Short Communication

Physiological factors of growth and susceptibility to virus regulating Vero cells for optimum yield of vaccinia and cloned gene product (β-hCG)

Asok Mukhopadhyay a,*, S.N. Mukhopadhyay b, G.P. Talwar a

a National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi-110 067, India
b Department of Biochemical Engineering & Biotechnology, Indian Institute of Technology, Hauz Khas, New Delhi-110 016, India

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Abstract

Replication of vaccinia virus carrying β-hCG gene at the 'tk' locus was studied in microcarrier culture of Vero cell. The yields of recombinant vaccinia virus and its expressed product (β-hCG) were influenced by the physiological state of the host cell, at which it was infected. Cells infected with the virus between the 3rd and 6th day gave maximum amount of virus and β-hCG in a parallel manner, the yields of which were $2.3 \times 10^9$ pfu per $10^8$ cells and 450 ng per $10^8$ cells, respectively. The yields were reduced by 33–36% in the virus-infected stationary culture of the 8th day. The physiological state of the host cell has been quantified by the cellular activity parameter $P$ ($P(t)/P_{max}$) in the scale of 1 to 0. The value of $P$ was highest in the exponentially growing culture, which reduced with decline in growth rate. The cellular energy generation has been related with the parameter $P$. ATP generation was reduced to 55% in the stationary culture on the 8th day ($P = 0$). Also, the adsorption of virus was reduced by nearly 55% in extremely slow growing or stationary culture. Host cells at moderate growth rate ($P > 0.15$) were found to be optimum for maximizing the yield of viral products.

Key words: Vero; Cellular activity; Vaccinia; β-hCG

1. Introduction

Viruses are obligatory intracellular parasites, and require suitable host cells for replication. Synthesis of host cell macromolecules is turned-off within a few hours of viral infection (Moss, 1968; Dales and Pogo, 1981) and the cell machinery gets directed in favour of the synthesis of virus particles (Holland and Kiehn, 1970). In practical bioprocessing, growing and maintenance are two physiologically important states of the cell. In case of anchorage-dependent cells, the proliferation is stopped beyond the limit of confluence and the culture reaches a state of maintenance. It has been reported that viruses are replicated efficiently in the growing culture (Scherer, 1953; Smith and Sharp, 1960). Dales and Pogo (1981) first reported certain metabolic and cellu-
lar functions of host cells important for infection and replication of the viruses. However, reports are scanty on the nature of these metabolic and cellular activities, and their relationship with the growth of the host cells and modulations influencing the yield of viral products.

In this study, microcarrier culture of Vero-76 cell line was employed to understand the physiological functions of the host cells for the replication of recombinant vaccinia virus and the expression of \( \beta \)-hCG. The yield of viral products vis-a-vis the physiological status of the host cells at the time of infection has been investigated. The manner in which the cellular energy generation, responsible for the virus adsorption and product synthesis, is changed with the physiological state of the cells is discussed.

2. Materials and methods

Cell culture and medium

Vero-76 cell line (ATCC CRL 1587) was cultivated on Cytodex-1 microcarriers (5 g l\(^{-1}\)) in a 500-ml Spinner flask (Techne, UK) for a period of 12 d. About 10–50% (v/v) of spent medium was replaced every day (from 2nd day onwards) with fresh medium. Details of the culture procedure were described earlier (Mukhopadhyay et al., 1993). Dulbecco’s modified eagle medium (DMEM) supplemented with 5% (v/v) newborn calf serum (NBCS) was used for the cultivation. The concentration of NBCS was reduced to 2% during infection.

Virus

Recombinant vaccinia virus expressing \( \beta \)-subunit of human chorionic gonadotropin (\( \beta \)-hCG) hormone was used for this study (Chakrobarty et al., 1989).

Isolation of virus

5 ml of infected cell suspension was taken in a 10-ml sterile tube, and medium aspirated. The contents were resuspended in 2 ml of 10 mM Tris-HCl (pH 9) containing 1 mM EDTA and subjected to two cycles of freezing and thawing followed by sonication for 2 min in a cup-horn probe (Heat Systems-Ultrasonics, Inc.) at 120 W output. Microcarrier particles and cell debris were pelleted by brief centrifugation (1000 rpm, 5 min) and the supernatant was used for plaque assay.
Virus adsorption

1.0 ml of microcarrier culture suspension was taken in each of two sterile siliconized Eppendorf tubes at regular time intervals (2nd–10th day of cultivation). The cells were infected with virus of multiplicity of infection (MOI) of 10. The virus adsorption was continued for 1.5 h at 37°C under continuous rocking. The unadsorbed virus was titrated by plaque assay on CVt cell line (Mackett et al., 1985).

