

Evaluating kinetic and physiological features of rCHO-K1 cells cultured on microcarriers for production of a recombinant metalloprotease/disintegrin

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Abbreviations: ACLD: *Agkistrodon contortrix laticinctus*
ADC: anchorage-dependent cells
SE: standard error

We present kinetic and physiological data regarding the culturing of rCHO-K1 cells on various microcarriers, to evaluate the potential of this culture strategy for mass production of these cells and expression of a recombinant disintegrin. Cultures were performed in 500 mL spinner flasks in DMEM culture medium with 10% v/v fetal calf serum, gently shaken at 37°C, pH 7.4, in a 10% v/v CO₂ atmosphere. The following values were obtained, respectively, for the adhesion time-constant K_a (h) and specific growth rate μ_{max} (d⁻¹) on each microcarrier: Cytodex 1 (0.91, 0.45), Cultispher S (0.28, 0.34), Immobasil FS (0.85, 0.52) and Pronectin F (5.12, 0.67). Metabolic characteristics showed some variation among the cultures with the four microcarriers, the most significant being the higher production of ammonia with microcarriers coated with adhesive molecules (Cultispher S and Pronectin F) relative to the uncoated carriers (Cytodex 1 and Immobasil FS). Experiments where the DMEM medium was gradually replaced by the serum-free medium (CHO-SFM-II) revealed important advantages over media containing serum, not only for assay purposes, but also for purification of the disintegrin. Altogether these results demonstrate that cultures on microcarriers, especially on Pronectin F, show good potential for larger scale cultures of rCHO-K1 cell.

A significant number of animal cell lines with industrial potential have an absolute requirement for adhesion to a solid substratum for growth and optimal synthesis of the product of interest. Such anchorage-dependent cells (ADC) grow in monolayers, and yields in biomass and product are thus limited by the size of the available adhesion area. To maintain high yields in large-scale production, the concept of culture on microcarriers introduced by van Wezel (1967) is being used with great success in research and industry. With this technique, the ADC attaches to and grows on the surface of small particles that are kept in suspension in the culture medium by gentle agitation. Fabricated from a variety of substrates, such particles are commercially available and supplied with diverse technological properties (surface charge, density, hydrophilicity, size, porosity, and adhesion-promoting coatings), for optimization of adhesion and cell growth, since many key cell processes and properties, such as the synthesis of DNA, RNA, and proteins, depend on correct cell adhesion and spreading (Varani et al. 1985; Nilsson et al. 1988; Varani et al. 1992).

The selection of the most suitable microcarrier for a new bioprocess involving the use of animal cells is an important stage, not only for technical reasons, since there is no microcarrier which is optimal for use in all applications, but also for economic reasons, since the cost of the microcarrier contributes significantly to the cost of the bioprocess. This rational approach is resulting in easier, more efficient and economical large-scale culture of animal cells for the manufacture of novel therapeutic proteins. Therefore, we decided to test microcarrier technology in the production of a biologically-active disintegrin from *Agkistrodon contortrix laticinctus* snake venom (Selistre-de-Araujo et al. 1997). Disintegrins were demonstrated to interfere with high affinity for integrins, cell-surface receptors that play crucial roles in physiological processes such as development and angiogenesis as well as in some diseases such as inflammation and cancer metastasis (Gould et al. 1990; Ramos and Selistre-de-Araujo, 2006). Therefore, disintegrins may be useful as integrin inhibitors in the treatment of cancer and inflammation. Snake venom disintegrins are synthesized *in vivo* as multimodular proteins that comprise a signal peptide, a pro-domain, a metalloprotease domain, a disintegrin domain, and a cysteine-rich domain (Bjarnason and Fox, 1994; Fox and Serrano, 2005; Ramos and Selistre-de-Araujo, 2006).

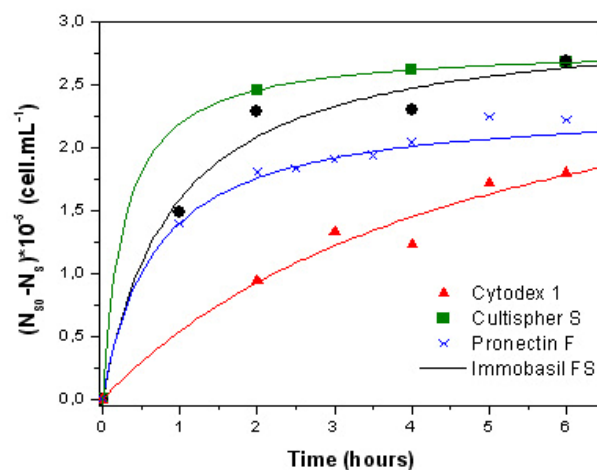


Figure 1. Density of attached CHOZMD cells during the adhesion phase in cultures with the four microcarriers. The curves were plotted by fitting equation 1 to experimental data of $(N - N_0) \text{ vrs } t$.

