Microcarrier Culture of Lepidopteran Cell Lines: Implications for Growth and Recombinant Protein Production

Laertis Ikonomou,† Jean-Christophe Drugmand,† Georges Bastin,‡ Yves-Jacques Schneider,§ and Spiros N. Agathos*,†

Unit of Bioengineering, Place Croix du Sud 2/19, Centre for Systems Engineering and Applied Mechanics, Av. George Lemaitre 4, and Laboratory of Cell Biochemistry, Place L. Pasteur 1, Cell Bioengineering Group, University of Louvain, B-1348 Louvain-la-Neuve, Belgium

Several microcarrier systems were screened with Sf-9 and High-Five cell lines as to their ability to support cell growth and recombinant (β-galactosidase) protein production. Growth of both cell lines on compact microcarriers, such as Cytodex-1 and glass beads, was minimal, as cells detached easily from the microcarrier surface and grew as single cells in the medium. Cell growth was also problematic on Cytopore-1 and -2 porous microcarriers. Cells remained attached for several days inside the microcarrier pores, but no cell division and proliferation were observed. On the contrary, insect cells grew well in the interior of Fibra-Cel disks mainly as aggregates at points of fiber intersection, reaching final (plateau) densities of about 4 × 10^6 (Sf-9) and 2.7 × 10^6 (High-Five) cells mL^-1 (8 × 10^6 and 5.5 × 10^6 cells per cm^2 of projected disk area, respectively). Their growth was described well by the logistic equation, which takes into account possible inhibition effects. β-Galactosidase (β-gal) production of Sf-9 cells on Fibra-Cel disks (infected at 3.3 × 10^6 cells mL^-1) was prolonged (192 h), and specific protein production was similar to that of high-density free cell infection. Cultispher-S microcarriers were found to be a very efficient system for the growth of High-Five cells, whereas no growth of Sf-9 cells took place for the same system. Concentrations of about 9 × 10^6 cells mL^-1 were reached within 120 h, with cell growth in both microcarriers and aggregates, appearance of cellular bridges between microcarriers and aggregates, and eventual formation of macroaggregates incorporating several microcarriers. Specific protein productions after β-gal baculovirus infection at increasing cell concentrations were almost constant, thus leading to elevated volumetric protein production: final β-gal titers of 946, 1728, and 1484 U mL^-1 were obtained for infection densities of 3.4, 7.2, and 8.9 × 10^6 cells mL^-1, respectively.

Introduction

The increasing demand for therapeutic proteins with correct posttranslational modifications has led in the past 20 years to the elaboration of several expression systems and to significant advances in the areas of expression vectors, scale-up, and medium development (1–3). One of the most popular systems is the Baculovirus Expression Vector System (BEVS), which uses insect cells infected with a recombinant baculovirus to express abundantly recombinant proteins. Like other expression systems involving eukaryotic cells, its successful scale-up depends heavily on the use of stable, highly productive fast-growing cell lines, the minimization of various stresses in the bioreactor environment and the possibility of high-density cell culture without dramatic decrease in specific protein production (4, 5).

The use of microcarriers (macroporous or solid) in different bioreactor types is a good way to increase the available growth area for cells per unit of volume and to attain some of the above goals (6, 7). Microcarriers are used routinely in animal cell culture for the growth of anchorage-dependent cells and are among the established technological platforms for industrial production (8). Yet, there are only sporadic reports on their use in insect cell culture mainly because of the anchorage-independent nature of most cell lines and the one-shot, lytic nature of the BEVS. This technology was first used for the large-scale production of mosquito cells. Lazar et al. (9) screened several microcarriers to optimize the growth of Aedes aegypti (AA) cells and the production of arthropod-borne viruses. Cylindrical cellulose-based microcarriers (DE-53) were found to provide the best substratum. On the other hand, Agathos et al. (10) obtained promising results from the scale-up point of view with another mosquito cell line (C7-10) immobilized in open-pore collagen-based supports.

A few reports exist also on the use of microcarriers for lepidopteran cell lines. Three-millimeter glass beads gave the best performance for Trichoplusia ni (T. ni) cells immobilized in a split-flows, airlift bioreactor (11). The use of collagen-coated microcarriers with T. ni cells in suspension culture gave good growth and levels of the expressed protein (gp105) close to those produced in other immobilization systems, such as T-flasks and roller
bottles (12). As for attachment-independent insect cells, a Spodoptera frugiperda cell line (SF-21) was cultured in a packed bed reactor with Fibra-Cel disks (13). Cells reached high densities by growing along the fibers of the disks, and β-galactosidase and wild-type baculovirus production levels were similar in serum-supplemented and serum-free media (13).

