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Further Characterization of the Gonad-Specific Virus of Corn Earworm, *Helicoverpa zea*[1]

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The gonad-specific virus (GSV) is a DNA virus infecting the reproductive tracts of adults of both sexes of the corn earworm, *Helicoverpa zea*, causing severe tissue deformities leading to sterility. Atypical occlusion bodies containing large concentrations of virions embedded in a granular matrix were seen in the lumen of the oviduct and the bursa copulatrix of infected females. The virus, transmitted by both sexes, was successfully propagated *in vivo* and in tissue culture. The GSV genome is about 225 kb in size, with no apparent similarity to the nucleopolyhedrovirus type species, AcMNPV, genomic DNA, as determined by Southern hybridization. PCR amplification of GSV genomic DNA with primers derived from the highly conserved polyhedra gene of several baculoviruses indicated no similarity. GSV at 10^{-2} female equivalents (based on virus obtained from the bursa copulatrix and oviducts of one infected female) injected into a newly emerged female and mated to a normal male resulted in >95% agonadal progeny. However, at lower doses, some of the adult progeny looked normal but apparently carried a low level of the virus that could be responsible for sustenance of infection in a given colony, as well as in nature.

Key Words: **nonoccluded virus; DNA virus; *Helicoverpa zea;* reproductive system; sterility.**

INTRODUCTION

Moths of the group *Helicoverpa/Heliothis* constitute some of the most injurious crop pests throughout the world. The corn earworm, *Helicoverpa zea*, is a serious pest of corn, cotton, and soybean in the United States, and a related species, *H. armigera*, causes severe damage to cotton and grain legumes in Asia and Africa. Herzog and Phillip (1982) reported atrophy of the reproductive organs in adults of a diapause strain of *H. zea* and attributed the abnormality to a genetic trait. In 1993, we detected similar abnormalities in *H. zea* shipped from a laboratory colony maintained at the Southern Insect Management Laboratory in Stoneville, Mississippi. Seven additional shipments of *H. zea* pupae received in 1994 revealed 17–57% females and 15–58% males with highly deformed reproductive systems.

In a preliminary report, Raina and Adams (1995) showed that the abnormality was caused by a virus with atypical occlusion bodies. The virus did not appear to affect growth and survival of the infected insects, and the adults looked normal, except for a hypertrophied ovipositor in the females. This finding was corroborated by Hamm *et al.* (1996), using insects from the same source as ours. Recently, Burand and Lu (1997) reported successful replication of this virus in TN-368 tissue culture cells. Since this virus is confined to the tissues of the reproductive system, we designated it as a gonad-specific virus (GSV). We describe here further details about atrophy of the reproductive system, ultrastructure and preliminary molecular characterization of GSV, *in vivo* and *in vitro* culture of the virus, and dose response of the crude viral preparation. Based on laboratory observations, we also discuss the possibility of using this virus as a potential biological agent for the control of the corn earworm.

MATERIALS AND METHODS

Source of Insects

Initially, GSV-infected *H. zea* were obtained as pupae from the Southern Insect Management Laboratory of the USDA, Stoneville, Mississippi. Upon emergence,

[1] Mention of a commercial product does not imply endorsement by the U.S. Department of Agriculture. The sequences reported in this paper have been deposited in the GenBank database under Accession No. AF245396 and AF245397.

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the adults were dissected to determine whether they had normal or atrophied reproductive systems (hereafter referred to as agonadal or AG adults). Comparative drawings of male and female reproductive systems in normal and AG adults were prepared. Eggs of an uninfected laboratory colony of *H. zea* were shipped each week from the Crop Science Research Laboratory of the USDA in Mississippi State, Mississippi. Larvae were reared on artificial diet in environmental chambers maintained under L:D 15:9 h and temperatures of 26 and 21°C during the photophase and scotophase, respectively.

