Attachment characteristics of normal human cells and virus-infected cells on microcarriers

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Abstract

The attachment kinetics of normal and virus-infected LuMA cells were studied to improve the production of live attenuated varicella viruses in human embryonic lung (LuMA) cells. Normal LuMA cells and LuMA cells infected by varicella virus at various cytopathic effects (CPE) were grown on microcarriers. Ninety-three percent of suspended LuMA cells attached to the solid surface microcarriers within fifteen minutes and cell viability was greater than 95% when the cell suspension was stirred. Low serum levels did not affect the attachment rate of virus-infected cells in the microcarrier culture system. Kinetic studies showed that varicella infected cells had a lower attachment rate than normal LuMA cells. Virus inoculum (= infected cells) at low CPE showed a relatively better attachment rate on cell-laden microcarriers than virus inoculum at a higher CPE. Maximum titers were obtained at 2 days post-infection. Based on cell densities, the use of viral inoculum showing a 40% CPE led to an approximately 2- and 1.2-fold increase in the cell associated and in cell free viruses, respectively, than a virus inoculum with a CPE of 10%. However, the ratio of cell-free to cell-associated virus in a microcarrier culture was very low, approximately 0.04–0.06. These studies demonstrate that the virus inoculum resulting in a high CPE yielded a high production of cell-associated and cell-free virus in microcarrier cultures because of the high cellular affinity of the varicella virus.

Introduction

Animal cells are grown *in vitro* either as anchorage-dependent or as anchorage-independent cells. As anchorage-dependent cells in culture requires cell attachment to either specific ligands (substrate) or a charged surface, the majority of anchorage-dependent cells cultured *in vitro* has been grown as monolayer on a substrate covering the available surface area of a tissue culture flask. Subsequent introduction of microcarriers with larger surface areas has made it possible to grow adherent cells on a large scale for vaccine production (van Wezel, 1967). Human diploid fibroblasts were grown and cellular production of various biological products, glycoproteins, cytokines, growth factors etc. was performed in microcarrier culture systems (Morandi and Valeri, 1982; Griffiths and

Thornton, 1982; Nahapetian, 1986; Forestell et al., 1992).

The inoculation of the microcarrier culture is critical to maximizing final cell density and product yield of the culture (Clark et al., 1980; Pharmacia, 1981; Nillson, 1989). Despite early studies, problems associated with the inoculum stage, such as poor attachment efficiency and non-homogeneous cell distribution were not clearly understood. Studies on the attachment kinetics of anchorage-dependent cell lines on microcarriers (Levine et al., 1979; Himes and Hu, 1987; Tao et al., 1988; Lim et al., 1992) and optimization of culture conditions for the attachment of human embryonic lung cells on microcarriers (Clark and Hirtenstein, 1981) have been reported. High attachment efficiency combined with effective utilization of the surface area available resulted in the highest multiplication ratio possible from the inoculum in human

Figure 1. Comparison of human embryonic lung (LuMA) cell attachment to different types of microcarrier. The microcarrier concentrations were 5 mg ml^{-1} in solid microcarrier, Cytodex 1 and Cytodex 3 and 1 mg ml^{-1} in macroporous microcarrier, CultiSphere GL.

cells on a microcarrier system. The initial distribution of attached cells had a significant effect on the overall efficiency of adherent cell cultures and was used to evaluate attachment efficiency.

We have previously (Chun et al., 1996) evaluated a microcarrier culture system for the production of cell-associated and cell-free varicella virus using human diploid fibroblast cells. Thus far, it has been difficult

to obtain a high yield of infectious cell-free varicella virus for human vaccine production from cell culture. Therefore, an understanding of the attachment kinetics of both normal cells on microcarriers and virus-infected cells on cell-grown microcarriers is necessary to develop strategies that will enhance production of varicella virus in a high-density, large-volume microcarrier culture. The first objective in this study is to

Figure 2. Suspension culture of LuMA cells without microcarriers. The cells were inoculated at concentration of 2.4×10^5 cells ml^{-1} (a) and 4.8×10^5 cells ml^{-1} (b) in 50 ml of culture in 250 ml spinner flasks and incubated with stirring at 30 rpm.

identify the attachment characteristics of both normal human embryonic lung cells on microcarriers and virus-infected cells on cell-grown microcarriers. The second objective is to improve virus production by studying the attachment kinetics of both normal cells and virus-infected cells and by determining the CPE level of virus inoculum.