Most commonly used lepidopteran cell lines are currently being cultured in suspension (14–19). Nevertheless, the above-cited results indicate that cultivation of insect cells on microcarriers can be an attractive alternative to free cell suspension culture even for anchorage-independent cell lines. A comprehensive research on the immobilization of insect cells could identify the best substratum for the their growth and demonstrate the possible extent on protein production after infection with recombinant baculovirus. We undertook a comparative study aiming at the screening of several microcarrier types for the widely used High-Five and SF-9 cell lines. The microcarriers tested included Cytodex-1 and glass beads (solid microcarriers), Cytopore-1 and -2 and Cultispher-S (macroporous microcarriers), and Fibra-Cel disks (nonwoven polyester fabric). Cells grown on the microcarriers were infected at very high cell densities with a recombinant β-gal baculovirus, which resulted in high volumetric productions.

Materials and Methods

Cell Lines and Media. The SF-9 and High-Five cells were a gift from Els Roode, Laboratory of Virology, Wageningen University, The Netherlands. The SF-9 cells were cultured initially in Grace’s medium supplemented with 10% fetal bovine serum (FBS). They were sequentially adapted to SF-900 II serum-free medium in T-flasks and, afterward, to suspension by gradual increase of the agitation rate. Subsequent adaptation to other serum-free media was quite easy, with cells being completely adapted after two to three passages in the new medium. The High-Five cell line initially grew in aggregate form in serum-free culture. The cells were adapted to single-cell growth in suspension. The whole procedure lasted 1 month and resulted in full adaptation to single-cell growth with occasional appearance of small (5–10 cells) aggregates. The maintenance conditions for the cells used in the microcarrier studies were, briefly, as follows: SF-9 and High-Five cells were maintained in 125-mL Erlenmeyer polycarbonate flasks with 25 mL liquid volume. Flasks were kept at 27 °C on an orbital shaker (model C-24, New Brunswick Scientific, Edison, NJ) and agitated at 120–130 rpm. Cells were passed every 3–4 days at a density of (2–3) × 10^6 cells mL^{-1}. Cell counts were performed with a Neubauer improved hemocytometer, and cell viability was determined with the trypan blue exclusion method (0.2% w/v final concentration).

More details on the maintenance of the cell lines, the media used, and the adaptation procedures in serum-free suspension culture can be found in ref 19. Growth and protein production studies were performed in either Insect-XPRESS (BioWhittaker Europe, Verrières, Belgium) or YPR, a new medium based on the use of yeastolate ultrafiltrate and Primatone RL and supplemented with glucose (55.5 mM final concentration) and glutamine (15–17 mM final concentration) (19).

Microcarrier Systems. Solid Microcarriers. The solid supports tested included glass microcarrier beads (G-2892) and Pharmacia’s Cytodex-1, both purchased from Sigma (St. Louis, MO). For both types of microcarriers, stock solutions of 20 g L^{-1} were prepared before use. Briefly, 1 g of microcarriers were added in 50 mL of PBS (phosphate-buffered saline) in a pre-sterilized bottle and left to hydrate. The PBS was then replaced with fresh buffer, and the microcarriers were autoclaved. Stock solutions were kept at 4 °C before use. The preparation of the microcarriers for inoculation involved the removal of PBS, addition of fresh filter-sterilized medium, and soaking of the microcarriers overnight at room temperature. The inoculation step was done in one-third of the final volume over 4 h at 27 °C with periodical agitation of the flasks to ensure uniform distribution of the cells. A small sample of the cell suspension was withdrawn in order to determine the seeding efficiency, and the final volume was reached with addition of fresh medium. The seeding efficiency is defined as follows:

\[
\text{seeding efficiency} = \frac{V X_0 - V X_f}{X_0 V_0} \times 100
\]

where \(X_0\) and \(X_f\) are, respectively, the viable cell concentrations for the liquid phase (10^6 cells mL^{-1}) at time 0 and at the end of inoculation and \(V_0\) and \(V_f\) are the liquid volumes (mL) at time 0 and at the end of the inoculation.

Inoculation densities were 2 × 10^6 and 4 × 10^6 cells mL^{-1} (with respect to the final volume) for High-Five and SF-9 cells, respectively. Microcarrier concentrations were 5 and 20 g L^{-1} for Cytopore-1 and glass beads, respectively.

Macroporous Microcarriers. Cytopore-1 and -2 were donated to us by Amersham Pharmacia Biotech Benelux (Roosendaal, The Netherlands), and Cultispher-S was a gift from Hydnone Europe (presently Perbio Science NV, Erembodegem-Aalst, Belgium). The former are DEAE (N,N-diethylaminoethyl)-coated microcarriers based on a cross-linked cellulose matrix with a mean diameter of 230 μm, average pore size of 30 μm, and high porosity (>85%). The latter are gelatin microcarriers with a hydrated diameter range of 130–380 μm, average pore size of 20 μm and porosity of about 50%. The preparation of these macroporous microcarriers for cell culture was performed according to the manufacturers’ instructions and was similar to that for solid microcarriers. The macroporous supports were rinsed at least once with sterile PBS and twice with medium before inoculation. Seeding densities were the same as for the solid microcarriers, and support concentration was 2 g L^{-1} for Cytopore-1 and -2, and 1 g L^{-1} for Cultispher-S microcarriers.

