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Biochemical Analysis of *Anagrapha falcifera* NPV Attachment to *Spodoptera frugiperda* 21 Cells

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Abstract The binding characteristics of *Anagrapha falcifera* nuclear polyhedrosis virus (AfNPV) to *Spodoptera frugiperda* 21 (Sf21) cells were investigated. The cells displayed an affinity of 4.7×10^{10} M^{-1} with about 3,300 binding sites per cell. The biochemical nature of the AfNPV-binding sites on the cell surface was also partially identified. Our findings suggest that the binding-site moiety has a glycoprotein component, but that the direct involvement of oligosacccharides containing N-acetylglucosamine or sialic acid residues in binding is unlikely, and that AfNPV entry into Sf21 cells may be via receptor-mediated endocytosis.

Key words: *Anagrapha falcifera* nuclear polyhedrosis virus, *Spodoptera frugiperda* 21 cells, binding site, attachment

Insect viruses have received considerable attention, because of their potential and effective biological control agents. Nuclear polyhedrosis viruses (NPVs) are an important subgroup of pathogenic baculoviruses that may prove to be valuable insect control agents. AfNPVs were used to infect *Spodoptera frugiperda* 21 (Sf21) cells *in vitro* [1]. It is a rod-shaped virus with a double-stranded and circular DNA genome [6]. There are two forms of the AfNPV virus: occluded virus (OV) and non-occluded virus (NOV). In a tissue culture, NOV is more infectious than OV. The majority of NOVs in NPV have been observed to enter cells *in vivo* by fusion at the cell surface [12]. However, the infectious entry route into tissue culture cells has been shown to be primarily through receptor-mediated endocytosis [14] and a smaller percentage of cases appear to enter through some other routes, possibly by fusion on the cell surface [11].

The attachment of virus particles to specific receptors on the plasma membrane is one of the initial events in the interaction of NPV with insect cells. The attachment kinetics of baculoviruses *Lymantria dispar* NPV (LdNPV) [5] and *Trichoplusia ni* NPV (TnNPV) [12] have been investigated. However, at present, the attachment process of AfNPV has not yet been studied extensively. As an important characteristic in the attachment processes, the number or affinity of AfNPV binding sites may be quite different from those of LdNPV and TnNPV. In addition, it is unclear whether the biochemical nature of AfNPV binding sites is identical among the insect cell species. To answer these questions, we investigated the binding characteristics of AfNPV to Sf21 cells and the biochemical nature of the binding sites on the cell surface.

The virus used in this work was the *Anagrapha falcifera* nuclear polyhedrosis virus, which was described by Hostetter and Puttler [6]. The virus inoculum used was NOV, derived from cell culture media according to the procedure described previously [2]. The *Spodoptera frugiperda* cell line (IPLB-SF-21) (Sf21) was obtained from L.K. Miller (University of Georgia, Athens, U.S.A.). The Sf21 cells were routinely maintained in a TNM-FH (Sigma, St. Louis, U.S.A.) medium supplemented with 0.35 g/l sodium bicarbonate, 50 μg/ml gentamycin (Sigma, St. Louis, U.S.A.), 2.5 μg/ml fungizone, and 10% fetal bovine serum (Gibco, Grand Island, U.S.A.). For ^{32}P-labeled AfNPV, Sf 21 cells were infected with AfNPV and the inoculum was replaced by the TNM-FH medium minus phosphate containing 10% serum and 10 μCi/ml of ^{32}P-orthophosphate (Amersham, Buckinghamshire, U.K.). The cells were then incubated at 27°C and the virus was harvested after two days before cell lysis occurred. The solution was centrifuged at 1,000 ×g for 20 min to remove cells and cell debris. Then, the supernatant was concentrated and purified by centrifugation at 8,000×g through a 35% (w/w) sucrose cushion. The virus pellet was resuspended overnight in the TNM-FH medium.

AfNPV binding was measured by adding progressively higher concentrations of the labeled virus to the cells. ^{32}P-Labeled AfNPV was incubated with a constant number (4×10^5 cells/well) of Sf21 cells at 4°C for 1 h in 24-well

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plates. The Sf21 cells were heated at 90°C for 10 min and these cells were used as a nonpermissive control for the nonspecific binding. All the experiments were performed in triplicate for a Scatchard analysis. The radioactivity was measured using a scintillation counter (Wallac, 1209 RackBeta, Turku, Finland). The number of bound/free particles against bound particles were plotted for a Scatchard analysis. The virus particle concentration was determined by measuring the DNA concentration. Phage λ DNA at a known concentration was used as a standard. The virus particle concentration was calculated from the molecular weight of the viral genome and the measured AfNPV DNA concentration using a spectrophotometer (Varian DMS-300, U.S.A.). The concentration of the infectious virus was determined by $TCID_{50}$ [10], which was then converted into an infectious particle number [3]. The virus concentration in the virus particles per liter was divided by Avogadro's number to obtain the molar concentration [15].

