Enzyme Immunoassays for Detection of Gypsy Moth Nuclear Polyhedrosis Virus

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Abstract

Enzyme-linked immunosorbent assays (ELISA) were developed for detecting gypsy moth (Lymantria dispar L.) nuclear polyhedrosis virus (NPV). They were used to detect the presence of NPV in hemoplymph samples collected from infected larvae. The incorporation of hybridoma antibodies with these procedures would make them even more specific for gypsy moth NPV detection. A pilot study of evaluating NPV spray with ELISA is also presented here. Potentially, these immunochemical methods have important applications in an IPM approach toward the control of gypsy moth.

Introduction

Nuclear polyhedrosis virus of the gypsy moth has received considerable attention as a biological control agent because it is (1) selective for lymantriids, (2) environmentally safe, (3) endemic among natural populations, and (4) persistent in the field after its introduction into a population (Doane 1970, Podgewaite and Campbell 1971, Lewis and Etter 1978, and Lewis 1981). With the recent registration of the European sawfly virus (Neochek) by the Environmental Protection Agency, there are four insect viruses (gypsy moth, Douglas-fir tussock moth and Heliothis NPVs) that need to be examined carefully as how they could fit into integrated pest management schemes of these important pests.

Detection of the gypsy moth nuclear polyhedrosis virus

In order to evaluate effectively the potential of these NPVs for controlling insects, reliable bioassays and analytical methods are needed for both laboratory and field studies. Numerous bioassay procedures have been developed for assaying the presence and the virulence of gypsy moth NPV. One of the disadvantage of bioassay is the long incubation time, usually 10-15 days, required to show any disease symptoms. During this time, polyhedral inclusion bodies (PIBs) can be seen as refractile particles, 1-10 microns in diameter, in the larval hemolymph. Microscopic identification of PIBs could shorten the diagnostic time. However, this method is only reliable in cases of acute infection. When microscopic diagnosis is performed in early stages (viruses present mostly in the non-occluded form) and low levels of larval infection, there is the possible difficulty of distinguishing a few PIBs from other particulate materials in the hemolymph.

Dubois (1981) reported that refractometer measurements of the hemolymph could be used to detect the presence of virus. Quantitative prediction of virus mortality could be made with accuracy in third instar larvae. However, this method is rather stadium-specific, since accuracy cannot be assured with fourth and fifth instars. Larvae just prior and after ecdysis and sample size of the first two instars also present problems for analysis.

Enzyme-linked immunosorbent assay (ELISA), since its development by Engvall and Perlmann (1971, 1972), has received wide application in the biological sciences. This method involves the detection of an antigen with enzyme labelled antibodies. The antigen-antibody complex is then detected by the enzyme label which changes the color of added substrate. The results are presented in the form of a quantitative optical density measurement. The advantages of ELISA over other immunochemical methods include: (1) fast test results (3-4 hrs), (2) simple colorimetric end point, (3) specificity for a given antigen, and (4) extreme sensitivity and reliability. ELISA methods have already received wide applications in modern agricultural research, particularly in diagnostics and epidemiological studies of plant and animal diseases. In recent years, different ELISA methods have been employed for the detection of insect baculoviruses (Kelly et al. 1978, Crook and Payne 1980, Longworth and Carey 1980, Langridge et al. 1981, Evans et al. 1981, Volkman and Falcon 1982). ELISA will undoubtedly be used more extensively in insect control programs involving microbials in the future.

Development of ELISA for gypsy moth NPV detection

Preparation of Specific Antibodies

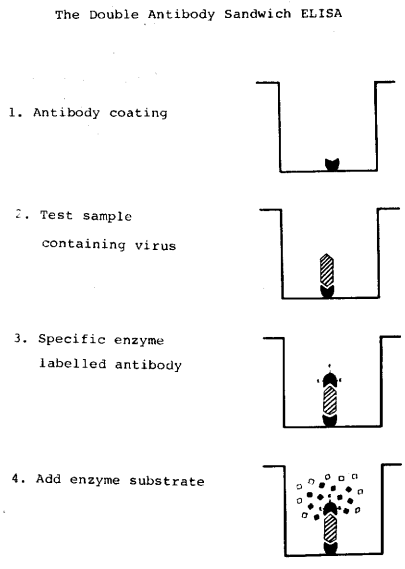
The most important requirement for an ELISA method is the development of a high titer antiserum against a purified preparation of antigen. Gypsy moth NPV PIBs were purified from moribund larvae following the procedure of Tompkins et al. (1981). Whole PIBs were dissociated in 0.1M Na_2CO_3 at pH 11 for 30 minutes, followed by pH adjustment to 7.3 with 2N HCl. New Zealand white rabbits and guinea pigs were immunized for antisera production. Antisera were only accepted if they detect 1 ug/ml of NPV at $1:10^5$

