Equilibrium and kinetic analysis of *Autographa californica* nuclear polyhedrosis virus attachment to different insect cell lines


The kinetic and equilibrium attachment of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) to seven insect cell lines was evaluated. Kinetic experiments revealed differences of up to 10-fold in the infection rates among cell lines. Equilibrium binding also varied between cell lines and was saturable. The Tn 5B1-4 and Tn F cell lines had the highest virus binding affinities and infection rates and exhibited diffusion-limited attachment. The rate of infection appears to be limited by the rate of attachment. For the Tn 5B1-4 cells the physical to infective particle ratio for AcMNPV was 5-3. From the Scatchard analyses, the cell lines Tn 5B1-4 and Tn F displayed affinities of $2 \times 10^{10}$ M$^{-1}$ and $1 \times 10^{10}$ M$^{-1}$, respectively, with 6000 and 13700 binding sites per cell. The insect cell line Hz 1075, which is not susceptible to AcMNPV infection, displayed a much lower, but saturable, binding of AcMNPV with 900 sites/cell and an affinity of $1 \times 10^{10}$ M$^{-1}$. Unlabelled AcMNPV, but not *Lymantria dispar* MNPV could compete with labelled AcMNPV for binding sites. There were 93 to 96% reductions in virus cell binding following pretreatments of cells with three proteases, suggesting the involvement of a cellular protein component in virus binding. Tunicamycin, an inhibitor of N-linked glycosylation and expression of some membrane proteins on the cell surface, reduced virus binding in a dose-dependent manner suggesting a role for glycoprotein(s) in binding. However there was no evidence for the direct involvement of oligosaccharides in attachment. Metabolic inhibitors of oligosaccharide trimming and competition binding assays using simple sugars caused no measurable reductions in virus binding. These findings suggest that AcMNPV attachment to insect cells is receptor-mediated via a glycoprotein component(s); the direct involvement of oligosaccharide moieties in binding is unlikely.

Introduction

The baculovirus *Autographa californica* nuclear polyhedrosis virus (AcMNPV) is used widely as a vector for the expression of heterologous proteins in insect cells (Luckow, 1990). It is a rod-shaped virus with a double-stranded, circular DNA genome of 125 kb. AcMNPV is capable of infecting a number of cell lines, including ones from *Spodoptera frugiperda* (IPLB-21AE) and *Trichoplusia ni* (Tn-368). There are two forms of the virus. The first is occluded within crystallized protein polyhedra that are formed in the nucleus late in infection. The occluded virus (OV) is liberated from polyhedra following ingestion by insect larvae and is responsible for the primary infection of insect midgut cells. The second form of the virus is non-occluded. It buds from infected cells early in the infection process and is responsible for the secondary infection of cells in *vivo*. In tissue culture, the non-occluded virus (NOV) is 1500 times more infectious than the OV (Volkman et al., 1976). The majority of the NOVs have been observed to enter cells in *vivo* by fusion at the cell surface (Wang & Kelly, 1985). However the infectious entry route into tissue culture cells has been shown to be primarily through receptor-mediated endocytosis; a minority appear to enter through some other route, possibly by fusion at the cell surface (Volkman et al., 1986). The 64K glycoprotein present in large amounts in the NOV envelope is involved in fusion and appears to be activated below a pH of approximately 5-5 (Volkman et al., 1986). The NOV attachment and entry kinetics of a closely related virus, *T. ni* MNPV, have been measured (Wang & Kelly, 1985); however attachment, the earliest step in the AcMNPV NOV infection process, has not been studied in detail. The existence of receptor-mediated attachment, the type of receptor, and the physical parameters such as the number and affinity...
of virus-binding sites involved in attachment, have not
been investigated for AcMNPV.

For most viruses, attachment studies have involved
the identification of the viral receptor because its
identity and expression are often key to determining
tissue tropism and host range. Although receptor
expression is not the sole determinant of susceptibility,
infection is initiated through receptor-mediated binding
which thus represents a necessary step in the infection
process. Specific cellular proteins have been identified as
viral receptors, such as the CD4 receptor for human
immunodeficiency virus (HIV) (Dalgleish et al., 1984;
Klatzmann et al., 1984). However others utilize more
ubiquitous cell surface molecules, such as the sialic acid
residues bound by influenza virus (Paulson et al., 1986).
Numerous studies on virus–cell interaction have used
Scatchard analysis to examine the virus binding to cells
to obtain parameters such as the virus–cell affinity and
the number of viral sites on the cell (Alcami et al., 1989;
Basak & Compans, 1989; Epstein et al., 1984; Fries &
Helenius, 1979; Roberts, 1989; Schlegel et al., 1982;
Schwarz & Datema, 1984; Taylor & Cooper, 1989;
Schlegel et al., 1982; Schwarz & Datema, 1984; Taylor &
Cooper, 1989; Trimble & Maley, 1984; Verdin et al.,
1989; Volkman et al., 1984; Wunner et al., 1984).
Additionally, a theoretical study of virus attachment has
investigated the different possible interpretations of
viral Scatchard analyses with respect to the virus site
number obtained and the relationship of the overall virus
affinity to the individual receptor affinity (Wickham et
al., 1990). Metabolic inhibitors, receptor analogues, and
receptor-cleaving enzymes have also been used to
identify and determine the receptor for a particular virus
(Mettenleiter et al., 1990; Paulson et al., 1986; WuDunn &
Spear, 1989).