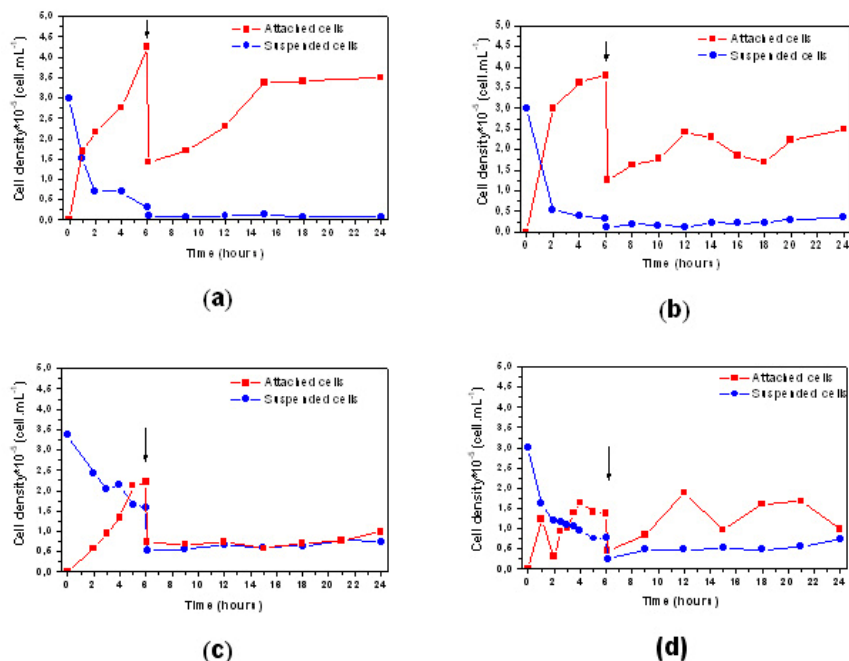


Figure 2. CHOZMD cell adhesion to the microcarriers Cytodex 1 (a), Cultispher S (b), PronectinF (c) and Immobasil FS (d) during the 24 hrs of culture. The arrows indicate the instant of 1:3 dilution with fresh medium.

Disintegrins can be released by proteolysis from their precursors, yielding soluble proteins with biological activity.

Recently, several disintegrins have been proposed as therapeutic drugs, in view of their antitumor, antiangiogenic and antithrombotic activities (Braud et al. 2000; Matsui et al. 2000; Zhou et al. 2000; Schmitmeier et al. 2003; Swenson et al. 2004; Markland et al. 2005; Yang et al. 2005). For such applications, a few cDNAs encoding disintegrins, originally from snake venom glands, have been cloned and expressed both in bacteria and in insect cells (Park et al. 1998; Fan et al. 1999; Jeon and Kim, 1999; Wang et al. 2003; Wang et al. 2004) by genetic engineering techniques. However, due to the structural complexity and high number of disulfide bonds of this type of protein, the yield and activity of the recombinant protein from most production processes are very low. Also, the expression of a metalloprotease in an active form has been shown to be very unstable (Selistre-de-Araujo et al. 2000). To overcome these difficulties, we decided to express the zymogen form of a snake venom metalloprotease with DCD-disintegrin and cysteine-rich domains from the snake *Agkistrodon contortrix laticinctus* named ACLD (Selistre-de-Araujo et al. 1997). The main focus in the present study is on the utilization of a genetically modified CHO-K1 cell-line to express ACLD stably in a large-scale mammalian system. As no information is available in the literature on the development of a cell culture process with microcarrier technology for production of that sort of biomolecule, the objective was to evaluate kinetic and physiological features

of a rCHO-K1 cell line when grown on four types of microcarriers with the purpose of identifying culture conditions that lead to high densities of active cells expressing the recombinant disintegrin. The cell performance was characterized by determination of adhesion, growth and metabolic parameters. The presence of the disintegrin was detected by immunoblotting.