dilution. Immunoglobulin (IgGs) was purified by Protein A chromatography (Goding 1978) and the IgG concentration estimated spectrophotometrically with an extinction coefficient of 1.8 at 280 nm (McGuigan and Eisen 1968). The purification procedure is important for the production of antibody-enzyme conjugates with high specific activity and precison in the development of ELISA methods.

The Double Antibody Sandwich Method

Purified antibodies specific to the virus are coated or adsorbed onto a solid surface such as the well of a polystyrene or polyvinyl 96-well microplate (Fig. 1). A test sample, suspected to contain the viral antigen, is incubated with the antibody sensitized wells. All the hemolymph samples collected are first treated with pH 11 buffer followed by neutralization with acid. The viral antigens, polyhedrin and virions, are bound to antibodies developed with dissolved polyhedra. Enzyme-labelled specific antibodies are added after the test sample is removed. The antigen is sandwiched between two antibodies and the whole complex is bound to the surface of the well non-covalently. After a period of incubation, the enzyme conjugate is removed and is followed by the addition of the substrate. The color intensity is directly proportional to the amount of enzyme present in the sandwich complex, which in turn is proportional to the amount of virus present.

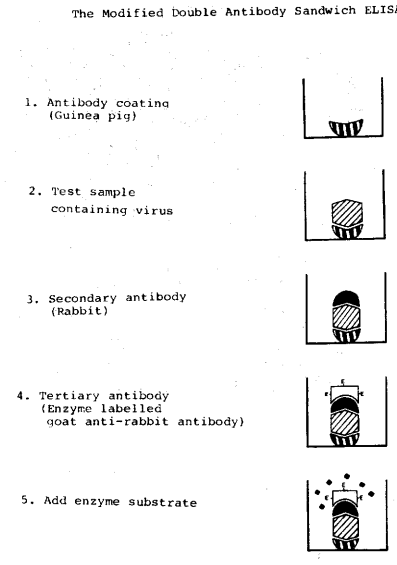
FIGURE 1



The Modified Double Antibody Sandwich Method

This method involves the development of antisera from 2 different animals (Fig. 2). The antibody used to sensitize the well is referred to as the primary antibody. After the addition of the test sample, antisera developed against the virus in a different animal is introduced. This is referred to as a secondary antibody. The virus is sandwiched between antibodies from two different animals. In the current studies, guinea pigs and rabbits were used. At this stage, either antiserum could be used as the primary antibody so long as the enzyme-antibody conjugate is directed at the secondary antibody. The antibody conjugated to the enzyme is referred to as the tertiary antibody which can be either goat anti-guinea pig or anti-rabbit antibody. These tertiary antibodies can be conveniently obtained from many commercial sources. When the sensitivity of the two ELISA methods are compared, the modified double antibody sandwich method is shown to be more sensitive. Detection limit of 50 ng/ml can be obtained consistently with the modified double antibody sandwich method. Since a secondary antibody is used, more than one enzyme-labelled tertiary antibody would link to the sandwich complex. With the presence of more enzymes present in the complex, the detection sensitivity is increased as a result.

