Organisation de Coopération et de Développement Economiques
Organisation for Economic Co-operation and Development

08-Jan-2002

English - Or. English

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

ENV/JM/MONO(2002)1
Unclassified

Series on Harmonization of Regulatory Oversight in Biotechnology, No.20

CONSENSUS DOCUMENT ON INFORMATION USED IN THE ASSESSMENT OF ENVIRONMENTAL APPLICATIONS INVOLVING BACULOVIRUSES

JT00119078

English - Or. English

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Series on Harmonization of Regulatory Oversight in Biotechnology

No. 20

Consensus Document on Information used in the Assessment of Environmental Applications involving Baculovirus

Environment Directorate

Organisation for Economic Co-operation and Development

Paris 2002

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FOREWORD

The OECD's Working[1] Group on Harmonization of Regulatory Oversight in Biotechnology decided at its first session, in June 1995, to focus its work on the development of ***consensus documents*** which are mutually acceptable among Member countries. These consensus documents contain information for use during the regulatory assessment of a particular product.

This document contains general information on baculoviruses such as organism characteristics, behavior in the environment, their history of use and interactions, as well as environmental safety considerations.

Germany served as lead country in the preparation of this document. It has been revised on a number of occasions based on the input from other Member countries. It is intended for use by regulatory authorities and others who have responsibility for assessments and by those who are actively involved with genetic improvement and intensive management of the genus.

[1] In August 1998, following a decision by OECD Council to rationalise the names of Committees and Working Groups across the OECD, the name of the "Expert Group on Harmonization of Regulatory Oversight in Biotechnology" became the "Working Group on Harmonization of Regulatory Oversight in Biotechnology."

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PREAMBLE

OECD Member countries are now commercialising and marketing agricultural and industrial products of modern biotechnology. They identified the need for harmonization of regulatory approaches to assess these products in order to avoid unnecessary trade barriers.

At the end of 1992, a project entitled Environmental *Applications of Modern Biotechnology* (formerly known as *Industrial Products of Modern Biotechnology*) was initiated under the auspices of the Environment Policy Committee (EPOC). The scope of the project includes micro-organisms for use in applications such as bioremediation, bioprevention, biomining and bioleaching. Its objective is to assist countries in their regulatory assessment of such applications and to facilitate international harmonization.

The first step in the project was to identify the information used by regulatory authorities in Member countries when assessing these applications. The results, which show considerable commonality among OECD Member countries, are described in OECD Environment Monograph No. 100, *Analysis of Information Elements Used in the Assessment of Certain Products of Modern Biotechnology* (OECD, Paris 1995).

Building on this work, the ***types*** of information used to address the elements in Environment Monograph No. 100 were identified (see Environment Monograph No. 117, *Industrial Products of Modern Biotechnology Intended for Release to the Environment: The Proceedings of the Fribourg Workshop).* This document shows that much of the information used in regulatory assessments is not case-specific, but would be equally applicable to many assessments involving the same or similar host organisms. It was also found that much of this information, such as that related to the biological properties of the host organism, is available in the scientific literature.

At its first session, in June 1995, the Working Group on the Harmonization of Regulatory Oversight in Biotechnology instituted the development of ***consensus documents***, which are ***mutually acceptable*** among member countries, as an initial step in efforts to facilitate harmonization. The goal is to identify common elements in the safety assessment of environmental application of modern biotechnology, to encourage information sharing and prevent duplication of efforts among OECD member countries. In order to reflect the most current scientific and technical developments, it was agreed that the consensus documents would be "living documents", developed in a "modular" fashion. This allows for flexibility through updating the consensus documents and adding components relevant to the subject addressed in the initial consensus document. Therefore, users of the consensus documents are invited to inform the OECD Secretariat of such relevant new scientific and technical information and proposals for additional components.

In this consensus document, the focus is on non-case-specific information, which is readily available from the scientific literature, related to Baculoviruses.

A. GENERAL CONSIDERATIONS

1. Subject of Document: species included and taxonomic considerations

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, 1986b; 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 *Nucleopoly-hedrovirus*) 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 (Fig. 1 in A 2.1).

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 califonica* is termed *Autographa califonica* 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. Characteristics of the organism which permit identification, and the methods used to identify the organism

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.

2.1 Morphological and physicochemical characteristics

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 immunodiffusion, 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).

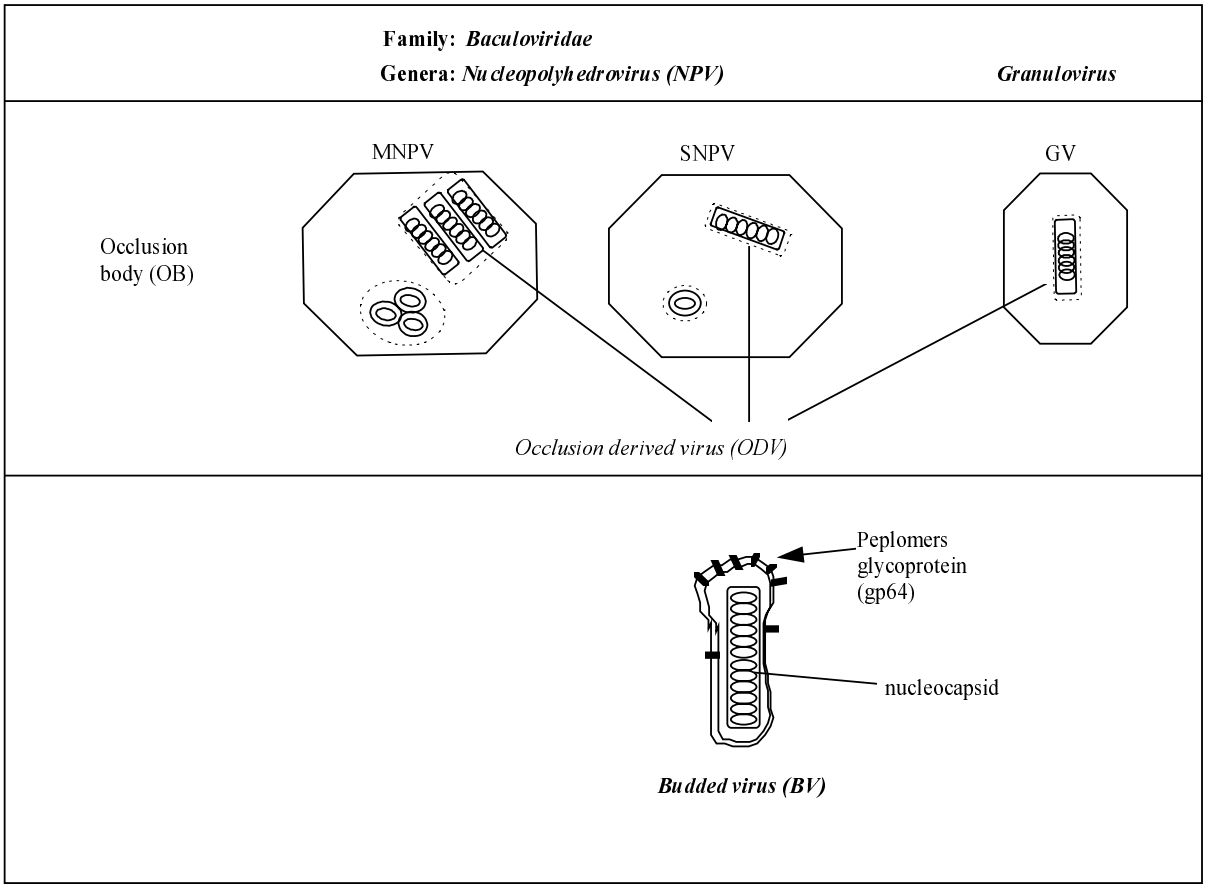

Fig. 1: Morphological characteristics of nucleopolyhedroviruses and granuloviruses

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. (see also C 23.3)

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).

2.4 Genetic characteristics

2.4.1 Restriction mapping

Early studies of the size and GV content of baculovirus DNA were based on a number of different methods, such as electron microscopy, melting point determination and reassociation kinetics, ultra-centrifugation in sucrose gradients and chemical analyses.

Restriction endonuclease analysis of isolated DNA has been for many years one of the most important and powerful tools for characterisation and identification of DNA viruses including baculoviruses (Rohrmann et al., 1978). This method allows investigators (i) to identify unequivocally the infecting agent, e.g. when a latent baculovirus is activated (Jurcovicova, 1979), (ii) to distinguish among different viruses or virus isolates, which infect the same host species and show similar biological properties (Vlak, 1980), and (iii) to recognise the identity of a virus infecting different host species (Miller and Dawes, 1978). Furthermore, restriction endonuclease analysis is indispensable for the construction of

physical maps, which assign the position of the restriction fragments to each other. By convention, the smallest restriction fragment containing the gene encoding polyhedrin or granulin was chosen as the zero point of baculovirus physical maps (Vlak and Smith, 1982). To date restriction maps have been constructed for 18 NPV and 5 GV (Table 2).

Table 2: Restriction maps of different nucleopolyhedroviruses and granuloviruses

Virus species	**References**
Nucleopolyhedroviruses	
AcMNPV	Miller and Dawes, 1979; Smith and Summers, 1979; and 1980; Cochran et al., 1982; Brown et al., 1984
AgMNPV	Vlak, Johnson and Maruniak, 1989
Amsacta albistriga NPV	Anuradha et al., 1995
Anagrapha falcifera NPV	Chen et al., 1996
BmNPV	Maeda and Majima, 1990
Buzura suppressaria SNPV	Qi and Huang, 1987; Liu et al., 1993
CfMNPV	Arif et al., 1984
HzSNPV	Knell and Summers, 1984
Helicoverpa (=Heliothis) armigera NPV	Jin and Cai, 1987
LdMNPV	Smith et al., 1988; McClintock and Dougherty, 1988
MbMNPV	Possee and Kelly, 1988
OpMNPV	Chen et al., 1988
Panolis flammea NPV	Possee and Kelly, 1988; Weitzmann et al., 1992
SeMNPV	Wiegers and Vlak, 1984; Heldens et al., 1996
SfMNPV	Maruniak et al., 1984
Spodoptera littoralis NPV	Croizier et al., 1989
Spodoptera litura NPV	Meizhen and Yiquan, 1990
Granuloviruses	
ArGV	Smith and Crook, 1988
CpGV	Crook et al., 1985; Crook et al., 1997
Cryptophlebia leucotreta GV	Jehle et al., 1992
TnGV	Hashimoto et al., 1996
Xestia c-nigrum GV	Goto et al., 1992

2.4.2 DNA homology

Early homology studies of baculovirus strains have been accomplished by comparative restriction analysis and by the estimation of sequence relationships from the number of co-migrating restriction fragments in agarose gels by using the formula of Upholdt (1977). However, this method is only useful for closely related virus strains with more than 90% sequence homology. It does not take into consideration that many genomic variations are due to small sequence insertions and deletions which result in differences of the restriction profiles even of very closely related virus strains.

DNA hybridisation techniques, such as Southern blot, dot blot and cross blot analysis, have been widely applied for identification and quantification of the intergenomic relationship of many baculoviruses. These methods permit the identification of heteroduplex formation of two different DNAs if they show more than 67% sequence identity (low stringency) or more than 85% identity (high stringency) (Howley et al., 1979).

Smith and Summers (1982) analysed the genomic interrelationship of 18 baculoviruses by restriction analysis, Southern hybridisation and semi-quantitative dot blot hybridisation. They found that under low stringency conditions all viral DNAs showed detectable cross hybridisation. Their results also corroborated the earlier classification of baculoviruses into three subgroups (nuclear polyhedrosis viruses, granulosis viruses and non-occluded baculoviruses, see A 1.1).

The combination of physical mapping and cross hybridisation indicated that the genomes of baculoviruses are similarly arranged. Leisy et al. (1984) showed that OpMNPV and AcMNPV are two distinct viruses with a colinear genomic arrangement. Similar observations were made for the genomes of CfMNPV and AcMNPV (Arif et al., 1984), MbMNPV and *Panolis flammea* NPV (Possee and Kelly, 1988) as well as for ArGV and CpGV (Crook et al., 1997) and for *Cryptophlebia leucotreta* GV and CpGV (Jehle et al., 1992).

3. Information on the recipient organism's reproductive cycle

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 The different temporal phases of gene expression

The following stages and secondary infections initiated by budded virions (BV) of host tissues or cultured cells are thought to be similar. In the early stages (8 hr p. i.) of infection the nucleus becomes hypertrophic and a virogenic stroma is formed where DNA replication and nucleocapsid assembly take place. Host cell protein synthesis is completely shut off by 24 hr p.i.

Viral gene transcription and expression follows a temporally co-ordinated cascade. Early and delayed early genes (α and β genes) are transcribed by a host dependent RNA polymerase II, which is sensitive to alpha-amanitin. These genes are necessary for regulation of viral gene transcription, for viral DNA replication, and late gene expression. Their promoters resemble those of host genes in having a CAGT transcription initiation site which is 25-31 bp downstream of TATA box and are recognized by nuclear extracts of uninfected cells.

Transcription and expression of late genes (or γ genes) involved in DNA replication, production of structural proteins and budded virions (BV) occurs between 8 and 24 hr p.i.

These genes as well as the very late (or δ genes) (see below) have a universal and invariant (A/G/T)TAAG transcription initiation site and are transcribed by a viral encoded RNA polymerae insensitive to alpha-amanitin. Five essential (*p143, ie-1, lef-1, lef-2,* and *lef-3*) and five stimulatory genes (*dnapol, p35, ie-2, lef7,* and *pe38*) were identified to be involved in AcMNPV DNA replication. In addition, transient expression studies using the chloramphenicol acetyl transferase (*cat*) reporter gene under the control of the late and very late promoters (*vp39, p6.9, polyhedrin, p10*) showed that eighteen AcMNPV genes (those necessary for DNA replication *and lef-4, lef-5, lef-6, lef-8, lef-9, lef-10, lef-11,*

p47, and 39K) were essential for optimal expression from these promoters (Lu and Miller, 1995a). DNA replication and late viral gene transcription can be blocked by aphidicolin (Miller et al., 1981; Rice and Miller, 1986).

Expression of very late genes (or δ genes) begins about 18 - 24 hr p.i. and is characterised by a dramatic increase of *polh* and *p10* transcription and expression. *Polh* encodes the polyhedrin (its homologue in granuloviruses is called granulin gene), which is the major component of the occlusion body matrix. The *p10* gene product is associated with the formation of extensive fibrillar structures in the nucleus and cytoplasm of infected cells (Quant-Russell et al., 1987; van der Wilk, et al., 1987). Although the biological function of *p10* has not been convincingly elucidated so far, it was suggested that it might contribute to the disruption of the nuclear membrane and to the release of occlusion bodies from infected cells (van Oers et al., 1993; Williams et al., 1989).

Since both *polh* and *p10* are not required for DNA replication and are hyperexpressed during cell infection, their strong promoters were exploited for the development of the baculovirus expression vector systems. The coding region of these genes can be replaced by exogenous genes resulting in the production of high levels of the foreign protein (Smith et al., 1983; Vlak et al., 1990).