Materials and methods

Cell line and cell culture

Anchorage-dependent human diploid embryonic lung fibroblasts, LuMA cells, were isolated from the lung tissue of a human fetus and were subcultured in 175- cm^2 tissue culture flasks (T-flask; Nunc, Denmark)

Figure 3. Attachment kinetics of virus-infected cells on cell-laden Cytodex 1 microcarriers. Cells were inoculated at different concentrations of 4 cells/bead, 8 cells/bead, and 12 cells/bead in 50 ml of culture in 250 ml spinner flasks.

in Dulbecco's Modified Eagle's (DME; Gibco BRL, U.S.A.) high glucose medium in a 37 °C humidified atmosphere containing 10% CO₂. The LuMA cells were free from mycoplasmas and other adventitious agents. The culture medium was supplemented with 5% (v/v) fetal bovine serum (FBS; Gibco BRL, U.S.A.) for cell growth and was adjusted to 2% (v/v)

FBS for cell maintenance. The cells for experimental use were at a population doubling level (PDL) of 20 to 25.

Virus and virus culture

The MAV/06 strain of varicella virus obtained from

Figure 4. Attachment kinetics of normal LuMA cells on cell-laden Cytodex microcarriers. Cells were inoculated at different concentrations of 4 cells/microcarrier, 8 cells/microcarrier, and 12 cells/microcarrier in 50 ml of culture in 250 ml spinner flasks.

vesicles of varicella virus-infected Korean patients was used for this study. Four or six days post infection, round refractile cells, a typical cytopathic effect (CPE) of virus infection of normal cells, were detected under the microscope. When the inoculated LuMA cells showed 40–50% CPE, viruses were harvested using 0.25% trypsin in a Ca^{2+} and Mg^{2+} -free phosphate-buffered saline (PBS) and passed into T-flasks contain-

ing confluent LuMA cells (approximately $6\text{--}7 \times 10^5$ cells cm^{-2}) at 1:10 split ratio of virus-infected cells to normal LuMA cells. The cells infected with varicella virus strain were incubated at 36 °C in a humidified CO_2 incubator and used as virus inoculum for microcarrier cultures. The seed-lot system was adopted for maintenance of virus infectivity, and viruses between the 4th and the 7th passage from a working virus bank

Figure 5. Attachment kinetics of virus-infected LuMA cell attachment on Cytodex 1 microcarriers at different CPE levels: 10, 20 and 40%. Cells were inoculated at a concentration of 8 cells/microcarrier in 50 ml of culture in 250 ml spinner flasks.

were used. The working virus bank was prepared under conditions of cell-free virus, and the virus titer was approximately 10 000 plaque forming units (pfu) per vial.

Microcarrier culture

Cytodex 1 microcarriers (Pharmacia Fine Chemicals, Sweden) were added into 250 ml spinner flasks (Bellco, U.S.A.) containing 30 ml DME medium without serum, and the spinner flasks were placed in a CO_2 incubator to allow the pH and temperature to

reach 7.3–7.4 and 37 °C, respectively. Cell suspensions at a concentration of $2\text{--}3 \times 10^5$ cells ml⁻¹, obtained directly from T-flasks were used as the inocula. A cell suspension containing appropriate cell concentration was centrifuged at 1000 rpm for 5 min and resuspended in 20 ml DME medium without serum and subsequently transferred to Cytodex 1 microcarriers in spinner flasks. The microcarrier concentration used was 5 mg ml⁻¹, and the corresponding surface area was 22 cm² ml⁻¹ (Pharmacia, 1991). Microcarriers were hydrated in PBS and washed with DME medium after autoclaving at 121 °C for 20 min. All spinner flasks used with microcarriers were siliconized with Sigmacote® (Sigma, U.S.A.) prior to use to prevent microcarriers from sticking to the vessel walls. Initial attachment of cells was performed in reduced volume (50% of working volume of 100 ml), and the suspension was agitated continuously at 30 rpm on a biological stirrer (Thermolyne, U.S.A.). After cell attachment on microcarriers, 50 ml of culture medium containing serum was added, and the stirring speed was adjusted to 40 rpm. After 3 days incubation, the medium exchange was performed as follows: the microcarriers were allowed to settle to the bottom of the spinner flask, and 50% of culture supernatant was removed and replaced with a fresh serum-containing DME medium. The culture medium was supplemented with Pluronic F68® for suspension cultivation.