Here the physical parameters involved in the kinetic
and equilibrium attachment of the non-occluded form
of AcMNPV to insect cell lines are examined. Experiments
designed to determine the existence and nature of an
AcMNPV receptor, whether it is a specific protein or a
more common oligosaccharide component, are reported.

Methods

Cells, media and virus. The following cell lines were used. T. ni BTI-
TN-F (Tn F) and BTI-TN-MG1 (Tn M) are novel cell lines (R. R.
Granados et al., unpublished results) established from T. ni fat body
and midgut tissues, respectively. Another novel cell line, BTI-TN-SB1-
4 (Tn 5Bl-4; R. R. Granados, unpublished results) was established
from T. ni eggs. BTI-EA-88 (Ea 88; R. R. Granados et al., unpublished
results) is an attached cell line derived from Estigmene acrea BTI-EAA
(Granados & Naughton, 1976). Other cell lines used were TN-368
(Tn 368; Hink, 1970), IPLB-SF21AE (SF-21; Vaughn et al., 1977), SF-9
(Summers & Smith, 1987) and Heliothis zea 1075 (Hz 1075; Goodwin et
al., 1982). All cells were grown in TNNM-FH medium [Grace's insect
cell medium (Gibco) plus lactalbumin and yeastolate] supplemented
with 10% foetal bovine serum (FBS, Hyclone) (complete medium).
Fourth tissue culture passage AcMNPV clone 1A (Wood, 1980) was
used in all binding experiments. The G isolate of Lymantria dispar
multiple nuclear polyhedrosis virus (LdMNPV) was also used in
competition experiments (Smith et al., 1988).

Growth of labelled and unlabelled virus particles. Unlabelled virus was
obtained by inoculating SF-21 cells at an m.o.i. of 10 p.f.u./cell for 1 h.
The inoculum was then removed, replaced with fresh medium, and the
cells were incubated at 28 °C. AcMNPV NOV was harvested at 2 days
post-infection (p.i.) LdMNPV NOV was harvested at 8 days p.i.

For 32P-labelled virus, cells were infected as described above except
that the inoculum was replaced by Grace's medium minus phosphate
containing 10% dialysed FBS (Hyclone) and 100 µCi/ml of 32Pi
(NEN). Cells were then incubated at 28 °C and at 6 h p.i. 0.1 volume
of complete medium was added to replenish phosphate. Virus was
harvested from the culture supernatant at 2 days p.i., which is before
cell lysis normally occurs.

Both unlabelled and labelled virus was concentrated and purified by
centrifugation at 500 g for 15 min to remove cells. The supernatant
was then centrifuged at 50000 g through a 35% (w/w) sucrose cushion.
The virus pellet was allowed to resuspend overnight in Grace's medium.
Negative staining of the resuspended pellet showed that over 90% of
the virus particles were intact. The radioactive virus activity was
typically 10^-1 c.p.m./virus particle.

Measurement of virus particle and infectious particle concentration.
Unlabelled virus particle concentration was determined by measuring
DNA concentration. Purified virus was digested with 2 mg/ml
protease K (Boehringer-Mannheim) overnight at 55 °C to release
viral DNA. Digested samples were added in 1 to 100 µ volumes to 2.0
ml of TNE buffer pH 7.4 containing 100 ng/ml of Hoechst 33258 dye
(Polysciences). Background from free DNA or broken viral particles
measured before protease K treatment was always less than 10% of
the total. Phage λ DNA at a known concentration was used as a
standard. Using calf thymus or Escherichia coli DNA also gave the
same standard curve as the λ DNA. The virus particle concentration
was calculated from the Mv of the viral genome and the measured
AcMNPV DNA concentration using a TKO 100 fluorometer (Hoefer
Scientific) (Labarca & Paigen, 1980). The concentration of infectious
virus was determined by a centrifugation plaque assay (Wood, 1977)
using Tn 5Bl-4 cells (described below under measurement of infection
kinetics). The ratio of physical to infectious particles could then be
calculated.