3.1.3 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 (see Fig. 1 in A 2.1). 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 Behaviour in semi- and non-permissive insect cells

Baculovirus gene expression, replication and reproduction is only possible if one of the virion phenotypes (BV or ODV) is able to enter a permissive host cell. There are a number of viral factors and host responses which are necessary for a productive infection. In a non-compatible baculovirus-host cell interaction, the baculovirus gene expression and replication is blocked at a early stage. In order to analyse the genetic factors involved in host specificity and baculovirus-host cell interaction, a number of studies on the effect of virus on semi- and non-permissive insect cell lines were conducted. Infection studies with vertebrate and mammalian cells will be discussed in Section B.

When *T. ni* cells (TN-368) were infected with SfMNPV, typical early cytopathological effects such as nuclear hypertrophy and the formation of a virogenic stroma could be observed. However, only a few early transcripts and two proteins, an early 97 kD and a late 29 kD polypeptide, were synthesised. Virion assembly could not be detected. This result suggested that the block of infection occurred during the early phase of gene expression (Carpenter and Bilimoria, 1983; Bilimoria, 1991).

AcMNPV infection of *Choristoneura fumiferana* cell lines Cf124T and CF-203 is blocked after DNA replication and causes apoptotic cell death, respectively (Liu and Carstens, 1993; Palli et al., 1996). An apoptotic response to AcMNPV infection has also been observed for *Spodoptera littoralis* cell line SL2 (Chejanovsky and Gershburg, 1995).

By using recombinant AcMNPV expressing chloramphenicol acetyltransferase (*cat*) reporter gene under control of different temporally regulated promoters such as early, late and very late promoters, Morris and Miller (1992; 1993) showed that the activity of late and very late promoters is significantly reduced in semi- and nonpermissive cell lines of *Bombyx mori* (BmN-4), *Choristoneura fumiferana* (CF-1), *Lymantria dispar* (Ld652Y), *Mamestra brassicae* (MaBr-3) and *Drosophila melanogaster* (Dm) compared to permissive cell lines of *Spodoptera frugiperda* (Sf-21). Since there was some evidence for viral DNA replication but not for virus assembly, it was suggested that in these cell lines progression of infection is blocked during DNA replication. By infecting different cell lines with AcMNPV expressing *cat* reporter gene controlled by the Rous sarcoma terminal repeat promoter and β-galactosidase controlled by the very late polyhedrin promoter, it was shown that nonpermissive *Drosophila* cells and permissive Sf-21 had a similar *cat* expression, whereas very late promoter activity was only observed with Sf-21 (Carbonell et al., 1985, Carbonell and Miller, 1987).

3.3 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.

Although genetically closely related, AcMNPV and BmNPV have distinct host range specificities. Kondo and Maeda (1991) demonstrated that an AcMNPV mutant containing part of the BmNPV *p143* gene became infective to the normally refractile BmN cell line of *Bombyx mori*. This mutant, called eh2-AcMNPV, was obtained after coinfection of Sf-21 cells with AcMNPV (OT2) and BmNPV(T3), which are not infective for BmN and Sf21 cells, respectively. The progeny virus was subsequently passaged through BmN and Sf-21 cells thereby isolating eh2-AcMNPV which was found to be infective for both cell lines. In further experiments, the genome region responsible for host range extension was more precisely localised on a 572-bp fragment of BmNPV *p143*, which differed in 14 out of 109 amino acids as compared to AcMNPV (OT2) (Maeda et al., 1993). Similar results were obtained by Mori et al. (1992) who performed the co-transfection experiments with fragments of the BmNPV genome instead of BmNPV virions. This finding was corroborated by studies of Croizier et al. (1994) who demonstrated that the exchange of only three amino acids within *p143* was sufficient to expand the host range of AcMNPV to *B. mori* cells. It appears that the substitution of a single serine residue to an asparagine residue of AcMNPV *p143* is sufficient for this host range extension (Kamita and Maeda, 1997).

Infection of the *Lymantria dispar* cell line Ld652Y with AcMNPV is characterised by early cytopathic effects, transcription from all temporal classes of promoters and DNA replication but with a very low level of protein translation and no formation of infectious virions (McClintock et al., 1986; Guzo et al., 1991; 1992). However, when Ld625Y cells were coinfected with AcMNPV and LdMNPV, replication and production of AcMNPV was observed suggesting that LdMNPV encodes a trans-acting factor which rescues an abortive AcMNPV infection (McClintock and Dougherty, 1987). This trans-acting factor encoded by LdMNPV was finally mapped and identified by co-transfecting Ld625Y cells with AcMNPV genomic DNA and single cosmids of the LdMNPV genome. It was called *host range factor 1* (*hrf-1*) and was shown to extend the host range of recombinant AcMNPV expressing the factor to Ld652Y cells and to *L. dispar* larvae. This indicates that *hrf-1* determines the host range at the cell culture and larval levels (Thiem et al., 1996).

Transient expression studies using the *cat* reporter gene revealed that eighteen AcMNPV genes, so-called *lef* genes, were essential for optimal expression of late and very late genes in Sf21 cells (see also Chapter 3.1.2). For the permissive *T. ni* cell line TN-368, it was found that in addition to these 18 *lef* genes an additional gene, called *host cell-specific factor-1* (*hcf-1*) was required for efficient late gene expression. AcMNPV mutants lacking *hcf*-1 replicate normally in Sf21 cells and *S. frugiperda* larvae but are unable to productively infect TN-368 cells and *T. ni* larvae (Lu and Miller, 1995b; 1996; reviewed by Miller and Lu, 1997). This observation clearly indicated that *hcf*-1 has tissue specific as well as species-specific effects on the replication of AcMNPV in cultured insect cells and in insect larvae.

Abortive replication in the permissive cell lines SF-21 (and SF-9) was observed with AcMNPV mutants lacking a functional *p35* gene. *p35* is an inhibitor of programmed cell death (apoptosis) of insect cells, which might be a defence reaction of insects against baculovirus infection at the organismal level (Clem et al., 1991; for review Friesen, 1997). It appears that *p35* also exerts host range function since this gene is essential for replication in SF-21-cells and *S. frugiperda* larvae but not in TN-368 and *T ni* larvae. AcMNPV *p35* mutants were 1000-fold less infective for *S. frugiperda* larvae than wildtype-AcMNPV when the virus is injected into the hemocoel and they were about 25-fold less infective in peroral infections (Clem and Miller, 1993; Clem et al., 1994).

4. Biological features and environmental conditions which affect survival, reproduction, growth, multiplication or dissemination

4.1. Formation of occlusion body

NPV and GV form polyhedral and granular occlusion bodies, respectively, into which the occlusion derived virions (ODV) are embedded. The occlusion bodies serve to protect the embedded virions against damaging environmental conditions and allow the virions to remain viable for many years. The occlusion bodies are solubilized in alkaline conditions of the midgut and thus deliver the ODV to susceptible columnar epithelial cells.

The crystalline matrix of the occlusion body mainly consists of a single protein, called polyhedrin and granulin, respectively. These proteins of about 245 amino acids (29 kDa) are hyperexpressed during the very late phase of virus infection and are not required for virus replication (for review see Rohrmann, 1992; Funk et al., 1997). To date, polyhedrin and granulin genes of about 35 different lepidopteran- and one hymenopteran-specific baculovirus species have been sequenced. The polyhedrin and granulin genes are highly conserved with at least 70% amino acid identity among lepidopteran NPV and about 50% amino acid sequence identity between NPV polyhedrins and GV granulins.

The importance of the occlusion body for the stability and maintenance of infectivity of baculoviruses in the environment has been clearly demonstrated by field tests using polyhedrin deficient AcMNPV mutants. In 1987, a field test was performed where insect larvae were infected with an AcMNPV polyhedrin minus mutant and then released into enclosed field plots. The non-occluded virus progeny rapidly lost its activity in the decaying larval carcass and no virus activity could be detected on cabbage leaves or in soil samples within two weeks after the release (Bishop et al., 1988a;1988b). Wood and co-workers applied a slightly different approach by co-occluding polyhedrin-minus AcMNPV into the polyhedrin matrix of wild-type virus (Hamblin et al., 1990). In laboratory experiments it was shown that the persistence of such a co-occluded polyhedrin-minus mutant was significantly reduced at inoculum levels below a 100% dose. This observation was corroborated in a three-year field test in which AcMNPV polyhedra containing polyhedrin minus and wild-type AcMNPV in a ratio of 48:52 were applied on *T. ni* larvae, and the amount of polyhedrin minus mutants was analysed in the following years. It was found that the amount of polyhedrin minus mutant dropped below 20% of the virus progeny in the second and third year (Hamblin et al., 1990; Wood et al., 1994).

The occlusion bodies are not solely composed of polyhedrin. They are surrounded by an envelope, called a polyhedron calyx or polyhedron envelope (PE). Minion et al. (1979) reported that the PE of *Helicoverpa (=Heliothis) virescens* NPV was composed of hexose and pentose carbohydrates. Whitt and Mannig (1988) showed that the PE of AcMNPV consisted of a phosphorylated protein which might be covalently linked to the carbohydrate component. The PE protein is encoded by a late and very late gene (*pp34*) which has been sequenced for several NPV (Gombart et al., 1989; Bjornson and Rohrmann, 1992). OpMNPV mutants lacking the PE gene produced unstable polyhedra with a rugged and pitted surface (Gross et al., 1994a).

Another protein which is normally associated with polyhedra in the infected cells is the *p10* protein. Evidence suggests that it is involved in the formation of PE and lysis of infected nuclei. AcMNPV mutants with inactivated *p10* genes failed to release the polyhedra from infected cells. *p10* negative OpMNPV and AcMNPV mutants also failed to form an intact polyhedron envelope generating fragile

polyhedra with significantly reduced stability but with the peculiarity to form polyhedral aggregates (Williams et al., 1989; van Oers et al., 1993). Based on these observations, it was suggested that the function of *p10* and PE is twofold: to protect the polyhedra from mechanical damage by sealing their surface and to prevent their aggregation. Hence, these properties could be important to maximise the number of intact virions per occlusion body and to optimise virus dissemination (Gross et al., 1994a).

4.2 Enhancing of host susceptibility

Synergism between *Pseudaletia unipuncta* NPV and GV, which resulted in an increased susceptibility of *P. unipuncta* larvae for the NPV, has been described (Tanada, 1959). A protein component in the granule of PsunGV, termed synergistic factor, which interacts with the microvillar membrane of midgut cells and facilitates adsorption of the NPV virions was identified (Tanada et al., 1975; Zhu et al., 1989).

Similar factors have been found in TnGV, *Xestia c-nigrum* GV and *Helicoverpa (= Heliothis) armigera* GV (Derksen and Granados, 1988; Goto, 1990; Roelvink et al., 1995). These proteins are commonly known as enhancing factors or enhancin. TnGV enhancin was shown to increase the infectivity of AcMNPV to different noctuid larvae. The predicted amino acid sequences of TnGV enhancin and the synergistic factor of PSunGV-H are almost identical. TnGV enhancin is a protein of 104 kDa which most probably functions as a metalloprotease, since it can be reversibly inactivated by metal chelators (Lepore et al., 1996). TnGV enhancin is located in the occlusion body and causes specific degradation of the intestinal mucin component of the peritrophic membrane (Wang and Granados, 1997). The disruption of the peritrophic membrane of the larval midgut facilitates the virions access to the midgut columnar cells.

Although such a function was also found with a component of NPV polyhedra, no gene homologue to the TnGV enhancin could be identified in NPV (Derksen and Granados, 1988; Hashimoto et al., 1991). Observations of the synergistic enhancement of infectivity by the activity of components from different NPV, and the isolation of another synergistic factor from *Pseudaletia unipuncta* GV indicate that a somewhat heterogeneous set of genes and functions may contribute to this modulation of the infectious process (Arne and Nordin, 1995; Ding et al., 1995).

4.3 Inhibition of cellular apoptosis

Programmed cell death or apoptosis is a cellular pathway during which a cascade of responses is activated resulting in a well regulated cellular suicide. Apoptosis is the normal fate of many cells during development and metamorphosis, cellular turnover of renewing tissues and other dynamic cellular processes. There are also many examples of viral infections whose progression is blocked during early stages of replication by an apoptotic response of the infected host cell. It appears that apoptosis is a very powerful mechanism of many vertebrate and invertebrate cells to prevent viruses from replicating and becoming persistent. On the other hand, viruses have evolved mechanisms that overcome or block this apoptotic response and so establish an infection (for review see Clem, 1997).

In baculoviruses two classes of proteins, the *p35* protein and the IAP (inhibitor of apoptosis) proteins, have been identified and characterised as anti-apoptotic agents. Expression of *p35* was found to be essential for AcMNPV replication in *S. frugiperda* cells (Clem et al., 1991). SF-21 cells infected with an AcMNPV 'annihilator' mutant, which was shown to contain a deletion in *p35* gene, underwent apoptotic cell death within 24 hours. The antiapoptotic effect of *p35* has been demonstrated not only in insect cells but also in many heterologous systems, such as *Caenorhabditis elegans, Drosophila*

melanogaster, mammalian and human cells (Sugimoto et al., 1994; Hay et al., 1994; Rabizadeh et al., 1993; Beidler et al., 1995). The competence of *p35* to prevent apoptosis in many different cell types pointed to the possibility that *p35* acts on a universal step in the apoptotic cascade. Xue and Horvitz (1995) and Bump et al. (1995) demonstrated that *p35* is able to inhibit cystein proteases, so-called ICE-like proteases, which play a key role in the highly conserved effector pathway of apoptotic response.

Compared to wild-type viruses, the infectivity of *p35* minus AcMNPV mutants to *S. frugiperda* larvae was dramatically reduced (by a factor of 1000 if injected into the hemocoel and by a factor of 25 if perorally applied). The yield of virus progeny from infected larvae was decreased 900-fold suggesting that the anti-apoptotic effect of *p35* may play an important role during *in vivo* infection of host larvae. In contrast to SF-21 cells, *p35* is not essential for AcMNPV to establish infection in TN-368 nor is there any difference between *p35* minus and wildtype AcMNPV in the infectivity for *T. ni* larvae. This suggests that *p35* is also involved in determining a baculovirus host range (Clem and Miller, 1993; Clem et al., 1994). A *p35* homologue has been also identified in BmNPV but is not present in OpMNPV (Maeda, 1994; Ahrens et al., 1997).

A second class of antiapoptotic genes of baculoviruses are *iap* genes, which were identified in CpGV and OpMNPV because of their ability to rescue replication and polyhedra formation of *p35* minus AcMNPV mutants in SF-21 cells (Crook et al., 1993; Birnbaum et al., 1994). All baculovirus *iap* proteins contain two cysteine-rich repeats known as BIR (baculovirus *iap* repeat) at their N-termini and a RING finger motif at the C-terminus. Further *iap* genes were identified upon genome sequencing in AcMNPV (*iap*1, *iap*2), BmNPV (*iap*1, *iap*2) and OpMNPV (*iap*1-*iap*4) (Ayres et al., 1994; Maeda, 1994; Ahrens et al., 1997). Cellular *iap* homologues were identified in humans and insects and might be ubiquitous in a wide variety of organisms (for review see Clem, 1997). Although the mode of action of *iap* is not well understood, there is strong evidence that both *iap* and *p35* inhibit defence mechanisms at the cellular and organismal levels, thus allowing baculoviruses to replicate and reproduce.

