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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

JUL 12 1994

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Review of Human Cell Culture Infectivity/Toxicity Assay for Granulosis Virus of *Cydia pomonella* (CYD-X) from Crop Genetics International

TO: Linda Hollis (PM-18)
Insecticide-Rodenticide Branch
Registration Division (7505C)

FROM: John L. Kough, Ph.D., Biologist
Biological Pesticides Section
Science Analysis Branch
Health Effects Division (7509C)

THROUGH: Roy D. Sjoblad, Ph.D., Section Head
Biological Pesticides Section
Science Analysis Branch
Health Effects Division (7509C)

DATA REVIEW RECORD

Active Ingredients:	Codling moth granulosis virus
Product Name:	CYD-X
ID No:	058788-u CYD-X
Submission No:	S463882
Chemical No:	122201 Codling moth granulosis virus
DP Barcode:	D202428
MRID:	431905-01- Human Cell Culture ..Assay for Codling Moth Granulosis Virus Replication

ACTION REQUESTED.
Review the toxicity/infectivity study of CYD-X on human cell lines to determine if it is adequate to support the registration of this viral insecticide.

BACKGROUND
Crop Genetics International (CGI) has continued to seek the registration of CYD-X originally submitted by Espro, Inc. The data reviewed to date include product chemistry, confidential statement of formula and manufacturing process as well as waivers for the required mammalian toxicology studies. The majority of these waivers were acceptable or rendered acceptable by SAB's rewriting of the original waiver request (memoranda from J.T. McClintock to L. Hollis, June 1, 1993 and August 16, 1993). The waiver requests for the human cell culture assays were denied. This submission is intended to satisify the required cell culture studies.

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SUMMARY OF REVIEW

No evidence of cytopathic or toxic effects were seen in microscopic examination of human cell cultures exposed to freed virions of the Granulosis Virus of *Cydia pomonella*. The human cell cultures obtained from the ATCC were derived from embyronic lung tissue (WI-38) or from normal skin (Detroit 551). Each cell was exposed to approximately 60 LD_{90}'s of granula derived virus suspension. Insect bioassays of the treatments indicated that the viruses that were not rinsed away after inoculation remained infective for neonate *C. pomonella* larvae and only slightly decreased in infectivity over the 7 days incubation at 37°C.

CLASSIFICATION: Supplementary. This study can be upgraded to acceptable with data demonstrating a lack of significant effect on the cell generation time between inoculated and uninoculated cultures and data to confirm that 2 nl was the average amount of suspension imbibed by the test insect larvae.

SAB COMMENTS AND CONCLUSIONS

The results indicate an absence of overt cytopathological or morphological effects resulting from inoculation of 2 human cell lines, WI-38 and Detroit 551. There is an indication that the original inoculum could be declining and is probably not replicating in cells incubated at 37°C. The endpoints used to determine infectivity and toxicity were the appearance of granules of CpGV in the cell lines and results from insect bioassays. Another appropriate endpoint for overall toxicity or infectivity would be similar cell generation times to indicate a lack of effect due to viral inoculation. This could be indicated by similar cell numbers between inoculated and control treatments at the final harvest or calculating the generation times GDV treated and control cell cultures kept at 37°C.

The data generated with diamondback moth larvae to justify the assumption of 2 nl being imbibed by the codling moth larvae should be presented.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, SAB/HED
Secondary Reviewer: Roy D. Sjoblad, Ph.D., Microbiologist, SAB/HED

STUDY TYPE:	Human Cell Culture Assay for Infectivity/Toxicity (152A-15)
MRID NO:	431905-01
CHEMICAL NO:	122201 Codling moth granulosis virus
TEST MATERIAL:	CYD-X
STUDY NO:	240IVR
SPONSOR:	Crop Genetics International, Columbia, MD
TESTING FACILITY:	Crop Genetics International, Columbia, MD
TITLE OF REPORT:	Human Cell Culture Assay for Codling Moth Granulosis Virus Replication
AUTHOR:	Nikolai van Beek, Ph.D.
STUDY COMPLETED:	April 4, 1994
CONCLUSION:	No evidence of cytopathic or toxic effects were seen in microscopic examination of human cell cultures exposed to freed virions of the Granulosis Virus of *Cydia pomonella*. The human cell cultures were derived from embyronic lung tissue (WI-38) and fibroblasts from normal skin (Detroit 551). Each cell was exposed to approximately 60 LD_{90}'s. Insect bioassays of the treatments indicated that the viruses that were not rinsed away after inoculation remained infective for neonate *C. pomonella* larvae and only slightly decreased in infectivity over the 7 days incubation at 37°C..
CLASSIFICATION:	Supplementary. This study can be upgraded to acceptable with data demonstrating a lack of significant effect on the cell generation time between inoculated and uninoculated cultures and data to confirm that 2 nl was the average amount of suspension imbibed by the test insect larvae.

