Detection of Entomopoxvirus Proteins in Insect Cell Culture by Enzyme-linked Immunosorbent Assay (ELISA)

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SUMMARY

An indirect enzyme-linked immunosorbent assay (ELISA), using antibodies made against gradient-purified *Amsacta moorei* entomopoxvirus (EPV), detected down to 13 ng of virus protein. Little antigenic relatedness was detected by ELISA between the structural proteins of *Amsacta* EPV, *Euxoa* EPV, *Melanoplus* EPV and vaccinia virus. Antibodies made against *Amsacta* EPV occlusion body matrix protein cross-reacted extensively with the occlusion body protein of *Euxoa* EPV. A rapid increase in the biosynthesis of *Amsacta* EPV structural proteins in *Estigmene acrea* (BTI-EAA) cells was detected from 12 to 50 h after virus infection. Low concentrations of virus-specific proteins were detected by ELISA in extracts of *Amsacta* EPV-infected *Trichoplusia ni* (Tn-368) cells from 1 to 96 h after virus inoculation.

The entomopoxviruses are large (235 × 400 nm) occluded insect pathogenic viruses which multiply in the cytoplasm of invertebrate cells (Matthews, 1979). Both vertebrate and insect poxviruses contain double-stranded, linear DNA of two size classes (135 × 10^6 daltons and 200 × 10^6 daltons) (Langridge & Roberts, 1977) and have similar enzyme complements (McCarthy et al., 1975; Pogo et al., 1971).

In this report, we have used an enzyme-linked immunosorbent assay (ELISA) to detect *Amsacta* EPV structural protein synthesis in *Estigmene acrea* cells (BTI-EAA) which permit virus multiplication and *Trichoplusia ni* cells (Tn-368) which are restrictive for virus multiplication.

*Amsacta moorei* entomopoxvirus-containing occlusion bodies were isolated from virus-infected *E. acrea* larvae as described by Roberts & Bergoin (1970). Occlusion bodies of another lepidopteran, poxvirus from the army cutworm *Euxoa auxiliaris* were obtained from Robert Burton, Oklahoma State University, Stillwater, Oklahoma, U.S.A. Entomopoxvirus occlusion bodies from the grasshopper *Melanoplus sanguinipes* were obtained from John Henry, United States Department of Agriculture, Bozeman, Montana.

Virus was isolated from occlusion bodies of all three entomopoxviruses by treatment of the occlusion bodies with 0·4 M-Na₂CO₃ and 0·025 M-mercaptoacetic acid pH 10·6 (McCarthy et al., 1974). Vaccinia virus, strain WR was obtained from Dr E. Paoletti, New York State Department of Health, Empire State Plaza, Albany, New York, U.S.A. and was produced in mouse L-929 fibroblast cells. The virus was isolated from infected cells as reported by Dales & Mosbach (1968).

Cells of the salt marsh caterpillar, *E. acrea* (BTI-EAA) and of the cabbage looper, *T. ni* (Tn-368) were grown at 28 °C in BML-TC/10 medium (Gardiner & Stockdale, 1975). *Amsacta* EPV occlusion body protein was obtained by dissolving virus-containing occlusion bodies in dilute alkali as described. The virus was removed from the preparation by centrifugation in a Sorvall SS-34 rotor at 10000 rev/min for 30 min at 4 °C. Occlusion body protein was precipitated from the supernatant by the addition of an equal volume of saturated ammonium sulphate at 0 °C. The precipitate was centrifuged at 10000 rev/min, for 10 min at 4 °C and the pellet was resuspended in 4 ml 0·01 M-tris–HCl pH 8·5, containing 10⁻³...
m-phenylmethylsulphonyl fluoride. After dialysis of the preparation against 2.10-0.01 M-tris-HCl buffer containing 0.001 M-EDTA pH 8 at 4 °C, the occlusion body protein was separated by preparative polyacrylamide slab gel electrophoresis (Laemmli, 1970; Studier, 1973). After electrophoresis, the band of occlusion body protein was excised from the gel and eluted from the polyacrylamide in 5 ml 0-01 M-tris-HCl pH 8.5, for 12 h at 4 °C. The occlusion body protein concentration was determined prior to ELISA (Bradford, 1976).