MATERIALS AND METHODS

Cell and culture media

The mammalian cell-line CHO-K1 (Chinese hamster ovary) was genetically modified by Selistre-de-Araujo et al. (1997), who cloned and expressed the cDNA encoding a metalloprotease/disintegrin (ACLD) from the venom gland of the snake *Agkistrodon contortrix laticinctus*. The cDNA was cloned into the plasmid vector pCDNA3 (Invitrogen), which was in turn stably transfected into the CHO-K1 cells. The genetically modified CHO-K1 cell line, here denominated CHOZMD (for zymogen of metalloprotease/disintegrin), was cultured in Dulbecco's modified Eagle medium (DMEM, Gibco/BRL) supplemented with 110 IU.mL⁻¹ penicillin (Sigma), 0.1 mg.mL⁻¹ streptomycin (Sigma), 0.146 g.L⁻¹ glutamine (Ajinomoto) and 10% v/v fetal calf serum (Gibco), at 37°C in a 10% v/v CO₂ incubator. In some experiments, this medium was gradually replaced by the serum-free medium CHO-SFM-II, in order to avoid interference caused by serum proteins in detection of the disintegrin. Details of the stepwise replacement are given in Disintegrin detection.

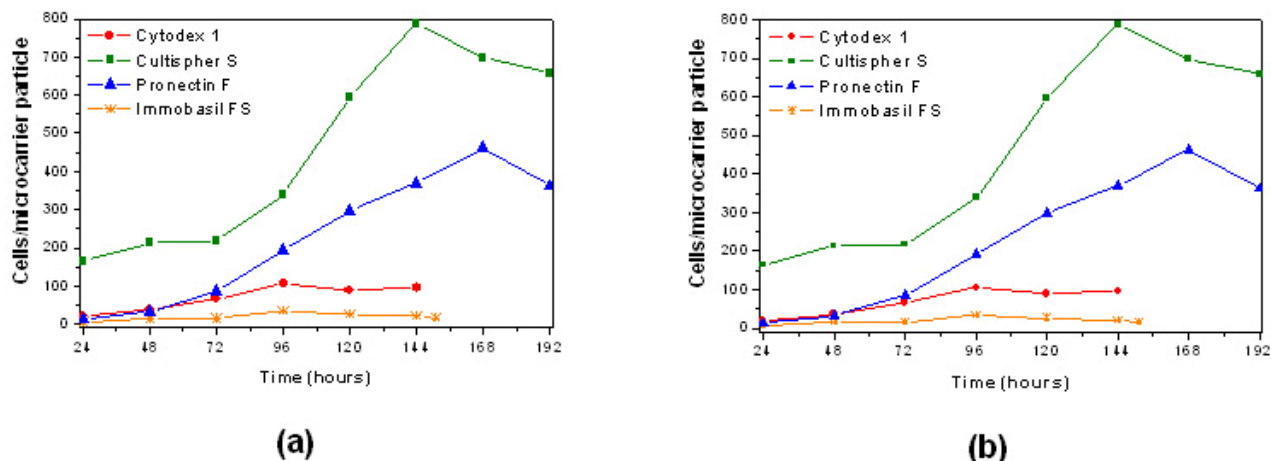


Figure 3. Growth of CHOZMD cell cultures beyond 24 hrs in spinner flasks with the four microcarriers, expressed in two ways: (a) number of anchored cells per mL of culture medium and (b) number of anchored cells per particle (microcarrier). Based on manufacturer information, the estimated number of particles per gram of each microcarrier was: 4.3×10^6 particles/g for Cytodex 1, 8.0×10^5 particles/g for Cultispher S, 8.6×10^3 particles/g for Immobasil FS and 3.9×10^5 particles/g for Pronectin F. Notice that the microcarriers are of different sizes as described in Table 1.

Microcarriers

Four microcarriers were selected on account of the outstanding technical features claimed by the manufacturers to enhance their performance in animal cell culture. The solid microcarriers Cytodex 1 (Pharmacia) and Pronectin F (Solohill) and the macroporous Cultispher S (PerCELL Biolytica) and Immobasil FS (Ashby Scientific) were tested under conditions as close as possible to those recommended by their manufacturers. The most noteworthy features of these microcarriers, their densities and the inoculum level used in the CHOZMD cell culture are presented in Table 1.

Spinner flask cultures

After attaining a sufficient quantity of cells in 150 cm² T-flask cultures (Corning), through one passage in 25 and 75 cm² T-flasks, 100 mL of culture medium, equilibrated with a predefined amount of microcarriers in a 500 mL glass spinner flask (Wheaton) were inoculated as specified in Table 1. During the first six hrs, in order to achieve a uniform and efficient cell attachment, the culture was performed with only 1/3 of the final volume and with intermittent stirring (5 min of stirring at 25-30 rpm every 30 min). After cell attachment, the volume of culture was made up to 300 mL and the stirring was kept constant at 60 rpm until the end of the experiment. Medium was replaced according to the requirements of each individual experiment; as soon as the pH of the medium decreased to approximately 7.1, 50% of its working volume was replaced by fresh medium.