Propagation of GSV in Vivo

The deformed oviduct and bursa copulatrix of an AG female were dissected in phosphate-buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na_2PO_4, and 0.24 g KH_2PO_4 per liter) and homogenized for 1 min by a Microson ultrasonic cell disruptor (Heat Systems, Farmingdale, NY). The homogenate was centrifuged for 2 min at 8000 rpm, and the supernatant was diluted with PBS to 500 μl. The preparation was stored at −80°C until further use. Female *H. zea* moths were injected with 0.02 female equivalents (FE) (10 μl of the above preparation) from the ventral side of the abdomen within 2 h of emergence. The injected females were held singly in paper cups (11 cm diameter, 8 cm high) for 24 h and then mated to normal males. Eggs laid from 3rd to 5th day of oviposition were allowed to hatch and the larvae were placed on artificial diet in individual 30-ml plastic cups. Pupae were sexed and males and females placed in cages in separate chambers. Over 95% of the resulting progeny were AG, and the oviduct and bursa of these females provided an excellent source of the virus. Several newly emerged male moths were also injected with 0.02 FE of GSV and after 24 h mated to normal females. The resultant progeny was examined for AG condition just as in the case of females.

Electron Microscopy

Tissues from infected *H. zea* adults were dissected in PBS and fixed in 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer according to previously described procedures (Adams *et al.,* 1977; Adams and Bonami, 1991). GSV was also obtained by squeezing the bursa of an AG female directly into the fixative. Tissues and the virus pellet were embedded in an Epon 812 embedding medium (Bozzola and Russell, 1992). Specimens were sectioned with an LKB Utramicrotome IV, stained with an alcoholic solution of uranyl acetate and lead citrate, and examined in a Philips 400 T electron microscope.

In Vitro Culture

GSV-infected *H. zea* adults were surface-disinfected by submersion in 70% ethanol for 5 min followed by two washes with sterile demineralized (MilliQ) water. Reproductive tissues from these adults were dissected into modified TNM-FH (Hink and Strauss, 1976) supplemented with 50 μg gentamicin sulfate (Sigma, St. Louis, MO). Pieces of common oviduct, bursa, and testes were transferred to IPLB-HvT1 cells (Lynn *et al.*, 1988), in 24-well plates. The cell line has been maintained at room temperature in modified TNM-FH and subcultured at a 1:2 to 1:3 split ratio each week by trypsinization. After 4 weeks, plaques which formed in some wells were collected by scraping with Rainin pipet tips and transferred to additional IPLB-HvT1 cells. Subsequently, these cultures were subcultured at 1:2 to 1:4 split ratios at 2-week intervals. Some of the cells were processed for electron microscopy as follows. Cells were pelleted by centrifuging at 1000 rpm for 5 min. The pellet was resuspended in 4 ml of 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer + 80 μl acrolein for 24 h at 4°C. The procedures described by Adams and Bonami (1991, Appendix) were then followed, except that centrifugations were performed at each step, followed by resuspension in the next solution of the wash buffer, dehydration, and infiltration before final embedding in Araldite 506 epoxy resin. Samples from cultures were also bioassayed by injection into newly emerged females.

Molecular Characterization

The size of the GSV genome was estimated by pulse field–gel electrophoresis (PFGE) using a CHEF II Bio-Rad apparatus (Bio-Rad, Hercules, CA). Approximately 3 μg of GSV DNA, purified on CsCl, was loaded onto a 1% agarose gel in 0.5× TBE (45 mM Tris–borate, 1 mM EDTA) buffer. Electrophoresis was carried out at 200 V for 19.4 h at 1°C in 0.5× TBE buffer. The conditions for electrophoresis were as follows: initial time 10 s, final time 20 s, included angle of 120°. For size standards, the λ DNA ladder (Carle and Olson, 1984) was used. GSV genomic DNA was cloned and sequenced as previously described (Lupiani *et al.*, 1999). The sequence obtained was analyzed using the Wisconsin Package Version 10.0 (Genetics Computer Group, Madison, WI).

