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Short Communication

Multi-peak Phenomenon of Insect Cell Infection with Baculovirus at Low Multiplicity of Infection

You-Hong ZHANG[1,2]*

[1] *Hubei Key Laboratory of Novel Chemical Reactor & Green Chemical Technology, Wuhan Institute of Technology, Wuhan 430073, China;*
[2] *Department of Biotechnology Engineering, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel*

Abstract In this communication we report the infection of armyworm *Spodoptera frugiperda* IPLB-Sf-21 cells with *Anticarsia gemmatalis* multicapsid nucleopolyhedrovirus at low multiplicity of infection (MOI). The temporal variation of the extra-cellular virus and of the unstained cell was followed. The series of peaks in the virus concentration and the unstained cells count were used in order to infer the dynamic mechanism of the infection at low MOI. This mechanism can be used as the basis for the future formulation of a mathematical model of the process.

Key words insect cell; *Anticarsia gemmatalis* multicapsid nucleopolyhedrovirus (AgMNPV); infection; multiplicity of infection (MOI); dynamics

The baculovirus expression vector system was developed as a powerful tool for eukaryotic recombinant protein production and biopesticide production due to its strong polyhedron promoter and toxin to host insects [1–4]. There is a growing need for the development of large-scale production for recombinant protein and biopesticide in suspended insect cell culture [3,5]. Therefore, studies on the kinetic process of the suspended insect cell infection with baculoviruses are necessary [6,7].

There are two ways to infect insect cells in suspension culture, classified into high multiplicity of infection (MOI) and low MOI. In the case of high MOI, a synchronous infection of the insect cells is achieved, the kinetics of which is not complex. However, the design of large-scale implementations involves the scaling up of two parallel processes, one for the cells and one for the infecting virus, as high MOI requires large amounts of viruses [8]. In addition, the problem of the "passage effect" is bound to appear during virus multiplication [8,9]. On the other hand, recombinant protein and biopesticide production using low MOI can be carried out on an industrial scale to shorten the production process, reduce the cost of equipment and diminish the possibility of contamination [8]. The main consideration in this approach is that inoculation of the viral stock can be done directly from a well-characterized master bank into a single scaled-up bioreactor [8]. Little effort has been made on insect cell infection with baculoviruses at low MOI [8,10]. The mathematical description of such a system is more complex than in the case of synchronic infection, basically because the process itself is more complex.

In the case of low MOI, the infection kinetics is quite complicated [8]. Only a small fraction of the cells are initially infected in the first infection cycle. The remainder of the cells will continue to grow as non-infected cells. These cells and their progeny will be exposed at a later point in time to the viruses that bud from the cells infected in the first infection cycle. This is called the second infection (SI) cycle. In a similar manner, a third infection cycle can exist when uninfected cells are infected by viruses that bud from the cells infected in the SI cycle, and so on. Eventually, all cells will be either infected or be at a physiological stage in which their capacity to attach to viruses is negligible. At any moment, the extracellular virus concentration in the culture will result from a balance between the rate of virus attachment to the cells and the rate of virus budding from the infected cells. The virus attachment rate depends on the virus concentration, the

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*Corresponding author: Tel, 86-27-62330622; Fax, 86-27-87194465; E-mail, youhong64@yahoo.com.cn

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cell concentration and the virus-binding capacity of cells, which varies along the growth curve. This dependence on the growth phase is supported by several sources. It was reported that cells in the S-phase show a higher affinity for baculovirus infection, and during the exponential phase the percentage of S-phase cells is higher [11,12]. It is suggested that during the S-phase of the cell cycle the increased DNA synthesis would help the virus to replicate its genome at a faster rate, due to the availability of precursors and enzymes involved in this replication [13]. The rate of virus budding, on the other hand, depends on the amount of infected cells and the time of infection. This is therefore a "history-dependent" term. The balance between these two processes results in the dynamic behavior of the non-occluded virus (NOV) concentration, which can be measured in the culture. A mathematical description of the process of insect cell infection at low MOI, which is a substantial element in the scale-up of the process, requires the understanding of this dynamic behavior. Nevertheless, published reports on this matter are scarce.

Here we report our experimental results on the dynamic process of insect cell infection with baculovirus at low MOI through the temporal variation of extracellular viruses, that is, NOV and unstained insect cells.

