Construction of genetically engineered baculovirus insecticides containing the *Bacillus thuringiensis* subsp. *kurstaki* HD-73 delta endotoxin

Alison T. Merryweather, Ulrike Weyer, Mark P. G. Harris,† Mark Hirst, Timothy Booth and Robert D. Possee*

1Institute of Virology and Environmental Microbiology, Mansfield Road, Oxford OX1 3SR, U.K.

The δ-endotoxin gene from *Bacillus thuringiensis* subsp. *kurstaki* HD-73 was inserted into *Autographa californica* nuclear polyhedrosis virus (AcMNPV) using two transfer vector systems. In the first, the δ-endotoxin gene was placed under the control of the polyhedrin gene promoter in lieu of the polyhedrin coding sequences, thus deriving a polyhedrin-negative virus. In the second, it was inserted under the control of a copy of the AcMNPV p10 promoter positioned upstream of the polyhedrin gene to produce a polyhedrin-positive virus. Analysis of infected cell extracts showed that the δ-endotoxin was expressed in insect cells as 130K, 62K and 44K proteins, with peak syntheses at 18 h post-infection. Each of these products reacted with antisera specific for the complete protoxin and the cleaved, active form. When extracts from the cells infected with the polyhedrin-negative virus were fed to *Trichoplusia ni* larvae, feeding by the insects was inhibited and deaths occurred that were inconsistent with virus infection. This effect was also observed after the inoculum had been treated with detergents to inactivate virus particles prior to feeding to the larvae. These data indicate that the expression of the *B. thuringiensis* δ-endotoxin gene by a baculovirus in insect cells produces material with insecticidal activity. The biological activities of the two recombinant viruses were assessed in conventional bioassay tests by feeding virus particles or occlusion bodies to the insects. The polyhedrin-negative virus preparation appeared to be contaminated with endotoxin which inhibited feeding of the insects and prevented determination of the LD$_{50}$ value. The polyhedrin-positive virus had an LD$_{50}$ value about twofold higher than that of unmodified AcMNPV. The significance of these data for the genetic engineering of virus insecticides is discussed.

Introduction

Baculoviruses have been evaluated as biological insecticides because their replication is confined to certain invertebrates, particularly Lepidoptera, Hymenoptera, Diptera, Coleoptera and Crustacea. No baculovirus has been demonstrated to infect mammals, reptiles, birds, other invertebrates such as earthworms, or plants. This limited host range has resulted in their successful use as insecticides on a number of occasions to control specific insect pests (Cunningham, 1982; Entwistle & Evans, 1985; Huber, 1986; Evans & Entwistle, 1987).

A disadvantage with baculoviruses as commercial insecticides is that they can take several days to kill the pest species, whereas chemical insecticides may work in a matter of hours. Furthermore, during this time the insect may continue to feed, causing damage to the crop (Entwistle *et al.*, 1988). This undesirable feature of baculoviruses has restricted their use in agriculture.

One possible way to improve the effectiveness of baculoviruses as insecticides is to insert genes into the genome that encode insect-specific toxins, hormones, or other gene products. When these products are expressed in the target insect, they may affect it physiologically or cause an earlier demise of the host. The development of baculoviruses as expression vectors of foreign genes allows such investigations to be undertaken. The primary example of this system is *Autographa californica* nuclear polyhedrosis virus (AcMNPV) (Smith *et al.*, 1983b; Pennock *et al.*, 1984; Matsuura *et al.*, 1987).