For cell adhesion measurements, 1 mL samples were taken from the spinner flask every 2 hrs during the first 20 hrs of

culture. 3 mL samples for monitoring of cell density and viability, as well as glucose, lactate, amino acid and ammonium concentrations were taken daily. The sample volume taken from the cultures was the same in all experiments to avoid differences in the remaining working volume.

Analytical methods

The viability and density of cells in suspension were measured by the Trypan blue dye exclusion method (Doyle and Griffiths, 1998). Adhered cell numbers were estimated by counting in haemocytometer the stained nuclei with crystal violet (GE Healthcare, 2005). The two ruled areas of the haemocytometer were counted two times. Glucose, amino acid and ammonium ion concentrations were measured by high performance liquid chromatography (HPLC). For glucose assays, a Waters chromatograph with a Lonpack KS 803 (Shodex) column was used. For amino acid and ammonium analysis a Shimadzu chromatograph with Amino Na and ISC-3050504 Na Shim-pack columns from Shimadzu were used. The lactate concentration was determined with an enzymatic kit from Sigma Diagnostics.

Analysis of cell growth, metabolism and product

Cell adhesion was characterized by estimating the adhesion constants of a hyperbolic model which correlates the adhered cell density with time as follows:

$$N_a = N_{so} - N_s = N_{af} \frac{t}{K_a + t} \quad (1)$$

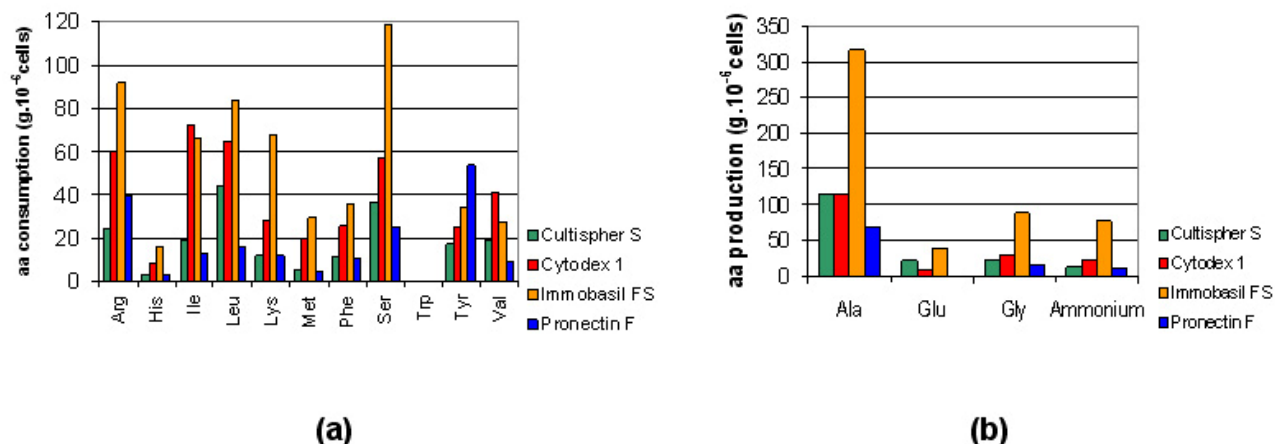


Figure 4. (a) Specific amino acid consumption profiles and (b) specific production of alanine, glycine, glutamate and ammonia in the cultures with CHOZMD cells conducted with the four microcarriers.

where N_a is the number of attached cells at time t (cell.mL^{-1}), N_s is the suspended cell density at time t (cell.mL^{-1}), N_{so} and N_{sf} are the initial measured and fitted, cell densities in suspension (cell.mL^{-1}), respectively, and K_a is the adhesion time constant (h). The parameters N_{af} (final number of attached cells (cell.mL^{-1})) and K_a were estimated by nonlinear curve fitting of experimental measurements of $(N_{so} - N_s)$ at various t utilizing the program Origin 6.0, assuming no dependency between the parameters. Determinations of cells in suspension were preferred because its experimental error was much lower than the measurements of adhered cells.

To estimate the maximum specific growth-rate in the exponential growth phase, the following equation was utilized:

$$\ln N_a = \ln N_{ao} + \mu_{\max} \cdot t \quad (2)$$

where μ_{\max} is maximum specific growth-rate, N_a is the attached cell density at time t (cell.mL^{-1}) and N_{ao} is the initial attached cell density (cell.mL^{-1}).