4.4 Arresting host development by ecdysteroid-UDP-glucosyltransferase

One of the most remarkable features of baculoviruses is their ability to impair larval moulting. In normal development, larval moulting is a physiological process regulated by ecdysteroid hormones. It was found that AcMNPV is able to inhibit larval moulting by expressing the enzyme ecdysteroid-UDP-glucosyltransferase (EGT). EGT inactivates insect ecdysteroid hormones by conjugation with glucose or galactose residues (O´Reilly and Miller, 1989). Whereas, the moulting process in healthy larvae is characterised by a cessation of feeding, baculovirus infected larvae do not show this behaviour. By generating an EGT minus AcMNPV mutant and comparing its pathogenicity with wild-type virus, O'Reilly and Miller (1989) found that EGT expression is responsible for the suppression of the host development during infection. Molecular studies showed that EGT is transcribed and expressed early during infection (O'Reilly and Miller, 1990). To date, homologous EGT genes have been identified and sequenced from 12 nucleopolyhedroviruses and it seems very likely that it is a conserved ancestral gene present in all baculoviruses (Chen et al., 1997).

Since EGT minus AcMNPV mutants yielded about one fourth less virus progeny in late *S. frugiperda* instars than wild-type AcMNPV, it was proposed that EGT expression might have an important role in maximising the amount of virus progeny from an infected larva (O'Reilly and Miller, 1991, for review see O'Reilly, 1997). A further intriguing observation was that *egt* minus AcMNPV mutants killed host larvae faster than wild-type virus. The mean survival time (ST_{50}) of EGT minus AcMNPV-infected *S. frugiperda* and *T. ni* larvae was about 20-30% shorter than that of wild-type AcMNPV (O'Reilly and Miller, 1991; Eldridge et al., 1992). Although the reason for this increased

virulence is not yet elucidated, there is apparently a correlation with the decreased amount of virus progeny. In the faster dying insects, the virus does not have enough time to produce the maximum amount of virus progeny.

The accelerated mortality of the EGT minus AcMNPV mutant accompanied by reduced feeding of the infected larvae led to a new concept of genetically engineered baculoviruses with improved insecticidal properties (O'Reilly and Miller, 1991). In recent years, EGT minus baculoviruses have been tested in the field and they are the most likely candidates to become the first registered genetically engineered baculovirus control agents.

4.5 Facilitating the release of occlusion bodies from larval cadavers

The final step of baculovirus infection is the breakdown of the larval cuticle and the release of the occlusion bodies into the environment. The cuticles of insect larvae consist mainly of chitin fibres embedded into a proteinacous matrix. Two baculovirus genes, encoding a chitinase (*chiA*) and cathepsin (*cath*), have been described to contribute to the liquefaction of the larval carcass and the release of occlusion bodies. Chitinase is a chitin-degrading enzyme with endo- and exomolecular specificity, whereas cathepsin has cysteine proteinase activity (Slack et al., 1995; Hawtin et al., 1995).

The *chiA* and *cath* genes appear to be located next to each other in many baculovirus genomes and are expressed late in infection (for review see O'Reilly, 1997). The functional role of *chiA* and *cath* was elucidated by constructing AcMNPV and BmNPV mutants lacking either *chiA* or *cath* or both. Both single *chiA*- or *cath*-minus mutants were not able to cause liquefaction of infected larvae (Slack et al., 1995; Hawtin et al., 1995). Hence, the co-operative action of chitinase and cathepsin appears to exert an effect on the release of viruses from succumbed larvae and to facilitate the dissemination of occlusion bodies in the environment.

An additional role of *cath* was proposed by Lanier et al. (1996) who found that cathepsin is associated with BV of AcMNPV and is able to cleave actin in an *in vitro* assay. Whether cathepsin is also involved in the rearrangement of actin filaments during early stages of infection needs further investigation

4.6 Latent virus infections

Latency is the ability of a virus to persist in a host without causing disease symptoms, thereby providing the virus the possibility of vertical transmission from one generation to another. There are a number of early publications reporting circumstantial evidence of latent baculovirus infections. UV light and treatment with chemicals, rearing conditions, or superinfection with a different virus have been described as reasons for activating latent infections (Krieg, 1956; David and Gardiner, 1965; Longworth and Cunningham, 1968; Biever and Wilkinson, 1978; Podgwaite and Mazzone, 1986). However, most of these early studies were made when molecular tools for identification such as hybridisation techniques or restriction endonuclease analysis were not available. Hence, it cannot be excluded that at least some of these reports were biased by cross-infections and contamination. Ponsen and de Jong (1964) and Jurcovicova (1979) reported activation of latent *Adoxophyes orana* SNPV by infecting *A. orana* larvae with *Barathra brassicae* MNPV and vice versa activation of latent *B. brassicae* MNPV by infection of *B. brassicae* larvae with *A. orana* SNPV. An increase in larval deaths from granulovirus infection correlated to stress from dehydrated diet or low temperature was observed in *Pieris rapae* (Biever and Wilkinson, 1978). Virulence testing indicated the identity of the stress induced virus with laboratory grown virus

preparations. It was noted that larvae demonstrating symptoms of stress induced virus infection could recover to a healthy condition after relief of the stress by providing a fresh diet.

More recently, Hughes et al. (1993) provided first molecular evidence for activation of a latent baculovirus in *M. brassicae*. Activation of a MbMNPV was observed after feeding *Panolis flammea* NPV or AcMNPV to the laboratory culture of *M. brassicae* termed MbLC. Other (environmental) stress factors such as high and low temperatures, starvation or crowding were not effective in triggering apparent infections in this culture (Goulson unpublished data, Goulson and Cory, 1995a). Contamination of the virus inoculum was excluded by control infections of another *M. brassicae* culture (MbWS). Challenging this insect culture, established from a novel environmental isolate and adapted to laboratory growth conditions, did not result in multiplication of MbMNPV when infected with the same inoculum. Furthermore, MbMNPV-specific sequences were detected by PCR in each stage of insect development, i.e. in eggs, larvae, pupae and adults. When dissected larval tissues were analysed, latent virus sequences were only detected in the fat body of MbLC larvae. Cell lines established from fat body cells also harboured MbMNPV-specific sequences.

The occult state of the virus in the MbLC culture was further characterised by analysis of m-RNA in larvae, demonstrating the presence of polyhedrin specific m-RNA. Assays of transient reporter gene expression (CAT, chloramphenicol acetyl transferase) after transfection of primary cultures of MbLC fat body cells with constructs containing early, late and very late promoters, demonstrated the presence of expression factors for all of these, albeit at low levels. Furthermore, inoculation of MbWS larvae with MbLV fat body cells resulting in larval deaths from MbMNPV infection, strongly suggested the presence of viable virus particles in these cells (Hughes et al., 1997). The latent or occult status of MbMNPV in the insect can thus be described as a persistent infection, with a continuous production of virus proteins at a low level. Whether this state of the virus is controlled by host functions exclusively or to which degree viral regulatory functions are engaged in its establishment and support, remains an intriguing and challenging question.

A more detailed knowledge of the possibility and extent of virus persistence as a latent infection with vertical transmission to insect progeny and the potential to induce an infectious cycle through environmental conditions or stress factors, is crucial for an improved understanding of baculovirus ecology and population dynamics. So far, the contribution of this feature to the persistence of baculoviruses in natural populations, and its occurrence among insect and virus species and genotypes, is largely unknown. The perception of baculovirus population dynamics as, for example, represented in the modelling of natural and induced epizootics, almost exclusively considers the multiplication of viruses as a result of infections initiated by horizontal virus transmission.

4.7 Environmental factors influencing virus persistence

Virus persistence is an important factor affecting the potential of baculoviruses to interact with their hosts in the environment. Biotic and abiotic persistence mechanisms allow the virus to overcome situations of varying host density without running the risk of extinction (Evans, 1986). Baculoviruses may persist in the host population through vertical transmission of disease or by a latent infection (see A 4.6) or in non-target organisms which may serve as vectors of virus dispersal (see C 26.2). This biotic persistence complements the maintenance of virus activity outside living organisms (abiotic persistence). In the open environment UV radiation of the sunlight has the most significant impact on abiotic persistence which also may be influenced by temperature, relative humidity and precipitation (for review see Jacques, 1975). Half life time may be as short as 1.3 days for a purified preparation of the *Phthorimaea operculella* granulovirus (Kroschel et al., 1996) or, more typically, about 5 - 10 days for

many NPV and GV (Bell and Hayes, 1994; Kolodny-Hirsch et al., 1993). Hence, a variety of formulations and additives have been tested and applied to improve the life time of preparations for pest control applications (Ignoffo et al., 1989; Ignoffo and Garcia, 1996; see also A.6.3). On the other hand, after spray applications of *Panolis flammea* NPV on pine foliage, only a slight decrease of infectivity was monitored over several months (Carruthers et al., 1988). This observation suggests that the application time of PaflNPV for control of the pine beauty moth, *Panolis flammea*, does not necessarily need a precise optimisation (Cory and Entwistle, 1990). Agricultural operations can affect the distribution of the baculoviruses in soil. Following *A. gemmatalis* NPV spraying on soybeans and subsequent epizootics, it is transported into the soil and decreases rapidly due to overwinter weathering, but enough remains near the soil surface at the beginning of the next growing season to initiate new epizootics. Persistence of the NPV in soil is not adversely affected by discing, cultivating, or other agricultural soil operations (Fuxa and Richter, 1996).

Furthermore, plant surfaces can have a distinct influence on viability and virulence as discussed in section C. 23.4.1. In soil, which can also be a potential reservoir of baculoviruses, the inactivation rate not only depends on soil type and pH but also on microbial activity (Thompson and Scott, 1979; Ignoffo and Garcia, 1966; Jaques and Huston, 1969; Undorf, 1991). Baculoviruses may persist in soil for long periods. Soil samples taken from a field treated with *Trichoplusia ni* NPV showed 15% of the original virus activity 6 years after application (Jacques, 1964). Similar results were obtained for other GVs and NPVs (for review see Jaques, 1975).

In summary, these observations denote that the physical environment, chemical conditions and the (micro)-biological composition of micro-habitats have a very significant impact on the persistence and activity of baculoviruses. The validity of comparisons of the persistence of different virus types depends on the degree of control of experimental variables. In particular cases the determination of the abundance of genotypes in artificial mixtures could be used as an approach. Parameters of persistence used in calculations of virus spread and modelling have either to account for the detailed structure of the respective environment or must include wide margins for the variation of median values. Biotic and abiotic persistence are prerequisites for mechanisms and routes of dispersal (summarised in section C.26).

5. Behaviour in simulated natural environments such as microcosms, growth rooms, greenhouses, insectaries, etc.

5.1 Monitoring of baculoviruses in closed environments and ecological modelling

Experiments such as bioassays (C.23.1) test well defined characteristics of baculovirus phenotypes in the laboratory. The use of microcosms or similar facilities may supplement these investigations by monitoring virus interactions in more complex environments. Microcosms may represent the variables of natural settings to a widely divergent degree, depending on their particular objective. Fraser and Keddy (1997) describe the “manipulation of an individual environmental axis” to explore its function in structuring the community as a common factor of microcosm research in ecology. Research of this kind may contribute to an improved understanding of baculovirus ecology by investigating mechanisms of fate and interactions in the environment in some detail (Cory et al., 1997; Cory and Hails, 1997). Increased knowledge of ecological behaviour will also generate a better predictability and may improve safety assessments. Perspectives of safety may also trigger microcosm experimentation because some degree of confinement from the open environment is provided.

Traditionally, such testing was not usually performed with natural strains during the development of baculovirus insecticides. Baculovirus behaviour (e.g. pathogenicity, virulence, host range, and toxicity for particular non-target organism) is inferred from laboratory testing on one side. On the other, the world-wide application of baculoviruses for pest control together with scientific investigations on baculovirus population dynamics in the open environment contribute to the present understanding of baculovirus ecology. This background of knowledge about the mechanisms driving the dynamics of effects on insect species and about the ability to manipulate it, mostly is considered to be adequate for judgements about applications and safety in the environment. To some extent this view is reflected in the criteria of registration procedures (e.g. Andersen et al., 1989).

Conventional testing procedures using soil columns as a section of the natural environment were used to test the leaching behaviour of baculovirus preparations as a component of environmental fate in order to predict potential exposition rates of the groundwater. Rates were tested under conditions similar to pesticide registration procedures (C.26).

The perspective of using genetically modified baculoviruses seems to have changed the perception with respect to testing in "closed" environments to some degree. The first "deliberate release" of a genetically engineered baculovirus can be viewed as an experiment performed in a confined "simulated natural environment" (Bishop et al., 1988). Modified versions of microcosms introduced for environmental transport and fate studies in chemical risk assessment were used in this context to monitor essentially similar parameters of baculovirus fate. Modelsystems of this kind also provided some simulation of meteorological conditions (sunlight, rainfall, and air movement). Investigations included the comparison of modelsystem and field data, and of the persistence and spread of natural and genetically modified virus types. The potential of monitoring individual genotypes was demonstrated to some extent. A reduced spread and multiplication of the modified genotype in some contrast to similar biotest data was noted (Undorf, 1991). However, systematic comparisons of biotest, modelsystem, and field data, in order to analyse their correspondence, are not well documented in the scientific literature. The potentials as well as the limitations of experimentation in an intermediate scale of complexity have not been explored in great detail. This is in contrast to its use for the evaluation and modelling of the fate and effects of chemicals in the environment. The interrelationship of an improved understanding of baculovirus ecology on one side and their biosafety on the other is stressed in recent publications (Cory et al., 1997). Limited

relevant modelling supported by experimentation is cited there to exemplify the need to analyse the correspondence between theory, closed environment experimentation and field data for an improved understanding of baculovirus population dynamics (Begon and Bowers, 1994; Begon et al., 1996).

For inferences, empirical data must be interpreted by theoretical structuring of knowledge in some kind of model, representing the essential components and the relations assumed to exist between them. Explicit modelling of baculovirus ecology given a mathematical form tries to calculate the dynamics of viruses and of their arthropod hosts under a given set of parameter variations. Results of such calculations are then compared with empirical data from modelsystem experimentation, field testing, or natural epizootics, to explore their present contribution to an understanding of mechanisms and to a limited degree of predictive potential, eventually. Simulated natural environments have been employed to a limited extent for testing environmental variables, to verify the formulation of interactions, and to analyse parameter values. This type of research is described in the following to account for common experimental approaches and for the correspondence with objectives to improve (predictive) knowledge of baculovirus ecology.