Within AcMNPV-infected cells, virus gene expression can be subdivided into four phases, namely immediate early, delayed early, late and very late (Kelly & Lescott, 1981). In the late phase, rod-shaped nucleocapsid structures containing a copy of the DNA genome (about 128 kbp) are assembled within the nucleus (see Kelly, 1982 for review). These are released from the cell by budding via the plasma membrane and acquisition of an envelope and a virus-encoded glycoprotein to produce...
infectious virus particles. Enveloped virus particles are also formed within the nucleus but these are packaged into occlusion bodies or polyhedra in the very late phase. These structures are formed almost entirely from a single polypeptide (28K) called polyhedrin that serves to protect the virus within the environment. After ingestion by a susceptible caterpillar, polyhedra dissolve within the alkaline midgut and release virus particles which initiate infection. In cell culture, polyhedra are redundant since only plasma membrane-derived virus is able to infect other cells. Therefore, the polyhedrin coding sequences may be deleted and replaced with others provided they do not affect virus replication (Smith et al., 1983a). The foreign protein is highly expressed by the efficient functioning of the polyhedrin promoter sequences (Smith et al., 1983; Pennock et al., 1984; Matsuura et al., 1987). The promoter of the AcMNPV p10 very late gene has also been used to express foreign genes such as the Escherichia coli lacZ gene (Vlak et al., 1988; Williams et al., 1989).

A candidate gene for incorporation into a baculovirus insecticide is the insect-specific δ-endotoxin from the Gram-positive bacterium Bacillus thuringiensis. During sporulation, the bacterium produces crystalline inclusions containing the toxin. There are B. thuringiensis strains with activities against a wide range of insect species (Burges, 1981). Many strains produce a toxin that is active against lepidopteran larvae. Some strains produce toxins that affect dipteran (Goldberg &Margalit, 1977; Hall et al., 1977), or coleopteran larvae (Krieg et al., 1983). Natural isolates of B. thuringiensis have been used commercially as insecticides (Luthy et al., 1982; Cunningham, 1988). The presence of the active toxin within the gut of the insect appears to cause an immediate reduction in feeding (Heimpel &Angus, 1959). The toxin is thought to generate pores in cell membranes leading to disruption of the osmotic balance and cell lysis (Knowles &Ellar, 1987; reviewed by Hofte &Whiteley, 1989). Specific binding sites for some B. thuringiensis toxins have been identified on the midgut epithelium of susceptible insects (Hoffman et al., 1988, a, b). The gene(s) for the protein may be either encoded on plasmid(s) or in the bacterial chromosomal DNA (Gonzalez et al., 1981; Schnepf &Whiteley, 1981; Kronstad et al., 1983; Held et al., 1982; Adang et al., 1985). In the case of B. thuringiensis subsp. kurstaki HD-73, the gene is encoded by a 75 kbp plasmid (Gonzalez et al., 1982; Whiteley et al., 1982). It encodes a 130K protoxin which is proteolytically cleaved to an active toxin of 62K in the insect gut (Adang et al., 1985).

In this study we have inserted the protoxin gene from B. thuringiensis subsp. kurstaki HD-73 into the genome of AcMNPV to derive polyhedrin-negative and polyhedrin-positive viruses and have made a preliminary assessment of their effectiveness.

Methods

Viruses and cells. AcMNPV C6 (Possee, 1986) and other recombinant viruses were propagated in Spodoptera frugiperda cells (IPLB-Sf-21) (Vaughn et al., 1977) at 28 °C in TC100 medium supplemented with 5% foetal calf serum (FCS) and titrated for infectivity as described by Brown & Faulkner (1977).

Virus purification. Polyhedra were purified from infected cells or insects as described by Harrap et al. (1977), but with the addition of 0.1% (w/v) SDS to all solutions. The SDS was removed by washing the polyhedra with water at the end of the purification. Polyhedra were counted using the dry counting method of Wigley (1976).