To infect the confluent LuMA cells grown on microcarriers with virus, virus-infected cells showing CPE of 10, 20, and 40%, respectively, in T-flasks were harvested and centrifuged at 1000 rpm for 5 min. The estimate of CPE level is based on a microscopic examination of round refractile cells present in the confluent cells grown on T-flasks. The cell pellets were resuspended with DME medium containing 2% FBS. The confluent microcarriers were allowed to settle to the bottom of the spinner flasks, and the supernatant was removed. The cell-laden microcarriers were washed with serum-free DME medium, and again the microcarriers were allowed to settle and the wash medium was removed. The microcarriers were resuspended with 30 ml of DME medium without serum. Serum did not influence cell attachment to microcarriers. Then the resuspended virus-infected cells were inoculated into the spinner flasks. The initial virus infection volume was 50 ml and the suspension was agitated continuously at 30 rpm for attachment of virus-infected cells on the cell-laden microcarriers. After cell attachment, 50 ml of DME medium supple-

mented with 2% FBS was added, and the stirring speed was adjusted to 40 rpm. Cell growth was monitored according to the nuclei count method reported by van Wezel (1967).

Cell attachment to microcarriers

Cultures for measuring attachment kinetics of normal cells and virus-infected cells on microcarriers or cell-laden microcarriers were performed in reduced volume in 250 ml spinner flasks. The rate of cell attachment was estimated by measuring the concentration of unattached cells after inoculation. Cell suspensions obtained directly from 175-cm² T-flasks showing near confluence were used as inoculum for cell culture on microcarriers, and virus-infected cell suspension harvested from T-flasks showing CPE were used as inoculum for virus infection of cells growth on microcarriers. After the cell suspension containing appropriate cell concentrations was centrifuged, the cell pellet was resuspended in 20 ml DME medium without serum and added to the microcarrier suspension. The suspension was agitated continuously at 30 rpm. Microcarrier cell cultures (approximately 3.4×10^5 cells ml⁻¹) cultured for 5 days after inoculation with 16 cells per microcarrier were also inoculated with the appropriate normal cells or virus-infected cells. To measure the concentration of unattached normal cells or virus-infected cells, the agitation of spinner flasks was periodically stopped to allow microcarriers to settle. The supernatant from a microcarrier-free zone to appear near the top of the cultures was carefully withdrawn, and the number of unattached cells in the supernatant was determined by counting in a hemocytometer. During the incubation period, samples from cell cultures were taken periodically for microscopic examination and for cell concentration determination. Viable cells were counted by using the dye exclusion method using trypan blue. All experiments were performed in duplicate.

Assay of virus titer

The titration of virus was performed by the plaque assay using 6-well cell culture plates (Nunc, Denmark) containing confluent LuMA cells (Chun et al., 1996). Two days before performing the plaque assay, confluent LuMA cells were used for inoculating the 6-well cultures. The sampling of the microcarrier cultures was done as follows: 0.5 ml of culture supernatants were withdrawn from well-mixed spinner flasks or

from a microcarrier-free zone after setting of the carriers and were centrifuged at 1000 rpm for 5 min. The supernatant was removed for virus titration, the pellet of cell-laden microcarriers were washed with Ca^{2+} , Mg^{2+} -free PBS, and cells on the washed microcarriers were induced to change their morphology to a spherical shape using 0.02% ethylenediaminetetraacetic acid (EDTA) in Ca^{2+} , Mg^{2+} -free PBS. The cell population harvested was used for assay of cell-associated virus, and a part of this suspension was further used as preparation for assay of cell-free virus. Cell-free viruses were prepared from virus-infected cells by ultra-sonication.