FIGURE 2

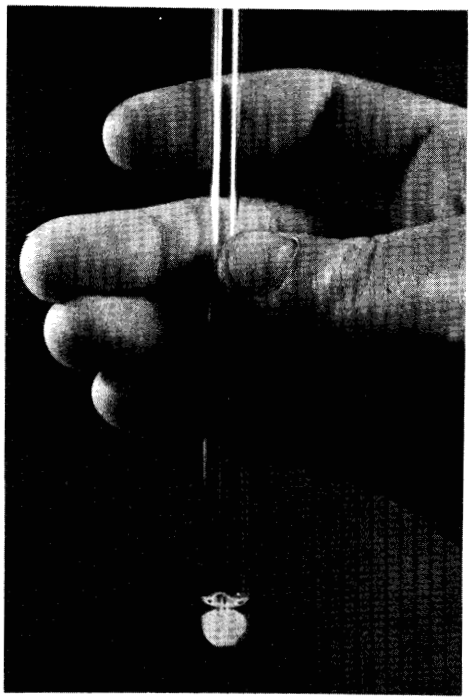


Plastic Bead ELISA

Despite the advantages of ELISA, it has not received wide acceptance in virus epizootiological studies because most field researchers usually lack the equipment or expertise to perform microplate ELISA

procedures. Hendry and Herrman (1980) have employed plastic beads as adsorptive solid phases for protein antigens. In the laboratory for this study (Ma et al. 1984), the relative merits of polystyrene and nylon beads were compared. The polystyrene bead was used in place of a microplate for ELISA detection of gypsy moth NPV (Fig. 3). The protocols developed for double antibody sandwich and modified double antibody sandwich ELISA can be easily adapted to the plastic bead procedure. There are several advantages in using plastic beads: (1) simple protocol which does not involve specialized equipment, (2) plastic beads are easily washed and ready for reuse. Cost is reduced considerably compared to that of disposable microplates. However, this method is not able to handle a large number of test samples which might be a disadvantage in certain situations.

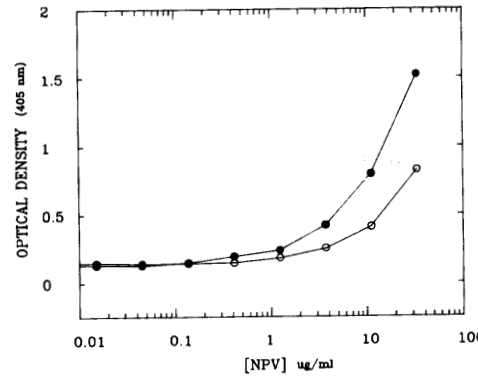
FIGURE 3



Detection of NPV in Infected Gypsy Moth Larvae

When we compared the sensitivity of the double antibody sandwich ELISA between the dissociated virus and whole polyhedra as antigenic preparations, the results showed that better sensivity can be achieved when the virus test sample is subjected to alkaline dissociation followed by pH neutralization (Fig. 4). There are two possible resons for this result. (1) When the antigen is in the dissociated

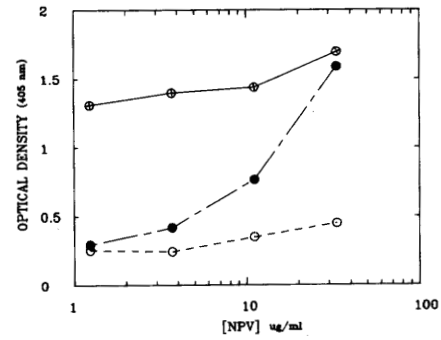
FIGURE 4



form, the antibodies recognize the epitopes of both the polyhedral and viral proteins. However, only those antibodies that recognize the surface epitopes of the polyhedra will react with the whole inclusions. (2) The affinity of proteins to the microplates is mainly a hydrophobic-hydrophilic type interaction. Since there are repeated washings in an ELISA protocol, some of the denser polyhedra may be washed off. A dissociated virus preparation consists of smaller protein fragments which would result in stronger attachments.

In order to look at the feasibility of ELISA for the detection of NPV in insects, the following experiment was performed. Fourth instar gypsy moth averaging 100 mg were used as experimenal animals. Lewis et al (1981) reported that the LC_{50} for early 4th instar larvae was found to be approximately 10^5 PIBs for the Pennsylvannia strain larvae. Ten 4th instar larvae were fed with a lethal dose of 10^6 PIBs/larva and all the hemolymph

FIGURE 5



samples were collected and checked for NPV with the double antibody sandwich method after one week. A negative control was provided by taking hemolymph from non-infected larvae at about the same age and assaying them for virus. The colorimetric reading of infected hemolymph, at 1:3 dilution, was found to be approximately one optical unit higher than the non-infected samples (Fig. 5). When the larvae were monitored at different times after ingesting a lethal dose of PIBs, the double antibody sandwich method can detect NPV in the hemolymph as early as three days post infection.