Experimental Procedures

The IPLB-Sf-21 cell line from the pupal ovaries of the fall armyworm *Spodoptera frugiperda* [14] was maintained in 25 cm^2 T-flasks as adherent cultures in TC-100 medium (Sigma, St. Louis, USA) [15] supplemented with 10% fetal bovine serum (Sigma). Subcultures were made every 4–5 d to maintain the cells in the exponential phase. When the cells were grown in suspension, 0.2% (*W*/*V*) Pluronic F-68 (Sigma) was added to the medium. A strain of *Anticarsia gemmatalis* multicapsid nucleopolyhedrovirus (AgMNPV) isolated from the infected larva [14] was used. The cell line and original virus stock were obtained from INTEBIO (Universidad Nacional del Litoral, Santa Fe, Argentina). The virus inoculum was prepared by amplifying the original strain virus stock in suspension cultures of IPLB-Sf-21 cells at an MOI of 0.01.

The number of cells was measured microscopically using a Neubauer hemocytometer. Cell viability was determined using the 0.04% Trypan blue exclusion method. Cells that excluded the colorant were considered viable. The infectivity of the NOVs was measured using an end-point dilution assay [16].

Four days later, 50 ml cell culture from the T-flasks was transferred to 250 ml Erlenmeyer flasks to diminish the lag phase. When cells in the first Erlenmeyer flask grew to the late exponential phase (after approximately 4 d), the cells were transferred to Erlenmeyer flasks and diluted with fresh medium to a cell concentration of 1.9×10^5 cells/ml. After 24 h, the cultures, which were considered to be in the early exponential phase, were centrifuged and diluted with fresh medium into six Erlenmeyer flasks (two for mock infection and four for infection) to an initial cell density of approximately 4×10^5 cell/ml. Cells in these Erlenmeyer flasks were infected by AgMNPV at an MOI of 0.10 and 0.01. The flasks were rocked at a frequency of 70 rpm and kept in a constant temperature chamber at 27 ºC.

Sampling for measurement of NOV was performed once every hour for the first 6 h to investigate the kinetics of virus attachment. Considering that the process of budding *in vitro* starts at 10–12 h post-infection (hpi) and is gradually completed from 24 hpi to 30 hpi, according to Volkman and Keddie [17], samples were taken at 8, 10, 12, 18, 24, 36, 42 and 48 hpi. Mock infection was used as a control and sampled every 24 h.

Results and Discussion

Fig. 1 shows the profile of NOV concentration at an MOI of 0.10 or 0.01 during the first 6 h of the first infection cycle. In both cases, the disappearance of viruses from the medium seems to follow first order kinetics on NOV concentration. It should be taken into account that in all

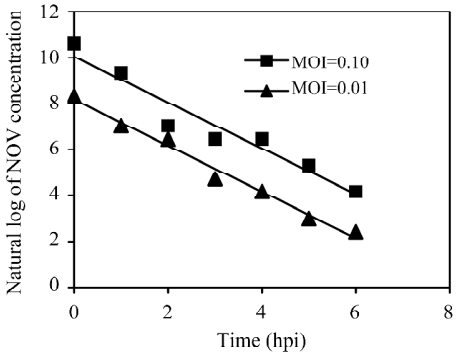

Fig. 1 Unbound virus concentration in Sf-21 cell cultures infected by *Anticarsia gemmatalis* multicapsid nucleopolyhedrovirus at a multiplicity of infection (MOI) of 0.10 and 0.01

The cell density in each culture was approximately 4×10^5 cells/ml. hpi, hours post-infection; NOV, non-occluded virus.

experiments the initial cell concentration was the same, and because of the low MOI the cell concentration remained practically constant during the first 6 h. It can be assumed that the decreasing rate of virus concentration in the medium is equal to the attaching rate of the virus to the cells. The NOV concentration drops to practically zero for both MOIs during this first period.

At an MOI of 0.10, the NOV concentration curve peaks at approximately 24 hpi, then decreases to almost zero again at 36 hpi [**Fig. 2(A)**]. This profile of viral concentration can be understood as a result of a balance between the release of viral particles that bud from the cells infected in the first infection cycle and the attachment of the produced viral particles to the cells. At this stage, the cells are growing vigorously in their early exponential phase, as shown in **Fig. 3**. In the infected group, uninfected cells also remain unaware of the infection and behave in the same way as in the mock group.