The work of Anderson and May (1980, 1981), cited in reviews of baculovirus ecology with some emphasis, is the key reference for the modelling of the dynamics between pathogens and their invertebrate hosts (Evans, 1986; Cory et al., 1997). In the Anderson and May model, the variations of forest insect pest abundances with low densities between regular extensive population increases were triggered exclusively by the dynamic relations between an insect pest population and pathogens persisting in the environment. Their model assumptions, describing the rate of disease transmission by a mass-action law with a linear relationship between pathogen/host densities and productive infection, have been criticised for incorrectness, oversimplification and lack of correspondence to reality (Bowers et al., 1993; D´Amico et al., 1996; Berryman, 1997). Whereas the statement that "it is no longer possible to claim that mathematical models provide a basis for believing that forest insect cycles might be generated by host-pathogen interactions alone" (Bowers et al., 1993) may be accepted or be viewed as a matter of discussion, the basic structure of the model is further employed as a starting point for modifications and adaptations of parameters and interactions. Cory et al. (1997) describe in some detail the modifications of the terms of transmission, virulence, yield, and persistence that are introduced or should be incorporated to generate correspondence with actual knowledge about baculovirus biology, as summarised in different sections of this paper.

Modelsystems, not intending to simulate natural environments to any significant degree, were used by Sait et al. (1994) and Begon et al. (1996) to study fluctuations in population densities of the Indian meal moth *Plodia interpunctella* including a baculovirus pathogen and a parasite. Systems varied with respect to composition: virus-free populations, infected populations continuously exposed to a granulovirus, or three-species systems including the interaction with the ichneumonid parasitoid *Venturia canescens* were used. A cyclic dynamic of abundance in all systems was recorded, with a very significant dependence of cycle periods upon the composition of the system. The correspondence of the details of the dynamical patterns with predictions from mathematical modelling was assessed only qualitatively.

The variation of viral disease transmission was analysed using different larval instars in laboratory and field studies. Modelling was used to account for different susceptibilities, feeding habits and times of larval death after infection (Goulson et al., 1995). The influence of the parameter of larval population density on transmission of gypsy moth NPV was investigated by D´Amico et al. (1996). In order to control the "environmental axis" under investigation, meshs were used to prevent the movement of larvae from their place on red oak foliage. Different numbers of larvae were used to investigate the influence on the transmission coefficient. Its density dependent variability did not correspond to predictions of different modelling approaches, demonstrating the need to redefine this process. A

correspondence with data of large scale investigations (Woods and Elkinton, 1987) suggested a common driving force for the type of variability. It was concluded that the large-scale dynamics of the virus may be determined by interactions at the small scale. Further experimentation at a large scale was considered necessary in order to test this hypothesis.

Observations of virus distribution following artificially induced epizootics failed to correspond with any of the predictions included in mathematical modelling of spatial spread (Dwyer and Elkinton, 1995). The mechanism of dispersal in addition to larval movement, including ballooning of first instars, remained a matter of speculation.

In the type of research as described above, field experimentation or observations in the open environment were sometimes used directly to compare model predictions with data. The need for particular data and the convenience of their generation were the main factors determining the level of complexity for investigations. A special role of microcosm experimentation was not appreciated explicitly. Altogether, empirical testing challenges theoretical modelling, to improve its accuracy and correspondence to reality, in this way increasing its predictive value. In general, the correspondence with data from the open environment is considered the crucial test for the validity of models.

Whereas theoretical investigations have their own merits - in particular by shaping the reflection about essential components and relations, their value for the description of real systems is that of an untested hypothesis. This character compromises the direct applicability for microbial pest control or the prediction of the fate of non-target organisms with a low susceptibility for virus infection (Bowers and Begon, 1991; Begon and Bowers, 1994).

The interrelationship of an improved understanding of baculovirus ecology on one side and their biosafety on the other is stressed in recent publications (Cory et al., 1997; Cory and Hails, 1997). The importance of ecological testing and modelling is highlighted and its use for inferences on the fate of novel baculovirus genotypes (is the genotype prone to extinction in time? or will it be established as a member of natural populations?) and on the fate of hosts with varying sensitivities is briefly considered. Molecular biology techniques not only offer new insights into the mechanisms of virus host interactions, they also provide methods of its modification with some impact on safety perception. The potential of strain detection and identification also provides for novel details of monitoring in space and time suitable for direct comparisons of different genotypes, differing in subtle biological parameters.

The generation of a novel quality of (predictive) knowledge about baculovirus fate and effects seems to represent a significant scientific challenge in baculovirus research. The potentials and inherent limits of an intermediate stage of microcosm research still have to be explored in response to such a challenge. There is no doubt that strategies of baculovirus use as insecticides will profit from improvements in ecological knowledge in the long run.

5.2 Experimentation and uses in greenhouses

Experimentation in closed environments is not generally used to improve control over experimental parameters, to confine experimentation from the open environment, or to generate predictive knowledge about the environmental behaviour of viruses. The development of a virus application in greenhouses is cited here to illustrate the stepwise extension of experience and knowledge during the conventional steps for the commercialisation as a biocide product. In this case, greenhouses merely represent the intended localisation for the application and do not constitute “simulated environments”. The

design of experimentation thus is not adapted to generate novel potentials of inferences about the fate and behaviour of the virus in the open environment.

The intended use in greenhouse cultures triggered experiments studying the efficacy of a nucleopolyhedrovirus, SeMNPV, for controlling larval populations of the noctuid *Spodoptera exigua*. Application of $1x10^8$ OBs per m^2 on ornamental plants and tomatoes resulted in 95% - 100% larval mortality. Mortality of early and late instar larvae was similar, but feeding damage was more pronounced when larvae were in late instars at the time of application. This is referred to as "maturation resistance". It was concluded that the virus was an effective potential control agent for *S. exigua* in greenhouses also in comparison with a use of chemical insecticides (Smits et al., 1987b). Superior activity of baculoviruses against pests of ornamental plants in comparison to chemical treatments has also been reported by others (Geissler and Schiephake, 1991).

Different virus strains, known or suspected to be infective to specific target insects, have been screened with respect to their virulence (lethal dose, LD_{50}). Selection of a strain for application may then include other criteria like the knowledge about host range and the possibility of cost effective production (Smits and Vlak, 1988a). Monitoring included the observation of movement and feeding behaviour of the target organism to adapt application techniques (Smits et al., 1987a). After optimisation of production conditions, the product was characterised for its biological activity and its microbiological composition and purity (Smits and Vlak, 1988b). After the accumulated information was considered to be satisfactory by all parties, registration of the product was the outcome (Smits and Vlak, 1994). Data of virus dispersal, virulence, and replication together with features of the host organism (development, susceptibility of instar stages, movement) provided input for modelling. Understanding viral population dynamics in generating predictive knowledge was considered to be useful for safety assessment in general and to contribute to the optimisation of application strategies (Moed et al., 1990; van der Werf et al. 1991).

6. History of use (examples of environmental applications of the organism and information derived from these examples)

6.1 Brief historical account of baculovirus detection and application

Observations of silkworm (*Bombyx mori)* cultures with descriptions of jaundiced larvae due to paralysis, as described in ancient Chinese literature, appear to be the earliest accounts describing the effect of baculovirus infections. In western literature, this is credited to the verses of the poem "De Bombyce" by the Italian bishop Marco Vida of Cremona, 1527 (Benz, 1986). Liquefaction of diseased larvae occurs in the process of converting insect biomass into progeny virus and the older literature refers to these symptoms as "melting" or "wilting".

The breakdown of the European silk industry and virus infections threatening the emerging shrimp aquaculture industry provide two of the very few examples of negative consequences from baculovirus infectivity as perceived by mankind (Chen et al., 1989; Chou et al., 1995; Momoyama and Sano, 1996). Favourable conditions for epizootics in artificial mass cultures of arthropods contribute to this type of observation. Considering present knowledge of the widespread occurrence of baculoviruses in insects, their persistence and spread by a variety of vectors which exposes soil, crop plants and plant debris with great numbers of virus particles (Heimpel et al., 1973), all day life can be regarded as natural "nontarget-species testing". This occurence demonstrates to some extent the lack of infectivity and pathogenicity of baculoviruses for organisms other than arthropods, in particular vertebrates including humans.

The first attempts to use baculoviruses for biological control can be dated back to the year 1892. During massive population increases of nun moths (*Lymantria monocha*, L.), a severe pine pest in Europe, the use of the infectious agent causing the so called "Wipfelkrankheit" was intended to combat the insect pest. But the "insecticide" was prepared in a manner corresponding to its identification as a bacterium at that time. Thus, the observation of diseased larvae and a final collapse of the population short after application must be attributed to a common natural outbreak of an epizootic, not to a successful application strategy (Huber, 1986).

The history of integrating novel experimental techniques and increasing knowledge in biology to finally identify the nature and features of the viruses did not proceed in a continuous and straightforward manner (Benz, 1986). The systematic exploration of the potential of baculoviruses for the control of insect pests is described by Benz (1986) and Huber (1986). Yearian and Young (1982) and Cunningham (1982) documented the use of about 50 different baculoviruses. The successful biological control of insect pests in field crops, plantation, orchard crops, and forests was demonstrated by field experimentation and applications of widely different scales all over the world. These experimentations and applications resulted in the development of fully registered baculovirus insecticides in several countries. However, registration and commercialisation of viral pesticides did not keep pace with the development, although, it was estimated that about 30% of the major insect pest species could potentially be controlled by baculoviruses (Falcon, 1978).

A substantial increase in the use world-wide did not fundamentally change the situation regarding the registration and commercial production of baculovirus insecticides and their economic success. Although environmental policy and public awareness demand an increasingly rigorous testing for the registration of chemicals, a trend that should generally favour biologicals, the situation regarding the registration and commercial production of baculovirus insecticides has not changed fundamentally.

Baculoviruses, due to their character as biologicals, are endowed with a variety of application constraints. Among these, the narrow host range, a limited life time and the slow speed of action resulting in demands on application strategies, are important factors in their failure to effectively compete with chemicals (Huber, 1986; Bohmfalk, 1986). Two noticeable developments might, in the future, contribute to a more favourable situation for baculovirus insecticides on the market.

(1) The progress in the development of techniques for virus production may significantly improve their economic competitiveness. Supported by the development of cell culturing techniques and a widespread use of baculovirus systems for the expression and production of a multitude of heterologous proteins, a cost effective multiplication of viruses by insect cell cultures in fermenters has become a realistic perspective (Rhodes, 1996).

(2) Principles and methods of molecular biology contribute to an enhanced understanding of the interaction of baculoviruses with their hosts and offer a variety of potentials to modify this interaction with the objective to relieve some of the (biological) constraints on their convenient applicability (Bonning et al., 1992; Crook and Winstanley, 1995).

The first trend can clearly be seen in an increasing number of publications on technical aspects of baculovirus insecticide production. Limitations on maintaining infectivity following growth in insect cell culture are becoming better understood and overcome. The exclusive emphasis given to the registration of genetically modified baculoviruses in a recent contribution on commercialisation of baculovirus insecticides, appears to underpin the importance given to the latter trend internationally (Black et al., 1997).

6.2 Types of pest control and strategies of virus selection for application

Any intended use of baculoviruses for insect pest management includes the screening for a virus strain virulent for the particular species. Isolates from diseased insects in the application area frequently are the first choice and are used as a promising and convenient starting point. If available, such isolates are in general included in the screening, but the testing for suitable viruses is not conventionally limited to those indigenous agents (Smits and Vlak, 1988a; Shapiro and Robertson, 1991). Both, Benz and Huber (1986) give a special credit to the control of the European spruce sawfly, *Gilpinia hercyniae*, introduced in the US and Canada long before the 1930s, when it became a serious pest of spruce. Population gradations frequently collapsed after the dissemination of a NPV which was probably accidentally introduced with imported parasites. Later, in the following, planned applications successfully supported the natural epizootics and the establishment of the virus finally controlled areas of minor infestations. The CpGV-isolate which is registered and commonly used for the control of the codling moth in different European countries, was originally isolated from diseased insects found in Mexico (Tanada, 1964).

This instance can be taken as an example of two types of strategies of biological control: the **introduction and establishment of a novel agent from a different geographic region**, however not planned in this case, and **inoculative augmentation**, used to enhance a natural antagonist, in order to achieve control of the pest species. The history of baculovirus introductions or uses does not contain any documented observations of negative or unintended consequences. This is in contrast to other introductions for biological control (Ehler, 1991) and reflects the biological properties of these viruses to some extent.

The application of baculoviruses in forests can most often be characterised as an **augmentation** of an indigenous pathogen reservoir to improve natural control over high rates of insect multiplication

(radiations). This type of application represents the most impressive record of the use of baculoviruses with respect to extent and success (Huber, 1986). The control of insect pests in orchards, greenhouses and annual crops, in general, demands a third type of application, **inundative augmentation**, by inoculating these areas with an amount of viruses that productively infects the number of pest organisms that must be controlled to keep the damage below economic threshold. Such applications do not rely on virus multiplication and spread to achieve the desired effect - and thus are more comparable to the use of topically applied chemicals. However, the slope of the rate effect curve and economic reasons will not allow management of the efficacy of applications by modifying (increasing) the application dose in the same way as is sometimes the case with chemicals (Huber, 1986).

The screening for virus isolates suitable for a pest management objective is generally not adapted to the intended type of control as categorised above. Infectivity and virulence, encompassing the dose-response function of the virus interaction with the pest species (C.23) make up the most important and frequently exclusive criteria for the selection of a virus and the planning of field application. Whereas bioassays serve in the analysis of the potency of a baculovirus preparation, field testing is essential to evaluate whether its use is feasible and to investigate the effect of formulations and the application conditions of the efficacy of the insecticide. World-wide, many improvements and modifications in production, formulation and application of baculovirus insecticides have been achieved and tested in recent years.

Before the era of molecular biology, the potential to modify infectivity and persistence of baculoviruses was frequently tested by conventional means of selection, eventually including mutagenesis (e.g. Reichelderfer and Benton, 1973). Eventually, selection resulted in a virus mutant analogous to genetically modified strains carrying a deletion of a gene for host interaction with the same objective. The description of a mutant, selected by Wood and co-workers (1981), which demonstrated a significantly reduced mean lethal time, suggests such a coherence (with genetically modified viruses lacking the *egt*-gene, see A 4.4). From experiments intending to modify host range or to adapt baculoviruses to alternate hosts, no clear cut picture has yet emerged. Experimentation before the introduction of molecular methods suffered from the difficulty of differentiating inoculum viruses from viruses persistent in the insect culture and potentially activated during the experiment. An adaptation to novel hosts has been described in several cases and/or an enhanced virulence was observed after virus multiplication in an alternative host (Shapiro et al., 1982; Martignoni and Iwai, 1986a; Stairs, 1990).

The testing of virus multiplication in alternative hosts may also be triggered by the objective to improve virus production with respect to cost effectiveness. For example, salt marsh caterpillars (*Estigmene acraea*) appeared to be a suitable production host for several NPVs of forest pests (*Orygia pseudotsugata* SNPV, *Choristoneura fumiferana* MNPV) by not only improving virus yield per larvae, but also by enhancing virulence (Shapiro et al., 1982). From experiments analysing the virulence of viruses obtained from different instar stages of larvae, it was recommended the virulence in and yield of occlusion bodies (OB) per weight of larvae for the optimisation of production yields be observed (Shapiro et al., 1986). Considering the potential of virus evolution during multiplication in an unnatural environment with novel selective constraints, an observation of the biological activity of production lots obviously is advisable, especially after propagation in alternative hosts (Reiser et al., 1993; Maracaja et al., 1994). The optimisation of production in cell culture may represent an additional challenge in this respect (Lynn et al., 1993; Tompkins et al., 1988).