Antisera to both Amsacta EPV and polyacrylamide gel-purified Amsacta EPV occlusion body protein were prepared in New Zealand white rabbits by multiple subcutaneous injection of virus or occlusion body protein antigen (10 μg/ml) in an equal volume of Freund's complete adjuvant (Vaitukaitis et al., 1971). Immunoglobulin was obtained from virus or occlusion body protein antisera by ammonium sulphate precipitation according to Garvey et al. (1977). The protein concentrations of Amsacta EPV immunoglobulin which gave an optimum reaction with Amsacta EPV was determined by checkerboard titration to be 10 μg immunoglobulin/ml. This Amsacta EPV immunoglobulin concentration was used in all subsequent experiments. The specificity and sensitivity of Amsacta EPV immunoglobulin was determined by indirect ELISA according to the procedure described by Voller et al. (1976).

In the assay, Amsacta EPV, Melanoplus EPV and vaccinia virus were titrated against an optimum concentration of Amsacta EPV immunoglobulin (10 μg/ml).

The ELISA was performed by binding serial dilutions (4 μg to 12.5 ng) of virus or occlusion body protein dissolved in 0-05 M-carbonate coating buffer pH 9-6 to the wells (200 μl/well) of a polystyrene microtitre plate (Dynatech No. 1-223-29). The plate was incubated in a humid container for 5 h at room temperature. The plate was washed three times with phosphate-buffered saline (PBS) containing 0.5 ml Tween-20/l. Virus or occlusion body immunoglobulin (10 μg protein/ml) in PBS–Tween buffer was added to each well (200 μl/well) and incubated for 5 h at room temperature. The plate was washed three times with PBS–Tween and then goat anti-rabbit immunoglobulin (Miles Laboratories, Elkhart, Ind., U.S.A.) was added (200 μl/well) at a dilution of 1:1000 in PBS–Tween and incubated for 12 h at 4 °C. The plates were washed three times with PBS–Tween and 200 μl amounts of 10% diethanolamine substrate buffer pH 9-8, containing 1 mg/ml paranitrophenyl phosphate (Sigma) was added to each well and incubated at room temperature. The substrate buffer contained 97 ml diethanolamine and 0.2 g NaN₃ per litre. Upon the development of a yellow colour (between 30 min and 60 min) the reaction was terminated with 50 μl 3 M-NaOH/well and the absorbance measured at 405 nm in a Gilford model 2400 spectrophotometer.

Dissociation of the poxvirus antigen was accomplished by freezing and thawing the virus three times, followed by sonication of the thawed preparation at 20 W for 30 s at 0 °C with a Labline Ultratip Sonicator. A sample of each preparation was stained for electron microscopy with 2% phosphotungstic acid. No intact virus was detected in the virus preparations.