Because the first infection takes place during the first 6 h, the budding process is "smeared" in this period. The budding starts at 10–12 hpi and is gradually completed from 24 to 30 hpi, so the peak at approximately 24 hpi can be easily understood. The cells infected at time 0.0 hpi would start to bud at approximately 10 hpi and gradually finish budding from 24 hpi to 30 hpi. But cells infected at approximately 6 hpi would end budding at 36 hpi. Indeed, the NOV concentration profile shows that at this point the second infection cycle is complete and free viruses are very scarce, indicating that most viruses have attached to cells.

During the second infection cycle, the number of infected cells is much larger than in the original infection, as indicated by the peak of NOV at approximately 72 hpi in **Fig. 2(B)**, which comes down to approximately 84 hpi. During this period, the shape of the peak is influenced not only by the concentration of viruses in the medium, but also by the amount and physiological state of viable cells. As shown in **Fig. 3**, the viable cells are decreasing in number and the culture has finished the exponential phase and entered the death phase; the cell affinity for viruses has diminished and therefore the rate of attachment is lower. It is originated because the rate of virus budding is much higher than the rate of attachment at this stage, therefore the concentration of NOV increases sharply. This explains the temporal accumulation of viruses in the medium.

When the budding ends, there are still enough cells to attach to most of the viruses in the medium, and the concentration of NOV decreases again. The peak at approximately 96 hpi, produced by the third infection cycle, is much lower. The affinity of the cells for baculovirus is now low, as the cells are well past the exponential growth phase, but the budding rate of new virus is also low.

The concentration of the viruses produced after the third infection cycle, on the other hand, does eventually change a little, indicating that either the insect cells have lost both their capacity for attaching and budding virus, or that the release of viruses from the last budding cells balances the attachment of the suspended viruses to the cells, both processes being relatively slow. This is also seen in the fact that the unstained cell concentration remains unchanged

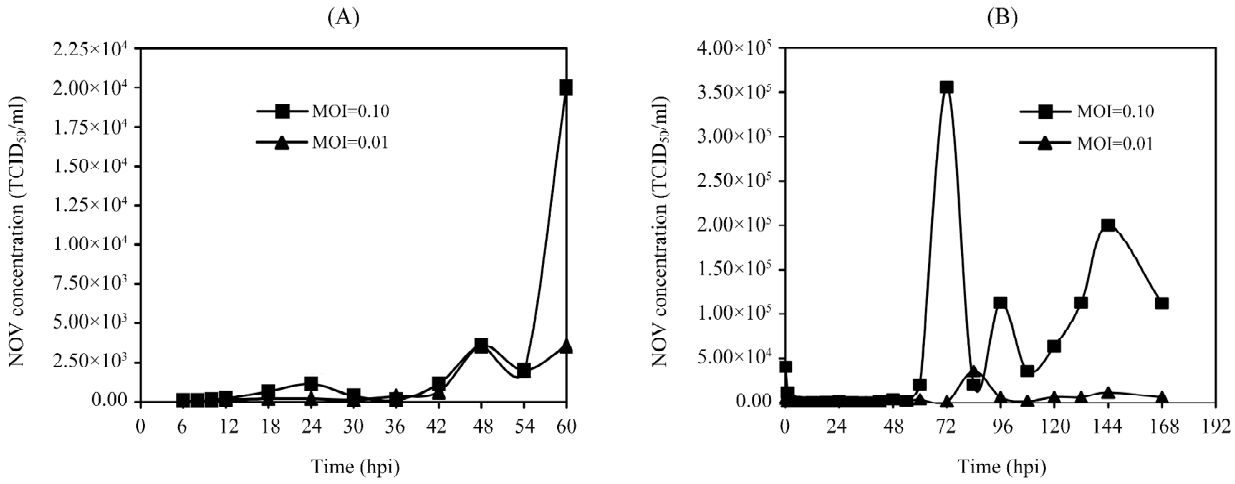

Fig. 2 Non-occluded virus (NOV) concentration for Sf-21 cells infected at a multiplicity of infection (MOI) of 0.10 and 0.01
(A) The NOV concentration from 6 h post-infection (hpi) to 60 hpi. (B) The NOV concentration from 0 hpi to 168 hpi. The initial cell density was around 4×10^5 cells/ml.