The genetic relationship of GSV and *Autographa californica* nucleopolyhedrovirus (AcMNPV) was studied by Southern hybridization. *Eco*RI- and *Pst*I-digested GSV and AcMNPV genomic DNA were separated on a 0.7% agarose gel and transferred to a nylon membrane (Southern, 1975). Hybridization was carried out at 42°C without formamide and in the presence of 5× SSC (1× SSC = 10 mM sodium chloride, 15 mM sodium citrate, pH 7.0) using *Eco*RI-digested GSV and AcMNPV DNA, labeled with digoxigenin–dUTP (Boehringer Mannheim, Indianapolis, IN), as probes. The genetic relationship of GSV and some of the common baculoviruses was also studied by PCR amplification. Sets of primers were designed from the highly

conserved polyhedrin gene of AcMNPV, *Lymantria dispar* NPV (LdMNPV), and *H. zea* NPV (HzSNPV) (polyhedra forward: 5′ C/AG/AT/ACCTAC/T GTGTACGA 3′; polyhedra reverse: 5′ TTGTAGA AGTTG/CTCCC 3′) and from the available sequence of GSV (P13 forward: 5′ TCGA TGCCGTAATACC 3′; P13 reverse: 5′ GTCGCTGAATCAAGTCTG 3′). PCR was done using the GeneAmp PCR reagent kit (Perkin–Elmer, Branchburg, NY) with Ampli*Taq* DNA polymerase. Reaction was carried out in 25-μl volume containing 1× concentration of the appropriate buffer (10 mM Tris–HCl, 50 mM KCl, pH 8.3), 200 μM dNTPs, 1 mM $MgCl_2$, 0.6 units Ampli*Taq* DNA polymerase, 0.5 μM each primer, and 1–10 ng of template DNA. Template DNA was obtained from purified AcNPV, LdNPV, HzNPV, and GSV using standard procedures. Samples were denatured for 2 min at 94°C, followed by 30 cycles consisting of a DNA melting step of 94°C for 1 min, a primer annealing step at 55°C for 1 min, and a primer extension step at 72°C for 1 min. After PCR, 10 μl of each sample was separated on a 1.2% agarose gel containing 1 μg/ml of ethidium bromide. ϕX174 RF DNA, digested with *Hae* III (Life Technologies, Gaithersburg, MD), was used as a DNA size marker.

PCR analysis was also used to determine whether GSV and Hz-1V were genetically related. These reactions were carried out as outlined above using DNA samples extracted from TN-368 cells infected with each of these viruses and either the GSV primer set P13 or a set of primers derived from the Hz-1V P34 gene sequence (P34 forward: 5′ CTTATATTGAAACATGCG 3′; P34 reverse: 5′ AACAAGATGCACTTTAGG 3′) (Guttieri and Burand, 1996).

Dose Response and Transmission

One FE GSV was serially diluted with PBS to obtain 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, and 10^{-10} equivalents. Three newly emerged *H. zea* females were injected with 10 μl of each GSV concentration. After 1 day, the females were mated singly and allowed to oviposit for 3 days. Upon hatching, 30 larvae from each treatment replicate were placed on diet in individual cups and reared to the adult stage. The resulting adults were examined for condition of the reproductive system. Newly emerged males were also injected with 10^{-2} FE GSV and after 1 day mated to normal females. The resulting progeny were checked for agonadal condition. In another experiment, 5 newly emerged females were injected with 10^{-8} FE GSV. After 24 h the females were mated to normal males. Larvae hatched from eggs laid by 3 of the females (2 females died before laying eggs) were placed on diet. From the resulting adults, 10 randomly selected males and 10 normal-looking females were mass-mated. The remaining adults were examined for condition of their reproductive system. Normal-looking reproductive organs were subjected to a PCR assay (Lupiani *et al.,* 1999) to detect the presence of GSV. Progeny of the mass-mated adults were examined for agonadal condition.