All experiments for the AfNPV-binding studies were performed in triplicate. The Sf21 cells were treated with enzymes and lectin as follows. The effective concentrations of enzymes and lectin were determined from published data [7, 13]. The cells were treated with 1 mg/ml α-chymotrypsin (Sigma, St. Louis, U.S.A.), 1 mg/ml proteinase K (Sigma, St. Louis, U.S.A.), or neuraminidase (0.03 U, Sigma, St. Louis, U.S.A.) in a PBS buffer (pH 6.2) for 1 h at 27°C, and then washed with PBS to remove any residual activity. The cells were also treated with 2 mg/ml WGA (Sigma, St. Louis, U.S.A.) both with and without proteinase K in a PBS buffer for 1 h at 27°C. In the experiments using a glycosylation inhibitor, the cells were preincubated for 48 h with 0.1, 1, or 4 μg/ml tunicamycin (Sigma, St. Louis, U.S.A.). To study the effect of the energy inhibitors, the cells were preincubated with 10 mM sodium azide or 1 mM dinitrophenol for 48 h at 27°C. The effective concentrations for tunicamycin, sodium azide, and dinitrophenol were also determined from previously published data from other AcNPV binding studies [13-14]. The same amount of radio-labeled AfNPV was added to the control and treated cell layer. The cell layer was designed to contain about 4×10^5 cells/well in 24-well plates or 1×10^5 cells/well in 96-well plates. After 1 h of incubation at 4°C, the cells and supernatant were separated. The radioactivity was measured using a scintillation counter.

The time-course of AfNPV adsorption to Sf21 cells was studied using a virus binding assay at 4°C. Heat-treated Sf21 cells were used as a nonpermissive control. This nonspecific binding value of the control was subtracted from that of total binding. As shown in Fig. 1, the AfNPV binding to Sf21 cells occurred notably between 0 and 30 min, however, the maximum value of virus-binding to the cells was obtained after about 60 min.

Specific binding data for Sf21 cells was obtained with ^{32}P-labeled AfNPV. When this data was transformed into a

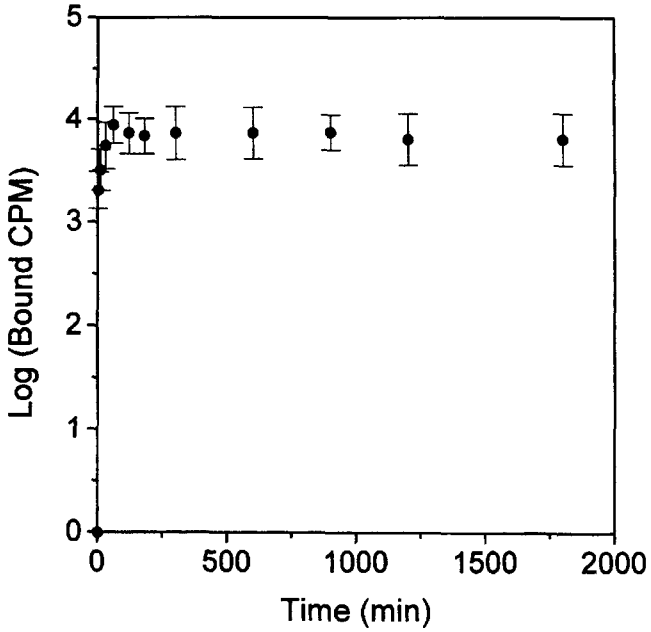

Fig. 1. Time-course of specific AfNPV binding to *Spodoptera frugiperda* 21 cells.
Data points represent average of triplicate results.

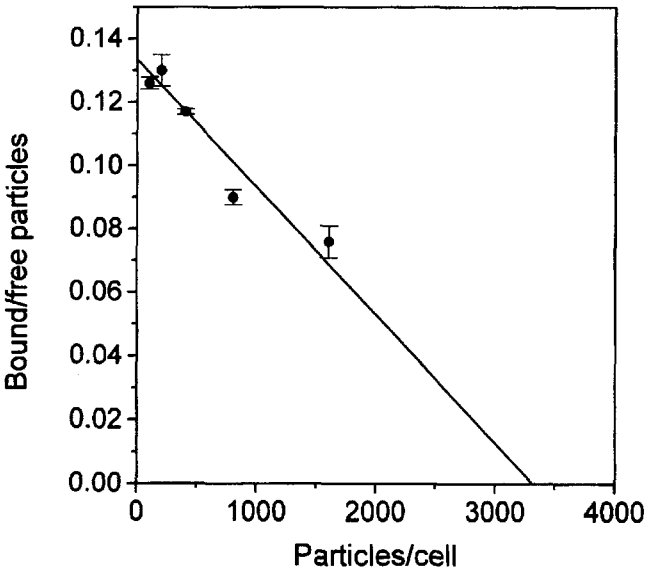

Fig. 2. Scatchard plot of specific AfNPV binding to *Spodoptera frugiperda* 21 cells.
Specific binding data for Sf21 cells were obtained with ^{32}P-labeled AfNPV. Data points represent average of triplicate results. The solid line was obtained using the linear regression.