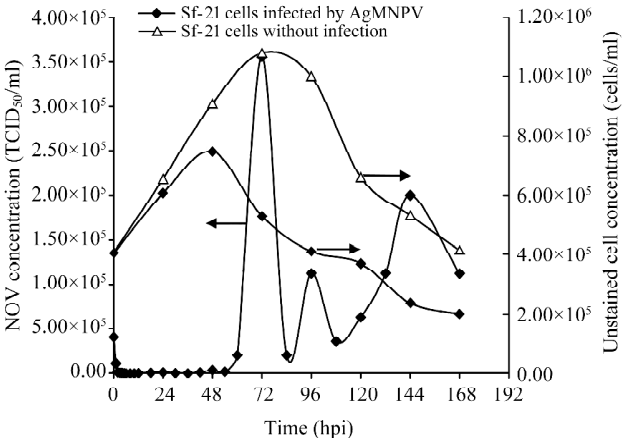

Fig. 3 The evolution of non-occluded virus (NOV) and unstained cell concentrations of Sf-21 cells infected at a multiplicity of infection (MOI) of 0.10

AgMNPV, *Anticarsia gemmatalis* multicapsid nucleopolyhedrovirus; hpi, hours post-infection.

from 144 to 168 hpi (**Fig. 3**). These small changes in virus concentration may be errors resulting from the viral titer of the end-point method.

At an MOI of 0.01, the curve of NOV concentration follows the infection of the cell culture, as can be seen in **Fig. 2**. The corresponding unstained cell concentration and control viable cell density curves are shown in **Fig. 4**. It can be seen that the increase in NOV concentration due to the budding of infected cells during the first infection cycle is almost undetectable at an MOI of 0.01. On the other hand, the smearing is felt less. This concurs with the measured data of initial attachment, shown in **Fig. 1**. At an MOI of 0.01, the NOVs are depleted much faster. For example, for an MOI of 0.10 the NOV concentration drops to 35.6 $TCID_{50}$/ml after 6 h, but this level is reached in the case of MOI 0.01 after only 4.5 h. Consequently the peaks will be less smeared over time. The peak at approximately 72 hpi at an MOI of 0.10 is replaced by two neat peaks at approximately 72 hpi at an MOI of 0.01. These two peaks are seen to be overlapped for the MOI 0.10 data and slightly shift later. This can be explained by the presence, initially, of a larger amount of viruses, which badly smeared the peaks.

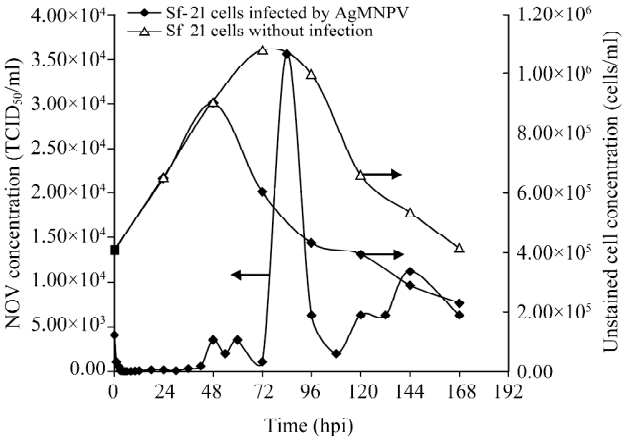

Fig. 4 Evolution of non-occluded virus (NOV) and unstained cell concentrations for Sf-21 cells infected at a multiplicity of infection (MOI) of 0.01

AgMNPV, *Anticarsia gemmatalis* multicapsid nucleopolyhedrovirus; hpi, hours post-infection.

The present observations will serve as indications of the mechanism of infection at low MOI in the process of mathematical modeling of the system. An accurate description of the system should also consider the different stages of the infected cells, but these stages are not clearly defined during simple observations. NOV and unstained cells, on the other hand, are easy to detect. Our objective should be, therefore, to express the whole system as a function of these observable variables, which will make possible the calibration of the future mathematical model.

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Yi-Peng QI