The disintegrin present in the medium was detected by two methods, SDS-PAGE (Laemmli, 1970) and dot blotting. For the gel electrophoresis, samples were taken just after the end of the exponential growth phase, from a culture in which a high final yield of cell was attained with the microcarrier Cytodex 1. At this stage of the culture, the DMEM medium with 10% v/v fetal bovine serum was replaced by similar medium with only 1% v/v serum to diminish the protein content. The cell culture was prolonged in this low serum medium for 24 hrs, samples being taken at 0, 3, 6, 9, 12 and 24 hrs. Samples of 30 mL were filtered (Millipore membrane, 0.45 μm) and concentrated by ultrafiltration in an Amicon system

(MWCO 10 kDa, Millipore) followed by additional concentration in Centriprep (MWCO 30 kDa, Millipore) and Centricon (MWCO 30 kDa, Millipore), to give a final volume of 500 μL . Samples of 10 μL were then resolved by electrophoresis in a 15% polyacrylamide gel.

For the dot blotting technique, samples of 30 mL were taken from a culture of CHOZMD cells adapted to the serum-free medium CHO-SFM-II, growing on the microcarrier Pronectin F, which was the one showing the highest cell density in CHOZMD cultures. These samples were passed through a 0.45 μm filter (Millipore), concentrated by ultrafiltration to a final volume of 500 μL . Samples (200 μL) were then applied on a nitrocellulose membrane (Immobilon-Millipore) in Bio Dot equipment (BioRad). After a first washing with TBST (25 mM Tris-HCl, pH 8.0, 125 mM NaCl, 0.1% Tween 20), and blocking with non-fat milk in TBS, the membrane was incubated for 2 hrs, first with a 1:500 dilution of mouse anti-MD (metalloprotease/disintegrin) primary antibody, and afterwards with phosphate-labeled mouse anti-IgG (Sigma) secondary antibody, diluted 1:5000. After washing with TBST, colour was developed by incubating the membrane in a solution of chromogenic substrates, BCIP/NBT (alkaline phosphatase conjugate substrate kit-BioRad). The positive control consisted of a solution of recombinant ACLD expressed in K562 cells. After transfection, the K562 cells were cultivated in DMEM with 10% v/v serum. Subsequently, the cells were re-suspended in DMEM without serum and incubated for 48 hrs at 37°C in 5% v/v CO_2 atmosphere. After filtration (Millipore membrane, 0.45 μm), 5 μL of medium were used for immunodetection as described above for the samples.

RESULTS AND DISCUSSION

Cell adhesion on microcarriers

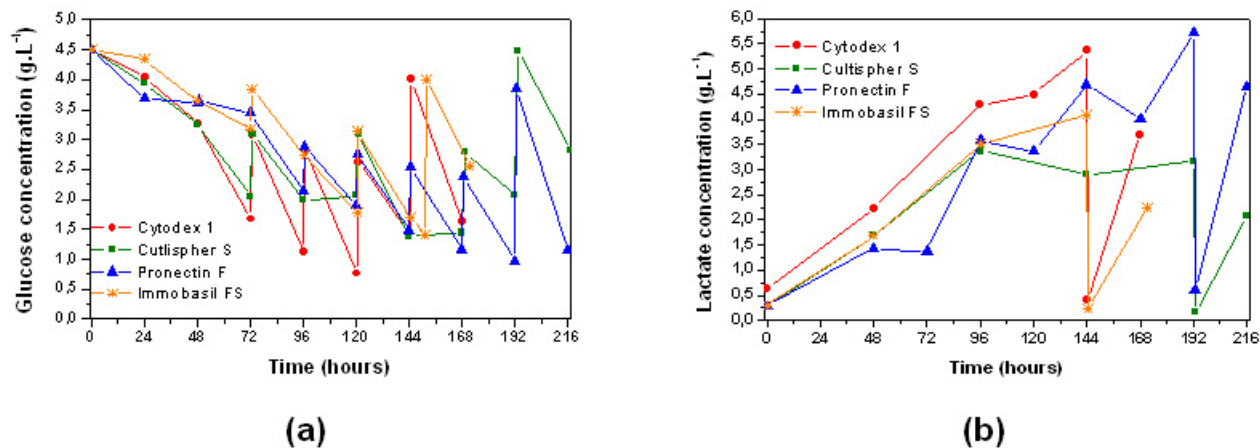


Figure 5. Glucose consumption (a) and lactate production (b), in the experiments conducted with the four microcarriers. The sudden drops in concentration are due to the replenishment of culture media.