Cell Growth Studies on Microcarriers. All experiments were performed in either 125- or 250-mL polycarbonate shake-flasks (Corning, Acton, MA) agitated on an orbital shaker (model C24, New Brunswick Scientific, Edison, NJ) at 150 rpm unless otherwise indicated. The flasks were siliconized with Sigmacote (Sigma, St Louis, MO) and autoclaved before use. Suspension volume (with microcarriers) was one-fifth of the total flask volume to ensure good aeration and agitation of the cultures. One-milliliter samples were withdrawn daily to measure the concentration of free and immobilized cells as well as the consumption of metabolites and the accumulation of metabolic byproducts. Microcarriers were left to settle for about 3 min, and 100 μL was withdrawn to be used for free cell count with a Neubauer hemocytometer and cell viability determination by trypan blue exclusion (0.2% w/v final concentration). Another 400 μL of microcarrier-free supernatant was employed for metabolite analysis using a BioProfile 100 analyzer (Nova Biomedical, Waltham, MA). The rest was mixed with 400 μL of crystal violet.
solution (0.1% w/v in 0.1 M citric acid) and incubated for 4 h at 27 °C with vigorous vortexing every 30 min. Enumeration of nuclei was performed with a Neubauer hemocytometer, and the concentration of cells on the microcarriers was calculated by taking into account the total free cell concentration in the medium.

**Protein Production Studies on Microcarriers.** A recombinant AcMNPV baculovirus carrying the E. coli β-galactosidase (β-gal) gene under the control of the polyhedrin promoter was purchased from Invitrogen (San Diego, CA). Amplification of the virus was performed in either 100-mm Petri dishes or in shake-flasks by infecting SF-9 cells with a MOI (multiplicity of infection) of 0.1 plaque forming units (pfu) cell⁻¹. Virus titer was determined by the end-point dilution assay in 60-well (Terasaki) plates (Nalge Nunc International, Rochester, NY). The virus stock was diluted from 10⁻¹ to 10⁻⁹; 90 μL of each dilution was mixed with 90 μL of a SF-9 cell suspension (1.5 × 10⁶ cells mL⁻¹); and 10 μL of this mixture were added to each well of the Terasaki plate (six wells per dilution). Six wells with uninfected SF-9 served as negative control. After a 4-day incubation, 10 μL of a 0.4% (w/v) Bluo-gal (Life Technologies SA, Merekeke, Belgium) solution were added to each well. Infected wells were identified by the presence of blue color. The TCID₅₀/mL and the virus titer were calculated using standard methods (20).

Baculovirus infections of cells grown on microcarriers were performed in duplicate in shake-flasks with a MOI of 2. Medium change was done just before infection. Sampling (2 mL) was performed daily. One milliliter was used for cell count as described above. The other 1 mL was used for the determination of β-gal in the supernatant, in the free cells, and in the immobilized cells. After sedimentation for 3 min to separate the microcarriers from the liquid phase, 250 μL of this sample was centrifuged and the supernatant was recovered for the assay of the extracellular β-gal. One microlitre of lysis solution (1/3 v/v Triton X-100) was added to another 250 μL of the sample, and the supernatant, after removal of the cell debris, was used for the determination of the β-gal in the free cells by taking into account the result of the first measurement. Finally, 500 μL of lysis solution was added to the remaining 500 μL of the sample that contained the microcarriers, and the debris-free supernatant was used for the determination of the β-gal in the immobilized cells. All of the treated samples were kept at –20 °C until the β-galactosidase assay. The latter was a modification of Miller’s method (21). Briefly, 10 μL of appropriately diluted sample was mixed with 1000 μL of Z-buffer in a cuvette at 37 °C. Afterward, 200 μL of an ONPG solution (4 mg mL⁻¹ in 0.1 M phosphate buffer) was added. The reaction was stopped after 10–15 min with addition of 500 μL of Na₂CO₃ (1 M). The absorbance was measured at 420 nm, and the concentration of the β-gal (U mL⁻¹) was calculated by using the calibration curve of a β-gal standard (X grade, G 2513, Sigma, St Louis, MO).