Measurement of attachment kinetics. Radioactive virus particles in
Gracey's medium plus 10%, FBS were added to confluent Tn 5Bl-4 cells
(2 x 10^6/35 mm well) at 4 °C in a volume of 1.0 ml. The virus-
containing medium was then removed from each sample at various
times up to 10 h. The cells were washed twice with Grace's medium and
then removed by scraping to measure the number of bound TCA-
precipitable counts.

Measurement of infection kinetics. Tn 5Bl-4 cells were added to 35
mm wells at 1 x 10^6 cells/well and allowed to become firmly attached.
This is an optimum density of Tn 5Bl-4 cells, selected to be low enough
to support high amounts of polyhedra (Wood et al., 1982), but
sufficiently high for cells to reach confluence, preventing the formation
of overly large plaques. This density gave rise to small plaques with
large numbers of polyhedra which could be detected easily using phase-
contrast microscopy. Virus stock was diluted to 5500 p.f.u./ml in
complete TNM-FH medium and 1.5 ml was added to duplicate wells
and incubated at 22 °C. The inoculum then was removed, the cells were
washed twice with Grace's medium, and overlaid with 1.5% agarose
(FMC Bioproducts) in TNM-FH medium containing 5% FBS.
Infected foci were counted under a phase-contrast microscope.
equipped with a grid reticle to determine the number of infected cells at each time point. For the other cell lines, 100 cells were added per well in six-multiwell tissue culture plates. Virus stock diluted to 5000 p.f.u./ml in Grace’s medium plus 10% FBS was added (1-0 ml) to the wells for 15 min after which the medium was removed and the cells were washed twice with Grace’s medium. The cells were overlaid and the number of plaques determined in the same way as for Tn 5B1-4 cells. The time course of Tn 5B1-4 infection showed that 15 min was a sufficient time in which to determine the initial infection rate. Fifteen min was used for the other cell lines without concern about large decreases in rate due to depletion of infectious virus in the medium because their infection kinetics were all slower than Tn 5B1-4 cells. In measuring the effect of cell concentration on the number of infectious foci produced, 0-5, 0-75, 1-0 or 1-5 x 10^6 cells were added per 35 mm well, exposed to 500 p.f.u./ml in 1-0 ml for 15 min and the resulting infectious foci were then counted under a microscope. Cell density was kept below 1-5 x 10^6 cells to minimize cell density effects in the plaque assay, as already noted (Wood et al., 1982).

Equilibrium binding studies. Cells were added to Falcon 24-multiwell plates (Becton Dickinson). The cells were then allowed to attach and were centrifuged at 2000 g for 15 min to strengthen their attachment. The medium in pairs of wells was then successively removed and replaced with 0-2 ml of Grace’s medium at 4 °C containing a known virus concentration and c.p.m. per particle. (Inclusion of 10% FBS or 1% albumin in the medium had no effect on virus binding and these were not included in the binding medium.) The cells and virus were then incubated at 4 °C for 18 h to reach equilibrium. Following the incubation, the medium was removed and the cells were washed twice with Grace’s medium to remove unbound virus. The cells were then removed and the TCA-precipitable radioactivity could be measured to determine the number of bound viruses per cell. Non-specific binding was determined by measuring the percentage of bound c.p.m. in the presence of a 20-fold excess of unlabelled virus.

Competition studies with unlabelled baculoviruses. Unlabelled LdMNPV and AcMNPV preparations were added to 4 x 10^3 Tn 5B1-4 or Hz 1075 cells in 15 mm tissue culture wells at 4 °C. Ten min later a small amount of 32P-labelled AcMNPV was added to the wells. The contents of the wells were mixed gently and incubated at 4 °C for 2 h. The cells were then washed twice with PBS and the cell-bound TCA-precipitable c.p.m. was measured.

Pretreatment of cells with proteases. Three proteolytic enzymes were used to pretreat cells, followed by extensive washing of the cells and measurement of radiolabelled virus binding: proteinase K (Boehringer-Mannheim), 1-0 mg/ml, pH 7-0, 25 °C; trypsin (Boehringer-Mannheim), 0-5 mg/ml, 25 °C; chymotrypsin (Boehringer-Mannheim) 1-0 mg/ml, 25 °C. Cells were pretreated with the proteases in PBS at pH 7 and fixed with 4% glutaraldehyde at room temperature. The fixed cells were stained with 1% OsO4 in 0-1 M-sodium cacodylate buffer pH 7, and washed with distilled water. The sample was then dehydrated in ethanol and propylene oxide and embedded in epoxy plastic. Samples were then thin-sectioned. Negative staining of virus samples was accomplished by staining with 2% aqueous phosphotungstic acid, pH 7-2.