As can be seen in Figure 1, a high proportion of cells adhered during the first 6 hrs, in a reduced culture medium volume (1/3 of final volume) and with gentle stirring at 25-30 rpm. When the culture medium volume was made up to 300 mL and stirring increased to 60 rpm, the cell adhesion rate decreased. This behaviour is the result of a lower number of collisions between cells and microcarriers when both were at lower densities in addition to stronger hydrodynamic forces caused by higher stirring of the culture medium after the first 6 hrs.

From the results for the adhesion of cells to the microcarriers (first 6 hrs of culture), presented in Figure 1, it was possible to determine the adhesion parameters N_{af} and K_a of a hyperbolic model displayed in Table 2. A first order model suggested by Ng et al. (1996) for adhesion of Vero cells was also tested with these data, as can be verified in Table 2. The parameters that differentiate the two models are K_a for the hyperbolic model and k_a for the first order model. Each of these constants can be interpreted as a measure of affinity between cell and microcarrier, therefore can be used as a parameter for comparison.

The results obtained by the nonlinear fitting algorithm presented in Table 2 show that, in general, the hyperbolic model describes better the behaviour of the cellular adhesion onto the microcarriers than the first-order model suggested by Ng et al. (1996). Using the correlation coefficient, R , as a measure of the quality of the adjustment, the value of that coefficient is, on average, slightly larger than the one for the first-order model. Another statistical parameter that reflects the fitting quality is the standard error (SE) of the estimated parameters. The SEs of the hyperbolic parameters N_{af} and K_a are smaller than those of the first order model N_{af} and k_a . The mean of SEs (in %) of such parameters for the models is, respectively, 1.98 and 6.00 for the hyperbolic and 2.24 and 7.36 for the first order. An additional reason for the choice of the hyperbolic model

is that it predicts values of the parameter N_{af} closer to the experimental values of inoculated cells (N_{S0}) than those of the first order model (Figure 2).

A statistical analysis at a confidence level of 95% of the values of N_{af} in Table 2, reveal that only the microcarriers Cytodex 1 and Pronectin F have not significant difference. The results presented also indicate that the microcarrier with the highest expected number of adhered cells is Pronectin F, with $N_{af} = 330,855 \text{ cells.mL}^{-1}$, and in second place Cytodex 1 ($N_{af} = 303,726 \text{ cells.mL}^{-1}$), although it can take a long time to attain such asymptotic values. The higher rate of the Cytodex 1 is possibly due to the optimized positively-charged dextran matrix that enhances the binding between cells and particles. In third place is Cultispher S, with $N_{af} = 280,350 \text{ cells.mL}^{-1}$; this maximum value of adhered cells tends to be well below that initially inoculated in the suspension (N_{S0}), possibly due to the low amount of microcarrier utilized in culture. Immobasil FS tended to stabilize with a low adhesion of cells ($N_{af} = 250,763 \text{ cell.mL}^{-1}$). This behaviour can be explained by the physical-chemical nature of the surface of the microcarrier, as will be reinforced later, in the analysis of cell growth.

Referring to K_a , a statistical analysis at a confidence level of 95% shows that only the values of Cytodex 1 and Immobasil FS are not significantly different. The values of K_a , the parameter that differentiates better the adhesion characteristics of the microcarriers, can be interpreted as a measure of the affinity for the cell, having a low value when the adhesion rate is high. In this sense, the microcarrier Cultispher S showed the highest affinity, followed by Immobasil FS, Cytodex 1 and Pronectin F.

Cell growth on the microcarriers

Cell growth on the different types of adhesion substrate, presented in Figure 3a, can be compared through the maximum specific growth rate (μ_{\max}), estimated by means

Table 1. Characteristics of the microcarriers and conditions in which they were tested in spinner-flask cultures of the CHOZMD cell.

Microcarrier	Outstanding features ¹	Particle size (μm) and shape ¹	Densities (based on medium volume)	
			Microcarrier (g.L^{-1})	Cells (cell.mL^{-1})
Cytodex 1	Non-porous, dextran matrix positively charged, hydrophilic	Bead (190)	3.5	1.0×10^5
Cultispher S	Porous, gelatin matrix ² uncharged, hydrophilic	Bead (125 - 212)	2.0	1.0×10^5
Pronectin F	Non porous, coated with pronectin ³ uncharged, hydrophilic	Bead (130 - 380)	22.0	1.1×10^5
Immobilasil FS	Porous, silicone rubber matrix negatively charged ⁴ , hydrophobic ⁴ highly permeable to oxygen	Disc (800 x 250)	26.0	1.0×10^5

¹Information supplied by manufacturers.