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Fig. 1. Construction of a transfer vector containing the B. thuringiensis protoxin gene under the control of the polyhedrin promoter. The protoxin gene isolated from pIC4 was modified and inserted into the BamHI site of pAcRP25 to derive pAcRP25.Bt. Abbreviations: B, BamHI; E, EcoRI; Ev, EcoRV; H, HindIII; N, NdeI; Ns, NsiI; X, XhoI; CIP, calf intestinal phosphatase; LGT, low gelling temperature agarose gel; oligo 5′, synthetic oligonucleotide encoding the amino-terminal region of the protoxin gene. The lower part of the figure shows the nucleotide sequence of part of the B. thuringiensis protoxin gene and the AcMNPV polyhedrin promoter. The amino-terminal coding sequence of the protoxin gene is shown, with the appropriate amino acid residues above each codon. Upstream from the endotoxin sequences are nine nucleotides duplicated from the polyhedrin 5′ non-coding leader sequence, a BamHI restriction enzyme site, and the complete polyhedrin 5′ non-coding leader sequence (delineated by the arrowhead) with the transcription initiation site (T). The sequences underlined with a thin line represent the synthetic oligonucleotides used to modify pUCBt1.
Construction of transfer vectors containing the *B. thuringiensis* endotoxin. (i) pBt6. The plasmid pIC4, containing the δ-endotoxin gene from *B. thuringiensis* subspecies *kurstaki*, strain HD-73, was obtained from Dr Susan Ely, ICI Seeds, Jealotts Hill Research Station, Bracknell, U.K. The plasmid DNA was digested with *Nde*I to release a 3771 nucleotide fragment containing the endotoxin gene (Fig. 1). This was purified from a low gelling temperature agarose gel (Possee & Kelly, 1988) and inserted into the *Nde*I site of pUC18 using standard protocols (Maniatis et al., 1982) to generate pUCBt1. The amino-terminal coding sequences of the endotoxin gene were in close proximity to the vector polylinker. pUCBt1 was digested with *Bam*HI (in the polylinker) and *Nsi*I (in the endotoxin gene) to remove the 5'-non-coding sequences of the gene and the first 31 nucleotides of the coding region. This deletion was replaced with a complementary pair of synthetic oligonucleotides produced using an Applied Biosystems Automated Synthesizer. The oligonucleotides contained a *Bam*HI compatible 5' end, the first nine nucleotides of the polyhedrin 5'-non-coding region (upstream from the ATG) and the coding region of the endotoxin which had been removed with *Nsi*I and *Bam*HI (Fig. 1). This plasmid, designated pUCBt5, was digested with *Nde*I, the ends repaired with the Klenow fragment of *Escherichia coli* DNA polymerase in the presence of dNTPs, and ligated to another synthetic double-stranded oligonucleotide (5' TAGGATCCTA 3') to generate pBt6. The oligonucleotide served to insert a *Bam*HI restriction enzyme site about 160 nucleotides downstream from the translation stop codon of the endotoxin gene. (ii) pAcRP25.Bt. The plasmid pBt6 was digested with *Bam*HI to release the endotoxin gene as a 3691 nucleotide fragment which was then inserted into the baculovirus transfer vector pAcRP25 to produce pAcRP25.Bt. Plasmid pAcRP25, which is a derivative of pAcRP5 (Possee, 1986), contains the complete 5'-non-coding sequence of the polyhedrin gene, and the first two nucleotides of the translation initiation codon, but lacks all of the polyhedrin coding sequences. (iii) pAcUW2.Bt. The *EcoRV* site 92 nucleotides upstream from the polyhedrin gene in pUC8/6/8 (Possee, 1986) was converted to a *Bgl*II site by the addition of a synthetic linker. A 230 nucleotide fragment containing the p10 promoter (*Weyer & Possee, 1988, 1989*) was inserted at this *Bgl*II site in the opposite orientation to the polyhedrin promoter, together with the 3' end of the *lacZ* gene from pCH110 (Pharmacia) to provide simian virus 40 transcription termination signals (Possee & Howard, 1987). This plasmid was designated pAcUW2. The 3691 nucleotide fragment containing the endotoxin coding sequences was then inserted into the *Bgl*II site in pAcUW2 to generate pAcUW2.Bt (Fig. 2).