Scatchard plot, it exhibited a straight line, suggesting that a single class of AfNPV-binding sites is present on Sf21 cells. The abscissa intercept of the plot showed that there were about 3300 binding sites per cell (Fig. 2). The AfNPV binding receptor number on the Sf21 cell surface determined in this study was found to be higher than the

Table 1. Effect of enzymes and lectin on AfNPV binding to *Spodoptera frugiperda* 21 cells.

Treatment	Concentration	% Virus binding[1]
None		100
Proteinase K	1 mg/ml	59±5
α-Chymotrypsin	1 mg/ml	36±2
Neuraminidase[2]	0.03 unit	99±1
WGA[3]	2 mg/ml	99±2
WGA+Proteinase K	2 mg/ml+1 mg/ml	57±9

[1]Average of triplicate experiments ± standard deviation.
[2]*V. cholerae* neuraminidase.
[3]Wheat germ agglutinin.

reported value of 6000 binding sites per *T. ni* 5B1-4 cell for AcNPV [14] and lower than that of 10^6 binding sites per *L. dispar* cells for LdNPV [5]. The Sf21 cells had an affinity of 4.7×10^{10} M^{-1}. This affinity value was higher than the previously reported value (1.6×10^{10} M^{-1}) for the Tn cell line and AcNPV interaction [14].

The biochemical nature of the AfNPV-binding sites on the cell surface was examined by the incubation of Sf21 cells with two proteases. As shown in Table 1, the treatment with α-chymotrypsin and proteinase K resulted in 41 and 64% reductions in AfNPV binding to Sf21 cells, respectively. This indicates that an essential component of the AfNPV-binding moiety on Sf21 cells is a protein. AfNPV binding to the cells was also investigated using tunicamycin (TM). 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 [4]. The treatment of cells with TM resulted in 8-55% reductions in AfNPV binding (Table 2). The fact that the treatment of cells with a glycosylation inhibitor such as TM reduced AfNPV binding supports the hypothesis that the cell-surface receptor for AfNPV may have a glycoprotein structure. Alternatively, the treatment of Sf21 cells with wheat germ agglutinin or neuraminidase had no effect on the AfNPV binding. The pretreatment of Sf21 cells with wheat germ agglutinin (WGA), which specifically binds to oligosaccharides containing *N*-acetylglucosamine, did not affect AfNPV binding. WGA experiments combined with proteinase K treatment showed similar results. In addition, AfNPV binding is insensitive to Sf21 cells treated with neuraminidase, which has the function of desialyation activity. The collective results of WGA and neuraminidase treatment suggest that an oligosaccharide containing *N*-acetylglucosamine or sialic acid is not a necessary moiety for the attachment of AfNPV.

Table 2. Effect of metabolic inhibitors on AfNPV binding to *Spodoptera frugiperda* 21 cells.

Treatment	Concentration	% Virus binding[1]
None		100
Tunicamycin	0.1 μg/ml	92±0.2
	1 μg/ml	53±2
	4 μg/ml	45±4
Sodium azide	10 mM	58±8
2,4-Dinitrophenol	1 mM	52±3

[1]Average of triplicate experiments±standard deviation.

The effects of metabolic inhibitors on AfNPV binding are also summarized in Table 2. Endocytosis is an energy-requiring process, and thus, energy inhibitors such as sodium azide and dinitrophenol have been used as endocytosis inhibitors in the analysis of virus-cell interaction [8]. In our experiments, sodium azide and dinitrophenol strongly inhibited the AfNPV binding. Data on endocytosis inhibitors indicate that the AfNPV attachment to Sf21 cells may be closely linked to the virus entry via receptor-mediated endocytosis. This data agrees with a previous report on the *Bombyx mori* NPV entry mechanism of receptor-mediated endocytosis in *Bombyx mori* BmN-4 cells [9].

This study has provided an insight into the nature of the Sf21 cell surface receptor in the AfNPV-insect cell interaction and our findings provide fundamental data for the research on bioinsecticide development. However, the entire mechanisms of AfNPV host specificity are still unclear. Further research is needed to resolve the controversy surrounding the nature of insect cell receptors in baculovirus-insect cell interactions and to test whether the host range of baculoviruses is restricted to an initial level of attachment to insect cell receptors.

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