Case Study: Evaluation of Gypsy Moth NPV Field Application with ELISA

Dr. Tim Tignor (Department of Conservation and Economic Development, Division of Forestry, Virginia) conducted a pilot study on the evaluation of NPV spray applied to shade trees in 1983. Twenty white oaks (Quercus alba L.) on a small portion of the Conway-Robinson State Forest in Prince Williams County, Virginia were chosen for the evaluation of gypsy moth NPV spray. Trees ranged in size from 3.1 to 10.8 inches DBH and about 25 to 45 feet in height. Gypsy moth larvae were extremely difficult to find. Test trees were essentially uninfested. Larvae for spray treatment evaluation were reared from egg masses collected near the nothern Virginia/West Virginia border along U.S. Route 9 as the eggs were hatching on April 19. Larvae were held in the laboratory and fed with fresh oak leaves until most were second instars. On the afternoon of May 9, 200 larvae were placed about 20 feet above ground on each of the 20 test trees. Another 100 larvae per tree were added on the morning of May 12. Larvae were distributed among several branches.

Gypsy moth NPV was applied with a hydraulic sprayer to ten test trees on May 12th. Spray formulation consisted of 5.95 lbs of NPV powder blended well with a small portion of water and added to a larger volume of water containing 2 gallons of Rhoplex B60 A sticker, followed by 20 gallons of feed grade molasses and enough water to make 100 gallons. The virus concentration calculated to be approximately 5 X 10^7 PIBs/ml. Separation of treated and untreated trees ranged from about 50 to 500 feet.

TABLE 1. Numbers of larvae and pupae under burlap bands on NPV sprayed and unsprayed trees.

Date	5/18	5/23	5/26	6/3	6/9	6/16	6/23	6/30	7/7	7/15	7/21
Sprayed	23	32	28	0	0	0*	0*	1	0	0	2
Unsprayed	91	176	355	139	105	50	30	18	3	0	1

*Larvae on 6 of 10 trees; total number estimated.

Counts of larvae and pupae found under the burlap bands are shown in Table 1. Differences in larval numbers between sprayed and unsprayed trees were readily apparent from the first count on May 18. Larvae were noted on most sprayed trees throughout June, but none was found on sprayed trees during this period. After May 26, mean larval numbers declined steadily, and pupae were found on only a few trees. On May 26, all larvae under burlap bands were collected and frozen for later analysis by modified double antibody sandwich procedure described previously. One hundred larvae reared to the third instar were also tested for virus. Table 2 summarized the results. Larvae held in the laboratory yielded negative results. Only 18% of the larvae from untreated trees were infected compared to 64% of larvae from treated trees. When arbitrary categories were chosen to rank levels of infection, the difference between treatment and control groups was still greater. The heavily infected larvae have up to 50 ug/ml of virus. It is important to note that the survivors from the sprayed trees exhibit a higher level of infection. Most of these larvae would probably be dead and become a source of virus dissemination. The source of virus detected in insects from the unsprayed trees may be due to NPV drift from sprayed trees or possibly the breaking of "virus latency" under environmental stress.

TABLE 2. Virus infection levels of larvae from NPV sprayed and unsprayed trees as determined by the modified ELISA technique.

Infection Level [1]	Percent of Larvae [2] Sprayed	Unsprayed	Laboratory
None	36	82	100
Light	14	9	0
Moderate	11	2	0
Heavy	0	1	0
Very Heavy	39	6	0

[1] Positive infection levels arbitrarily assigned to consecutively increasing optical density ranges of .05 units each.

[2] Numbers of larvae sampled: sprayed = 28, unsprayed = 292, laboratory = 100.