Transfection and screening for recombinant viruses. (i) Polyhedrin-negative (PH⁻) virus. The *S. frugiperda* cells were cotransfected with AcMNPV DNA mixed with pAcRP25.Bt or pAcRP8 (Matsuura et al., 1987) and putative recombinants [Ac(PH⁻)Bt or AcRP8] lacking polyhedra were isolated as previously described (Smith et al., 1983a; Possee, 1986). (ii) Polyhedrin-positive (PH⁺) virus. Virus DNA was purified from AcRP8 (polyhedrin-negative), coprecipitated with pAcUW2. Bt and used to transfect cells. Progeny virus that produced polyhedra [Ac(PH⁺)Bt] were selected in a standard plaque assay.

Radiolabelling of cells and analysis of proteins. *S. frugiperda* cells were infected with virus as described by Possee & Howard (1987). At intervals, cells were radiolabelled for 1 h in leucine-deficient TC100 medium containing 2% dialysed FCS and 10 µCi [14C]leucine. Protein extracts were analysed in denaturing 10 to 30% polyacrylamide gradient gels (Cook et al., 1979) which were stained with Coomassie blue and treated with EN³HANCE (Dupont) prior to drying and exposure for fluorography.

Immunoblot analysis of infected cell proteins. Protein extracts were fractionated on 10% polyacrylamide gels (Possee & Howard, 1987) and then transferred to nitrocellulose filters using a Bio-Rad electrophotob apparatus for 17 h at 30 V; 20 mM-Tris, 100 mM-glycine was used as a transfer buffer. The filter was incubated in 2% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h, treated with washing buffer (0.5% (w/v) BSA, 0.2% (v/v) Triton X-100) for 30 min (twice) and then incubated for 1.5 h at 21 °C with rabbit antiserum diluted 1/500 in washing buffer. The sera used were specific for the protoxin or toxin forms of the *B. thuringiensis* δ-endotoxin and were generous gifts from Professor Bruce Hammock (University of California at Davis, U.S.A.). The filter was washed several times in the same buffer without antiserum to remove unbound antibody. Immune complexes were detected with an anti-rabbit IgG-alkaline phosphatase conjugate.

**Immunogold labelling of infected cells.** *S. frugiperda* cells infected with Ac(PH⁺)Bt were harvested at 17 h post-infection (p.i.), washed with PBS, fixed in 1% (w/v) paraformaldehyde/phosphate buffer, pH 7.5 and finally washed in 0.1 M-glycine pH 7.5. After dehydration, cells were embedded in LR white resin (Agar Scientific) (Newman et al., 1982). Sections on nickel grids were incubated for 1 h at 21 °C in 0.1% (w/v) BSA/PBS containing antiserum to the protoxin or active toxin, or control rabbit antiserum to an unrelated antigen. The sections were washed three times by floating grids on PBS followed by another 5 min blocking step with PBS/BSA. Grids were incubated with goat anti-rabbit colloidal gold conjugate (Biocoll). After extensive washing with PBS/BSA, grids were stained in lead citrate, uranyl acetate and 50% (v/v) ethanol and examined using a Jeol 100S electron microscope.

**Insects and bioassay of endotoxin and viruses.** *Trichoplusia ni* insects were maintained in the laboratory on a semi-synthetic diet (see Hunter et al., 1984). *S. frugiperda* cells were infected with Ac(PH⁺)Bt or AcRP8 (10 p.f.u./cell), harvested at 24 h, pelleted and resuspended in 1 ml TE (10 mM-Tris–HCl pH 8.0, 0.1 mM-EDTA). Aliquots were sonicated for 2 min in a Sonibath (Dawee Instruments) and further treated with 1% (v/v) Triton X-100 and 0.5% (w/v) sodium deoxycholate (TDOC). Extracts were fed to second instar *T. ni* larvae on small plugs of diet for...
18 h. The insects that consumed the plugs were then given fresh diet and monitored until death or pupation. To assess the biological activity of the polyhedrin-positive virus, 500 polyhedra purified from Ac(PH+)Bt-infected cells were fed to T. ni larvae. The larvae that succumbed were pooled and the polyhedra purified. The infectivity was assessed by feeding defined doses to T. ni larvae as described above.