**Growth and Protein Production Studies on Fibra-Cel Disks.** The optimized inoculation procedure for insect cells has been described elsewhere (22). Growth of both cell lines was evaluated for two different Fibra-Cel concentrations, namely, 5 and 10 g L⁻¹, whereas disk concentration for infection studies was 10 g L⁻¹. To determine cell numbers and β-gal concentration in the medium, samples were treated as described above for macroporous and solid microcarriers. For growth studies, three Fibra-Cel disks were removed daily from the culture flask and put in 1 mL of lysis solution (0.1% w/v crystal violet in 0.1 M citric acid). The released nuclei were counted after appropriate dilution with an improved Neubauer hemocytometer. To determine the β-gal content of immobilized cells, four Fibra-Cel disks were put in lysis solution for 30 min and the supernatant, after centrifugation, was used as described above. The sampling of four disks per day without a proportional decrease of medium volume dictated the need for correcting the extracellular and immobilized cell β-gal concentrations. For each time point we took into account the β-gal that could have been produced by the disks that had been removed up to this time point. The corrected extracellular β-gal at time i, i.e., β-gal of cells in the Fibra-Cel disks, was simply calculated from the rule of three. The corrected extracellular β-gal at time i had been calculated by taking into account the β-gal that would have been produced in the time interval from i – 1 to i by the disks removed at time i – 1. This correction was based on the assumption that all of the disks have more or less the same productivity and is described by the following two equations:

extracellular:

\[ \beta\text{-gal}(i) = \beta\text{-gal}(m) + \frac{(N_{\text{disks}} - N_{\text{disks}}(i))}{N_{\text{disks}}} [\beta\text{-gal}(m) - \beta\text{-gal}(i)] \]  

intracellular (Fibra-Cel):

\[ \beta\text{-gal}(i) = \beta\text{-gal}(m) \times \frac{N_{\text{disks}}}{N_{\text{disks}}(i)} \]

where \( \beta\text{-gal}(i) \) is the corrected β-gal concentration for time i (U mL⁻¹), \( \beta\text{-gal}(m) \) and \( \beta\text{-gal}(i) \) are the measured β-gal concentrations at time i – 1 and i (U mL⁻¹), and \( N_{\text{disks}} \), \( N_{\text{disks}}(i) \), and \( N_{\text{disks}}(i) \) are the number of Fibra-Cel disks at time zero, i – 1, and i, respectively.

**Analytical Methods.** Metabolite concentration, pH, and osmolality of medium samples were measured with a Bioprofile-100 analyzer (Nova Biomedicals, Waltham, MA). Immobilized cells were observed using either phase-contrast microscopy (Leitz Labovert, Leitz, Germany) or fluorescent microscopy (Labophot-2, Nikon, Japan). In the case of macroporous microcarriers we have found that addition of trypan blue dye (diluted 2×) would result, at the same time, in the coloration of dead cells and slight coloration of the microcarriers, rendering easier the examination of the immobilized cells. The fluorescent microscopy was combined with the Live/Dead cytotoxicity kit (Molecular Probes, Leiden, The Netherlands) to evaluate the viability and the distribution of cells on the microcarriers. In the case of Fibra-Cel disks where direct microscopic observation was difficult because of the disks’ size, confocal microscopy was employed. The system consisted of an emission source (Bio-Rad MRC 1024, Bio-Rad, U.K.) and a microscope (Zeiss Axiosvert 135 M, Zeiss, Germany) coupled with a color video camera (TK-1280E, Rad, U.K.) and a microscope (Zeiss Axiovert 135 M, Zeiss, Germany) coupled with a color video camera (TK-1280E, Rad, U.K.).

**Results**

**Growth Studies on Microcarriers.** A variety of microcarriers with different surface and matrix composition (Table 1) were tested with both High-Five and SF-9 cell lines. The growth studies of insect cells on solid
microcarriers gave rather disappointing results. Although seeding efficiencies were high for both microcarrier types (100% for Cytodex-1 and 85% for glass beads), cells detached easily from the supports during culture and grew freely in the medium (Figure 1). Thus compact microcarriers were not considered for infection studies of immobilized cells.

Growth of insect cells on Fibra-Cel disks was then evaluated in shake-flask culture. As this system is heterogeneous we chose to report cell numbers per cm² of projected disk area and not per mL of medium. We had previously found that 5 × 10⁵ and 7.5 × 10⁵ cells cm⁻² projected area, respectively, were the optimal inoculation densities for High-Five and Sf-9 cells (22). We studied the growth of insect cells at two different disk concentrations and two different agitation rates. Thus, the conditions for this study were 10 g L⁻¹ and 150 rpm, whereas for Exp.2 the respective conditions were 5 g L⁻¹ and 100 rpm. The model fit of cell growth according to the logistic equation is the continuous line for Exp.1 and the dashed line for Exp.2.

\[
\frac{dX}{dt} = \mu X (1 - \beta X) \tag{4}
\]

where \(X\) is the immobilized cell concentration (10⁶ cells).

\[
X = \frac{X₀ e^{\mu t}}{1 - \beta X₀(1 - e^{\mu t})} \tag{5}
\]
The unobstructed formation of aggregates and cellular bridges between microcarriers and aggregates (Figure 3D) resulted in the formation of macroaggregates that incorporated several microcarriers (Figure 3E). The viability was high inside these macroaggregates, as can be seen in Figure 3E. This phenomenon coincided with an increase of attached cell concentration that rapidly exceeded $6 \times 10^6$ cells mL$^{-1}$. The total cell concentration at the end of the fed-batch culture was above $10 \times 10^6$ cells mL$^{-1}$ with 80–85% viability. To verify that the formation of macroaggregates was necessary for achieving a high attached cell concentration, the experiment was repeated without dispersing any aggregates formed (Figure 4B). High-Five cells grew rapidly and reached a total viable cell concentration of $10 \times 10^6$ cells mL$^{-1}$ within 120 h, out of which $7.5 \times 10^6$ cells mL$^{-1}$ represented cells growing in aggregates, microcarriers, or macroaggregates incorporating microcarriers (Figure 4B).