²Polypeptide extracted from animal tissue where it functions as a promoter of cell adhesion.

³Recombinant polypeptide with multiple copies of the sequence RGD (Arg-Gly-Asp), a common motif recognized by specific cell-surface receptors during adhesion.

⁴Data from Nakari-Setälä et al. (2002).

of equation 2, for the exponential growth phase of each culture. For Cytodex 1, Cultispher S and Immobilasil FS, μ_{max} was estimated in the period from 24 to 96 hrs. For Pronectin F, the period chosen was 48 to 120 hrs. The calculated values of μ_{max} are presented in Table 3. After the anchored cell concentration fell at an advanced growth stage, probably due to key nutrient exhaustion, the experiments were stopped. Another parameter that can be used for comparison of cell growth on the microcarriers is the maximum cell density achieved in the cultures. Estimation of the error carried out with data obtained in our laboratories for adhered cell quantification by nuclei counting has shown standard deviation around 7% of the mean. The maximum values of cell density accomplished in the cultures with microcarriers were 2.76×10^6 cells.mL⁻¹ for Pronectin F, 1.47×10^6 cells.mL⁻¹ for Cytodex 1, 1.34×10^6 cells.mL⁻¹ for Cultispher S and 3.84×10^5 cells.mL⁻¹ for Immobilasil FS. Each of these values was determined as the mean of a quadruplicate counting of nuclei and their statistical comparison at a confidence level of 95% gave as results: no significant difference between the cultures with Cytodex 1 and Cultispher S, the highest cell density being produced by Pronectin F and the lowest by Immobilasil FS.

Analyzing the specific cell adhesion in terms of cells per particle, as plotted in Figure 3b, it may be noted that microcarriers coated with adhesion promoter substrates, namely collagen for Cultispher S and Pronectin™ for Pronectin F, showed a higher attached cell number than

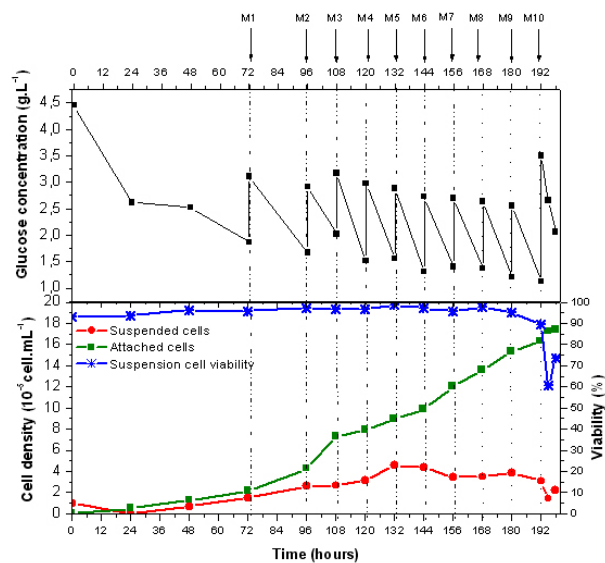


Figure 6. Culture for adaptation of CHOZMD cells on microcarrier Pronectin F for production of the recombinant disintegrin in serum free medium (CHO-SFMII). The culture was initiated with medium DMEM + 10% v/v of serum. The arrows indicate the times of medium replacement with increasing concentrations (% v/v) of CHO-SFMII: M1 (5%), M2 (12.5%), M3 (15%), M4 (20%), M5 (25%), M6 (30%), M7 (40%), M8 (50%), M9 (50%) and M10 (100%).

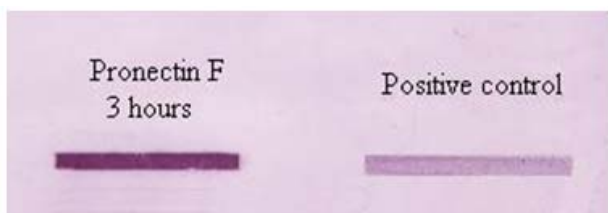


Figure 7. Immunodetection of the recombinant disintegrin in serum-free medium. The sample was collected at the end of the culture of CHOZMD cells with gradual medium replacement, after 3 hrs of incubation in medium CHO-SFMII (M10 medium in Figure 6). Positive control: 5 μ L of culture medium of ACLD-transfected K562 cells.

uncoated microcarriers such as Cytodex 1 and Immobasil FS. Immobasil FS, even though showing good adhesion and growth, did not attain a high cell density. The culture performed with the microcarrier Immobasil FS exhibited the lowest anchored cell density. The superior performance of Cultispher S may be attributed to its higher specific area. As Cultispher S has a very porous structure, cells grow on the inner as well as the outer surfaces, and the culture with this microcarrier produced approximately 780 cells/particle. The Pronectin F microcarrier produced 450 cells/particle, followed by Cytodex 1 (70 cells/particle) and Immobasil FS (15 cells/particle).