RESULTS

Atrophy of Reproductive System

Morphology of the reproductive system of normal *H. zea* adults has been described by Callahan (1958). As reported earlier (Raina and Adams, 1995; Hamm *et al.,* 1996), the infected adults do not show any external abnormality except a hypertrophied ovipositor extruding a whitish material in the females. The pheromone gland (located in the ovipositor) of infected females contained almost two to three times more extractable sex pheromone compared to normal females (data not shown), and yet these females did not mate. When approached by normal males, the AG females aggressively avoided copulation. Internally, the AG females had a completely deformed bursa copulatrix. It could be identified by the presence of a small corpus bursa that bore the remanents of sclerotized spines (Fig. 1A). The copulatory opening was closed. The spermatheca was absent and the accessory glands were highly reduced. The common oviduct was directly connected to the bursa copulatrix and not through a duct, as seen in normal females (Fig. 1B). The oviporus or the opening for egg laying was partially closed. The lateral oviducts were enormous and did not present any definite shape or form. There were no ovaries (except in rare cases in which one or two small structures were attached to the distal end of the oviducts and may represent undeveloped larval ovaries). The anal opening was functional. The AG males had a normal-looking endophallus, aedeagus, and ductus ejaculatorius simplex. The testes were very small (about the size found in 3rd instar larvae) and not fused. Seminal vesicles, vasa deferentia, duplexes, and accessory glands were entirely absent. Most of the infected adults had a swollen rectum filled with brownish fluid.

Ultrastructure of GSV

The bursa copulatrix of AG females was full of a white buttery mass of viral atypical occlusion bodies (AtOB). Ultrastructural examination of various tissues, including trachea, hypodermis, fat bodies, Malpighian tubules, and reproductive tract of an AG moth, suggests that GSV is confined to the reproductive tissue. In the female, the lumen of the oviduct was full of AtOB with no visible cytopathology of the adjoining cells. The AtOB differ from the occlusion bodies of NPVs in that they contain high concentrations of virions and have a granular matrix rather than a typical polyhedrin protein matrix (Fig. 2). The AtOB also appear to have a host-derived “membrane” rather than a virus-produced or polyhedron-coded membrane. No occlusion bodies typical of *H. zea* SNPV, as described by

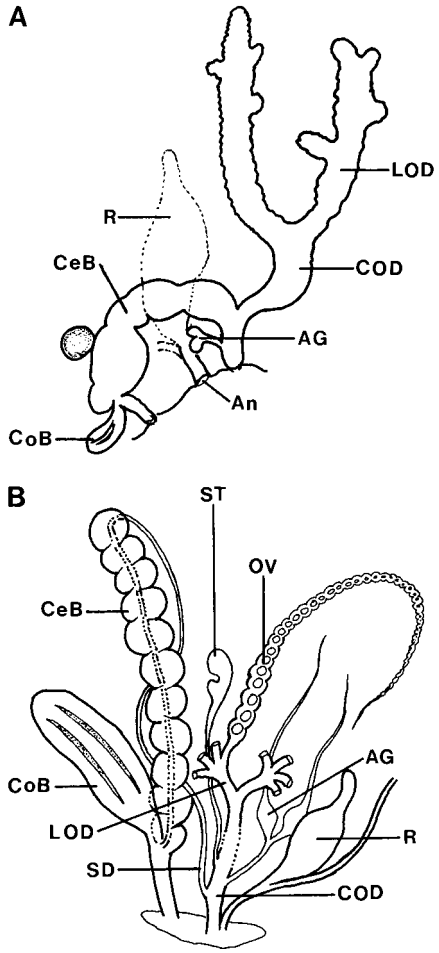

FIG. 1. Comparison of the female reproductive organs of *H. zea* adult. (A) Infected with GSV; (B) Normal. AG, accessory gland; An, anus; CeB, cervix bursa; CoB, corpus bursa; COD, common oviduct; LOD, lateral oviduct; OV, ovariole; R, rectum; SD, spermathecal duct; ST, spermatheca.

Ignoffo (1973), were observed in any of the tissues examined. Large numbers of virions were found in nuclei of the cells in the distal part of the oviduct (Fig. 3). Virions were also seen in the cytoplasm from which clumps of these virions budded into the lumen of the oviduct. The nucleocaspids (unenveloped virions) in thin sections measured 382 ± 30 × 77 ± 3 nm (n = 50).