Results

Measurement of physical to infectious particle ratio

The physical to infectious particle ratio for AcMNPV when measured with Tn 5B1-4 cells was 5-3 and corresponded to 19% of the particles being infectious. This ratio is lower than in previous reports (Volkman et al., 1986; Wang & Kelly, 1985); the differences are probably due to differences in assay methodology (see Discussion). Negative staining of AcMNPV NOV following purification and concentration showed that over 90% of the virus particles were intact with complete viral envelopes.

Infection rates of different cell lines

The infection rates of cell lines were determined by exposing attached cells to a dilute concentration of virus for various times, followed by washing the cells and overlaying with agarose. The resulting number of plaques then corresponded to the number of cells that had been infected during a given exposure time to the virus. The infection kinetics of Tn 5B1-4 cells were studied in detail because these had the highest infection rates. The time course of infection of these cells is shown in Fig. 1. The rate remained nearly constant for the first
hour of exposure and then started to decline as the remaining virus in the inoculum became depleted. The rate measured at 15 min was taken as the initial infection rate and was found to depend linearly on cell concentration ($r^2 = 0.99$) in a range between $0.5 \times 10^9$ and $1.5 \times 10^9$ cells/35 mm well. There was also a linear dependence on virus concentration ($r^2 = 0.97$) under the condition of dilute virus concentration ($< 1$ p.f.u./cell) that was used for these measurements (data not shown). Thus the infection rate could be expressed in the form $r_i = k_i CV$ where $r_i$ is the infection rate (infections/ml.min), $C$ is the cell concentration (cells/ml), $V$ is the radiolabelled virus concentration (c.p.m./ml), and $k_i$ is the infection rate constant (ml/cell.min), which depends on the cell line. This expression for the infection rate is a more general form than originally given by de Gooijer et al. (1989), who assumed viral infection was first order with cell concentration when non-occluded infectious virus was in excess. When virus is not in excess, the infection rate also depends first order on viral concentration. Based on this dependence the infection rate constant, $k_i$, could be calculated from the cell concentration, the initial infection rate and the virus concentration. The initial infection rates and infection rate constants of the other cell lines were similarly measured to determine which cell lines were most susceptible to AcMNPV (Table 1). Tn 5B1-4 and Tn F cells showed the highest infection rates ($k_i = 4.4 \times 10^{-9}$ cell/ml.min) compared to the cell lines Sf-21, Sf-9 and Tn 368 which showed infection rates five- to 10-fold lower. Detailed comparison between our measured infection rates and those determined by de Gooijer et al. (1989) is not possible without detailed knowledge of viral concentrations in their experiments.

## Table 1. Infection rate constants and percentage of virus bound at equilibrium measured for various cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Infection rate constant* (ml/cell.min × 10^-9)</th>
<th>Binding at 4 °C† (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sf-9</td>
<td>0.44 (± 0.9)</td>
<td>14 (± 1)</td>
</tr>
<tr>
<td>Sf-21</td>
<td>0.48 (± 0.6)</td>
<td>18 (± 1)</td>
</tr>
<tr>
<td>Tn 368</td>
<td>1.65 (± 0.1)</td>
<td>22 (± 5)</td>
</tr>
<tr>
<td>Tn M</td>
<td>2.02 (± 0.3)</td>
<td>34 (± 2)</td>
</tr>
<tr>
<td>Ea 88</td>
<td>2.27 (± 0.1)</td>
<td>34 (± 4)</td>
</tr>
<tr>
<td>Tn F</td>
<td>4.35 (± 0.3)</td>
<td>50 (± 3)</td>
</tr>
<tr>
<td>Tn 5B1-4</td>
<td>4.40 (± 0.3)</td>
<td>51 (± 2)</td>
</tr>
</tbody>
</table>

* Measured using the number of plaques formed at 5 days after a 15 min incubation at 22 °C with 5000 p.f.u./ml and 10^6 cells/ml.
† Measured using TCA-precipitable c.p.m. following incubation of 32P-labelled virus with 5 × 10^5 cells/15 mm tissue culture well (0.2 ml).

### Attachment rates to Tn 5B1-4 cells

The attachment kinetics of AcMNPV to Tn 5B1-4 cells were examined in detail. The attachment rate differs from the infection rate; clearly attachment is a prerequisite for infection, and we expect that the infection rate to be slower than the attachment rate. The same linear dependence on cell concentration and virus concentration were found for the initial attachment rate as was found for the infection rate. This dependence held only at initial times since the binding would approach equilibrium as time progressed. The initial attachment rate could be expressed as $r_a = k_a CV$, where $r_a$ is the attachment rate (c.p.m./ml.min), $C$ is the cell concentration (cells/ml), $V$ is the radiolabelled virus concentration (c.p.m./ml), and $k_a$ is the attachment rate constant (ml/cell.min) which has the same units as the infection rate constant, $k_i$. From the initial slope of the line in Fig. 2 $k_a$ for Tn 5B1-4 cells was $5.2 \times 10^{-9}$ ml/cell.min, satisfying the requirement that since attachment precedes infection, $k_a > k_i$. This value was very similar to the measured $k_i$, which was $4.4 \times 10^{-9}$ ml/cell.min. Since $k_i$ is only slightly greater than $k_a$, this suggests attachment is the rate-controlling step for infection, and that post-attachment steps occur quickly on the time-scale of attachment. Rates of attachment and infection for HIV also appear to be related similarly, which has led to the development of a mathematical model in which infection kinetics are adequately treated by attachment kinetics (Layne et al., 1989).