Results

Construction of recombinant viruses

The δ-endotoxin gene from B. thuringiensis (subsp. kurstaki, strain HD-73) was modified to reduce the untranslated sequences at each end of the coding region (see Methods and Fig. 1). At the 5' end of the gene, a synthetic oligonucleotide was inserted to provide a BamHI restriction enzyme site, a repeat of the nine nucleotides upstream from the ATG of the normal polyhedrin gene, and to replace coding sequences removed during the construction. The endotoxin coding sequences were removed from pBt6 with BamHI and inserted into pAcRP25 to produce pAcRP25.Bt. The endotoxin coding sequences were also inserted into the transfer vector pAcUW2 to produce pAcUW2.Bt. This vector has a copy of the AcMNPV p10 promoter inserted upstream of the polyhedrin gene to facilitate the expression of foreign genes while retaining the normal production of both very late gene products (Fig. 2). The sequences of the polyhedrin and p10 promoters relative to the endotoxin coding sequences are illustrated in Fig. 1 and 2. The transfer vector pAcRP25.Bt was cotransfected with infectious AcMNPV DNA to produce a polyhedrin-negative mutant [Ac(PH-)Bt], with the endotoxin gene in place of the polyhedrin gene. The transfer vector pAcUW2.Bt was cotransfected with DNA from AcRP8 (a polyhedrin-negative virus) to produce polyhedrin-positive recombinants [Ac(PH+)Bt], with the endotoxin gene placed upstream of the polyhedrin gene. These were plaque-purified to genetic homogeneity and further amplified to provide stocks of virus with titres similar to those of normal AcMNPV or other recombinant viruses containing foreign genes (10^7 to 10^8 p.f.u./ml). Southern blot hybridization analysis of DNA extracted from cells infected with Ac(PH-)Bt or Ac(PH+)Bt confirmed the insertion of the endotoxin gene in the correct position within the virus genome (data not shown).

Expression of the B. thuringiensis δ-endotoxin in insect cells

In a preliminary analysis, mock-infected cells, or those infected with Ac(PH-)Bt or AcMNPV were pulse-labelled at 18 h p.i. Proteins were analysed using a 10% polyacrylamide gel and fluorography (Fig. 3a). This showed that a 130K polypeptide was unique to the Ac(PH-)Bt-infected cells. A more comprehensive study was performed by infecting cells with Ac(PH-)Bt, Ac(PH+)Bt, or AcMNPV and pulse-labelling at various times after infection. Cell extracts were analysed by electrophoresis in 10 to 30% polyacrylamide gels, Coomassie blue staining and fluorography (Fig. 3b, c). In the cells infected with Ac(PH-)Bt or Ac(PH+)Bt, the 130K polypeptide was detected in increasing quantities from about 12 h p.i. The autoradiograph (Fig. 3b) shows that in cells infected with either recombinant virus the putative endotoxin was produced most prolifically at about 18 h p.i. By 24 h p.i. the synthesis of endotoxin had decreased and by 36 h p.i. it was very poorly labelled. In contrast, AcMNPV produced polyhedrin and p10 protein maximally between 24 h p.i. and 36 h p.i. The final levels of putative endotoxin expressed by the p10 promoter in Ac(PH+)Bt and the polyhedrin promoter in Ac(PH-)Bt appeared to be similar (Fig. 3c). However, the synthesis of the foreign gene product by Ac(PH+)Bt was slightly higher at 12 h p.i. than at the same time in Ac(PH-)Bt-infected cells. It is also interesting to note that in cells infected by either recombinant virus the amounts of p10 and polyhedrin proteins were markedly less than the amounts detected in AcMNPV-infected cells. Densitometric scans of the autoradiograph in Fig. 3(b) (data not shown) demonstrated that the amount of polyhedrin synthesized between 24 and 25 h p.i. in cells infected with Ac(PH+)Bt was reduced by a factor of seven compared to cells infected with AcMNPV. Similarly, the amount of p10 synthesized in cells infected with Ac(PH+)Bt and Ac(PH-)Bt was reduced by a factor of six compared to the amount of p10 synthesized in AcMNPV-infected cells between 24 and 25 h p.i.