In Vitro Culture

Obvious cytopathic effects were not seen in any of the cell cultures inoculated with fragments from testes of AG adults. Even though the initial inoculations of cell cultures with material from infected insects was performed in normal, liquid tissue culture medium (rather than a solid medium for plaque assays), areas that appeared to be plaques formed in the cell monolayers in wells inoculated with oviduct fluid or pieces of oviduct tissue. These cells in these plaques were scraped with a Rainin pipet tip, collected, and transferred to 24-well plates containing additional IPLB-HvT1 cells. Bioassay of the plaques by the female injection method yielded 100% AG progeny. When some of these cells were examined by electron microscopy, we found virus particles similar to those seen in GSV-infected adult *H. zea* (Fig. 4). Mitochondria were very

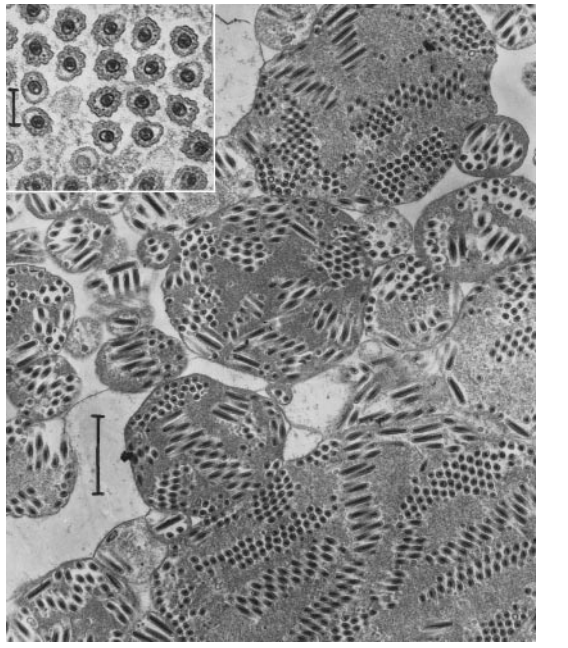

FIG. 2. Electron micrograph of a thin section through the mass of atypical occlusion bodies obtained from the bursa of an infected female. Scale bar, 1 μm. Inset: cross section of the virions. Scale bar, 0.1 μm.

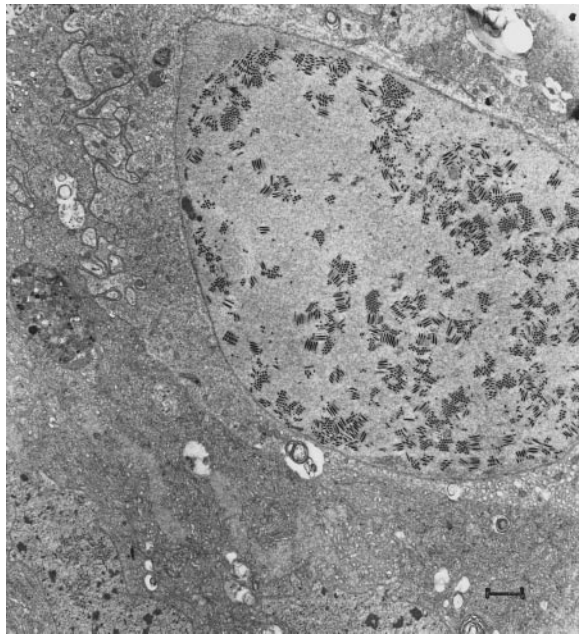

FIG. 3. Virions of GSV in the nucleus of a cell in the distal portion of the lateral oviduct from an infected female. Scale bar, 1 μm.