**Infection Studies on Fibra-Cel Disks and Cultispher-S Microcarriers.** The screening of several types of microcarriers for the growth of the two cell lines resulted in the selection of two immobilization systems, namely, the Fibra-Cel disks for SF-9 cells and the Cultispher-S microcarriers for the High-Five cells. Both systems ensured a high and sustained cell concentration with high viability when employed in fed-batch culture. The effect of the cell immobilization and very high local cell concentrations (aggregates inside the Fibra-Cel disks and macroaggregates on, in, and around Cultispher-S carriers) on recombinant protein production was investigated by infecting growing immobilized cells with a recombinant baculovirus expressing $\beta$-gal (Figures 5 and 6). SF-9 cells were allowed to grow on the Fibra-Cel disks to a density of $6.6 \times 10^6$ cells cm$^{-2}$ (corresponding to a concentration of $3.3 \times 10^6$ cells mL$^{-1}$), and they were subsequently infected with a MOI of 2. Production of $\beta$-gal was prolonged compared to that of cells infected in suspension (data not shown) and peaked at 192 h postinfection, with a $\beta$-gal concentration of about 620 U mL$^{-1}$ (Figure 5). As can be seen from this figure, at almost any time point after infection, extracellular $\beta$-gal made up for at least 75% of the total $\beta$-gal. This fact combined with the low $\beta$-gal content of free cells suggest that $\beta$-gal in the medium was coming primarily from immobilized cells that were either lysed or had compromised membranes. Although specific protein production was lower for the infection on the Fibra-Cel disks compared to that of a low-density infection of suspension cells it was similar to the one from a similar high-density infection (Table 3).

**Table 2. Kinetic Parameters of Logistic Equation for Insect Cell Growth on Fibra-Cel Disks**

<table>
<thead>
<tr>
<th>cell line and culture conditions</th>
<th>$X_0$ ($10^6$ cells cm$^{-2}$)</th>
<th>specific growth rate ($\beta$) (h$^{-1}$)</th>
<th>$\gamma$ ($10^6$ cells cm$^{-2}$)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF-9 (Exp.1)</td>
<td>0.28 (0.09)</td>
<td>0.018 (0.001)</td>
<td>0.12 (0.004)</td>
<td>0.98</td>
</tr>
<tr>
<td>SF-9 (Exp.2)</td>
<td>0.55 (0.15)</td>
<td>0.015 (0.002)</td>
<td>0.135 (0.011)</td>
<td>0.97</td>
</tr>
<tr>
<td>High-Five (Exp.1)</td>
<td>0.30 (0.09)</td>
<td>0.027 (0.003)</td>
<td>0.19 (0.004)</td>
<td>0.98</td>
</tr>
<tr>
<td>High-Five (Exp.2)</td>
<td>0.33 (0.19)</td>
<td>0.022 (0.005)</td>
<td>0.175 (0.012)</td>
<td>0.93</td>
</tr>
</tbody>
</table>

*The conditions for Exp.1 were a disk concentration of $10$ g L$^{-1}$ and an agitation rate of 150 rpm whereas for Exp.2 the respective conditions were $5$ g L$^{-1}$ and 100 rpm. The errors for the different calculated parameters are given in parentheses beside the respective parameter.
Further increase of the cell concentration to $8.9 \times 10^6$ cells mL$^{-1}$ did not lead to further increase of the final $\beta$-gal titer (Figure 6B and C). In the latter case, almost two-thirds of the cells were freely suspended at the time of infection and even at 96 h p.i. there were $\approx 2 \times 10^6$ viable cells mL$^{-1}$ in suspension (Figure 7C), implying that infection was not as efficient as at the other two densities (Figure 7A and B). Specific production decreased with increasing time of infection (TOI) for the free cell infections, despite a complete medium change before infection (Table 3). When cells were infected on Cultispher-S at $3.4 \times 10^6$ cells mL$^{-1}$ (concentration similar to that of the high-density free cell infection) specific protein production was restored to the low-density free cell infection levels (Table 3). Specific production remained at the same level upon further increase of the infection density on Cultispher-S microcarriers, resulting in a doubling of the volumetric production (Table 3 and Figure 6A and B). Only at a very high-density infection of $8.9 \times 10^6$ cells mL$^{-1}$ did specific
production decrease, reaching the level of the high-density free cell infection (Figure 6C and Table 3).