Apart from the modification or selection for host range or infectivity parameters, another biological feature of baculoviruses compromising their applicability in the environment seems to be prone to direct selection: that is their sensitivity for UV-radiation, being the most significant factor for the limited life time of baculovirus insecticides. Although the mechanism of improved UV adaptation is not

elucidated, some reports of successful selections were published (e.g. Brassel and Benz, 1979; Witt and Hink, 1979; Shapiro and Bell, 1984). However, the use of such variants in field experimentation was not reported. Selective procedures were also used to isolate variants of *Spodoptera frugiperda* MNPV with an enhanced rate of vertical transmission of baculovirus infections from adults to progeny, for example, by egg contamination (Fuxa and Richter, 1991). Such variants are likely to modify the dynamics of the spread of infections in natural populations and might improve the control of pests in particular situations.

Baculoviruses have also been used in combinations of different virus strains, in order to achieve control of different pest species (Harper, 1986; Dhandapini et al., 1992). The synergistic effect known from certain double infections (Arne and Nordin, 1995; Ding et al., 1995, see section A.4.2) has not been exploited in field experimentation.

6.3 Modifications of chemical composition of formulations and of application techniques

In addition to approaches intending to improve the use of baculoviruses by modifying some of their biological parameters, formulations were adapted to achieve an economically feasible control. In competition with chemical insecticides, field testing and applications world-wide are evaluated for the optimisation of pest control.

The limited lifetime of baculovirus insecticides in the environment, to a great extent caused by their sensitivity for sunlight (UV), can be increased by the addition of a variety of UV-protectants. Optical brighteners, originally only tested as radioprotectants also proved to very significantly enhance virulence of virus host interactions in the laboratory (Shapiro, 1992; Shapiro and Robertson, 1992). The median lethal dose was reduced up to 4 orders of magnitude, the median lethal time was reduced for about 50% (7 instead of 14 days), and non-susceptibility was converted to susceptibility in some cases (Shapiro and Dougherty, 1994; Zou and Young, 1996). Laboratory testing was also supplemented by field testing with qualitatively corresponding results (Webb et al., 1994; Zou and Young, 1996). The phenomenon obviously requires the combined action of brighteners and viruses. At the highest dosage, the application of brighteners alone does not induce larval death. In field tests the increase of mortality of *Lymantria dispar* caused by the indigenous natural virus was observed (Webb et al., 1994).

In order to enhance the frequency and effectiveness of exposition of insect larvae, which predominantly become infected by feeding on plant material, spraying techniques have to be adapted and optimised (Smits et al., 1988; Payne et al., 1996). The addition of detergents and stickers serves for modifications of the distribution of spray droplets, and feeding attractants (e.g. crude sugar) are tested in order to enhance productive encounters of larvae with this distribution. The requirement to carefully test the environmental safety of formulation additives is highlighted by a field observation that molasses added as an UV protectant proved to be phytotoxic for soybean plants (Im et al., 1990).

A variety of approaches describe the combined use of baculoviruses and chemical insecticides at significantly reduced application dosages (1/3 - 1/10). Apparently, the chemicals reduce the tolerance of insects for other stress factors, thereby enhancing their susceptibility for baculovirus infection. Alternatively, an impact of the virus infection on the insects ability to degrade the chemical has also been suggested as a mechanism (Huang and Dai, 1991). A synergistic (more than additive) enhancement in effectiveness of a combined use has been described (Jacques et al., 1988, 1989; Salama and Moawed, 1988; Peters and Coaker, 1993). This strategy was particularly recommended to compensate unsatisfactory control due to heavy insect infestations or climatic conditions (Moscardi and Corso, 1988; Ding et al., 1989).

6.4 Field testing and commercial use of baculovirus insecticides

A detailed continent-by-continent survey on the developmental, experimental and commercial use of baculovirus insecticides was recently compiled in "Insect Viruses and Pest Management" (Hunter-Fujita et al., 1998). Some examples of the most important baculovirus insecticides tested and used in the field are:

- *Adoxophyes orana* GV has been extensively field tested and finally registered in Switzerland for control of the summerfruit tortix (Andermatt, 1991). This virus is also registered in Germany.

- *Agrotis segetum* GV and NPV have been tested in many countries for control of the common cutworm (*A. segetum*) and the greasy cutworm (*A. ipsilon*) They showed superior efficacy compared to chemical insecticides. *Agrotis segetum* GV-based bioinsecticides were registered in Denmark and the former Soviet Union (Huber, 1998; Lipa, 1998).

- *Anticarsia gemmatalis* MNPV has been used for control of the velvetbean caterpillar in soybean on a large scale in Brazil (Moscardi, 1990; Da Silva, 1992). The application of AgMNPV has increased from 2000 ha treated in 1982/ 1983 to 1 million ha in 1989/90. At a dose of 50 LE/ha (LE=Larval equivalents, the amount of virus prepared from one larvae) one application of the baculovirus was as efficient as chemical control (frequently requiring several applications) to maintain population densities of *Anticarsia gemmatalis* below economic threshold. The combination of the virus preparation with low dosages of chemical insecticides has also been tested and proved to enhance effectivity. It was recommended to be used in situations of heavy infestations when control cannot be achieved with the virus preparation alone (Moscardi and Corso, 1988; Da Silva, 1995). The widespread use of the viral insecticide was the reason to initiate the special monitoring of Brazilian *Anticarsia* populations for the selection of phenotypes with enhanced resistance to the virus (see C.24).

- *Cydia pomonella* GV has been extensively tested for control of the codling moth in apples, pears and walnut in the last 25 years. Meanwhile CpGV based products are registered in many European countries, e.g. Austria, France, Germany, Spain, Switzerland and the Netherlands and have been registered in the US since 1995. Field testing has also been conducted in Australia, Canada, Chile, the USA and other countries. Best results were obtained in cooler climates where the codling moth produces only one annual generation (Audemard et al., 1992, Huber, 1998).

- *Autographa californica* MNPV has a broad host range and has been tested for control of different pest insects, esp. of *H. virescens* and *T. ni.* AcMNPV has been registered in the USA since 1994. Experimental and commercial use of AcMNPV is reported from Central America, where it has been applied on several thousand hectares of cabbage and broccoli (Hunter-Fujita et al., 1998). In recent years, field tests have been performed using genetically engineered AcMNPV recombinants with improved speed of kill. These recombinants, which express insecticidal toxins and/or lack the *egt* gene, are the prototypes for other genetically engineered baculovirus insecticides (Cory et al., 1994, Black, 1997).

- *Heliothis (Helicoverpa) sp*. NPV. The cotton bollworm (*Helicoverpa zea*) and the tobacco budworm (*Heliothis virescens*) are major pests of cotton in southern/south-eastern regions of the US and serious pests of many food, fibre, and forage crops world-wide. *Heliothis* SNPV based insecticides were developed during the late 1960s - 70s, registered in 1975, and were a

commercial success until the early 1980s when synthetic pyrethroids were introduced. The total area in the USA sprayed with this virus was estimated to be more than 1 million hectares (C. M. Ignoffo cited in Cunningham, 1998). A novel strategy for control of *Helicoverpa ssp*. using *Heliothis* SNPV was investigated when it was found that introduced and native early season host plants (in particular the wild geranium, *Geranium dissectum)*, occupying only 5% of the Mississippi rural area, support the first generation of bollworm/budworm populations which subsequently invade the cotton fields. A control of the first and possibly second generation by applying HzSNPV to alternate hosts had been tested in small field tests as an effective potential management strategy. Large area testing followed these first experiments (Bell and Hardee, 1994; Bell and Hayes, 1994; Hayes and Bell, 1994).

- *Helicoverpa armigera* NPV has a great potential for the control of African cotton bollworm, *H. armigera*, which is one of the most deleterious insect pest in warmer climates of the Old World. Extensive studies including combinations with another biological, *Bacillus thuringiensis* (Bt), have been performed in China, India and African countries (Zhang et al., 1996a; 1996b, *Kunjeku* et al., *1998*). A significantly reduced mean lethal time and a high efficacy (97 % of larval deaths) were observed for the combination with Bt, which also had an effect on pupal mortality in the following year. The effectiveness was found to be at least equivalent to that of recommended chemical insecticides.

- *Lymantria dispar* NPV has been widely applied for control of gypsy moth *L. dispar* in the USA, where it is registered since 1978 and where more than 11 000 ha have been treated (for review see Lewis, 1981; Podgwaite, 1985; Cunningham, 1998). It also became the most important viral insecticide in the former Soviet Union (for review see Lipa, 1998). In order to improve the control of divergent densities of the gypsy moth, the commercial product Gypchek was modified with sunlight protectants, feeding stimulants and a sticker. Two applications of 1.25×10^{12} OB/ha resulted in a reduction of egg masses by 98% and 80% in comparison with control woodlots at different sites (Podgwaite et al., 1992). This application dose is currently recommended for the use of LdMNPV preparations in the USA.

- *Mamestra brassicae* NPV has been tested for efficacy in a number field trials for control of the cabbage looper, *M. brassicae.* This virus has a considerably broad host range and more than 30 susceptible insect species have been identified (Doyle *et al*., 1990). Reasonable success was reported for the control of *P. xylostella, Heliothis spp, Spodoptera spp., Trichoplusia ni* and others. Commercial products were developed and registered in France and the former Soviet Union (Hunter-Fujita et al., 1998).

- *Neodiprion sertifer* NPV has been successfully applied for control of the European pine sawfly, which is a serious pest on pine plantations (for review see Cunningham and Entwistle, 1981). Field work with NeseNPV has been conducted in Canada, Scandinavia, Poland, UK, the former Soviet Union and USA. Commercial products were registered in Finland (1983), UK (1985), USA (1983, but discontinued by the company in 1991) and the former Soviet Union.

- *O. psuedotsugata* NPV is used and has been registered since 1976 in the US and Canada for control of the Douglas fir tussock moth. It is recommended particularly for early control when population densities are relatively low because it takes five to eight weeks before the larvae stop feeding, during which time further defoliation occurs. (Defoliator Management

Guidebook, Ministry of Forests, British Colombia, Canada http://www.for.gov.bc.ca/tasb/legsregs/fpc/fpcguide/guidetoc.htm).

– *Spodoptera spp. NPV.* Several specific viruses have been isolated from different *Spodoptera* species, such as SeMNPV from the beet army worm *(S. exigua*), SpexNPV from the African armyworm *(S. exempta),* SfMNPV from the fall army worm (*S. frugiperda*), SpltNPV from the Tobacco cut worm *(S. litura),* SpliNPV from the Egyptian cotton leaf worm (S. littoralis) and others. Extensive research on these viruses and field tests resulted in the development of several commercial products (Hunter-Fujita et al., 1998). Field isolates of SeMNPV were found in the USA, Thailand and Spain (Caballero et al., 1992). A registered product, based on the US isolate, is registered and widely used in Dutch greenhouses and in the USA, where *S. exigua* was found to be tolerant to many chemical insecticides. SfMNPV is being used in Central and South America, where *S. frugiperda* occurs as a major pest of corn and rice. It was reported that more than 20 000 ha were treated with SfMNPV in Brazil by 1992 (Oliveira et al., 1998).

7. Characterisation of the genomes (e.g. open reading frames, insertion sequences), and stability of these characteristics

7.1. The structure of the baculovirus genome

The baculovirus genome is a double-stranded, circular DNA which varies between 90 - 160 kilo base pairs (kbp) for different members of the family Baculoviridae (Murphy et al., 1995). Based on the analysis of the entire genome sequence of AcMNPV, it was estimated that the genome contain between 140 - 160 genes. About half of these genes have been transcriptionally and functionally characterised (for review see Kool and Vlak, 1993, Possee and Rohrmann, 1997). The immense variation of genome size among different baculovirus species suggests that some baculovirus genomes contain significantly less genes than others. Apart of these interspecific differences of genome size, an intraspecific variation caused by natural variation (Chapter 7.2) and host transposon insertion (Chapter 7.3) can be observed. The ability of the baculovirus nucleocapsid to expand and to harbour additional genetic information was exploited and led to the development of the baculovirus expression vector system.

To date, the genomes of three baculoviruses isolated from noctuid hosts have been completely sequenced. These are AcMNPV (with a genome of 133,894 bp), BmNPV (128,413 bp) and OpMNPV (131,990 bp) (Ayres et al., 1994, Maeda, 1994; Ahrens et al., 1997). Other complete baculovirus genome sequences can be expected in the future. Partial sequence information of about 35 other baculovirus genomes is available on the GenBank database (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/).

In general, baculovirus genomes contain non-overlapping open reading frames (ORFs) with short intergenic regions. However, a few ORFs which partially overlapped adjacent ORFs were found. For example, in the genome of several NPV and that of *Cryptophlebia leucotreta* GV, the 5’ end of the adjacent, bidirectionally orientated ORFs encoding *p38* and *lef*-5 overlap to a considerable extent (Ayres et al., 1994, Jehle and Backhaus, 1994). Gene splicing has only been observed with the early activator *ie-0* of AcMNPV. Its coding region consists of a small exon and the ORF of *ie-1*, which are spliced together and expressed early in infection (Kovacs et al., 1991).

Interspersed homologous regions, known as hr regions, have been identified in many baculovirus genomes. The hr regions in the AcMNPV genome consist of multiple copies of 28-30-bp palindromic sequences flanked on either side by direct repeats of 20 bp (Cochran and Faulkner, 1983; Ayres et al., 1994). These regions function as cis-acting enhancing factors for the transcription of early genes by RNA polymerase II (Theilmann and Stewart, 1992, for review see Friesen 1997) and as origins of DNA replication in transient DNA replication assays (Pearson et al., 1992; for review see Kool et al., 1995).

7.2 Natural variability of baculovirus genomes

In the 1970s, progress of the analysis of baculovirus genomes was accelerated: (i) the development of *in vitro* and *in vivo* cloning procedures, which allowed researchers to isolate and to propagate single virus genotypes, and (ii) the introduction of restriction endonuclease analysis, which allowed isolated virus DNA to be cleaved into fragments of specific length.

The occurrence of submolar bands in the restriction enzyme profiles of isolated DNA of baculovirus field isolates provided first evidence of substantial genetic variation within the natural baculovirus population. When single genotypes were isolated using plaque purification assays, these differences were commonly mapped to point mutations, acquisition or deletion of restriction sites or to insertions or deletions of hundreds or thousands basepairs (Lee and Miller, 1978; Crook et al., 1985; Smith and Crook, 1993).