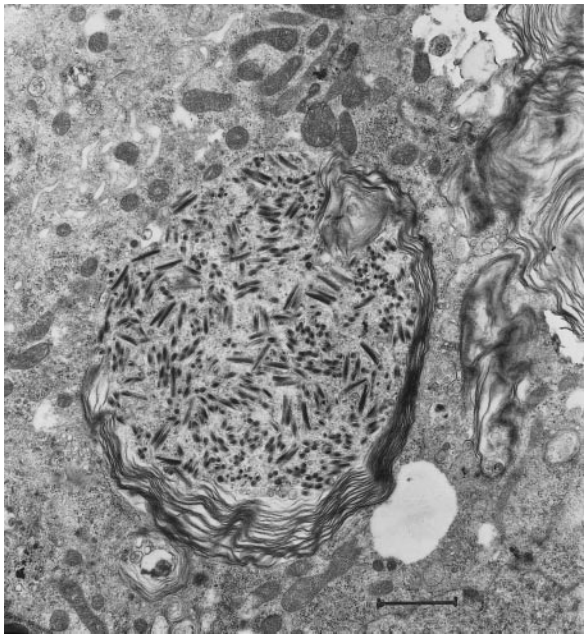

FIG. 4. Thin section of a GSV-infected IPLB-HvT1 cell in tissue culture. Large numbers of virions in the cytoplasm appear to be surrounded by membrane-like structures. Scale bar, 1 μm.

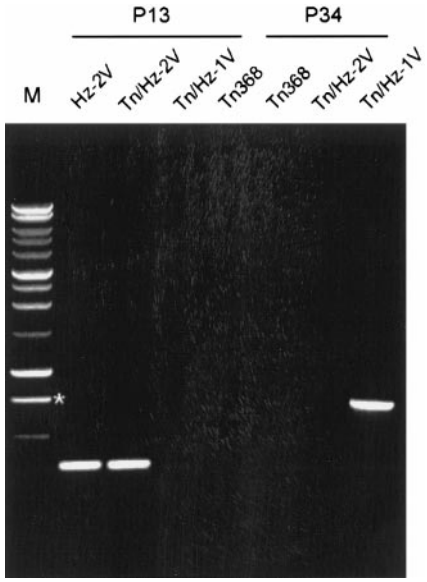

FIG. 6. PCR analysis of DNA samples from Hz-1V- and GSV-infected TN-368 cells using P13 and P34 primers. Each lane contains the PCR products obtained for DNA samples from GSV and both GSV- and Hz-1V-infected TN-368 cells using the primer sets as indicated. The lane labeled M contains a 1-kb DNA ladder marker. The (*) indicates the 750-bp band in the 1-kb marker lane and the 500-bp band is visible just below this band.

abundant in the cells, and the virions were partially enclosed by what looked like several concentric membranes.

Molecular Characterization

PFGE of GSV genome indicated a size of approximately 225 kb (Fig. 5). No hybridization was observed between GSV and AcMNPV genomic DNAs under the stringency conditions used (data not shown). When oligonucleotides designed from the highly conserved polyhedrin gene were used as primers, the expected 581-bp amplification product was obtained in reactions containing DNA from *A. californica, L. dispar*, and *H. zea* NPVs; however, no amplification product was observed in the reaction containing GSV genomic DNA. Similarly, when oligonucleotides designed from the sequence data available from GSV were used as primers, the expected 341-bp amplification product was observed only in the reaction containing GSV genomic DNA (data not shown). Using the P13 primer set, a 341-bp fragment was amplified only in DNA samples from TN-368 cells infected with GSV, whereas PCRs with the P34 primer set amplified only DNA in samples from Hz-1V-infected cells, producing a fragment of approximately 730 bp, as expected (Fig. 6).

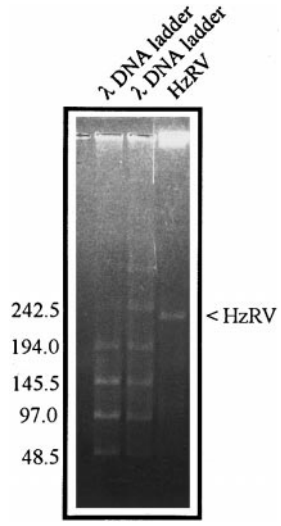

FIG. 5. Size determination of GSV (once referred to as HzRV) DNA by pulse field–gel electrophoresis on 1%/0.5× TBE agarose gel. λ DNA ladder was used as size standards.