Sample analysis

Total cell and viable cell numbers were enumerated using Crystal violet and Trypan blue solution, respectively (van Wezel, 1973; Phillips, 1973). Intracellular phosphate was measured spectrophotometrically (Chen et al., 1956). Before phosphate estimation, cells were washed five times with chilled saline (0.85% w/v), followed by extraction with 5% (v/v) trichloroacetic acid (TCA). Cellular adenylates (ATP and ADP) were assayed by luminometric technique (Lundin et al., 1986). The concentration of β-hCG in the culture supernatant was estimated by radio immunoassay (Chakrobarty et al., 1989).

3. Results

3.1. Cell growth and yield of viral products

Fig. 1 shows a typical growth profile of Vero-76 cells on Cytodex-1 microcarrier and the yields of virion and β-hCG at different phases of growth. Every 24 h, 10 × 10^6 cells were taken in 35-mm plate (duplicate) and infected with virus of 10 MOI. After 72 h postinfection, each sample was analyzed for intracellular virus titre and the concentration of β-hCG in the supernatant. The yield of β-hCG was constant in the exponential and initial reduced growth phase (up to the 6th day of culture). Whereas the virus yield was low on the 2nd day, which was followed by a constant yield until the initial reduced growth phase. The highest yields of virus and β-hCG were 2.3 × 10^9 pfu per 10^6 cells and 450 ng per 10^6 cells, respectively (Fig. 1). Both products started declining once the culture entered into stationary phase. The yields of virus and β-hCG were reduced to 43 and 50%, respectively, on the 12th day of culture (Fig. 1).

3.2. Growth kinetics

The growth curve of Vero-76 cell showed an initial exponential growth, followed by decreasing growth rate, until the culture entered into the stationary phase at the 7th day (Fig. 1). The viability of cells throughout the culture period was maintained between 93–95%. High viability of cells together with favourable nutrient levels and low concentrations of metabolites (data not shown) indicated that the growth reduced/halted due to non-availability of ‘free space’ on the microcarrier surface to accommodate daughter cells.

Considering the effect of diminished free space on growth, the specific growth rate of Vero-76 cell in microcarrier culture can be written as: \( \mu(t) = \mu_{\text{max}} \cdot A_{\phi}(t) \). The dimensionless quantity \( \mu(t)/\mu_{\text{max}} \) can be used as a parameter to quantify the cellular activities (Licari and Bailey, 1992). The absolute value of \( \mu(t)/\mu_{\text{max}} \) was high in actively proliferating cells, which tended to decrease in the reduced growth phase and finally approached ‘zero’ (Fig. 1). Therefore, normalized growth rate \( \mu(t)/\mu_{\text{max}} \) denoted by the parameter ‘P’ has been used as an indicator of physiological activity.

3.3. Adsorption of virus

The adsorption of vaccinia virus on the microcarrier culture of Vero-76 cells at different values of ‘P’ is shown in Fig. 2. The extent of virus adsorption was identical (47 ± 2%) up to the early stationary phase (P > 0.15). Virus adsorption in the stationary culture was reduced to 20%.

3.4. Energy metabolism and the physiological state

Fig. 3 depicts the change in ATP pool, intracellular phosphate (Pi) level, ATP/ADP ratio and phosphorylation potential of Vero-76 cells at different cellular activity levels. The specific ATP pool was highest and constant (3.4 × 10^{-3} \mu M
per $10^6$) during the exponential and early stage of reduced growth phase ($P = 1$ to 0.7). It was followed by a decrease in ATP pool with the reduction in cellular activity. This conformed with the reports of Ryll and Wagner (1992) and Sonderhoff et al. (1992) that the cell specific ATP for murine hybridoma and BHK cells was lowest in the stationary culture. Specific uptake rates of glucose, glutamine and oxygen became low in the early stationary growth phase (data not shown). Intracellular phosphate concentration was reduced linearly with the decrease in ‘P’. About 50% drop in cell specific Pi was found within 7 d of culture. ATP/ADP ratio was nearly constant at 6.0 during the exponential phase, which slowly increased to 11.0 in the reduced growth phase. In the stationary culture, ATP/ADP ratio was rapidly increased to 14.0. The decrease in Pi, together with the increasing trend of ATP/ADP ratio resulted continuous increase in phosphorylation potential. The phosphorylation potential was highest in the stationary culture ($P = 0$) at a value of 43 μM$^{-1}$ per $10^6$ cells.