Results and discussion

Comparison of cell attachment at different types of microcarriers

In order to achieve good attachment of anchorage-dependent human embryonic lung fibroblast cell and growth on the surface of the microcarriers, establishment of optimal culture conditions, i.e. interfacial tension, pH, inoculation level, concentration of microcarriers, and agitation rate, is an essential requirement (Suzuki and Sakai, 1992; Forestell et al., 1992). The initial pH of the culture media was kept at 7.3 and the stirring speed during initial attachment was maintained at 30 rpm of continuous agitation. The inoculum used was from a single cell suspension harvest out of the same PDL (23) that was in logarithmic growth phase and had a cell viability greater than 94%. Each culture was inoculated with LuMA cells at a concentration of 4.8×10^5 cells ml^{-1} using three different types of microcarriers. Both solid microcarriers (Cytodex 1 and Cytodex 3 with different surface charges, Pharmacia Fine Chemicals, Sweden) and the macroporous microcarriers (CultiSphere GL, Hyclone, U.S.A.) were used in 50 ml of culture media in 250 ml spinner flasks. The concentration of Cytodex 1 and Cytodex 3 microcarriers at each culture was 5 and 8.1 mg ml^{-1} , respectively, and the corresponding surface area per culture was 2200 cm^2 . The concentration of CultiSphere GL was 1 mg ml^{-1} .

The profiles of cell attachment on different types of microcarriers are shown in Figures 1a and b. There was a profound difference on cells attachment kinetics between the solid and the macroporous microcarriers. Greater than 90% of LuMA cells were attached on Cytodex 1 microcarriers within 15 min and 5% of cells

remained unattached after 30 min. Although no significant difference in the rate of cell attachment on the solid microcarriers as found, there was a slight enhancement of cell attachment kinetics on Cytodex 1 in comparison to the profile obtained with Cytodex 3. As reported earlier, a fast rate of cell attachment was essential for preventing cellular damage in an agitated suspension state and for ensuring subsequent growth of anchorage-dependent cells in the microcarrier culture (Hu and Peshwa, 1991). This high attachment rate on Cytodex 1 is an indication that the solid microcarrier substrate is sufficient for good cell growth in this culture system. On the other hand, the attachment kinetics of LuMA cells onto macroporous gelatin microcarrier, CultiSphere GL, was shown to be first order with respect to the unattached cell concentration, but the rate of attachment was far slower than those with the solid microcarriers. The concentration of unattached cells was about 3.5×10^5 cells ml^{-1} (72%) at 30 min after inoculation, and slowly decreased to 40 and 28% at 2 and 4 h, respectively. A similar slow cell attachment profile to macroporous microcarriers in comparison to solid microcarriers was reported by Nikolai and Hu (1992). Such results may reflect the limited number of cells available for attachment to the irregular porous surfaces of CultiSphere GL microcarriers.

Maintenance of cell viability during cell attachment

Cell viability during initial cell attachment step is known to influence the final outcome of cell growth. Adherent cells without a suitable substrate for attachment and growth could become more susceptible to cellular damage and would not withstand the exposure to physical abrasive forces incurred during stirring of culture fluid. Thus, cell attachment on microcarriers within short time period without loss of cell viability is an important factor to consider for achieving a good cell yield. To investigate the maintenance of cell viability under suspension culture conditions without substrate for cell attachment, LuMA cells were inoculated at a concentration of 2.4×10^5 cells ml^{-1} and 4.8×10^5 cells ml^{-1} in 50 ml of culture medium in 250-ml spinner flasks and stirred continuously at 30 rpm. As shown in Figure 2, about 10% of cells in both cultures lost its viability within 2 h of suspension, and after 16 h of incubation approximately only 15% of cells remained viable. Since an initial incubation period of 2 h was required for attachment of LuMA cells to microcarriers, the number of dead cells

Figure 6. Comparison of virus production in culture medium at CPE levels of 10, 20 and 40%. LuMA cells were inoculated at a concentration of 3.44×10^5 cells ml^{-1} in 100 ml of 250 ml spinner flasks.