The ELISA detected <25 ng of Amsacta EPV protein (Fig. 1a). No significant common antigenic determinants were detected between Amsacta EPV and the other three poxviruses (Fig. 1a). Thus, the entomopoxviruses are antigenically unrelated to each other and to vaccinia virus. Antibodies were prepared against Amsacta EPV occlusion body protein purified by polyacrylamide gel electrophoresis as described in the text. The immunoglobulin was titrated against polyacrylamide gel-purified occlusion body protein from Amsacta EPV, Euxoa EPV and Melanoplus EPV (Fig. 1b). Extensive cross-reaction was detected between Amsacta EPV immunoglobulin and Euxoa EPV occlusion body protein (Fig. 1b). The extent of cross-reaction between Amsacta EPV and Melanoplus EPV occlusion body protein was much less than that detected with Euxoa EPV occlusion body protein (Fig. 1b). Common antigenic determinants between the occlusion body proteins of entomopoxviruses might be expected, since considerable immunological and physicochemical homology exists between
I I I I I 1.2 (a) 1.0
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117x419
800 200 50 12.5 400 200 50 12.5 3
Protein concentration/well × 10^-9 g
Fig. 1. (a) ELISA of two entomopoxviruses and vaccinia virus. Amsacta EPV immunoglobulin, diluted with 0.01 M-phosphate buffer pH 7.5 to a final optimum concentration of 10 μg protein/ml, was titrated against serial dilutions of Amsacta EPV (O), Euxoa EPV (●) Melanoplus EPV (not shown) and vaccinia virus (Q). The initial virus protein concentrations were adjusted to 200 μg/ml before serial dilution. The ordinate shows absorbance of the paranitrophenol substrate at 405 nm; the abscissa shows the amount of virus protein/well. The control (A) was Amsacta EPV plus preimmune immunoglobulin. The maximum A_{405} values for Melanoplus EPV were <0.05 and therefore not included in the figure. Goat anti-rabbit antiserum conjugated to alkaline phosphatase was used at a dilution of 1:1000 with PBS–Tween. (b) ELISA of three entomopoxvirus occlusion body proteins. Amsacta EPV occlusion body protein immunoglobulin (10 μg protein/ml 0.1 M-phosphate buffer pH 7.5) was titrated against Amsacta EPV occlusion body protein (O), Euxoa EPV occlusion body protein (●) and Melanoplus EPV occlusion body protein (●). The initial occlusion body protein concentration was adjusted to 200 μg/ml before serial dilution. The ordinate shows absorbance of paranitrophenol substrate at 405 nm; the abscissa shows the amount of virus protein/well. The control (A) was Amsacta occlusion body protein preimmune immunoglobulin. Goat anti-rabbit antiserum conjugated to alkaline phosphatase was used at a dilution of 1:1000 with PBS–Tween.

the occlusion body proteins of other occluded insect pathogenic viruses (Krywienczyk & Bergold, 1961; Cibulsky et al., 1977). Amsacta EPV occlusion body protein immunoglobulin was detected down to 3 ng of homologous occlusion body protein (Fig. 1 b). At present, the nature of the common antigenic determinants between occlusion body proteins of different viral origins is not clearly understood.

The biosynthesis of Amsacta EPV proteins in invertebrate cells was detected by indirect ELISA. Virus-infected BTI-EAA or Tn-368 cells were obtained by filling each well of a No. 3008 Multiwell tissue culture plate (Falcon, Oxnard, Ca., U.S.A.) with 1 ml of medium containing 5 × 10^5 cells. First passage Amsacta EPV non-occluded virus produced in BTI-EAA cells was added to each well at an m.o.i. of 10. The multiwell plates were sealed with tape, balanced and centrifuged in microtiter plate carriers (Cooke Products, Alexandria, Va., U.S.A.) at 2100 rev/min for 60 min in a Sorvall GLC-1 centrifuge to synchronize virus infection. After centrifugation, the medium was removed and the cells were gently washed by the addition of 0.5 ml fresh medium containing 100 μg/ml gentamicin to each well. The plates were centrifuged at 1000 rev/min for 5 min at room temperature. The wash was removed and 0.5 ml fresh medium containing gentamicin (100 μg/ml) was added to each well and the
Fig. 2. (a) Detection of *Amsacta* EPV protein by ELISA in BTI-EAA cells. The ordinate shows absorption at 405 nm. Time intervals after infection with *Amsacta* EPV are plotted on the abscissa. Control, uninfected BTI-EAA cells (■); *Amsacta* EPV-infected BTI-EAA cells at an m.o.i. of 10 (○). The initial concentration of cellular protein before dilution was 200 µg/ml for both control and virus-infected cells. *Amsacta* EPV immunoglobulin concentration was 10 µg/ml in PBS–Tween. Goat anti-rabbit immunoglobulin-alkaline phosphatase conjugate was at a 1:1000 dilution with PBS–Tween. (b) Detection of *Amsacta* EPV proteins in Tn-368 cells. ○, Uninfected cells; ○, *Amsacta* EPV-inoculated Tn-368 cells at an m.o.i. of 10. The initial concentration of cellular protein before serial dilution was 200 µg/ml for both control and virus-inoculated cells. *Amsacta* EPV immunoglobulin concentration was 10 µg/ml. Goat anti-rabbit immunoglobulin–alkaline phosphatase conjugate was at a 1:100 dilution with PBS–Tween.

