oviposted in infected hosts have been shown to disseminate wild-type virus particles and generate infection in other susceptible larvae via oviposition or environment contamination (Iragbon and Brooks 1974, Levin *et al*. 1983, Brown *et al*. 1989, Caballero *et al*. 1991, Sait *et al*. 1996). Using PCR analysis, McCutchen *et al*. (1996) found that ~40% of *M. croceipes* emerging from recombinant virus-infected hosts in the laboratory tested positive for recombinant viral DNA. These studies indicate that parasitoids may be able to acquire and to move recombinant viruses out of the target area via parasitoid opposition as well.

For most parasitoid-virus studies discussed above, the host larvae were fed a high concentration of viral occlusion bodies on a small plug of artificial diet. This inoculation method insured severe infection of the hosts. Consequently, large doses of virus induce different reactions by the host larvae. In *Trichoplusia ni* (Hubner) (Lepidoptera: Noctuidae), a large dose of virus fed to larvae (<1,000 LD100 units) caused larvae to stop feeding, their gut tracts degraded, and their bodies shriveled soon after consumption (Volkman and Keddie 1990). High concentrations are not likely to be encountered by host pest larvae in the field. When larvae were fed a substantially smaller inoculum (~100 LD100 units), feeding and other behaviors were unaffected for at least 48 h (Volkman and Keddie 1990).

The effects of recombinant and wild-type viruses on a non-target beneficial wasp, the endoparasitoid *Microplitis croceipes*, have now been studied under field conditions in cotton using the noctuid *Heliothis virescens* as a host (Smith *et al*. 2000). Two scorpion toxin recombinant and their wild-type progenitor viruses had similar minimal impacts on emergent wasps and percentage emergence, sex ratio or size of the parasitoids did not differ between recombinant, wild-type or control treatments. None of the emergent *M. croceipes* had detectable levels of viral DNA and the probability of virus dispersal via parasitoids was judged low**. However, the possibility was not excluded that recombinant viruses could be vectored mechanically by parasitoids that emerged from or oviposted in diseased host larvae**.

Non-target Lepidopterans

Most baculoviruses have host ranges limited to a few closely related species within the same family of lepidopterous insects. The host range of AcMNPV appears to be much broader, infecting several dozen lepidopteran species in several different families. This host range for AcMNPV has been determined by laboratory bioassays where insects are exposed to high doses of virus under controlled conditions. The sensitivity of these various insect species to the virus varies greatly. Insect species that are efficiently infected only at high doses are often included in the host range of the virus as semi-permissive species. Thus, laboratory testing can overestimate the number of species that are infected under natural conditions. The effective host range of AcMNPV in nature is probably much more restrictive, with only the rare semi-permissive individual acquiring a high enough dose to become infected under field conditions.

It has been postulated that the expression of a highly active toxin by a recombinant baculovirus could increase the effective host range of the virus. In a practical sense, this would be a benefit, since rarely does only one insect pest species invade a farmer's field. However, any change in host range would need to be carefully evaluated. A comparison of the LD_{50} of recombinant AcMNPVs expressing AaIT to the wild-type parental virus has been made for a large number of lepidopteran species. In total, 48 species that exhibit varying degrees of sensitivity to AcMNPV have been tested for their relative sensitivity to an AaIT-expressing AcMNPV compared to wild-type AcMNPV. These species belong to nine separate families of the order Lepidoptera. The data from these studies do not support the hypothesis that expression of the toxin increases the inherent host range in recombinant baculoviruses. In fact, the addition of the AaIT gene to AcMNPV did not alter the relative infectivity of the baculovirus (Possee *et al.*, 1993b; American Cyanamid, 1994, 1996).

The data comparing the LC_{50} for the recombinant AaIT-expressing virus versus the wild-type virus indicate a four- to tenfold decrease in LC_{50} for the recombinant virus on the semi-permissive insect, *Helicoverpa zea.* Interestingly, a similar result was obtained for a second recombinant AcMNPV that expresses a different scorpion toxin gene, designated LqhIT2 (DuPont, 1996). In each case, determination of the LC_{50} was carried out at 12 and 6 days post-infection, respectively, time-points that give an accurate estimate of field performance. However, detailed observation of the course of AcMNPV infection in *Helicoverpa zea* larvae indicated that these early time-points may not be predictive of the final mortality for the wild-type virus on this and perhaps other semi-permissive species. A test was carried out on *Helicoverpa zea* where relative LC_{50}'s for the AaIT-expressing recombinant and the wild-type virus were calculated at various time-points up through emergence of the treated larvae as adult moths. Results show that the true LC_{50} was established early in the test for the recombinant due to its faster speed of kill. Larvae infected with the wild-type virus continued to die throughout the test. At 33 days, the time-point where all surviving adults had emerged, the LC_{50} for the wild-type virus reached the equivalent to that of the AaIT-expressing recombinant (American Cyanamid, 1996). These data indicate that, in some species, the addition of an insect-specific toxin gene may result in an incorrect LC_{50} calculation if care is not taken to determine mortality at a point where the wild-type infection process has run its full course in all of the test larvae.

Risk Assessment

With the exception of BT, most genetic modification has focused on the insect baculoviruses, which need to be ingested to initiate infection. However, the presence of the oclusion body allows them to persist outside the host for many years if not exposed to UV radiation.

As detailed above and expounded by Cory (2000) the risks to non-targets either lie in direct effects of the virus or indirectly through gene transfer to other viruses resulting in further non-target susceptibility. It can be concluded that baculoviruses are not infective to vertebrates or plants and essentially limited to the order that they have been isolated from within the Insecta. Since genetic modifications have been restricted to Lepidopteran isolates, species directly at risk are only other non-target lepidoptera.

Although host ranges are critical, host range testing is time-consuming and expensive with detailed studies being rare. However, as already indicated, AcNPV has been shown to infect a wide range of lepidopteran hosts from up to 13 different subfamilies (in Cory 2000). There was neither a discernible pattern to AcNPV host range nor any predictability in susceptibility. As most of NT species are likely to have intermediate susceptibility, more field-testing is required to establish whether responses vary to different or genetically modified baculoviruses. Nevertheless, it is practically difficult to:

i) test all potential NTs within a test area

ii) prevent baculoviruses spreading far beyond original release sites and encountering additional NTs.

iii) Account for persistence as baculoviruses can survive for many years in soil.

Thus, precaution is still necessary as little is known about baculovirus behaviour in alternate host species of varying susceptibility nor yet about the potentially wider natural range available to genetically modified forms such as AcNPV if released in the field.

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