Figure 7. Comparison of the maximum extents of cell-associated and cell-free virus production at CPE levels of 10, 20 and 40%.

unattached to the surface of Cytodex 1 microcarriers may be less than 5% during cell adhesion period as indicated from Figures 1 and 2. Having a good at-

tachment rate for cells, the suspension culture system using Cytodex 1 microcarriers is indeed adequate for growth of LuMA cells. Accordingly, the real attach-

ment rate of cells on microcarriers can be expressed as follows:

$$\text{Attachment rate of cells (AR}_{\text{cell}}) = \frac{N_i - N_{\text{unatt}} - N_{i, \text{dead}}}{N_i} \times 100 \quad (1)$$

where AR_{cell} is the attachment rate of normal cells on microcarriers; N_i is the cell concentration at inoculation; N_{unatt} is the unattached cell concentration; $N_{i, \text{dead}}$ is the dead cell concentrations during suspension for attachment.

Attachment of cells on cell-laden microcarriers

There are two types of viral infection methods of host cells for the production of human viral vaccines from animal cell culture. One is the use of cell-free virus and the other is the cell-to-cell infection, where a virus infection of normal cells was done with virus-infected cells. The former is used when the cell-free virus has a strong infectivity and when the virus is secreted into culture medium. The latter is mainly used when a virus can maintain its infectivity only in host cells or shows rapid loss of its infectivity in culture medium. The propagation of varicella virus was usually accomplished *in vitro* by addition of virus-infected cells to the non-infected normal cells. The reasons are that the varicella virus is closely cell-associated throughout the period of growth, that a great part of cell-free viruses having infectivity are lodged within cells and also that cell-free viruses secreted into the culture medium have low infectivity and are unstable during long-term incubation. In cell cultures using microcarriers, viral infection was also performed by direct addition of virus-infected cells to cell-grown microcarriers containing just confluent cells. Therefore, the attachment of virus-infected cells on cell-laden microcarriers was investigated for the optimum production of cell-associated viruses and for the maximum production of cell-free viruses (after sonication of virus-infected cells). This may be a critical factor for the large-scale production of cell-free viruses using a microcarrier culture system. The virus-infected LuMA cells were inoculated at concentrations of 4 cells per bead, 8 cells per bead, and 12 cells per bead in 50 ml of initial culture volume in 250 ml spinner flasks. The virus-infected cells harvested from T-flasks showing 40% CPE were used as viral inoculum. The attachment rate of virus-infected cells on cell-laden microcarriers can be expressed as follows:

Attachment rate of virus-infected cells ($\text{AR}_{\text{vi, cell}}$) =

$$\frac{N_{\text{vi}} - N_{\text{v, unatt}} - N_{\text{vi, dead}}}{N_{\text{vi}}} \times 100 \quad (2)$$

where $\text{AR}_{\text{vi, cell}}$ is the attachment rate of virus-infected cells on cell-laden microcarriers; N_{vi} is the virus-infected cell concentration at virus inoculum; $N_{\text{v, unatt}}$ is the unattached virus-infected cell concentration; $N_{\text{vi, dead}}$ is the dead cell concentration of virus-infected inoculum during suspension for the attachment of virus-infected cells on cell-laden microcarriers.

Regardless of inoculum size for viral infection, about 80% of viral inoculum including normal cells and virus-infected cells were attached to cell-laden microcarriers within 15 min, and close to 90% attachment rates were obtained after 90 min (Figure 3). The attachment rate was slightly lower than that of LuMA cells on Cytodex 1 microcarriers (about 93% at 15 min) (Figure 1). This result might be due to two reasons: one is a decrease of the attachment potential of the cells affected by the viral infection, and the other one is a difference of the attachment power between cell-to-cell-laden microcarrier and cell-to-microcarrier. Normal cells are generally thought to be attached directly to microcarriers by salt bridges between the negatively charged cell surface and the positively charged amino groups of the microcarriers. However, re-attachment on cell-grown microcarriers using cells or virus-infected cells will occur by cell-to-cell association response.

To verify the above possibilities, the attachment rate of normal LuMA cells onto cell-laden microcarrier was evaluated (Figure 4). LuMA cells harvested from T-flasks were inoculated at a concentration of 16 cells/microcarrier and cultured for 5 days with medium exchange. The cell growth on microcarriers was shown to reach the confluency. LuMA cells harvested from T-flasks at late log phase were inoculated at different concentrations of 4 cells per bead, 8 cells per bead, or 12 cells per bead onto cell-laden microcarriers in 50 ml of culture in 250 ml spinner flasks. More than 90% of normal LuMA cells were attached to cell-laden microcarriers within 15 min. This attachment rate was similar to cell attachment on microcarriers (Figure 1). The attachment rate of LuMA cells on cell-laden microcarriers was higher than that of viral inoculum (= virus-infected cells) onto cell-laden microcarriers and increased with the culture time regardless of the inoculum size. This result indicates that LuMA cells infected with virus showed a decrease in the attachment efficiency on cell-laden microcarriers and that there was little difference in

the attachment between cell-to-cell-laden microcarrier and cell-to-microcarrier.