**4. Discussion**

Vero-76 cells showed density-dependent retardation of growth in the late exponential phase, and finally entered into stationary phase or maintenance state at the 7th day (Fig. 1). Vero-76, being a clone of original Vero cell (ATCC CCL 81) has probably changed its characteristics and grew in monolayer in the agitated system. For the purpose of this study, the Vero-76 cell line has been found as an ideal host, since its growth kinetic follows three basic growth phases (exponential, reduced growth and stationary).

The adsorption of virus was reduced in the stationary culture, which means that the extent of infection was decreased at that phase (Fig. 2). Vaccinia is an intracellular virus, so the productivity depends on the extent of initial infection. The host cells, which are not infected initially, remain non-productive until infected by the secondary viruses released from lysed cells. Payne and Norrby (1978) have shown that the entry of enveloped and naked vaccinia virus particles oc-
ocurred by interaction with specific cell membrane receptors. Viral spike protein binds to the cell receptors and finally fuses with the cellular membrane before entry. The physiological state of the host cell has been found to play an important role for the presence of receptors on cell surface (Dulbecco, 1973). It seems that the number of specific receptors are greater in the active physiological state of Vero-76 cells; thus, more and more viruses are adsorbed. On the other hand, the low virus adsorption in the stationary culture could be due to the presence of a limited number of receptors. Again, animal cells of a non-growing population tend to accumulate more glycolipids in the cell surface (Dulbecco, 1973), which probably has unfavourable influence on the adsorption of vaccinia virus to the cell membrane.

The demand for ATP for various cellular processes was reduced at low growth rate, which reduced the generation of ATP. In the stationary culture ($P = 0$), the cell specific ATP pool was further reduced to $1.9 \times 10^{-3}$ $\mu$M per $10^6$ cells. The variation of the cell specific ATP pool with the culture age was probably due to the change in cellular energy metabolism (Lundin et al., 1986). Our presumption is supported by the decreasing trend of specific uptake rates of glucose, glutamine and oxygen with the culture age (data not shown).

The change in ATP/ADP ratio may be attributed to the pulsing effect of carbon source as well as to the effect of culture age. The 2-fold increase in ATP/ADP ratio was due to the fact that a decrease in ADP pool appears to occur faster than the decline in ATP pool. The decrease in ADP pool was either due to rapid degradation into AMP or to low energy demand in the slow growing and stationary culture. The decrease in 'Pi' pool was believed to be due to the reduction in phosphate uptake, caused by the low phosphorylation reactions in slow growing cells. The decrease in [ATP][ADP][Pi] $^{-1}$ was a combined effect of change in ADP and intracellular Pi pool size.

The cellular energy generation and turnover of precursors of macromolecules are expected to be optimum in the growing culture ($P \geq 0.15$). In infected cells, the synthesis of macromolecules related to virus are predominant, tapping energy and precursors from the host cells (Holland and Kiehn, 1970). Thus, host cells infected at the growing stage would provide adequate energy and precursors for adsorption and subsequent replication of viral DNA, which leads to maximize the yields of vaccinia and $\beta$-hCG. On the contrary, both the energy and precursors pool are reduced in stationary cells, thus culture infected at this stage reduces the yield.

5. Nomenclature

$\mu(t)$, specific growth rate at time 't' ($h^{-1}$)
$\mu_{\text{max}}$, maximum specific growth rate ($h^{-1}$)
$A_o$, dimensionless surface area
$P$, cellular activity parameter $[\mu(t)][\mu_{\text{max}}]^{-1}$
n, number of cells
$pfu$, plaque forming unit
Specific ATP pool, ATP per biomass ($\mu$M per $10^6$)
Phosphorylation potential, [ATP][ADP][Pi] $^{-1}$ ($\mu$M $^{-1}$ per $10^6$)
Yield of virus, virus per biomass (plaque forming unit per $10^6$)
Yield of $\beta$-hCG, $\beta$-hCG per biomass (ng per $10^6$)

References