The 130K protein expressed in infected cells was identified by performing immunoblot analysis with anti-endotoxin sera prepared against an intact 130K protoxin (Fig. 4a). The samples were from Ac(PH-)Bt-infected cells harvested at 24 h p.i. The antiserum detected a large protein of 130K, a faint component of 62K and a strong band of 44K. Similar results were obtained with the antiserum specific for the 62K active form of the endotoxin and with extracts from Ac(PH+)Bt-infected cells (data not shown). The endotoxin could not be detected in the medium from infected cell cultures. Immunoblot analysis of haemolymph extracted from fifth instar T. ni larvae following infection with Ac(PH+)Bt at the third instar revealed a 44K protein which was detected by antiserum raised against the intact endotoxin (Fig. 4b). The predominance of the 44K polypeptide in this sample of Ac(PH+)Bt-infected cells is a consequence of storage of the cell extract prior to electrophoresis. Southern blot analysis of virus DNA
from infected insects demonstrated that the endotoxin coding sequences were still intact (data not shown).

**Electron microscopy of infected cells**

When thin sections of Ac(PH−)Bt-infected cells were examined using an electron microscope, readily identifiable crystal structures analogous to those formed by the B. thuringiensis bacterium were not observed. The localization of the endotoxin within Ac(PH−)Bt-infected cells was examined using immunogold labelling of thin sections treated with antisera. Gold particles concentrated in dark foci were observed both within the nucleus (data not shown) and cytoplasm (Fig. 5) of...
Ac(PH-)Bt-infected cells. Control cells infected with AcMNPV or mock-infected cells did not bind significant quantities of gold particles (data not shown). Similar results were obtained with antisera raised to the protoxin and active form of the endotoxin.

**Biological activity of infected extracts**

The in vivo activity of the δ-endotoxin was assessed by feeding extracts from Ac(PH-)Bt-infected cells to second instar *T. ni* larvae. Uninfected cells, or cells infected with AcRP8 (a polyhedrin-negative recombinant) were used as controls. In order to isolate the effect of the toxin from the virus, the cell samples were sonicated and treated with TDOC, which reduced virus infectivity by almost 100%. The usual protocol with these bioassays was to feed the samples to insects on a small plug of semi-synthetic diet. To standardize the assay only insects that consumed the total diet plug within an 18 h period were studied. These were transferred to a fresh, uncontaminated diet and pupated normally. Insects fed the equivalent of 5000 cells infected with Ac(PH-)Bt refused to consume the contaminated diet, even after 4 days (Table 1). When this dose was reduced to 1250 cell equivalents 63% of the insects refused to feed, and when 312 cell equivalents were used, only 12% declined the diet. None of the insects that refused to feed were transferred to

Table 1. **Biological activity of cells infected with Ac(PH-)Bt or AcRP8**

<table>
<thead>
<tr>
<th>Sample†</th>
<th>Larvae* refusing diet (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5000‡</td>
</tr>
<tr>
<td>Sf cells</td>
<td>0</td>
</tr>
<tr>
<td>AcRP8§</td>
<td>4</td>
</tr>
<tr>
<td>Ac(PH-)Bt</td>
<td>100</td>
</tr>
</tbody>
</table>