Although YPR medium was used for the two high-density infections, with a glucose content of 10 g L\(^{-1}\) (55.5 mM) versus 6 g L\(^{-1}\) (33.3 mM) of Insect-XPRESS, glucose was rapidly consumed and completely exhausted by 72 h p.i. (Figure 8). As for the infection at 3.4 \(\times\) 10^6 cells mL\(^{-1}\), glucose was exhausted by 96 h p.i., but at that time almost all of the cells were dead and/or lysed (Figure 8). Although specific glucose consumption rates were almost the same for the infections at 3.4 and 7.2 \(\times\) 10^6 cells mL\(^{-1}\) there was a 23% decrease for the 8.9 \(\times\) 10^6 cells mL\(^{-1}\) infection (Table 4). Ammonia production, which reflects mainly the consumption of amino acids such as asparagine and glutamine, was higher in the 7.2 \(\times\) 10^6 cells mL\(^{-1}\) infection (final concentration of 20.7, 27, and 16.7 mM for infection densities of 8.9, 7.2, and 3.4 \(\times\) 10^6 cells mL\(^{-1}\), respectively; Figure 8). Specific ammonia production rate was 4.8 \(\times\) 10^{17} mol cell\(^{-1}\) s\(^{-1}\) for the 3.4 \(\times\) 10^6 cells mL\(^{-1}\) infection and increased about 43% and 31% for the 7.2 and 8.9 \(\times\) 10^6 cells mL\(^{-1}\) infections, respectively.

Discussion

Since their introduction in cell culture practice by van Wezel (27), microcarriers have been a valuable tool for the production of biologics and the scale-up of commercially viable processes. An important improvement of the system has been the conception of macroporous microcarriers (28), which increased considerably the available surface area for growth per unit volume. We undertook this study in order to screen several microcarrier types and to find out how the immobilization of insect (lepidopteran) cell lines could affect their growth and protein production after recombinant baculovirus infection.

The unsatisfactory results from compact microcarriers seem to contradict previous reports of good growth of insect cells on compact microcarriers in spinner flask cultures, namely, AA mosquito cells on Cytodex-1 and DE-53 (cylindrical cellulose-based) microcarriers (9) and Tn-5 cells on Cytodex-3 (collagen-coated) microcarriers (12). In the first case, the system had been easily scaled up to 8-L culture volume. However, this particular cell line is anchorage-prone. As for the second report, the lowest agitation rate capable of keeping microcarriers from settling was used (20–40 rpm) and no value for the maximal cell concentration was reported.

As for the macroporous microcarriers tested, the Cytopore-1 and -2 proved to be rather unsuitable for both Sf-9 and High-Five cells, which displayed virtually no growth even after serum supplementation, thus excluding the likelihood of nutrient limitation. Anchorage-independent cell lines such as CHO and hybridomas have been successfully cultivated on Cytopore microcarriers and cultures have been scaled up to 20 L (29). In our hands, CHO-K1 cells grew well on Cytopore-2 microcarriers, thus the difficulty to grow insect cells on this type of carrier appears to be cell-line-specific. It is probable that growth inhibition is due to the high porosity of the Cytopore microcarriers and their large pore size (30 \(\mu\)m). Cells cannot spread in the interior of the carriers and they rather have several points of contact. This, in turn, can result in stretching of the cell membrane and arrest of cell growth (30). In the case of the Cytopore-2 carriers, the high charge density can be an additional factor for the tight membrane–microcarrier interaction.
Table 3. Specific Production of Insect Cells Grown in Suspension and on Microcarrier Systems

<table>
<thead>
<tr>
<th>Mode of Culture and Infection Density</th>
<th>Specific Production of β-gal (10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF-9 suspension (1.2 x 10^6 cells mL^-1)</td>
<td>318 (144 h)</td>
</tr>
<tr>
<td>SF-9 suspension (2.9 x 10^6 cells mL^-1)</td>
<td>164 (144 h)</td>
</tr>
<tr>
<td>Fibra-Cel (3.3 x 10^6 cells mL^-1)</td>
<td>189 (192 h)</td>
</tr>
<tr>
<td>High-Five suspension (2.2 x 10^6 cells mL^-1)</td>
<td>246 (120 h)</td>
</tr>
<tr>
<td>High-Five suspension (3.7 x 10^6 cells mL^-1)</td>
<td>156 (120 h)</td>
</tr>
<tr>
<td>Cultispher-S (3.4 x 10^6 cells mL^-1)</td>
<td>225 (72 h)</td>
</tr>
<tr>
<td>Cultispher-S (7.2 x 10^6 cells mL^-1)</td>
<td>240 (96 h)</td>
</tr>
<tr>
<td>Cultispher-S (8.9 x 10^6 cells mL^-1)</td>
<td>166 (96 h)</td>
</tr>
</tbody>
</table>