Sequence analysis of about 3400 bp of GSV genomic DNA (submitted to GenBank under Accession Nos. AF245396 and AF245397) revealed the presence of a number of open reading frames ranging in size from 44 to 311 amino acids. However, no significant similarity was observed between these and those available in the databases.

Dose Response and Transmission

The highest dose tested (10^{-2} FE) caused the AG condition in about 95% of the progeny (Fig. 7), with the response being almost identical in both sexes. The effect dropped to about 50% at 10^{-8} and below 10% at the lowest test dose of 10^{-10} FE. On the other hand, when males were injected with GSV, the results were very

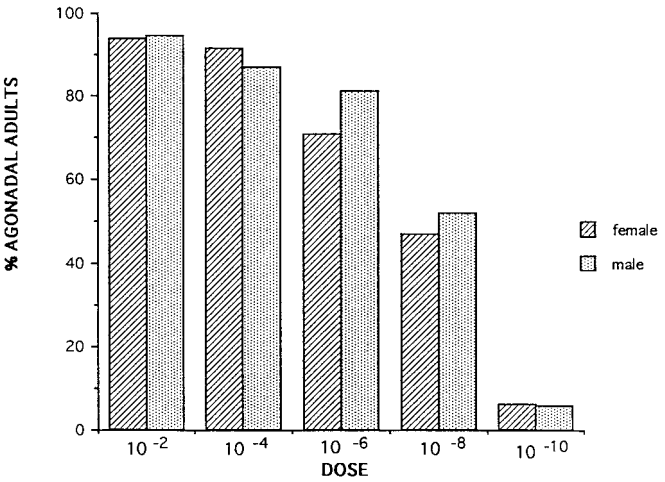

FIG. 7. Dose response of GSV. Virus obtained from the bursa and oviducts of an infected female represented 1 female equivalent. Adult progeny were examined for the agonadal condition. Data represent means of three replicates with 30 samples per replicate.

inconsistent. Progeny of only about 30% of the injected males showed the agonadal condition. However, when present, the condition was manifested in >75% of the adult progeny. When females were injected with a low dose (10^{-8} FE) of GSV and mated to normal males and had their progeny examined, an average of 53% females and 45% males were agonadal (Table 1). Examination of the reproductive organs of the remaining adults (after taking 10 each of females and males for mating) by a PCR assay showed that all of the females and 85% of the males carried GSV asymptomatically. Progeny of the mass-mated adults had a high level of infection with an unknown pathogen. Of the surviving adults (23), 17.4% were agonadal.

DISCUSSION

Normal *H. zea* males were attracted to agonadal females, perhaps due to the large amount of sex pheromone produced by the latter. However, because of the extreme deformation of internal reproductive organs and the fact that the copulatory opening in the agonadal female is almost blocked, the females vigorously avoided copulation. The replication and pathology of GSV appear to be limited to adult reproductive tissues which undergo maximal growth and differentiation in the late pupal stage. In agonadal adults, the ovaries and testes do not grow beyond what is found in 3rd instar larvae. However, most of the reproductive tissues of ectodermal origin do grow and differentiate, but the growth is abnormal. The disrupted growth is particularly pronounced in females. It appears that the virus stays latent in early instars and is induced into productive replication at sometime during the later stages of the insect's life cycle. It is likely that this induction of productive virus replication and the disruption of the growth of selected reproductive tissues at a specific developmental stage is linked to some type of hormonally regulated developmental signal or hormonal imbalance which is related to the differentiation of adult reproductive tissue. Disruption of normal growth in lepidopteran insects as a result of parasitization has been attributed to hormonal imbalance (Beckage, 1985). The exact mechanism through which GSV disrupts growth of selected tissues in the reproductive system of both female and male *H. zea* remains to be determined.