### Calculation of maximum attachment rate constant based on diffusion-limited attachment

The maximum possible attachment rate represents the case where attachment is diffusion-limited and every virus collision with the cell surface results in attachment. The attachment rate constant $k_a(max)$ in this case can be
AcMNPV attachment to insect cell lines

Fig. 2. Attachment kinetics of AcMNPV to Tn 5B1-4 cells at 4 °C. Radiolabelled virus particles (18 500 c.p.m.) were added to 2.0 × 10^6 Tn 5B1-4 cells in a 35 mm tissue culture plate in a total volume of 1.0 ml. Binding came to equilibrium by 10 h.

simply calculated from the virus diffusion coefficient $D_v$ and the radius of the cell $a$ such that $k_{\text{atmax}} = 4aD_v$ for an attached cell (Smoluchowski, 1917; Shoup & Szabo, 1982). By calculating this constant it is possible to set a maximum limit on the attachment rate of the virus and also to determine whether the binding of the virus to the cell is diffusion-limited. An attachment rate constant that is close to the maximum calculated rate indicates diffusion-limited attachment. Using the Stokes–Einstein relation and accounting for the rod shape of the virus (Atkins, 1982), the diffusion coefficient for AcMNPV was calculated to be $2.0 \times 10^{-9}$ cm^2/s at 22 °C. The radius of an attached cell is $13(\pm 2)$ μm so that the maximum possible attachment rate constant was calculated by the above equation to be $6.2 \times 10^{-9}$ cm^3/cell.min. Tn 5B1-4 cells had the highest attachment and infection rate constants which were $5.2 \times 10^{-9}$ cm^3/cell.min and $4.4 \times 10^{-9}$ cm^3/cell.min, respectively, indicating diffusion-limited attachment of the virus to these cells. Since the rate constant for infection is very close to the rate constant for attachment, this suggests the rate of infection of these cells by AcMNPV is limited by the diffusion of the virus to the cell surface. The infection rate constant for the Tn F cell line shows that it is also close to the diffusion-limited rate.

Equilibrium binding at 4 °C

The amount of radioactive virus that bound overnight to cells at 4 °C was measured for each cell line. Large differences in binding were observed between the cell lines as had been observed for their respective infection rates. Tn 5B1-4 cells displayed the highest binding whereas Sf-9 cells displayed the lowest (Table 1). A comparison of the equilibrium binding values with the infection rates for each cell line showed a correlation between affinity of the cell line for the virus and its infection rate (Table 1). Sf-9 and Sf-21 cell lines displayed both the lowest binding and infection rates whereas Tn F and Tn 5B1-4 cells both displayed the highest binding and infection rates.

Competition of unlabelled baculoviruses with labelled AcMNPV for binding

To test the specificity of binding of AcMNPV, competition experiments were performed using progressively higher concentrations of unlabelled AcMNPV and another nuclear polyhedrosis virus, LdMNPV. Unlabelled AcMNPV was able to compete with labelled AcMNPV for attachment to Tn 5B1-4 cells, whereas LdMNPV at the same concentrations as AcMNPV could not compete with labelled AcMNPV (Fig. 3).

Viral Scatchard analysis for different insect cell lines

The equilibrium binding of AcMNPV to different confluent insect cell lines was measured using standard Scatchard analysis to compare their relative viral affinities and number of saturable sites. Total binding was measured by adding progressively higher concentrations of unlabelled virus along with labelled virus. For selected virus concentrations, 20-fold amounts of excess unlabelled virus was added and the percentage of radiolabelled virus that continued to bind in the presence of this excess virus was measured. Specific binding was assessed by subtracting the binding values obtained in...
the presence of 20-fold excess from the binding values in the absence of excess virus (Schlegel et al., 1982; Taylor & Cooper, 1989). The percentage of radiolabelled particles that continued to bind in the presence of excess virus represents non-specific binding. Comparison of the total and specific binding showed that under non-saturating conditions specific binding of the virus accounted for 75% of the total binding for Tn 5B1-4 cells whereas for Hz 1075 cells specific binding accounted for only 25% of the total binding (Fig. 4). Specific binding data for Tn F, Tn 5B1-4 and Hz 1075 cells were converted into Scatchard plots (Fig. 5). The Hz 1075 insect cell line was used as a non-permissive control. Tn F and Tn 5B1-4 cells had affinities of $1.60 \times 10^{10}$ and $2.35 \times 10^{10} \text{M}^{-1}$, respectively, and specific binding sites per cell of 13700 and 6000, respectively. The Hz 1075 line, although not capable of supporting AcMNPV infection, displayed a lower, but saturable, binding of AcMNPV with approximately 900 sites/cell. The affinity of $1.1 \times 10^{10} \text{M}^{-1}$ was comparable to those of the Tn cell lines.