Effect of CPE level on cell attachment and virus propagation

After normal LuMA cells were infected with the varicella virus, round refractile cells, the typical CPE of viral infection, were observed under a microscope. The use of virus-infected cells containing normal cells as a virus inoculum had shown to influence the propagation of the virus in the culture. In order to understand the relationship between cell attachment and CPE level, the attachment characteristics of virus-infected cells on cell-grown microcarriers at various CPE levels were investigated. Virus inoculum, which consists of normal cells and virus-infected cells, harvested from T-flasks showing approximately 10% CPE, 20% CPE, and 40% CPE, respectively, were inoculated at a concentration of 8 cells per bead on cell-laden Cytodex 1 microcarriers. As shown in Figure 5, the lower the CPE level of virus inoculum, the higher the attachment rate of virus-infected cells on cell-laden microcarriers. In virus inoculum harvested from T-flasks showing 10% CPE, 90% attachment rate was obtained within 15 min, and on the other hand, 90% attachment rate of virus inoculum showing 20 and 40% CPE was obtained after approximately 90 and 180 min, respectively. This clearly indicated that the CPE level of viral inoculum affected the attachment of virus-infected cells to cell-laden microcarriers. Based on these results, it may be desirable to use the viral inoculum showing lower CPE to obtain higher attachment of virus-infected cells on cell-laden microcarriers, regardless of the final virus yield.

The effect of CPE level of virus inoculum on virus yield was measured to find the optimum level of CPE. The results shown in Figure 6 indicated that the yield of virus released into the culture medium was linearly dependent on the CPE level of viral inoculum between the three sets of CPE tested. However, the virus yield from culture medium was 1/50th of viruses harvested from cells on microcarriers. This result indicated that infectious viruses released into medium were detected at low levels in culture medium. After the confluent cells on microcarriers were infected with virus-infected cells, the cells that replicated viruses had a tendency to slough off the microcarriers with time, because of cell membrane alteration due to virus replication. Surprisingly, the attachment efficiency of virus-infected cells on cell-laden micro-

carriers decreased relatively little with the increase in the CPE level of the virus inoculum and the yields of cell-associated virus and cell-free virus were only moderately affected by the increase in the CPE level (Figure 7). The maximum titers of cell-associated and cell-free virus at a viral inoculum showing 40% CPE were about 2.6×10^5 pfu ml⁻¹ and 1×10^4 pfu ml⁻¹, respectively. These values were higher than those of cell-associated virus and cell-free virus (1.4×10^5 pfu ml⁻¹ and 8.4×10^3 pfu ml⁻¹, respectively) for an inoculum of 10% CPE. The maximum titers occurred at 2 days post-infection. Based on cell densities, the use of the viral inoculum showing 40% CPE led to about a 2- and 1.2-fold increase in the cell-specific viruses (cell-associated viruses and cell-free viruses, respectively) in comparison to yields obtained by using a CPE of 10% (Figure 7). The ratio of cell-free virus to cell-associated virus was very low (approximately 0.04–0.06), because varicella virus has a high cell affinity and the cell-free virus is unstable in solution. These studies demonstrated that the virus inoculum showing high CPE can result in a high yield production of cell-associated and cell-free virus in microcarrier cultures because of the characteristics of high cell affinity of varicella virus.

From these results it became obvious that the attachment of normal cells on microcarriers and of normal and virus-infected cells on cell-laden microcarriers was a very important factor for optimizing cell and virus inoculum in microcarrier cultures for human vaccine production. By understanding the attachment kinetics of normal cells on microcarriers and virus-infected cells on cell-laden microcarriers and by optimization of the CPE level of the virus inoculum, cell growth and virus production could both be enhanced and improved.

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