7.3 Transposon insertion into baculovirus genomes

Horizontal transfer of insect host transposable elements (TE) into baculovirus genomes is an occasionally observed event that contributes to genetic heterogeneity. Most TE insertion of AcMNPV or GmMNPV were associated with a specific alteration of plaque morphology of infected insect cells and characterised by a significantly reduced number of polyhedra per infected cell (Fraser and Hink, 1982; Fraser, 1986). These so-called “few polyhedra” or FP mutants were generated during serial passage and harboured transposon insertion within the 25K gene commonly known as FP locus (map unit 36.0 - 37.0). The role of the 25K gene has not yet been completely elucidated but it might be involved in the temporal regulation of BV and ODV production (Jarvis et al., 1992). AcMNPV with a mutated 25K gene was frequently impaired in virion occlusion and intranuclear nucleocapsid envelopment but released up to five-times more BV from infected SF-9 cells (Harrison and Summers, 1995). Disruption of the 25K gene apparently favours the production of budded viruses (BV). Since BV are about three orders of magnitude more infectious to cultured cell lines than ODVs, it is likely that transposon carrying FP-mutants have a strong selection advantage over wild-type viruses during the specific conditions of serial passaging in cultured cells.

A number of FP mutants of AcMNPV and GmMNPV harbouring transposons which belong to the class of (DNA)-transposons have been described (Table 3). These transposons originate from *T. ni* or *S. frugiperda* host cells. Although their sizes vary from 0.3 - 2.5 kbp, they share common structural characteristics as short terminal inverted repeats of 13-15 bp and a duplication of a TTAA target sequence (for review see Fraser, 1986; Friesen, 1993; Jehle, 1996). Most of these transposons lack open reading frames to encode a transposase suggesting that they are defective copies that cannot transpose autonomously. Only transposon piggyBac (formerly termed IFP2) was shown to contain an ORF which supports insertion and excision of this element (Fraser et al., 1995; Elick et al., 1996).

The conformation that transposon insertion into baculovirus genomes is not restricted to the specific conditions of cell culture but also occurs during normal infection of host larvae was provided by Jehle et al. (1995) who demonstrated the insertion of two different Tc1/mariner-like transposons into CpGV genome after infection of *C. pomonella* and *C. leucotreta* larvae.

Host transposon insertion into baculovirus genomes exerts drastic effects on the genomic integrity of the virus and has possibly far reaching consequences for baculovirus evolution. The observed effects are (i) acquisition of new functional genes and/or regulatory sequences, (ii) disruption of viral genes rendering them non functional, and (iii) deletion of extended portions of the genome by self-recombination or excision (Carstens, 1987). In most cases, transposon insertion can result in reduced fitness and competitiveness of the altered virus. However, the isolation of the FP-mutants and the CpGV mutants containing a transposon have demonstrated that these viruses are viable and that transposon insertion significantly increases genetic heterogeneity.

Table 3: Insect host transposons found in baculoviruses

Transposon	**Host Virus**	**Insect Origin**	**Reference**
TED (retrotransposon)	AcMNPV	*T. ni*	Miller and Miller, 1982; Friesen and Nissen, 1990
M5	AcMNPV	*S. frugiperda*	Carstens, 1987
IFP2 (piggyBac)	GmMNPV	*T. ni*	Cary et al., 1989
TFP3 (tagalong)	AcMNPV	*T. ni*	Wang et al., 1989
TFP3 (tagalong)	GmMNPV	*T. ni*	Wang et al., 1989
IFP2.2	AcMNPV	*S. frugiperda*	Beames and Summers, 1990
IFP1.6	AcMNPV	*S. frugiperda*	Beames and Summers, 1990
E	AcMNPV	*S. frugiperda*	Schetter et al., 1990
hitchhiker	AcMNPV	*T. ni*	Bauser et al., 1996
TCl4.7	CpGV	*C. leucotreta*	Jehle et al., 1995
TCp.3.2	CpGV	*C. pomonella*	Jehle, 1996

8. Genetic transfer capability

8.1. Recombination

Recombination is a decisive mechanism which is responsible for intergenomic exchange of information in all organisms, including baculoviruses. There are two different classes of recombination, a homologous and a non-homologous or illegitimate recombination. Homologous recombination means the genetic exchange of allelic sequences between two different genomes, whereas non-homologous recombination comprises all events of non-allelic sequences exchange.

8.1.1 Homologous recombination

With baculoviruses, extensive homologous recombination has been observed between the closely related AcMNPV, GmMNPV, RoMNPV and TnMNPV (Miller et al., 1980; Smith and Summers, 1980; Croizier and Quiot, 1981). After coinfection of TN-368 cells of *T. ni* with AcMNPV and RoMNPV seven out of 100 isolated plaques where identified as recombinants, each with at least one crossing over event (Summers et al., 1980). Intraspecific recombination was also observed when SF-9 cells were co-infected with different pairs of AgMNPV genotypes (Croizier and Ribeiro, 1992). These results indicated that the frequency of recombination among highly homologous baculoviruses is considerable.

When Croizier et al. (1988) co-transfected larvae of *Galleria mellonella* with isolated DNA of RoMNPV and restriction fragments of AcMNPV, a very high number of RoMNPV recombinants but no parental RoMNPV genotypes were recovered. Since replication of AcMNPV in *G. mellonella* is much more effective than that of RoMNPV, it was suggested that the RoMNPV might have acquired an unknown replication advantage by recombination with AcMNPV DNA fragments.

A high recombination rate was also found when *T. ni* larvae were co-infected with wild-type AcMNPV and a polyhedrin-negative, lacZ-expressing AcMNPV mutant. A recombination frequency of at least 6.6% was estimated by plaque assays of hemolymph of co-infected larvae and scoring for recombinant plaques (Merrywheather et al., 1994).

Further evidence for extensive homologous recombination among different variants of the same virus species is based on the isolation and restriction mapping of wild-type virus mixtures. By the method of *in vivo* cloning Smith and Crook (1988; 1993) isolated a number of different ArGV genotypes which differed in a few restriction fragments. Based on comparative physical mapping, it was suggested that the diversity of these variants was partly caused by recombination during natural coinfections. A rapid replacement of two closely related parental SeMNPV variants (Se-US and Se-SP2) by a recombinant (Se-SUR1) which differed from the parental strains by several cross over events was observed after co-infection of *S. exigua* larvae. This result suggested selection advantage of the new recombinant over the parental strains, though significant differences in the biological activities (LD_{50} and LT_{50}) were not observed (Munoz et al., 1997).

These findings underscore the fact that homologous recombination among virus variants is a normal process of genetic exchange which occurrs at a considerable frequency during *in vitro* and *in vivo* replication. The process of homologous recombination appears to be linked to and driven by the viral replicative machinery. Although the genetic factors involved in recombination have not been identified, it

was suggested that genes involved in DNA replication and possibly hr regions may play a central role in the events (Martin and Weber, 1997).

8.1.2 Heterologous recombination

The probability of recombination between baculovirus sequences decreases drastically when the baculoviruses are less closely related. So far, recombination between different baculovirus species has only been observed in laboratory experiments where the generation of recombinants was biased by selection or replication advantages. For example, the identification of genes encoding host range factors or apoptosis inhibitor was achieved using this approach (see A 3.3 and A 4.3).

Heterologous recombination has been forced by co-transfection of restricted DNA or cloned DNA fragments with intact viruses or virus DNA. In an experiment addressing the possibility of interspecific recombination Roosien et al. (1986) co-transfected *S. frugiperda* cells with a polyhedrin negative AcMNPV mutant and a plasmid containing the polyhedrin gene of MbMNPV. AcMNPV mutants were isolated and contained the MbMNPV polyhedrin gene insertion at different, non-specific sites. However, these recombinants contained less virions and showed reduced infectivity to *S. frugiperda* cells than wild-type AcMNPV. Similar results were obtained when a polyhedrin minus AcMNPV mutant was co-transfected with a cloned polyhedrin gene of SfMNPV (Gonzales et al., 1989). The recombinant isolated from this experiment expressed less than 25% of the level of wild-type AcMNPV polyhedrin gene.

In general, the competitiveness and biological fitness of heterologous recombinants is lower than that of wild-type viruses. One reason is that by non-specific recombination events, coding or regulatory sequence functions might become impaired resulting in a less viable virus.

B. HUMAN HEALTH CONSIDERATIONS

Summarizing Nos. 9 - 20

Baculoviruses are naturally occuring pathogens of arthropods. Their host range is exclusively restricted to arthropods. No member of this virus family is infective to plants or vertebrates. Baculoviruses are ubiquitously present 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.

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). Safety tests of more than 51 entomopathogenic viruses including 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.

Safety tests of baculoviruses included

In *vitro and* in vivo *replication of baculoviruses in vertebrate 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 intraperitoneal 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).

Subacute 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 as chromosomal aberrations or sister chromatid exchange to mammalian cell lines

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).

C. ENVIRONMENTAL AND AGRICULTURAL CONSIDERATIONS

21. Natural habitat and geographic distribution. Climatic characteristics of original habitats

Baculoviruses are ubiquitous in the environment, their prevalence depending on the frequency of occurrence of their arthropod hosts that inhabit terrestrial and marine ecosystems. By 1986, about 1100 viruses known to infect insects had been described, more than 60% of them being baculoviruses (Martignoni and Iwai, 1986b). Natural epizootics and their survival under a variety of environmental / transport conditions provides for particularly high densities in diverse locations in the environment which may act as infection sources for pathogen multiplication (cabbage leafs, dust: Heimpel et al., 1973; Olofsson, 1988). Viruses from the same arthropod species from different geographic regions of the world may be as closely related as their hosts as demonstrated by the similarity of their restriction pattern (Vickers et al., 1991). However, different baculoviruses can also be isolated from the same species in one geographical region. Restriction endonuclease digest patterns can be used to analyse the genetic heterogeneity of baculovirus strains and of strains obtained from different geographical regions (Laitinen et al., 1996).

22. Significant involvement in environmental processes, including biogeochemical cycles and potential for production of toxic metabolites

The ecological significance of baculoviruses is characterised by their impact on the population dynamics of their arthropod hosts. As deduced from experiences with epizootics of insects they can be regarded as a regulator of their host population density and multiplication. No report on a potential contribution of baculoviruses to the extinction of insect species is available. This feature does not seem to depend only on the usual evolutionary adaptation of the interaction of pathogens with their hosts, but might be a more fundamental property of their life style. Through international trade, and in the course of applications of baculoviruses for pest control to a considerable extent, strains of baculoviruses have been moved around the globe without negative observations on local faunas. The ecological traits of baculoviruses (in particular virulence and dispersal characteristics) in their combination are an apparent biological constraint on excessive population increases, potentially endangering the basis of their existence, and the survival of their arthropod hosts.

With the impact on population densities of their hosts, a secondary effect on predators, parasites and hyperparasites is evidently connected.

23. Pathogenicity - host range, infectivity, toxigenicity, virulence, vectors

23.1 Bioassays for the testing of infectivity and virulence

Parameters of baculovirus host range, infectivity, and virulence are tested by bioassays which measure the response (e.g. mortality) of test species ("subjects") to different doses of defined virus preparations ("stimulus"). The *in vivo* bioassay is the only means by which the combined effect of all factors determining the potency of a baculovirus preparation can be measured. It is also used as a very sensitive method of monitoring the fate of baculoviruses in the field (see C.28). The titration of occlusion bodies in the light microscope offers a convenient method to compare different virus preparations with respect to the concentration of their (potentially) active structures. In principle, any comparisons between two virus preparations (e.g., a test substance with a standard preparation) requires that the dose response curve for both have the same shape, i.e. the bioactive component of the preparations is essentially similar. An understanding of the basic biological and statistical concepts of bioassays is important to fully realise their potential for analysing baculovirus interactions with other organisms and for improving their application (Hughes and Wood, 1986).

To obtain statistically meaningful results with the desired precision bioassays have to be carefully designed with respect to the **number of subjects** challenged. Optimised strategies depending on previous knowledge and model assumptions have been described (e.g., Hughes and Wood, 1986). Bioassays determining the yes or no (quantal) response of larval death through virus infection result in dose response relationships with the median effective dose ED_{50} (or EC_{50} if expressed in virus particles per ml) and the slope of the response curve as characteristic parameters. In addition to counting dead individuals, response data may also include the determination of particle titers in the cadavers. If the time course of larval death is recorded (time-mortality assays), as conveniently done in one experimental approach, monitoring results yield median lethal time LT_{50} (continuous exposure to virus) or median survival time ST_{50} (one inoculum at start of experiment), respectively.

To obtain meaningful and reproducible results other conditions of bioassays must be carefully observed. With respect to the **test organism,** uniform instar stages of larvae are used and reared under identical conditions (temperature, light regime, diet, group size). The ability to hatch insect larvae reproducibly with minor premature deaths (e.g. by bacterial infections) is one major problem for testing a great number of different test species, as required for host spectrum analysis.

Infection is frequently performed by applying the virus preparation to the surface of the natural or artificial diet - or by incorporating virus suspensions to the components of the artificial diet. Depending on the experimental design, the test species are kept on inoculated material for the duration of the assay - or the regime includes a transfer to a noninoculated diet, in order to better control inoculum and infection time. A large variety of experimental protocols for controlled virus inoculation have been described. They all suffer from inaccuracies, to which the variation of feeding behaviour of the insects also contributes (Hughes and Wood, 1986). Direct administering of virus suspensions by microinjection into the mouth of larvae, or the "measured drop feeding" method are ways to improve control over dose and inoculation time (Kunimi and Fuxa, 1996). The incubation of eggs with virus suspensions to infect neonate larvae seems to be a method for a well controlled infection of large numbers of insects at an identical developmental stage. Some of the latter methods are time consuming and have other limits (size of larvae for injection) which prevent their widespread use. Experimental protocols in general have to seek a sensible compromise between accuracy and practicability. Modifications of the assays have to accommodate the habits and peculiarities of the test species.

Data interpretation follows model assumptions. If the only interest is an estimate of the median effective dose and no inferences with respect to the absolute number of virus particles can be made, the probit model may be used. It assumes a log-normal distribution of individual effective doses (IED) for each host which invariably induces the response. The model refers to mechanistic interpretation of chemical interactions and assumes some cooperativity to be necessary for infection. The necessity to overcome host defenses can be considered as an interpretation of such assumptions in light of present knowledge. However, unlike chemicals, replicating entities such as bacteria and viruses can act independently to produce a response. In these cases, and if the hosts are reasonably uniform in their susceptibility to the pathogen, the dose response curve can be described and interpreted more adequately by an exponential model. The model describes infection as a stochastic process in which the lethal dose is the same for all individuals whereas the response rates reflect the chance to receive this dose. Essentially, one infectious virus entering the replicative stage is able to manifest the response according to such a model. Predictions of quantitative features of the infection process according to the model can be tested experimentally and results do not conflict with its interpretation, stressing the general applicability. (Hughes et al., 1984; Hughes and Wood, 1986; Ridout et al., 1993).

The method of "*in vivo*-cloning" takes advantage from the capacity of single viruses to initiate a productive infection. Administering virus titers for infection far below the LD_{50} results in a relatively large fraction of surviving larvae. The fraction of larvae, which died from replication of a single infectious unit can then be calculated (Huber and Hughes, 1984). This method, eventually used in two consecutive steps to enhance probability of the selection, is conveniently used to obtain genetically homogeneous virus preparations (Smith and Crook, 1988).

23.2 Survey of 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 nontarget 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). In the following, the studies are only briefly summarised because they conclusively demonstrated the absence of any adverse effects.

(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 (section B).

(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.