* Second instar *T. ni* larvae; approximately 50 insects per dose.
† Cells were sonicated and treated with TDOC. The titre of virus was reduced by 99-99 to 100%.
‡ Number of cell equivalents per dose (1 μl per diet plug).
§ Polyhedrin-negative virus.
Fig. 5. Localization of *B. thuringiensis* δ-endotoxin in infected cells by immunogold labelling. (a) Immunogold-localized *B. thuringiensis* antiserum binds to cytoplasmic foci (large arrows) which appear slightly darker than the surrounding cytoplasam. Bar marker represents 500 nm. (b) Higher magnification of endotoxin-dense area. Bar marker represents 200 nm.
Table 2. Biological activity of AcMNPV and Ac(PH+)Bt in second instar larvae of T. ni*

<table>
<thead>
<tr>
<th>Virus</th>
<th>LD₅₀</th>
<th>95% confidence limits</th>
<th>Slope</th>
<th>CC†</th>
<th>DF‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcMNPV§</td>
<td>68</td>
<td>87 - 54</td>
<td>1.81</td>
<td>0.983</td>
<td>3</td>
</tr>
<tr>
<td>Ac(PH+)Bt‖</td>
<td>132</td>
<td>162 - 107</td>
<td>1.94</td>
<td>0.925</td>
<td>4</td>
</tr>
</tbody>
</table>

* Fifty second instar larvae per dose.
† Correlation coefficients.
‡ Degrees of freedom.
§ Doses: 27, 55, 109, 219, 438, 875 polyhedra.
‖ Doses: 31, 62, 125, 250, 500 polyhedra.

...f fresh diet. These insects died of undetermined causes, probably due to ingested toxin. The insects fed on AcRP8-infected cells consumed the dose within 18 h and were transferred to fresh diet. These individuals pupated normally. When the biological activity of Ac(PH-)Bt virus was assessed (by giving infectious virus particles from infected cell culture medium to insects), feeding was markedly inhibited. The endotoxin could not be removed from Ac(PH-)Bt virus particles by centrifugation in sucrose gradients, because the virus lost infectivity during these procedures.

The biological activity of Ac(PH+)Bt was assessed using conventional bioassays. Polyhedra derived from infected cell cultures were fed to T. ni larvae and those insects which subsequently succumbed to virus infection were pooled and processed to yield highly purified polyhedra. Second instar T. ni larvae were fed with defined doses of Ac(PH+)Bt or AcMNPV polyhedra to compare the LD₅₀ of each virus. The results are shown in Table 2. Following probit analysis, the correlation coefficients were calculated to be 0.9829 for AcMNPV with three degrees of freedom, and 0.9252 for Ac(PH+)Bt with four degrees of freedom. This is a highly significant fit of the bioassay data to a linear log dose/probit mortality response. The two viruses have similar slope values, 1.81 for AcMNPV and 1.94 for Ac(PH+)Bt. The LD₅₀ of AcMNPV was calculated to be 68 polyhedra (95% confidence limits of 54 and 87), and for Ac(PH+)Bt it was determined to be 132 polyhedra (95% confidence limits of 107 and 162). Furthermore, insects fed similar doses of each virus died at approximately the same time (i.e. they exhibited similar LT₅₀ values). Feeding inhibition of the type seen with the Ac(PH-)Bt recombinant was only observed when insects were fed very high doses of virus (more than 10⁵ polyhedra). Such inhibition may have been due to the endotoxin. This was not investigated further.