*Specific production was calculated by dividing the maximal total β-gal concentration for each system by the maximal viable cell concentration. All of the infections were performed with a MOI of 2. Complete change of medium was done before infection. Insect-XPRESS medium was used for all of the infections except for those at 7.2 and 8.9 x 10^6 cells mL^-1, which were performed in YPR medium. For each infection, the time p.i. where the maximal β-gal concentration was attained is indicated between parentheses.

with HepG2 cells (31). Formation of aggregates and cellular bridges between microcarriers was a precondition for the achievement of high attached cell concentration (Figure 4). The formatted structures often exceeded 500 μm (33). One could expect the presence of a necrotic core due to reduced oxygen and nutrient penetration (34). The size of the macrostructures is much greater than the estimated distance from the periphery where nutrient limitation typically occurs (100–220 μm) (35). The absence of such a necrotic center (Figure 3E) can be attributed to the loose connection of microcarriers and aggregates and the presence of some free space inside the macroaggregates, according to the aggregation mechanism proposed previously (33). The exceptional potential of this system to reach high attached cell densities with high viability is clearly illustrated in Figure 4B. Thus it guarantees the rapid growth of cells to high densities (>10 x 10^6 cells mL^-1) and facilitates baculovirus infection and protein production (see below).

Both cell lines were successfully immobilized on Fibra-Cel disks in shake-flask cultures. Although this support is primarily used in fixed bed configurations (36), it had been utilized at least once in suspension culture (37). Our results show that insect cells can grow to high densities inside the Fibra-Cel disks even in agitated systems. The predicted initial cell densities are somewhat lower than the ones calculated on the basis of the seeding density and efficiency, especially in the case of Exp.1 for SF-9 cells. Nevertheless, the other two parameters (μ and β) give valuable insights into the growth patterns of the two cell lines on the disks. Increase of agitation rate from 100 to 150 rpm did not affect significantly specific growth rate and maximum obtained density for the same cell line (Figure 2; logistic equation parameters in Table 2). Although High-Five cells grew faster than SF-9 (higher μ, Table 2) they eventually reached lower final densities (5.2 and 5.7 x 10^6 cells cm^-2 versus 8.3 and 7.4 x 10^6 cells cm^-2 for Exp.1 and Exp.2, calculated as the inverse of the β term). The good fitting of the experimental values by the logistic equation suggests that both High-Five and SF-9 cell growth may be inhibited beyond a certain cell density. Extensive cell leakage from the disks is unlikely as increase in free cell concentration was rather due to proliferation of nonattached cells (22). The profile of cell growth as seen in Figure 3B is in contrast with previous reports for insect (13) and MRC-5 cells (38), where cells grew along the fibers of these carriers. The different
growth patterns may be due to the total absence of serum at every step (fiber treatment, inoculation, and growth) in our case, contrary to the other reports where seeding and cell growth was in serum-supplemented media. The presence of serum proteins, such as fibronectin, could have favored the strong attachment of cells along the fibers and their growth as cell layer(s). This is further supported by our recent results from CHO cell growth on Fibra-Cel, where culture in the presence or absence of serum resulted in different growth patterns on the fibers (F. Verhoeye, unpublished data). Thus, pretreatment of disks with serum-containing medium might promote the attachment of insect cells on the fibers and increase the available area for growth. Our results compare favorably with previously published values. Sf-21 cells growing in a packed bed reactor (3 g of Fibra-Cel in a 50-mL volume) reached a cell density of $6 \times 10^6$ cells mL$^{-1}$ of packed carrier or $1 \times 10^8$ cells g$^{-1}$ of Fibra-Cel (13). This value is inferior to our own maximal attained densities of $4 \times 10^8$ (8 \times 10^6 cells cm$^{-2}$) and $2.9 \times 10^8$ cells g$^{-1}$ (5.8 \times 10^6 cells cm$^{-2}$) for Sf-9 and High-Five cells, respectively (Figure 2, taking into account that 1 g of Fibra-Cel has 50 cm$^2$ of projected area). Similarly, these maximal densities are much higher than that obtained from Sf-9 cells immobilized on sheets of non-woven polyester fibers in a 2-L bioreactor (2.47 \times 10^6 cells cm$^{-2}$ of gross surface area or 1.23 \times 10^6 cells cm$^{-2}$ of projected area) (39).

Infection of Sf-9 cells on Fibra-Cel disks at a density of $3.3 \times 10^6$ cells mL$^{-1}$ ($6.6 \times 10^6$ cells cm$^{-2}$) resulted in a rather prolonged $\beta$-gal production (Figure 5) compared to free cell infections where maximal $\beta$-gal concentration is reached within 96–120 h (unpublished results). Although the MOI was 2, only a small fraction of cells (most probably cells at the disk surface) appeared to be infected initially as indicated by the very low total $\beta$-gal content (Figure 5). The cells in the interior of the disks are most probably infected with a high MOI by the virus produced from the first cycle. This would explain the delayed $\beta$-gal production and the abrupt increase of $\beta$-gal concentration between 120 and 144 h (Figure 5). Fluorescent $\beta$-gal substrates (40) or GFP-expressing baculovirus (41, 42) could be very useful tools to visualize the progression of virus infection within the disks. Even though production of $\beta$-gal was delayed, the specific production was similar.
to that of a free cell infection at about the same viable cell density (Table 3).