Recently, toxic effects were observed with a larval test which was considered to be useful for assessing adverse effects of microbial pest control agents on nontarget 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. Similar mortality was observed

with a 10^{-4} dilution of a commercial mosquito larvicide based on *Bacillus thuringiensis* ssp. *israelensis* (Bti). No effect was observed with a molluscidal strain of *Bacillus alvei* or a broad-host-range fungal insect pathogen, *Metarhizium anisopliae.* Sodium dodecyl sulfate and a watersoluble fraction of a fuel oil were tested as a reference for comparison (Gormly et al., 1996).

(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 (see C.23/24).

(4) No genotoxic 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 (C.26).

One experimental approach to use vectorized transport by honey bees as a nonintrusive means for virus dispersal has been field tested and indicates the perception of safety and non-target-innocuousness of baculoviruses. 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).

23.3 Host range

The range of arthropods that can productively be infected by a given baculovirus can be regarded as its host range in a narrower sense. On one side, a limited host range is a feature of a rationally targeted biocontrol programme and an issue of biological safety. The control of defined pest species avoids side effects on the environment in general, and adverse effects on non-target insects including predatory and parasitoid species which provide additional control, in particular. On the other side, host range restrictions are also frequently regarded as an important application constraint if a complex of pest insects has to be managed. They determine limitations for the potential market volume of viral insecticides. Reports on particular host range testing and the selection strategies on virus strains for applications reflect this ambivalent view on host range restriction.

Among the approaches to relief application constraints for virus insecticides, prospects of modifying - and enhancing - host range by the use of molecular biology tools are being developed (Doyle *et al*., 1990; Thiem,1997). The realisation of this objective will challenge the validity of host range testing procedures and the interpretation of their results even more than the use of genetically modified viruses does in general. An increasing knowledge about the molecular interactions involved in host range determination and limitations is the basis for such an approach. In comparison to the use of selective

strategies on natural viruses such a basis may enable a design of modifications for more targeted and predictable effects on virus phenotype (Miller and Lu, 1997; see section A.3.3).

The genetic information of the virus genome, conserving evolutionary processes for perpetuation of its existence in time, is realised in the sequence of processes during an infectious cycle typically resulting in the release of high numbers of progeny virus from larval carcasses (see section A.3.1). Abortive or persistent infections indicate potential alternative strategies for persistence . A productive infection requires the specific interaction of host components with virus structure and virus encoded functions within a temporal and local arrangement of “key-lock fittings” in order to overcome host barriers and defenses and to exploit and transform host functions for viral replication. Overall, these specificities correlate with phylogenetic relationships, and the systematics of baculoviruses seems to represent the common evolutionary origin and concerted evolution with their arthropod hosts to some degree.

In detail however, evolution - and the variation and distribution of “keys” and “lockers” - follow different pathways in virus populations and in sexually reproducing animals. Genome modifications by mutations and genetic exchange are governed by different mechanisms and constraints. Thus, the degree of adaptation of a virus strain or “species” to a set of host organisms providing the requirements for productive interactions (specificities) presently is not easily discernible from its systematic position (which is defined by the degree of homology of (parts of) his genome to other viruses). In the future, the molecular analysis of the determinants (genes) for virus-host interactions and of their variability in natural populations may provide more power of inference with respect to the host range of a particular virus.

So far, the testing with host species *in vivo* provides the relevant empirical basis for judgements about host ranges. Methods of data generation with respect to the following cannot follow harmonised and accepted internationally standardised procedures:

- the control of the homogeneity of the virus inoculum,
- the administration of the virus and the range of concentrations to be used,
- the spectrum of insect or other arthropod species and cell types to be included, and
- the selection and control of end points (e.g., larval death, virus multiplication and yield, transcription of genes, DNA replication).

Parameters of influence on testing results include:

- the particular genotype of test species,
- the developmental stage of larvae during infection,
- the diet for host, and
- other conditions challenging the general stress tolerance of the test species (e.g., temperature, moisture).

Descriptions of a host range spectrum based on empirical testing will always suffer from ambiguities and incompleteness, due to apparent limitations in test species selection and experimental parameter variations. Studies of virus entry, DNA replication, transcription of genes and assembly of virus progeny together with the observation of cytopathic effects in cell cultures do substantially contribute to an understanding of host specificities at the level of molecular interactions (Miller and Lu, 1997). They complement but do not supplement, so far, in vivo testing of host range limitations (Danyluk and Maruniak, 1987; Castro et al., 1997). It may be stressed again, that an understanding of mechanisms is a prerequisite to improve inferences from genotype to host specificity phenotype.

Host range and parameters of virulence may be correlated to a different extent. An increase in virulence may appear as a host range extension in conventional testing using fixed doses of virus challenge. However, the spectrum of susceptible host species may not have changed to any extent. A maintenance of the order and quantitative relations of infectivity parameters (LD_{50}s) would indicate an unchanged host range. A precise definition of experimental methods and end points clearly is a prerequisite for a common understanding of host range and virulence parameters.

The modification of virulence/host range by a selective procedure in the laboratory, cited by Federici and Maddox (1996) to exemplify a natural ability to manipulate host range, can be used to illustrate the importance of both, virulence and host range determinations. Martignoni and Iwai (1986a) used large doses of the MNPV of *Orygia pseudotsugata* (tussock moth, family: Lymantriidae) to sequentially infect *Trichoplusia ni* (cabbage looper, family: Noctuidae) over a period of 12 generations. In the course of these experiments, representing a selective pressure which in its extent will not occur in natural settings with any realistic probability, the virus adapted to the novel host, the cabbage looper. Low mortalities and minor levels of infection in most tissues at the beginning were converted to increasing levels of tissue infection and a complete adaptation of the virus to the novel host by the seventh generation. The passaged virus strain, if not selected from a heterogeneous inoculum, apparently accumulated some (set of) unknown mutation(s) and also had increased its virulence for its original host, the tussock moth, by a factor of ten. This increase in virulence, however, would not in itself necessarily result in the expansion of the host range, as observed.

An overview of the host ranges of baculoviruses, as determined in cross infectivity studies in the laboratory is given by Gröner (1986). Results presented in tables are cited with the reservation that most studies did not control the identity with the inoculum virus after challenging of different hosts. Thus, the activation of a latent virus infection followed by its replication or the selective propagation of a minor contaminating virus type cannot be excluded in most cases. In general, NPVs infect only members of the genus, or in some cases the family, of their original host. A tendency that multiple embedded NPVs have a somewhat broader host range than SNPVs and an even more limited host range of granuloviruses was noted. Among the MNPVs, the prototype AcMNPV is known for its relatively broad host range and infectivity for more than ten families within the order Lepidoptera.

The identification of virus progeny by molecular methods is recommended and used as a standard tool for host range determinations in other publications as well. Predominantly a restricted range of infectivity is corroborated (Barber *et al*., 1993). Comprehensive studies on a broad selection of potential host insects were induced by the necessity to generate an adequate background for respective studies with genetically modified baculoviruses (Doyle et al., 1990; Doyle and Hirst, 1991). These studies resulted in the finding that MbMNPV is infective for both, butterflies and moths (Doyle and Hirst, 1991). MbMNPV of the cabbage moth *Mamestra brassicae* (Lepidoptera: Noctuidae) also infected the small tortoiseshell butterfly, *Aglais urticae*. However, a very high virus inoculum (106 OBs) was necessary for virus multiplication and larval death. MbMNPV also infects *Cynthia cardui* (painted lady) and *Vanessa indica* (Indian red admiral), also of the Nymphalidae, but does not seem to infect other butterflies of the families Satyridae, Lycaenidae, or Pieridae (Doyle et al., 1990). The cross infection studies showed a somewhat broader host range of MbMNPV than previously demonstrated. Other baculoviruses are also infective for Nymphalid butterflies.

The (quantitative) interpretation of biotest data with respect to potential consequences from exposing natural populations, has not been investigated in any detail but could be regarded as a key area of judgements about baculovirus biosafety (Doyle et al., 1990; Cory et al., 1997). It is conceivable that the non-susceptibility at lower doses might normally prevent epizootics from being initiated from weak cross infectivities, e.g. after spray applications for biological control. But an improved potential of extrapolation

from infectivity studies with respect to a potential of virus transmissions in heterologous populations is highly desirable, to more clearly define relevant borders of host range. Data from laboratory bioassays might be poor estimates for the prediction of nontarget impacts.

A multiple-embedded nucleopolyhedrovirus, AnfaMNPV, isolated from the celery looper, *Anagrapha falcipera* in central Missouri seems to have the broadest host range reported so far (Hostetter and Puttler, 1991). 31 susceptible species belong to ten lepidopteran families, and in addition to *Helicoverpa sp.*, *Heliothis sp*., and *Spodoptera sp*. include important pests like pink bollworm (*Pectinophora gossypiella*), fall webworm (*Hyphantria cunea*), cutworms (Noctuidae), velvet bean caterpillar (*Anticarsia gemmatalis*), codling moth (*Cydia pomonella*), navel orangeworm (*Amyelois transitella*), and the diamondback moth (*Plutella xylostella*). As compared to AcMNPV, known for its relatively broad host range, the virus demonstrated equal virulence for species differing in susceptibility for AcMNPV by one order of magnitude. Relatively narrow criteria were used for differentiation of susceptibility (max. 250 OB/mm^2 of diet). The list of nonsusceptible species as published (containing also Coleoptera and Diptera) might thus include some species which are susceptible, if challenged at higher doses. Propagation of virulent AfMNPV in tobacco hornworm (*Manduca sexta*) as untypical host demonstrated extensive possibilities for production in a variety of hosts and systems. Virulence against a great number of cosmopolitan economically important insect species was considered an encouraging result in this report and the virus became the first baculovirus patented by the US Government Patent and Trademark Office.

23.4 Variation of virulence and host range

By describing interactions between biological entities, virulence and host range are prone to modification by natural variability and by a variety of external “environmental” factors. Mutagenesis, recombination, and the (selective) propagation in different hosts have been analysed as causes of shifts in host range and virulence of baculoviruses, in the laboratory (sections A.3.3; A.6-8.). The same mechanisms are acting in natural or managed environments, however to an extent unknown in any detail and responding to different selection conditions. In the following, emphasis is given to some factors modulating insect responses to viral challenges that have not been described in other sections.

23.4.1 External (environmental) factors modulating insect response to baculoviruses

Chemicals

An additive or synergistic effect of chemicals on baculovirus infectivity has been exploited for control strategies, for example, by the use of combinations with insecticides or the addition of particular radiation protectants (section A.6).

Host plants

The plant material on which larvae are feeding may have a significant influence on their susceptibility for viral infections. Gypsy moths, feeding on *Quercus rubra* (red oak) or *Acer rubrum* (red maple) demonstrated a higher level of tolerance when infected with LdMNPV than larvae feeding on *Populus tremuloides* (quaking aspen) or *Pinus rigida* (pitch pine). The enhanced tolerance was correlated with an increased acidity and hydrolysable tannin content of leaf material (Keating and Yendol, 1987;

Keating et al., 1988). Different tolerances for *Spodoptera littoralis* NPV were also noted in the cotton leaf worm on castor bean, alfalfa, mulberry, cotton and potato. LD_{50} values differed by a factor of about 3 between castor bean (the most "protective" feeding source) and potatoes, and the LT_{50} was also reduced by more than 10% on the latter plant (Santiago-Alvarez and Ortiz-Garcia, 1992).

A different approach was followed by Rabindra et al. (1994), who investigated the influence of plant surface environments on the virulence of *Heliothis armigera* SNPV. The larvae of the American bollworm were challenged with identical (microscopically controlled) titers of virus suspensions which had been exposed to the surfaces of 5 host plants (chickpea (*Cicer arietinum*), pigeon pea([syn. *dhal*] *Cajanus cajan*), lablab bean (*Dolichos lablab*), sunflower, and cotton). Bioassays used the leaf dip method with shoots of chickpea as identical feeding material for the test species. The cotton leaf surface was the most detrimental for virus activity resulting in an increase of more than three orders of magnitude in the LC_{50} value in comparison to lablab bean -exposed viruses (5×10^6 OB/ml and 1.4×10^3 OB/ml, respectively). Lablab bean even had some protective or stimulating effect on virulence in comparison to untreated NPV preparations ($LC_{50} = 8 \times 10^4$ OB/ml). Median lethal time measurements corresponded with these observations. A mortality time expanded by 25% after cotton leaf - exposure in comparison to lablab bean exposed virus was observed. This experimental approach was also used to determine the effect of adjuvants added to the virus preparations because the study together with others indicates the need to develop suitable formulations which could protect the virus from inactivating factors on plant surfaces.

These examples demonstrate the modulation of baculovirus virulence by the plant environment on which target insect larvae are feeding. This environment either indirectly alters insect response and tolerance, or interferes with the first steps of virus infection through variation of the milieu of the midgut lumen (e.g. pH), and/or directly modulates the specific activity (the probability of an OB in the light microscope to enter the replicative state) of virus preparations. An understanding of these kinds of interferences is valuable to improve formulations for insect control and to adapt methods of *in vivo* baculovirus production. It can also be used to reassess control strategies if applied to novel plant varieties (Beach and Todd, 1988). In an advanced stage of development of modelling the variability introduced by the respective plant canopy may be included. (Foster et al., 1992).

Population density

A variety of environmental factors such as temperature, light, nutrition, and humidity may compromise the capability of insects to resist baculovirus infections (Briese, 1986). Among these, rearing density has a distinct effect on their susceptibility to viral infection. Together with other phenotypic modifications (development time, weight at moulting, degree of melanisation), enhanced susceptibility is observed as a reaction to the stress of high population density of lepidopteran larvae (Goulson and Cory, 1995b). This was interpreted as a consequence of the adaptive response of accelerated development at the expense of larval weight, which compromised the ability to express resistance functions. Interestingly, larvae which were reared singly exhibited many of the same characteristics, including enhanced susceptibility to virus infection. The benefit of spending resources for resistance development may be low, if a low density of conspecifics reduces infection risk. This phenotypic variation is among the factors modulating the relationship between virus density and disease transmission.

Other infectious agents, parasitoids

The exposition of insects with different infectious agents or parasitoids is a different kind of stress, eventually enhancing baculovirus induced mortality (C.24). Superinfection of insects with a latent or persistent infection may result in larval death due to the propagation of the latent virus (see A.4.6).

23.4.2 Insect resistance and immunity

The varying degree of tolerance of insect larvae for baculovirus infections at different instar stages, usually increasing with age and stage of development, is but one observation suggesting the existence of particular mechanisms of pathogen tolerance (Mikhailov et al., 1992; Engelhard and Volkman, 1995). To assess the short-term and long-term effectivity of control strategies with baculovirus insecticides an understanding of the adaptive potential of insect populations to baculovirus infections is of paramount importance. In a review Briese gives an account on developmental and environmental factors affecting resistance (1986), the knowledge about defence mechanisms and the genetic factors conferring increased virus tolerance. Dominant and recessive autosomal genes or a multigene family may form the genetic basis of tolerance in insects. E.g., McIntosh and Ignoffo (1989) demonstrated that the resistance of *Helicoverpa subflexa* against *Helicoverpa* zea NPV (HzSNPV) appears to be controlled by a single non sex-linked gene.