Development of Hybridoma Antibodies for the Gypsy Moth NPV

Limitions of Polyclonal Antibodies

ELISA may even be used as a quantitative tool in cases where the virus preparation is positively identified as gypsy moth NPV. Caution is advised because baculoviruses are shown to share similar antigenic characteristics using different serological methods such as radioimmunoassay, complement fixation and immunoprecipitin tests (Bilimoria et al 1974, Elliot et al 1977, and Carey et al. 1978). The presence of heterologous NPV virus in the test sample may be interpreted

as a small amount of gypsy moth NPV. However, there are examples of successes in using ELISA for diagnosis of viruses. Crook and Payne (1980) showed that ELISA can discriminate granulosis virus from Pieris brassicae , Agrotis segetum , and Cydis pomonella . ELISA is also capapble of distingusihing plant viruses which have been shown to have serological relationships by Ouchterlony double diffusion (Lister and Rochow, 1979, and Koenig 1978).

Since gypsy moth NPV is known to be specific for lymantriids only, it is therefore safe to assume that the hemolymph samples collected from infected larvae will yield positive readings only if the gypsy moth NPV is present. If we need to perform experiments such as the environmental persistence of NPV, an antibody preparation that is specific only to the gypsy moth is vital.

Rationale of Developing Hybridoma Antibodies to Gypsy Moth NPV

Conventional serological methods have been shown to be unreliable for the identification of Lepidoteran NPV (Hohmann and Faulkner 1983). The polyhedrin protein of these viruses share a lot of similar antigenic determinants. The antisera used in these studies involved the use of polyclonal antibodies which is a mixture of immunoglobulins to all the epitopes of an antigen. If two different antigens have a portion of the epitopes that are similar, crossreactivity would result.

Kohler and Milstein (1975) developed a method of making monoclonal antibodies that would only bind to one epitope. It is possible to develop specific biological probes for epitopes that are unique for that antigen. These antibodies are generated by somatic cell hybridization of mouse splenocytes and myeloma cells with polyethylene glycol which would result in the establishment of specific antibody producing cell lines. Each hybrid cell line synthesizes a homogeneous antibody that is highly specific for a single antigenic determinant or epitope (Yelton and Scharff 1981). This technology has the potential of locating antigenic determinants or epitopes that are unique for a particular antigen. In this case, monoclonal antibodies developed against these sites would be valuable for diagnostic work and the systematics of NPVs.

In our laboratory, 35 different hybridoma cell lines producing monoclonal antobodies have been developed to the purified polyhedra of gypsy moth. We are in the process of characterizing these hybriodoma antibodies as to their immunoglobulin subclasses and affinity to the antigen. To pick out the monoclonal antibodies specific to the gypsy moth, at least two crossreactivities tests against other lepidopteran NPVs found in the forest ecosystem will be performed.

The first test involves the use of indirect ELISA to screen all the gypsy moth NPV monoclonal antibodies against purified preparations of European sawefly, tussock moth, spruce budworm and other NPVs found in forest environment. The second method, referred to as Western blotting, involves the separation of the purified polyhedra proteins of other NPVs using gel electrophoresis, followed by horizontal transfer of proteins from gel to a nitrocellulose membrane. The membrane would then be incubated with the monclonal antibodies developed for the gypsy moth NPV followed by histochemical staining to visualize the actual binding sites. If a clone of gypsy moth monoclonal antibodies binds only to the gypsy moth viral protein, these tests would confirm that a specific biological probe for a type specific antigenic determinant is located for the gypsy moth NPV.

Prospectus

The study of the gypsy moth nuclear polyhedrosis is important because of its effect on population dynamics and the decision to use Gypcheck as a possible component in an integrated management program. ELISA offers immediate help in studies involving the surveying of virus in gypsy moth populations, quality control of virus formulation, and the evaluation of NPV sprays.

There are additional important questions that need to be answered such as the elucidation of epizootiology, the environmental persistence and dissemination pattern of this NPV. Intelligent decision involving virus applications would be difficult if these basic knowledge are lacking. The development of a reliable protocol for environmental sampling of soil, host plant and non-target hosts would be an important step towards answering these questions. Solid phase radioimmunoassay will have to be developed because it offers the sensitivity of detection down to a few polyhedral inclusion bodies. Hybridoma antibodies will be incorporated in both radioimmunoassay and ELISA procedures to ensure specific detection of gypsy moth NPV and not other NPVs that might be present in the forest ecosystem. I am a strong believer that a lot of the procedure developed in this gypsy moth program will become standard protocols in many insect control programs involving microbials in the future.

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