STUDY DESIGN

Test substance: *Cydia pomonella* Granulosis Virus (CpGV) as purified from CYD-X and released from the encapsulating matrix were used as the inoculum for the 2 human cell lines tested as well as the insect bioassay to determine the actual dose used in the cell cultures.

Viral inoculum preparation: Twenty-five ml with total of 2.7×10^{11} *Cydia pomonella* Granulosis Virus (CpGV) granules were placed in 25 ml of ice cold 0.1M Na_2CO_3 for 30 minutes to liberate the individual virus particles. The resulting suspension was centrifuged (4000g for 10 min @ 4°C) to remove the coarse debris from the virus suspension. This supernatant was layered onto a 2 ml cushion of 20% (w/v) sucrose in TE buffer (10mM Tris, 1mM EDTA, pH 7.2) and centrifuged again at 21,000 rpm in a AH628 rotor for 30 min @ 4°C.

The pellet was resuspended in 0.5 ml of PBS by incubation for 1 hour @ 4°C. This suspension of granula derived virus (GDV) was diluted to 5.0 ml and passed through a 0.45 μm membrane filter disc. The resulting 4.0 ml was used for inoculating the human cells (3.8 ml) and determining LD_{90} of the suspension in neonate codling moth larvae (0.2 ml).

TEST SYSTEM FOR CELL CULTURE

Cell lines: Two human cell cultures employed as the test system were obtained from ATCC: WI-38 and Detroit 551. WI-38 is a human diploid fibroblast cell-line derived from normal embryonic lung tissue of a 12-week female fetus. WI-38 has the normal complement of 46 chromosomes, a plating efficiency of about 10% and when inoculated at 2 $x10^4$ viable cells/ml will increase from 9-10 fold in 7 days. Detroit 551 is a human diploid fibroblast cell-line derived from the skin of a female Caucasian female fetus. Detroit 551 also has the normal complement of 46 chromosomes, a plating efficiency of less than 1% and when inoculated at 2 $x10^4$ viable cells/ml will increase 5-fold in 7 days.

Cell culture preparation: One day before inoculation with the viral preparations, eighteen T-25 flasks were seeded with 7.5 X 10^5 viable cells/flask of WI-38 (passage 17) and eighteen were seeded with 7.0 X 10^5 viable cells/flask of Detroit 551 (passage 17). The T-25 flasks for WI-38 contained minimal essential medium (Eagle) with Earle's BSS, 25mM HEPES and supplemented with 2mM L-glutamine and 10% fetal bovine serum (not inactivated). For the Detroit 551, the medium was minimal essential medium (Eagle) with Earle's BSS, non-essential amino acids and sodium pyruvate (1mM) supplemented with 10% fetal bovine serum.

Inoculation procedure: Twenty-four hours after seeding the T-25 flasks, the medium was removed from six flasks of each cell line and replaced with 10ml of fresh medium containing 300 μl of GDV suspension. The flasks were incubated @ 27°C for 90 minutes to facilitate viral attachment without increasing the inactivation known to occur at higher temperatures. The inoculated flasks were rinsed 3 times with 10 ml of fresh media. Three flasks were incubated at 37°C and 3 were maintained at 4°C to serve as controls for zero virus replication.

Two flasks from each cell line were not inoculated with GDV but were subjected to the triple rinsing procedure and served as treatment controls. The 6 other flasks of each cell line were used for the spiking and recovery procedures at the termination of the experiments.

Incubation and observation: All flasks (except the zero virus multiplication controls at 4°C) were maintained at 37°C and observed at 3, 5 and 7 days after inoculation using an inverted microscope at approximately 400X magnification. Observation included examination for abnormal cell morphology, indications of cytopathic effects or other differences in growth characteristics

between the GDV inoculated and control cells.