Observations of markedly expressed virus resistance are restricted to laboratory observations of insect populations obtained under high artificial selective pressure of virus challenges, for example, by propagating the surviving fraction of infections with an LD_{80} in several subsequent generations. Somewhat conflicting results with respect to the success of selection strategies may either be caused by the general potentials of the insect species under investigation or by the particular genotype of the strain as most obvious reasons (e.g. Kaomini and Roush, 1988). It is a general objection against the predictive value of laboratory selections, that insect cultures in the laboratory represent a minor fraction of the genetic heterogeneity of natural populations. The lack of documented cases of resistance in field populations of insects under control of a virus insecticide may reflect their less frequent and extensive use in comparison to chemicals, which have induced an exponentially increasing rate of resistance developments in insect populations.

There is some circumstantial evidence that the spectrum of tolerance and respective gene frequencies in natural populations may have been biased by natural virus epizootics or the application of virus insecticides (Fuxa et al., 1988). Laboratory selections in colonies of velvetbean caterpillar (*Anticarsia gemmatalis*) from the US and Brazil demonstrated the achievement of a significantly higher level (1000x) of resistance to AgMNPV after 13 - 15 generations in the Brazilian colonies than in the colony from Lousiana, the resistance of which levelled off at a ratio of 5x after 4 generations (Abot et al., 1995). AgMNPV, is used for biological control of the velvetbean caterpillar in soybean on a large scale in Brazil (see A.6.4).

Alleviation of the selection pressure by virus challenges seems to favour more competitive sensitive wild-type genotypes of insects, which are superior in their reproduction without the virus selection pressure (Fuxa and Richter, 1989). Such observations correspond to a more general perception that some cost in reduced fitness has to be paid for the expression of disease resistance. This hypothesis however, has not been tested rigorously in many cases (Gemmill and Read, 1998). Genes for virus resistance confer pleiotropic phenotypic effects including increased susceptibility to chemical insecticides (Fuxa and Richter, 1990). The exclusive restriction to the oral application route of the increased virus

tolerance and enhanced insecticide susceptibility phenotypes indicates a correlation of modifications to components of the midgut interfering with early stages of virus infection.

Using the genetically modified virus AcMNPV-hsp70/lacZ for the monitoring of virus activity by the lac-Z reporter gene, the physiological basis of resistance of *Helicoverpa zea* against AcMNPV was analysed by Washburn et al. (1996). The larval cells were actually very susceptible to AcMNPV infection, but infected cells were encapsulated by hemocytes and subsequently cleared from the midgut lumen. Cellular immune responses of larvae seem to be a significant factor in preventing the spread of the infection and a determinant of the functional host range of baculoviruses. Based on further experiments including chemical and biological immune suppression, it was suggested to use modified viruses to express immunosuppressive genes in order to compensate for this defence mechanism.

24. Interactions with and effects on other organisms in the environment

Direct effects of baculoviruses on other organisms in the environment are restricted to their host range, and most have been observed only in their original host or in the target species of biocontrol applications. Baculovirus infection interferes with other pathogens or parasitoids feeding on a shared host. Observations on the synergistic or additive effect of particular crossinfections with other pathogens as well as interferences with parasitoids have been summarised by Harper (1986). Section A 4.2 gives some account to the synergistic co-operation between some baculoviruses. Some of such pathogen combinations have been used in field experiments to improve control of pest species.

The analysis of the interference with parasitoid developments is used to assess the possibility of adverse effects on populations providing additional natural control of pest species. The observed detrimental effect on parasitoid development, fitness or reproductive success as well as an enhanced sensitivity of parasitised insect larvae for baculovirus infection can most adequately be described with the interspecific competition for a limited resource (Cossentine and Lewis, 1988; Hochberg, 1991; Al Fazairy et al., 1993; Nakai and Kunimi, 1997). The relative timings of baculovirus infection and parasitoid emergence determine the degree of interference to a large degree. No productive infection of parasitoids was observed but frequently a large number of viral occlusion bodies is present in their midgut lumen, indicating a potential mechanism of virus dispersal. A reduced level of a parasitoid population has been observed after aerial LdMNPV application (Webb et al., 1989).

25. Ability to form survival structures

The structure of baculovirus occlusion bodies provides for their potential to persist in the environment in a variety of abiotic and biotic conditions (A.4.1/4.7). This feature is described as a key factor dominating the dynamics of virus transmission and a prerequisite of baculovirus epizootiology by Evans (1986). Viability may extend for more than 40 years in forest soil. No special survival structures are formed.

26. Routes of dissemination, physical or biological

26.1 Physical dispersal

Transport and dispersal of baculoviruses by wind or water is considered to be of minor importance for the natural spread of baculovirus infections. Its contribution to mechanisms of primary dispersal in natural epizootics is not known in detail (Briese, 1986). In field experiments, testing the spatial spread of a baculovirus, ballooning of first instar larvae of the gypsy moth *Lymantria dispar* was a good predictor of viral spread in the first few weeks. The scale of spread and its lack of directionality at later times however, did not match the results of a mathematical modelling even when primary dispersal by ballooning and short distance larval dispersal with a high rate of disease transmission were included. Circumstantial evidence suggested that autonomous dispersal by wind of occlusion bodies did not significantly contribute to dispersal. Parasitoid vectoring of viruses was discussed as the potential relevant additional dispersal mechanism (whereas vectoring by other animals was not considered (Dwyer and Elkinton, 1995). Evidence for a dispersal of NPV from soil to pine foliage in dust was presented as a likely explanation for the observed distribution of diseased larval colonies near a forest road by Olofsson (1988).

Aerial dispersal is the predominant mechanism of distribution for insect control applications; techniques and timing have to consider meteorological conditions and other agricultural procedures like irrigation for optimal distribution (Young, 1990; Payne et al., 1996).

Reminiscent of traditional testing procedures for the prediction of exposition rates with chemicals, the leaching behaviour of baculoviruses in natural soil cores and columns filled with soil or sand has been tested to evaluate eventual rates of exposition of the groundwater table. Consistently, some experiments demonstrated the retention of baculoviruses in soil to be comparable or superior to that of other Viruses (Polio virus and bacteriophage f2 of *Escherichia coli* were used for comparison). Soil with higher levels of organic matter was less efficient than sand for the reference viruses but not for the baculoviruses (NPVs and GVs). Results were independent from the particular composition of percolating water. Lysimeter studies with baculoviruses did not result in any observation of positive samples of leachate taken at a depth of 1.5 m during 7 months of monitoring. The concentration of viruses applied in this experiment resulted in a surface exposition 3 - 4 orders of magnitude above concentrations used in agricultural applications (10^{14} particles of a Granulovirus in 20 L of tap water applied to a surface of 0.8 m^2). A good retention of baculoviruses by soils was concluded and this property was tentatively attributed to the particular protein envelope of virus particles consisting of polyhedrin and granulin, respectively (Lopez-Pila, 1988).

The behavioural shift of some insect larvae following baculovirus infection (i.e. the movement to higher and exposed positions on host plants: “Wipfelkrankheit”) effects the physical distribution of baculovirus particles following death. It might be an evolutionary adaptation of the virus to increase horizontal transmission, e.g. by contaminating more foliage following rainfall (Vasconcelos et al., 1996a; Goulson, 1997).

26.2 Vectorized dispersal by diseased larvae, predators and parasitoids

Virus infected larvae are an effective dispersal agent even before larval death and carcass lysis, which results in very high inoculum density. Viable virus particles are also dispersed in the environment through either defecation or regurgitation during late stages of infection (Vasconcelos, 1996). Cannibalism of infected larvae may be another possibility if it occurs at any relevant frequency. Transport and dispersal of virus particles also occurs by predators feeding on diseased larvae or by parasitoids ovipositioning on and developing within larvae (Briese, 1986; Boucias et al., 1987; Vasconcelos et al., 1996b). The viruses remain viable after passage of the alimentary canal of vertebrates and invertebrates, however to a widely divergent extent.

With respect to the speed and distance of the transfer of infectious viruses, the dispersal by birds deserves some special interest. Birds may take up infected or dead larvae directly or feed on non vertebrate or small vertebrate predators. Droppings may contain up to some 10^7 polyhedra. But the contribution of particular dispersal routes and mechanisms to virus epizootics is difficult to assess conclusively. Even a coincidence of novel areas with infected larvae and viruses in bird droppings is only circumstantial evidence of a significant contribution of avian transport to virus epizootics (Entwistle et al., 1977; Buse, 1977; Cory et al., 1988; Entwistle et al., 1993). Novel baculovirus infections appearing in *Gilpinia hercyniae* (European spruce sawfly) in a previously unexposed geographical area at a time, before any virus could be monitored in avian droppings, suggests a different mode of dispersal (Buse, 1977). Even more recent experiments including trials with different virus applications at different locations and the identification of virus types in bird droppings by molecular restriction-hybridisation methods must close with the statement, that “the actual role of birds and many other biotic dispersal agents in the spread of baculoviruses and other micro-organisms remains to be demonstrated” (Entwistle et al., 1993). Clearly, the knowledge of the intersection in space and time of insect behaviour and development and the ecological habit of particular bird species (including feeding - and defecation - behaviour and territoriality) must be included in such investigations.

Such intersections are also determinants of success for any approaches using insects as biotic dispersal agents for insect control. These experiments also can be regarded as a demonstration of a potential natural dispersal role of the engaged species (Biever et al., 1982; Young and Yearian, 1990, 1992; Gross et al., 1994b - see C.23.2). Any intentions to surmount the demonstrative character of these experiments in order to biologise application strategies for baculovirus insecticides would require the input of increased knowledge. It remains speculative whether such a manipulation of insect and baculovirus ecology for efficient pest control can be achieved in a practicable and effective manner.

27. Containment and decontamination

Special containment conditions in order to protect workers and the environment from baculoviruses are not usually required. The establishment of confinement measures is determined by the need to protect insect and cell cultures and the experiment from unintended infections. In part, depending on laboratory experiences with outbreaks of virosis in insectaries, the extension of technical installations and working procedures is adapted to these protection objectives. Small particle size and persistence of baculoviruses represent a challenge for effective measures. The performance of work with baculoviruses has to follow the principles of good microbiological practice. Local separation of cell culture and insect work is a minimum requirement. To change laboratory coats when beginning or leaving work with baculoviruses seems to be advisable.

Heating can be used for decontamination of laboratory material and media. Baculoviruses are inactivated at temperatures and times significantly below conventional microbiological sterilisation conditions (Martignoni and Iwai, 1977). Sodium hypochlorite and formaldehyde can be used for chemical decontamination. Sodium dodecyl sulfate was also tested for egg surface sterilisation to prevent ex ovarial (vertical) transmission. A lower impact on moth rearing and sufficient effectiveness of inactivation were noted (Ilsley et al., 1980).

No decontamination procedure seems to be recommendable for environmental contaminations, e.g. by spills from baculovirus insecticide containers. In fact, any procedure would compromise environmental quality to a greater extent than the baculovirus preparation. Soil decontamination by formaldehyde treatment as used after the first field trial with a genetically modified baculovirus or similar treatments will eventually be used according to the respective risk perception.

28. Description of detection and monitoring techniques, including specificity, sensitivity, and reliability

28.1 Detection

Detection of baculoviruses in the environment is frequently performed by collecting (diseased or dead) larvae and making microscopic observations of stained baculoviruses. While this technique is adequate to monitor an infectious process in an insect population, other techniques are required to analyse the abundance and activity of infectious particles in the environment. Methods may consist of biotests by feeding target larvae with potentially exposed (plant) material. In order to quantify virus particles and to additionally determine their infectivity, virus particles are separated from the habitat matrix by washing or sonication. Virus particles collected by centrifugation are stained and counted by microscopy (Taverner and Connor, 1992; Carruthers et al., 1988). Subsequent biotesting is then performed by applying an aquaous suspension to the insect diet.

Biotesting may amplify rather low concentrations of viruses, and does not seem to generally be inferior in its sensitivity in comparison to molecular methods which can be employed to directly detect baculovirus sequences by probe hybridisations or by amplifying specific sequences via PCR. The potential of these methods has not been evaluated for the monitoring of field trials in any significant extent. A method to detect and identify baculoviruses by a combination of PCR (amplifying a homologous region of the polyhedrin gene) and subsequent differentiation by restriction analysis has been described for 8

baculoviruses (De Moraes and Maruniak, 1997). However, PCR does not differentiate between viable and non-viable viruses.

28.2 Identification

For the identification and differentiation of Baculovirus species/strains, the restriction analysis and/or probe hybridizations has become an established method (Trzebitzky et al., 1988; Bensimon et al., 1987; Doyle et al., 1990; Barber et al., 1993; Entwistle et al., 1993; Hughes et al., 1997). The feasibility of the approach for ecological investigations was conclusively demonstrated by Laitinen et al. (1996) who used heterogeneities in restriction endonuclease patterns among geographic isolates to assess the origin and spread of genotypes. The number of restriction enzymes used and the cutting frequency determine the degree of differentiation of divergent genotypes. A quantification of the composition of heterogeneous mixtures is difficult to achieve by the analysis of banding heterogeneities and submolar bands. *In vivo* cloning of the genotypes of the mixture and quantification of their frequency would be a labour intensive solution.

In recent years, the PCR became an efficient tool with high sensitivity and specificity for the identification and diagnostics of micro-organisms. Primer specificities and reaction conditions determine the amplification of specific DNA fragments. De Moraes and Maruniak (1997) adopted this technique to amplify a highly conserved region of 575 bp within the polyhedrin gene of different NPVs with a specifically designed pair of primers. Restriction endonuclease digestion of the PCR product resulted in specific restriction patterns of the 8 analysed nucleopolyhedroviruses (*Anagrapha falcifera* NPV, AcMNPV, AgMNPV, BmNPV, and HzSNPV, OpMNPV, SfMNPV, and SeMNPV).

A similar approach was chosen to detect low levels of *Spodoptera littoralis* NPV from viral occlusion bodies and from infected host larvae and to differentiate between *S. littoralis* NPV and AcMNPV (Faktor and Raviv, 1996). These experiments demonstrate that PCR-based methods are useful for rapid identification and allow cost effective and sensitive monitoring of wild-type as well as genetically engineered baculoviruses.

APPENDIX I - ABBREVATIONS

BV	budded virus/virion
cat	chloramphenicol acetyl transferase
EGT	ecdysterorid-UDP-gluceoployhedrovirus
ELISA	enzyme linked immunosorbent assay
FP	few polyhedra
GV	granulovirus
hr	homologus regions
IAP	inhibitor of apoptosis
ICTV	International Committee on Taxonomy of Viruses
IED	individual effective dose
LC_{50}	lethal concentration (50%)
LD_{50}	lethal dose (50%)
LT_{50}	lethal time (50%)
MNPV	multiple nucleocapsid nucleopolyhedrovirus
NPV	nucleopolyhedrovirus
NOB	non-occluded baculovirus
OB	occlusion body
ODV	occlusion derived virus
ORF	open reading frame
PCR	polymerase chain reaction
PE	polyhedron envelope
p. i.	post-infection
SNPV	single nucleocapsid nucleopolyhedrovirus
TE	transposable element

APPENDIX II - REFERENCES

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