An estimate of the exposed area per cell of each line was calculated by dividing the total area of the tissue culture plate by the number of cells added to the plate that allowed a fully confluent monolayer to form. This area was used to calculate the number of binding sites per $\mu\text{m}^2$ of cell surface. The number of specific binding sites for Tn F, Tn 5B1-4 and Hz 1075 cells were 33, 20 and 2 per $\mu\text{m}^2$ of cell surface, respectively. The maximum number of virus particles that could fit as a monolayer over 1 $\mu\text{m}^2$ could be calculated from their dimensions. AcMNPV particles are 0.07 $\mu\text{m}$ in diameter and 0.33 $\mu\text{m}$ in length. Thus, approximately 200 particles/$\mu\text{m}^2$ could fit, tightly packed, onto the cell if oriented end-on or 43 particles could fit if oriented on their side. A comparison of these values with the measured number of sites per $\mu\text{m}^2$ indicates that between 17% and 76% of the surface of Tn F cells could be occupied by virus particles, depending upon the orientation of the particles.

Electron micrographs of AcMNPV particles binding to Tn 5B1-4 cells

Electron micrographs of virus particles binding near saturation to Tn 5B1-4 cells show that virus particles cover a large fraction of the cell surface (Fig. 6a). Virus particles appear to bind over the entire surface and some virions are bound to other cell-bound particles. A few particles are clearly visible in coated pits (Fig. 6b). The small degree of internalization seen here might be real, or might be an artefact due to warming the cells to room temperature to perform glutaraldehyde fixation. At 4 °C some particles were observed to be internalized by the cell as previously observed in Sf-21 cells (Volkman et al., 1986); however, the electron micrographs (Fig. 6a) show that the internalized (endocytosed or fused) virus represented less than 5% of the total particles. Additionally, endocytosed virus particles, as shown in Fig. 6(b), represented a small minority of the total cell-associated particles.
**Effect of pretreatment of cells with proteases on AcMNPV binding**

Protease pretreatments of cells were tested in an attempt to identify whether the potential AcMNPV receptor had a protein component. Table 2 summarizes the results of pretreatments with the proteases trypsin, chymotrypsin and proteinase K. All three pretreatments of Tn 5B1-4 cells caused a 93 to 96% decrease in binding.

**Effect of inhibitors of N-linked oligosaccharide addition and trimming**

The inhibitors of N-linked glycosylation dNM, SW and TM were used to investigate further the role of N-linked glycoproteins in binding (Table 3). These agents are routinely used to study the glycosylation of recombinant glycoproteins expressed in insect cells by using the AcMNPV vector (Jarvis & Summers, 1989; Oker-Blom et al., 1989). SW and dNM inhibit glycolytic trimming reactions of N-linked glycoproteins following the addition of the high-mannose structure. TM prevents the addition of the entire high-mannose oligosaccharide onto N-linked glycoproteins and can also inhibit the subsequent expression of glycoproteins on the cell surface (Elbein, 1987). Growth of Tn 5B1-4 cells for 3 days in the presence of dNM and SW had no effect on the binding of AcMNPV, compared to the control. Growth of the cells was unaffected by dNM, SW, and 0-2 and 1-0 μg/ml TM. TM at a concentration of 4 μg/ml caused slight vacuolation of the cells; however, the cells remained viable and growing. TM caused a dose-dependent reduction in the binding of AcMNPV to Tn 5B1-4 cells. At the highest concentration of TM, binding was reduced to 21% of the control. This residual binding represents non-specific binding.