Discussion

B. thuringiensis subsp kurstaki HD-73 produces a crystal protein of about 130K, otherwise known as the δ-

endotoxin. Cleavage in the gut of many lepidopteran larvae releases an active form of 62K which kills the host (see review by Hofte & Whiteley, 1989). We have inserted the complete coding sequence for this gene into two baculovirus expression vectors based on AcMNPV. The first involved replacing the polyhedrin coding sequences with those of the endotoxin, thus deriving a polyhedrin-negative virus where expression of the foreign gene was mediated by the polyhedrin promoter. The second virus made use of a duplicated p10 promoter to drive endotoxin expression in a recombinant which retained the normal polyhedrin and p10 functions. Analysis of cells infected with each recombinant virus demonstrated that a large polypeptide of about 130K was synthesized which reacted with antisera specific for the toxin. Furthermore, two additional polypeptides of 62K and 44K were detected. The significance of these smaller products is unclear but it is conceivable that the 62K product is the active (cleaved) form of the toxin. Insect cells have been shown to be capable of the correct proteolytic cleavages of certain proteins (reviewed by Miller, 1988), although the cleavage of the endotoxin usually occurs in the gut of the caterpillar. The toxin may be inherently unstable as, on storage, the 44K polypeptide increased in relative abundance. The peak time of synthesis for the 130K protein in insect cells was about 18 h p.i. In contrast, the normal polyhedrin gene product was not expressed at maximum levels until 24 h p.i. in AcMNPV-infected cells.

In cells infected with Ac(PH+)Bt, the levels of p10 and polyhedrin proteins produced appeared to be six and seven times lower, respectively, than those synthesized by AcMNPV-infected cells. A similar reduction in the level of p10 was observed in cells infected with Ac(PH-)Bt. This occurred in several experiments and might suggest that the intracellular endotoxin has a cytotoxic effect resulting in the depression of very late gene expression. The significance of this effect is currently under investigation.

In bioassays to determine the biological activity of the endotoxin, the insect larvae refused to consume diet contaminated with infected cell extracts treated with detergent to inactivate infectious virus. We attributed this to the presence of the endotoxin since feeding inhibition is a characteristic of insects that ingest the natural B. thuringiensis (Heimpel & Angus, 1959). These data indicate that the protein produced by the insect cells is biologically active. This anti-feeding phenomenon was also noted when virus particles from cell culture media were used in the bioassay tests, although immunoblotting failed to detect the endotoxin in the media from cultures of infected cells. Evidently, the very low concentration of the toxin in the virus preparations was sufficient to have a biological effect.
Future studies will have to use purified recombinant virus, free of protoxin material, which retains infectivity. This will permit definitive bioassays to be done where the insects consume the full virus dose. Although the use of a polyhedrin-negative virus producing the endotoxin may not appear to be a very encouraging prospect as an improved biological control agent, it does offer some advantages. In a cautious and regulated programme to investigate the use of genetically engineered viruses in the environment, it could be used in a controlled release, where the long-term persistence of the virus is not desired (Bishop et al., 1988). However, the fact that infected insects produce active endotoxin material will permit the effect on other insects to be determined.

The problems associated with the use of a polyhedrin-negative virus to control insect pests may be circumvented by employing the polyhedrin-positive virus which can express the endotoxin. Immunoblotting analysis detected a 44K protein in the haemolymph of T. ni larvae following infection with Ac(PH+)Bt, demonstrating that the toxin was expressed by the recombinant virus within the insects. The apparent lack of 130K and 62K products may be due to proteolytic cleavage of the endotoxin in the insect. It is unlikely to be a result of deletion of part of the endotoxin gene because Southern blot analysis confirmed the presence of the full-length gene within the virus genome. The LD50 value for this virus was apparently two-fold higher than that of the wild-type virus. This is probably within the experimental error of the bioassay technique used and we consider that the two viruses have essentially equal virulence for insect larvae. Similar variation has often been recorded when comparing the infectivity of different batches of AcMNPV (R. D. Possee, unpublished data). It is also conceivable that trace amounts of the endotoxin in the preparation of polyhedral used in the bioassay, may have affected the infection of cells in the gut by the virus. The bioassay results with the occluded recombinant were disappointing, but it must also be considered that the virus was producing endotoxin within the infected larvae. Therefore, after an initial round of virus replication within the insect, release of endotoxin onto the plant surface as the insect decays may have a significant effect on the larval population. Therefore, the efficacy of these agents as genetically engineered biological control agents should perhaps not be judged on the basis of preliminary laboratory studies. We are currently planning to test both viruses in enclosed systems using plants and insect larvae where their activity may be assessed in a situation more akin to their natural environment.

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References


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