Baculovirus Bioassay Not Dependent Upon Polyhedra Production

(Accepted 7 May 1981)

SUMMARY

An immunoperoxidase-based quantitative assay has been developed for infectious units of Autographa californica nuclear polyhedrosis virus infecting TN-368 cells. Major benefits of this assay over existing quantitative assays for baculoviruses are that it is not dependent upon polyhedra production, it is clear-cut and easy to score, and results are obtained within 24 h.

As with most viruses, an understanding of the biological properties of baculoviruses has been aided greatly by the development of in vitro cultivation (Faulkner & Henderson, 1972) and quantitative plaque assays (Hink & Vail, 1973). The in vitro bioassays which have been described so far for baculoviruses (Hink & Vail, 1973; Brown & Faulkner, 1977, 1978; Hink & Strauss, 1977; Knudson, 1979; Lee & Miller, 1978; Wood, 1977) appear to depend one way or another upon polyhedra production for virus quantification. It has been known for some time, however, that the formation of infectious progeny can take place in the absence of polyhedra production (Volkman & Summers, 1975; Volkman et al., 1976; Summers et al., 1978; Kimura & McIntosh, 1976) and that polyhedra production can be dependent upon the type of host cell infected (Summers et al., 1978). In addition, investigators are presently selecting for polyhedrin-deficient mutants which are difficult to quantify using the current methods of assay (Brown & Faulkner, 1980; M. Summers, personal communication).

We have developed a simple, clear-cut assay for infectious units of the baculovirus Autographa californica nuclear polyhedrosis virus (AcNPV) which is not dependent upon polyhedra production. The assay is immunological and involves specific staining mediated by horseradish peroxidase held in place by a series of specific antisera. The reaction site is marked by the enzyme's use of hydrogen peroxide as a substrate and diaminobenzidine (DAB) as an electron donor, resulting in the deposition of a golden-brown, insoluble, polymeric oxidation product. The horseradish peroxidase in this assay is not covalently attached to the immunoglobulin, but rather is bound as an antigen by rabbit anti-peroxidase IgG immunoglobulins (Taylor & Chir, 1978). The assay is conducted as follows.

1. Approximately $10^4$ exponential phase cells of the Trichoplusia ni ovarian cell line TN-368 (Hink, 1970) suspended in a 10 µl vol. of fresh TNM-FH medium are seeded on to each of 12 5 mm circles on tissue culture washed and sterilized printed slides (Carlson Scientific, Peotone, Ill., U.S.A.). The slides are placed within sterile Petri dishes and incubated for 1 to 2 h in a humid 27 °C atmosphere while the cells attach to the glass.
2. The medium is removed and the cells are inoculated with 10 µl samples of the E2 variant of AcNPV (courtesy of Dr M. D. Summers) diluted in TNM-FH medium. The slides are returned to the humid 27 °C environment and incubated for 16 h, in addition to a 1 h adsorption period (a total of 17 h).
3. After the incubation period the medium is carefully drawn off the circles, the slides are dipped into a solution of buffered formol–acetone pH 4.5 (20 mg Na$_2$HPO$_4$, 100 mg KH$_2$PO$_4$, 45 ml acetone, 25 ml concentrated formalin, 30 ml distilled water) (Mason et al., 1975) at room temperature for 30 s, rinsed briefly in distilled water and immediately processed through steps 4 to 10 without being allowed to dry. (All sera are diluted in 0.5 M-tris buffer pH 7.6, and all rinses are carried out in 0.05 M-tris-buffered saline pH 7.6.)
4. Endogenous peroxidases are blocked by adding methanol...
Fig. 1. Immunoperoxidase-stained AcNPV-infected TN-368 cells surrounded by unstained, uninfected cells. The procedure for the immunoperoxidase-based staining is described in the text.

containing 0.3% hydrogen peroxide (Streefkerk, 1972). Incubate for 30 min, then rinse once for 1 min. (5) Normal goat serum (10%) is added to help prevent non-specific immunoglobulin binding (Taylor & Chir, 1978). Incubate for 30 min then rinse once for 5 min. (6) Add rabbit antiviral antiserum at the appropriate dilution (determined empirically). The dilution we use is 1:400. Preparation of this antiserum has been described previously as the anti-LOVAL serum (larvae occluded virus, alkali-liberated) (Volkman et al., 1976). Incubate 30 min, rinse for 5 min, three times. (7) Add 10% goat anti-rabbit IgG antiserum (Miles Laboratories, Elkhart, Ind., U.S.A.) for 30 min. Rinse 5 min, three times. (8) Add peroxidase–anti-peroxidase complex (Litton Bionetics, Kensington, Md., U.S.A.) at the appropriate dilution (ours is 1:20). Incubate 30 min then rinse 5 min, three times. (9) Add freshly prepared diaminobenzidine tetrahydrochloride (DAB) reagent (Litton Bionetics or Sigma) (0.05% DAB in 0.5 M-tris pH 7.6, 0.01% hydrogen peroxide). Incubate 7.5 min. Rinse once for 3 min. (10) Dehydrate in increasing concentrations of ethanol and mount