An insect cell line, IMC-HZ-1, was reported to be infected with nonoccluded baculovirus-like particles (Ignoffo *et al.*, 1971; Granados *et al.*, 1978). This virus (IMC-HZ-1-NOV) had measurements ($363 \pm 62 \times 96 \pm 10$ nm, $n = 12$) that are similar to those of GSV. In contrast, nucleocaspids produced in the typical *H. zea* SNPV infection in the fat-body tissues measured $318 \pm 18 \times 42 \pm 4$ nm ($n = 50$) (Adams and McClintock, 1991), whereas alkali-liberated virions measured $336 \pm 22 \times 62 \pm 4$ nm (Gregory *et al.*, 1969). Burand and Lu (1997) reported ultrastructural differences between Hz-1V and GSV primarily in the appearance of the viral envelopes.

Burand and Lu (1997) reported replication of GSV in TN-368 tissue culture cells, with 90% of the infected cells showing cytopathic effects by 2 days postinfection with the release of the virus by cell lysis. GSV replication, assembly, and release in TN-368 cells appears very similar to those processes observed for Hz-1V (Burand *et al.*, 1986). Therefore, PCR analysis using primers derived from known viral DNA sequences was used to examine the genetic relatedness of these two viruses. Since amplification occurred only in DNA samples from virus-infected cells when the homologous primers were used, we concluded that these are two distinctly different viruses. A determination of the exact relationship between these two viruses, which have similar physical characteristics, will require a more complete genetic analysis. PFGE of the genome of GSV resulted in only one DNA band of approximately 225 kb, indicating that this virus is not a polyDNA virus, which is a virus that has multiple circular DNA genomes and is found associated with parasitic wasps.

TABLE 1

Effect of Injecting *H. zea* Females with Low Dose of GSV on Occurrence of Agonadal and Carrier Progeny

Progeny	N^a	Agonadal mean ± SE (%)	PCR assay	
			No. tested	No. positive
Female	41	53.7 ± 5.6	9	9
Male	36	44.7 ± 7.0	13	11

[a] Pooled data from the progeny of three females each injected with 10^{-8} FE GSV. In the case of female progeny, agonadal adults were first separated and, from the remaining, 10 were taken for mating. From the males, 10 randomly selected adults were taken for mating and the remaining examined for agonadal condition. PCR assays were conducted on normal-looking reproductive organs.

Because GSV was rod shaped, it had the appearance of a NPV. Thus, testing for the conserved polyhedrin gene would be normal (perhaps a mutation suppressed the expression). Lack of hybridization between GSV and AcMNPV DNAs indicated low or no genetic relationship among these two viruses. Further, lack of amplification products in reactions containing GSV genomic DNA and primers from the highly conserved polyhedra gene and absence of similarity by sequence analysis indicated that GSV either is unique among baculoviruses in this regard or is not a true baculovirus.

The fact that females, when injected with GSV, were consistently able to transmit the virus to their progeny indicates that the primary mode of transmission is transovarial. Inconsistency in transmission by males may indicate mechanical transfer of the virus during copulation, as against being carried within the sperm. It could also mean that the timing of the injection of the virus may be critical. This aspect needs further investigation.

Presence of GSV in asymptomatic adults confirmed by the PCR assay indicates that these adults perhaps act as carriers of the virus in nature. The test was reported to be very sensitive and able to detect as few as 175 copies of the viral DNA (Lupiani *et al.*, 1999). The presence of agonadal adults in a second generation, even though at low levels, shows how infection may have persisted in the Mississippi colony. It had been reported that the ovarian cell line with the Hz-1V infection was not susceptible to infection with HzSNPV (Burand *et al.*, 1986). HzSNPV has been used in area-wide suppression of *H. zea* and *Heliothis virescens* (Bell and Hardee, 1994). Recently, in laboratory experiments, *H. zea* larvae infected with GSV did not show any change in susceptibility to HzSNPV (Hamm, 1997). These results indicate that introduction of GSV into natural populations of *H. zea* may not impact negatively on the subsequent use of HzSNPV as a microbial-based pesticide.

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