GDV spiked sample preparation and cell harvest: Immediately before the cell harvest on day 7, 2 flasks of each cell line were spiked with 5, 50 or 500 μl of a 1:100 dilution of the original CYD-X stock solution used to generate the GDV inoculum. This solution contained 1.08×10^{10} granules/ml to yield the following doses of granules per spiking treatment 5.5×10^5, 5.5×10^6 or 5.5×10^7, respectively.

On this same day all flasks were subjected to an extraction procedure to concentrate the granules as follows. Cells were scraped from the bottom of the culture flasks, transferred to a centrifuge tube and subjected to 2 rounds of freezing to -80°C and thawing to disrupt the cells. This suspension was then centrifuged (10,000g for 30 min.) and the supernatant removed except for approximately 1 ml. which was used to resuspend the pellet. Samples were stored at 4°C until bioassayed.

TEST SYSTEM FOR INSECT BIOASSAY

Neonate larvae of codling moth (*C. pomonella*) to be used in the bioassay were shaken via a funnel onto a sheet of paper on a table covered with a plastic shelf paper. The viral dose is administered by a system that insures that larvae have the opportunity to feed on viral suspension. The system concentrates the larvae within a small circle using a funnel and surrounds them with a double ring of inoculum droplets dispensed from a syringe, forcing them to pass through inoculum when dispersing. Some larvae imbibe the viral solution while passing through the ring to reach a petri dish overhead. The larvae at risk by having ingested viral solution are determined by the presence of colored dye in the intestine. Only the colored larvae are transferred to 1 oz. paper cups containing insect diet and are maintained at 26°C in the dark for 7 days. Mortality is scored after 7 days.

Two different bioassays were run: one involved the original GDV suspension to determine the dose used to inoculate the human cell cultures and the other was used to determine the amount of virus in samples of the inoculated cell cultures, spiked cultures and uninoculated controls. All the test suspensions were diluted with concentrated food coloring to facilitate larval selection as indicated above.

RESULTS

The results from the insect bioassay with the original GDV inoculum indicates that each WI-38 cell was exposed to approximately 58 LD_{90}'s while each Detroit 551 cell received about 62 LD_{90}'s. The basis of this calculation are the assumptions that each larvae imbibed approximately 2 nl of GDV suspension when it fed and that the cells, seeded at 7.5×10^5 (WI-38) or 7.0×10^5 (Detroit 551)

cells per flask, doubled overnite.

The cells maintained at 37°C showed no differences between the inoculated and control treatments in cell morphology or evidence of cytopathic effects when examined microscopically at 3, 5 and 7 days. All cells kept at 37°C had normal morphology in contrast to those "zero virus replication" treatments kept at 4°C which were microscopically examined only once: immediately prior to harvest on day 7. These 4°C cells had a low density, did not adhere to the bottom of the flasks and appeared to have compromised cellular membrane integrity. All the effects seen in the human cells kept at 4°C can be attributed to the incubation temperature rather than virus inoculation.

The spiking and recovery experiments showed that a small percentage of the added viral granules could be recovered. The results of the insect bioassays done with the spiked samples were compared to a standard curve generated from a granule dilution series with probit analysis. The comparison indicates between 10 and 25% of the added granules could be recovered from the WI-38 cells and between 7 to 22% could be recovered from the Detroit 551 cells. No detection limit was stated for this endpoint.

The results from the GDV inoculated cell cultures maintained at 37°C compared to those maintained at 4°C indicate that the number of infective virus particles present probably declines at 37°C. This is shown by a slight decrease in the percent larval mortality between these 2 treatments: WI-38 @ 37°= 47, 85 & 92%; WI-38 @ 4°= 95 & 97%; Detroit 551 @ 37°= 78 & 18%; Detroit 551 @ 4°= 44 & 92%. While no clear difference in insect mortality between the 2 incubation temperatures is evident, the lower cell density at 4°C indicates the virus is probably not replicating at temperatures permissive for active human cell culture growth.

SAB COMMENTS
The results indicate an absence of overt cytopathological or morphological effects resulting from inoculation of 2 human cell lines, WI-38 and Detroit 551. There is an indication that the original inoculum could be declining and is probably not replicating at 37°C. The endpoints used to determine infectivity and toxicity were the appearance of granules of CpGV in the cell lines and results from insect bioassays. Another appropriate endpoint for overall toxicity or infectivity would be similar cell generation times to indicate a lack of effect due to viral inoculation. This could be indicated by similar cell numbers between inoculated and control treatments at the final harvest or calculating the generation times GDV treated and control cell cultures kept at 37°C.

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