Cell behavior and β-gal production profile were markedly different during infection of High-Five cells on Cultispher-S microcarriers compared to the SF-9 infection on Fibra-Cel disks (Figures 6 and 7). Examination of Figure 7 points to an efficient infection that led rapidly to aggregate dissolution and subsequent cell lysis. Despite the fact that the cells were in the form of macroaggregates at the time of infection, the system was quickly switched to single-cell suspension after infection. As cellular oxygen uptake rate (OUR) transiently increases after infection (40), dissolution of aggregates helps to avoid oxygen limitation that could otherwise occur in their center. The differences seen in Fibra-Cel and Cultispher-S infections can be partly attributed to different cell surface compositions. These differences appear to be due to the two different cell lines used (SF-9 for Fibra-Cel and High-Five for Cultispher-S) rather than to different culture conditions. SF-9 and High-Five cells have different membrane compositions, in terms of phospholipids and fatty acids (43). High-Five cells were found to be more prolific in terms of β-gal and SEAP production (44, 45). Passage number can also play a role in infection susceptibility, even for the same cell line. In our case, we think that virus diffusion may have played a more important role in the efficiency of the two infections. In the Fibra-Cel infection, diffusion of the virus may have been limited from the outer cell layer, whereas in the Cultispher-S infection baculovirus may have diffused more easily in the macroaggregates as a result of their loose nature, thus infecting more cells in the first place. In addition, cells are less likely to be released from the Fibra-Cel disks than from the dissolved Cultispher-S macroaggregates after baculovirus infection.

Comparison of the specific β-gal productions for the Cultispher-S infections with suspension culture infections gave unexpected results. Remarkably, the specific production of the infections at 3.4 and 7.2 × 10^6 cells mL^{-1} was about the same as that for a single-cell infection at 2.2 × 10^6 cells mL^{-1} (Table 3). This brought about a dramatic increase in volumetric production, which for the latter density was 1730 U mL^{-1}, one of the highest productions ever reported for insect cells. Similar results were also obtained with a SEAP-expressing baculovirus (33). The maintenance of high specific productions at very high infection densities strongly suggest that the so-called “cell density effect” can be overcome by medium change, in accordance with recent reports (5). Nevertheless, complete medium change can be only one of the reasons accounting for the restoration of specific production. It was recently suggested that cell cycle progression of insect cell cultures can have a significant effect on protein productivity (46) and that cell cycle can be arrested following baculovirus infection (47). Mammalian cells growing on microcarriers have been shown to be mainly in the G0 and G1 phases (48). Therefore, if the High-Five cell cycle is synchronized in G0 and G1 as a result of the cells’ immobilization, they may be more easily infected by the baculovirus and probably more productive (49). Unfortunately, the only existing (and few) data come from SF-9 infections (46, 49), making imperative the need for thorough studies of High-Five cell cycle and its relation to protein production. The exhaustion of glucose by 72 h p.i. for the two higher densities (Figure 8) and the unusually high ammonia production rates (Table 4) compared to previously reported values (16) point to a very active energetic metabolism during infection. As it has been pointed out before, insect cell metabolism is a substrate-concentration-dependent overflow metabolism (50). Thus, the high glucose and glutamine levels initially present in the YPR medium may have resulted in increased glycolysis and glutaminolysis fluxes. Ammonia production rate was similar to those of uninfected High-Five suspension cultures (16, 19), but it was severalfold higher compared to production rates of infected High-Five cells (16).

Overall, our results show that anchorage-independent cell lines, such as High-Five and SF-9, can be successfully cultivated on macroporous microcarriers and Fibra-Cel disks. These supports provide a microenvironment (6) that at the same time permits the entrapment or attachment of cells and their growth to high viable cell densities. The infection of our immobilized cells at high densities (the highest reported in the literature for High-Five cells, to our knowledge) led to elevated volumetric productions thanks to constant cell-specific production levels.

Acknowledgment

The support of a fellowship from FDS (Scientific Development Fund, Université catholique de Louvain, Belgium) to L.I. is gratefully acknowledged. We are thankful to Axel Jacobs for help with the confocal microscopy and to Geneviève Schmitz-Drevillon for technical assistance.

References and Notes

(13) Komper, R.; Kislev, N.; Segal, I.; Kadouri, A. Use of a stationary bed reactor and serum-free medium for the


(42) Marheineke, K.; Grünwald, S.; Christlieb, W.; Reißänder, H. Lipid composition of Spodoptera frugiperda (SF-9) and Trichoplusia ni (Tn) insect cells used for baculovirus infection. FEBS Lett. 1998, 441, 49–52.


Accepted for publication July 18, 2002.

BP2055107