Fig. 2. (a) Relative number of stained AcNPV-infected TN-368 cells per circle with increasing incubation time post-infection. Each point is the mean of four circles coincidentally infected with an identical inoculum of cell culture medium containing the E2 variant of AcNPV passed once in IPLB-SF-21 cells. (b) Dose-response curve of AcNPV on TN-368 cells with a 16 h incubation time. The inoculum was as in (a). Each datum point represents the mean count of four circles per dilution with the standard deviation of the mean also shown.
with Permount. (11) View slides and score total positive cells per circle. We use a Zeiss photomicroscope II with a blue conversion filter.

The clarity of staining of *T. ni* cells infected with *AcNPV* in contrast to the surrounding unstained, uninfected cells is shown in Fig. 1. Specificity controls for the assay include the absence of staining when the test is done on uninfected cells and when rabbit preimmune antiserum is substituted for the rabbit antiviral antiserum (step 6) on infected cells. Since there is no agar, agarose or antiserum in the medium to prevent secondary infection, it is important to determine the appropriate time of incubation to reveal the maximum number of cells infected from the inoculum, and not from progeny virions. The results illustrated in Fig. 2(a) show that a plateau in the number of stained TN-368 cells per circle is achieved when the incubation period is terminated after 14 to 16 h. Beyond 16 h incubation, a sharp increase in the number of stained cells per circle is noted. These results are in agreement with previous studies which showed that viral antigens could be detected by an indirect immunoperoxidase procedure 6 to 8 h post-infection, and progeny virions appeared in extracellular fluids about 10 h post-infection (Volkman *et al.*, 1976; Summers *et al.*, 1978).

The results of a dose-response experiment of *AcNPV* infecting TN-368 cells are presented in Fig. 2(b). The calculated regression line through the means exhibits a slope close to unity (1.04) which indicates that one virion (infectious particle) is sufficient to cause infection. The virus titre, as determined by this experiment, was $2.9 \times 10^7$ infectious units/ml which correlates well with the titre of $2.7 \times 10^7$ infectious units/ml determined by the endpoint dilution method (Knudson, 1979). This close agreement between the two methods attests to the quantitative accuracy of the immunoperoxidase-based procedure.

The immunoperoxidase assay is considerably different from any quantitative assay described so far for baculoviruses. The advantages of the assay, besides not being dependent upon polyhedra, are that it is rapid, clear-cut, easy, and requires few cells. Immunoperoxidase was chosen over immunofluorescence because the former requires no special microscope and because the permanence of the stain allows the slides to be kept as a record. Additionally, the peroxidase-anti-peroxidase method is reputedly of greater sensitivity than either direct or indirect immunochemical methods (Taylor & Chir, 1978).

Since the assay is not dependent either upon polyhedra production or cytopathic effect, but only on the formation of viral antigen, we are hopeful that it can be easily adapted to other systems where a convenient assay is not already available. Quantitative assays based on immunoperoxidase staining have been described for other systems (Vacquier & Cardiff, 1979), but to our knowledge none has been quite so rapid, clear-cut, and easy to score as this one. One drawback of the assay as it is now performed is that the cells used as indicator cells must be all equally susceptible to infection so that all primarily infected cells can be scored before any secondarily infected cells become apparent. While TN-368 cells appear to meet this requirement, other cell types may not. In such cases the addition of a methyl-cellulose or other type of easily removable virus-retarding overlay may be necessary.

We are indebted to Dr Max Summers of Texas A & M University for generously supplying the antiserum, the virus and the cell lines. This investigation was supported in part by BRSG Grant RR-7006 from the Biomedical Research Support Program, Division of Research Resources, National Institutes of Health. Some of the materials incorporated in this work were developed with the financial support of the National Science Foundation Grant DAR-8006421.

*Department of Entomological Sciences*
*University of California, Berkeley*
*California 94720 U.S.A.*

LOY E. VOLKMAN*  
PHYLLIS A. GOLDSMITH
REFERENCES


(Received 23 February 1981)