plates were resealed and incubated at 28 °C. At successive time intervals after infection, the cells were removed from four wells and transferred to polystyrene, conical centrifuge tubes. A haemocytometer cell count was obtained from a 0.1 ml sample taken from the tube and the cells were pelleted by centrifugation at 2000 rev/min, for 5 min in the Sorvall GLC-1 centrifuge. The cell pellet was resuspended in 1 ml 0.05 M-carbonate coating buffer pH 9·6 and stored at −20 °C.

Before ELISA, the frozen infected cell suspensions were thawed and sonicated for 15 s at 0 °C at a rheostat setting of 20 W. Insoluble material was removed by centrifugation at 2000 rev/min for 5 min at 4 °C in a Sorvall RC-5 centrifuge. The protein concentration in each sample was determined by the Bradford (1976) assay and all samples were adjusted to identical protein concentrations with 0·5 M-carbonate buffer. The amount of virus-specific protein present in the virus-infected cells was determined by indirect ELISA (Voller et al., 1976).

The *Amsacta* EPV protein concentration in virus-infected cell cultures was determined by comparing the absorbance (*A*405) of infected cell samples with an *Amsacta* EPV absorbance curve constructed by mixing known amounts of virus protein with uninfected cell protein prior to ELISA. *Amsacta* EPV structural protein was first detected by ELISA in virus-infected BTI-EAA cells between 12 and 15 h after infection (Fig. 2a). Synthesis of *Amsacta* EPV protein increased until 50 h after infection after which time no further increase in the amount of virus protein was detected. In comparison with the *Amsacta* EPV titration curve, the amount of virus protein detected 16 h after infection was approx. 15 ng (7·5 ×
10^{-15} \text{ g/cell}). The amount of virus structural protein detected in cell extracts 49 h after infection was 55 ng (2.75 \times 10^{-14} \text{ g/cell}).

No distinct morphological changes were detected in Tn-368 cells after inoculation with Amsacta EPV at an m.o.i. of 10. No evidence of occlusion body formation was detected in virus-inoculated cells by phase-contrast microscopy from 1 to 96 h after virus inoculation. The cell-doubling time in this experiment was 24 h, as determined by haemocytometer cell counts. Thus, if no virus protein synthesis occurred in the cells during the experiment, a decrease in the amount of virus-specific protein/cell would be expected with time as the amount of cellular protein in the assay was held constant at 200 \mu g/ml throughout the experiment. No significant decrease in virus-specific protein was detected from 10 h to 4 days after virus infection (Fig. 2 b).

Preliminary ELISA experiments, in which mouse L-929 cells were inoculated with Amsacta EPV at an m.o.i. of 10, showed that Amsacta EPV protein decreases and does not persist in the virus-infected cells beyond 60 h after virus inoculation. In alternative experiments (data not presented) in which BTI-EAA cells were inoculated with u.v.-inactivated vaccinia virus, the amount of virus protein detected by ELISA decreased to the level of the uninoculated control within the experimental period (60 h).

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Boyce Thompson Institute, Cornell University  
Tower Road, Ithaca, New York 14853, U.S.A.

W. H. R. LANGRIDGE  
J. F. GREENBERG

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