![Thin-section electron micrograph of AcMNPV particles (a) bound to Tn 5B1-4 cells under saturating conditions, and (b) bound and internalized by coated pits and vesicles, respectively.](image)

**Table 2. Effect of protease pretreatments on AcMNPV binding to Tn 5B1-4 cells**

<table>
<thead>
<tr>
<th>Protease</th>
<th>Radioactivity bound (c.p.m.)</th>
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<tbody>
<tr>
<td>Control (no protease)</td>
<td>1163 (± 73)</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>77 (± 28)</td>
</tr>
<tr>
<td>Trypsin</td>
<td>48 (± 24)</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>57 (± 16)</td>
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</table>
4 \times 10^6 \text{ cells/ml). The method for measuring infectious these cells under normal assay conditions (2 \times 10^6 to 4 \times 10^6 \text{ cells/ml}). The method for measuring infectious these cells under normal assay conditions (2 \times 10^6 to 4 \times 10^6 \text{ cells/ml}). The method for measuring infectious these cells under normal assay conditions (2 \times 10^6 to 4 \times 10^6 \text{ cells/ml}). The method for measuring infectious these cells under normal assay conditions (2 \times 10^6 to 4 \times 10^6 \text{ cells/ml}). The method for measuring infectious these cells under normal assay conditions (2 \times 10^6 to 4 \times 10^6 \text{ cells/ml}). The method for measuring infectious these cells under normal assay conditions (2 \times 10^6 to 4 \times 10^6 \text{ cells/ml}). The method for measuring infectious these cells under normal assay conditions (2 \times 10^6 to 4 \times 10^6 \text{ cells/ml}). The method for measuring infectious these cells under normal assay conditions (2 \times 10^6 to 4 \times 10^6 \text{ cells/ml}). The method for measuring infectious these cells under normal assay conditions (2 \times 10^6 to 4 \times 10^6 \text{ cells/ml}). The method for measuring infectious these cells under normal assay conditions (2 \times 10^6 to 4 \times 10^6 \text{ cells/ml}).

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Table 3. Effect of metabolic inhibitors on AcMNPV binding to TN 5B1-4 cells*

<table>
<thead>
<tr>
<th>Metabolic inhibitor</th>
<th>Inhibitory action</th>
<th>Concentration</th>
<th>Binding (percentage of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tunicamycin</td>
<td>N-linked glycosylation and transport of proteins to cell surface</td>
<td>0.2 \mu g/ml</td>
<td>54 (± 2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0 \mu g/ml</td>
<td>33 (± 1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.0 \mu g/ml</td>
<td>21 (± 3)</td>
</tr>
<tr>
<td>Swainsonine</td>
<td>Trimming to complex forms at an intermediate step†</td>
<td>10 \mu M</td>
<td>108 (± 12)</td>
</tr>
<tr>
<td>Deoxyxojirimycin</td>
<td>Early trimming steps producing high-mannose oligosaccharides†</td>
<td>4 mm</td>
<td>95 (± 4)</td>
</tr>
</tbody>
</table>

* Cells grown for 3 days in the presence of inhibitors and incubated with 12P-labelled virus for 2 h at 4 °C. Percentage of control bound is the average of triplicates.

† The effects represent those in mammalian cells since the exact mechanism of these inhibitors in insect cells is not known. They do, however, cause an increase in $M_f$ of N-linked glycoproteins in insect cells, suggesting that they function similarly.

Effect of monosaccharides on AcMNPV binding

Further competition experiments were conducted using the major sugar components that are incorporated into glycosaminoglycans, O-linked and N-linked oligosaccharides. The monosaccharides tested were glucose, mannose, N-acetylglucosamine, fucose and galactose. None of these was able to reduce binding significantly at the highest concentrations that could be tested (6 to 7% w/w). Higher concentrations could not be used because the osmolarity would affect cell viability and integrity.

Discussion

Measurements of the fraction of physical particles that are infectious revealed a higher proportion of infectious particles than previous reports (Volkman et al., 1976; Wang & Kelly, 1985). In previous studies, less than 1% of the particles were found to be infectious, whereas the present study found that at least 19% of the particles were infectious. This higher fraction is probably due to the higher concentration of particles employed in the present study involved concentrating the virus and cells onto the bottom of the tissue culture well, thereby increasing the probability of cell–virus interactions (Wood, 1977). The titres of virus measured using this method are typically five- to 10-fold higher than titres measured by other methods (unpublished observations).

A comparison of the attachment rate constant $k_a$ and infection rate constant $k_i$ of Tn 5B1-4 cells with the calculated maximum possible attachment rate constant $k_{a,\text{max}}$ shows that AcMNPV attachment to Tn 5B1-4 cells is very close to diffusion-limited, and that infection is very close to attachment-limited. This finding implies a strong binding interaction between the virus and cell since at the diffusion limit every collision of an unbound particle with the cell results in attachment. At 4 °C, attachment reached its equilibrium value between 3 and 5 h, and remained unchanged from that time to 18 h. This suggests that the small degree of internalization seen in the electron micrographs (Fig. 6) was not persistent or significant. Continued internalization would have led to an increase of cell-associated virus, which was not seen. Furthermore, the lack of change of cell-associated virus levels from 3 to 18 h suggested the minor internalization did not promote further attachment, or stimulate viral release. We conclude internalization has a minor effect, if any, on attachment at 4 °C.

The close agreement between $k_a$ and $k_i$ for Tn 5B1-4 cells also indicates that the viral attachment step may be a controlling factor in the establishment of infection in these cells. Thus, an unbound infectious virus particle will nearly always bind after its first collision with the cell, after which it will probably proceed to infect the cell productively rather than detaching, for instance. Additionally, the correlation between the virus binding of the cell lines at 4 °C and their respective infection rates demonstrates that cells with a higher affinity for the virus also become infected faster (Table 1).

Pretreatment of the cell surface with proteases decreases saturable binding by 93 to 96% implicating a protein in binding, rather than a non-specific interaction with lipid components in the cell membrane. These experiments were performed after 2 h of incubation, at which time virus binding was very close to its equilibrium value (90%), suggesting the virus was well bound during these protease experiments. Additionally the reductions in binding caused by tunicamycin also implicate a protein, or more specifically a glycoprotein. Tunicamycin inhibits the addition of the entire core oligosaccharide onto the protein. However the failure to glycosylate some proteins can also inhibit their expression on the cell surface due to altered folding, increased proteolysis, and/or inhibition of intracellular trafficking (Elbein, 1987). Because of these secondary effects, the reduction in binding cannot be taken to mean that an N-linked oligosaccharide is directly involved in binding. Rather, the reduction indicates that some component of
glycoprotein(s), and not necessarily the oligosaccharide moiety itself, may be involved in binding. A lack of direct participation of N-linked oligosaccharides in binding is supported by competition experiments using monosaccharides and inhibitors of N-linked glycosylation. Neither of the experiments was able to demonstrate any reductions in attachment. Although the exact mechanisms of the trimming inhibitors have not been elucidated in insect cells, they have been shown to be active in insect cells (Roberts, 1989; our unpublished observations) by increasing the apparent $M_r$ of N-linked glycosylated proteins, thus accomplishing the general alterations in the N-linked structures that were intended.

The competition of unlabelled AcMNPV, but not LdMNPV, with labelled AcMNPV indicates that the binding component has specificity. Electron micrographs of AcMNPV binding to cells also shows the virus localized in coated pits which are responsible for the internalization of ligands through receptor-mediated endocytosis. Although electron micrographs of cells at 4 °C show internalization, it is small in extent compared to levels of attachment (as determined by counting bound and internalized virus in Fig. 6 and similar electron micrographs). Therefore it appears valid to treat measurements of cell-associated virus as representing bound virus at 4 °C. Further evidence that internalization is minimal at 4 °C is the lack of increase in cell-associated virus from 4 to 18 h; active internalization would lead to a progressive increase in cell-associated virus. The significant variations in virus binding and infection rates between cell lines is suggestive of variations in the expression of a component or components mediating binding.

Calculations of the maximum number of AcMNPV particles that physically fit onto the surface of a cell reveal that a large fraction of the surface of some cell lines is occupied by virus particles. If AcMNPV (0.07 µm x 0.33 µm) were tightly packed end-on to cover the surface of Tn F cells completely (417 µm²), a maximum of only 85 000 virus particles could fit as a monolayer. If the virus particles were placed sideways onto the cell, a maximum of 18 100 could fit. Tn F cells were found from Fig. 5 to possess 13 700 specific sites enabling binding of 17% and 76% of the maximum that could fit tightly packed in end-on and sideways orientations, respectively. Electron micrographs of particles bound at near saturating conditions also show that a large fraction of the surface is occupied by virus particles. Theoretical treatments of virus attachment have shown that space can become limiting during virus binding even in the presence of receptor-mediated attachment if the receptor number is much greater than the number of spaces on the cell to which virus can bind (Wickham et al., 1990). When this occurs, the resultant Scatchard plot can have a constant slope with a site number approaching the maximum number of virus particles that can fit onto the cell surface, similar to what is seen for Tn F cells (Fig. 5). This interpretation of spatial saturation does not rule out the existence of a specific interaction of AcMNPV with the cell surface. In the above context, a virus ‘site’ is related to the area that a single particle occupies on the surface of a cell. Based on the measured number of sites and from electron micrographs of particles binding to these cells under saturating conditions, it appears that the amount of available binding space begins to become a limiting factor during AcMNPV attachment to Tn F cells. This interpretation of the binding data may be applicable to other virus systems with large virus site and receptor numbers.

AcMNPV is capable of infecting at least 34 insect species; however, most of these species require large doses of the virus to become infected (Evans, 1986). The inability of LdMNPV to compete with AcMNPV for binding, the differences in infection rates and binding between cell lines, and the reductions in binding caused by tunicamycin and protease pretreatment suggest specificity of binding. The discovery of an AcMNPV receptor will be important in discovering how its function relates to the relatively broad host range of AcMNPV and how differences in the sequences and expression of the receptor influence insect susceptibility.

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References


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