The statistical analysis at 95% of confidence level of the calculated μ_{max} values in Table 3 reveal no significant differences between the growth on Immobasil FS and

Cytodex 1 and also between Cytodex 1 and Cultispher S. In addition, Pronectin F was the microcarrier that gave the highest specific growth rate. With Immobasil FS, even though it showed a low density of adhered cells, a higher μ_{max} was achieved than with Cultispher S.

Metabolic patterns and amino acid consumption analysis

The profile of amino acids produced and consumed by the cultures with the four microcarriers followed patterns typical of animal cells in culture (Thomas, 1990), as can be observed in Figure 4a and Figure 4b. In all cultures the amino acids most consumed by the cells were arginine, isoleucine, leucine, lysine and serine and those most produced were alanine, glycine and glutamate. Appreciable differences in specific consumption of most of the amino acids occurred between the cultures with microcarriers coated with adhesion molecules and the uncoated microcarriers. The amount of amino acids consumed per 10^6 anchored cells was higher with the uncoated microcarriers. The same trend can be seen in Figure 4b, for amino acids produced by the cells: the cultures with uncoated microcarriers exhibited higher specific values of produced alanine and glycine.

Another remarkable feature of amino acid metabolism is the large quantity of ammonia produced in cultures with the uncoated microcarriers, namely, Cytodex 1 and Immobasil FS (21.5 and 77 $g \cdot 10^{-6}$ cells, respectively), when compared

Table 2. Values of the adhesion parameters of CHOZMD cells on the four microcarriers calculated by nonlinear fitting of two adhesion models to experimental data.

Microcarrier	Fitting results with the hyperbolic model Equation: $N_{af} = N_{af} \frac{t}{K_a + t}$ Parameters: N_{af} (cells.mL ⁻¹) and K_a (h)		Fitting results with Ng et al. (1996) first order model Equation: $N_a = N_{af}[1 - \exp(-k_a t)]$ Parameters: N_{af} (cells.mL ⁻¹) and k_a (h ⁻¹)	
	Parameter \pm SE	R	Parameter \pm SE	R
Cytodex 1	$N_{af} = 303,726 \pm 9,863$ $K_a = 0.909 \pm 0.114$	0.991	$N_{af} = 257,682 \pm 8,060$ $k_a = 0.918 \pm 0.104$	0.992
Cultispher S	$N_{af} = 280,350 \pm 105$ $K_a = 0.278 \pm 0.001$	1.000	$N_{af} = 265,988 \pm 1,170$ $k_a = 1.290 \pm 0.049$	1.000
Pronectin F	$N_{af} = 330,585 \pm 12,022$ $K_a = 5.125 \pm 0.340$	0.984	$N_{af} = 224,757 \pm 8,354$ $k_a = 0.261 \pm 0.018$	0.983
Immobasil FS	$N_{af} = 250,763 \pm 2,535$ $K_a = 0.846 \pm 0.038$	0.997	$N_{af} = 213,531 \pm 3,558$ $k_a = 0.915 \pm 0.068$	0.991

SE: Standard error.
R: Correlation coefficient.

to coated microcarriers, Cultispher S and Pronectin F (11.1 and 9.96 g.10⁻⁶ cells, respectively). Considering that the maximum coefficient of variation (standard deviation *100/mean) of this analysis is at most 10% (Bartolomeo and Maisano, 2006), the ammonia production in uncoated microcarriers is statistically higher than that of the coated ones. This result can be explained by the differing surface characteristics of the microcarriers, as the cells on the coated microcarriers are in contact with biomolecules similar to those that support attachment and growth in their original tissues. Considering the observation of Weber (1982) that animal cells *in vitro* tend to consume more glutamine than *in vivo*, the production of ammonia due to glutaminolysis in cultures with the uncoated microcarriers should be higher than with coated microcarriers. Ammonia was produced in largest amounts in the culture performed with Immobasil FS, with a maximum ammonium ion concentration of 5.14 mM (data not shown). The high ammonium concentration in this culture may be one of the reasons for the low cell growth on this microcarrier (Figure 3). Fassnacht and Pörtner (1999) found that an ammonium ion concentration of 5-6 mM resulted in a reduction of 50% in the maximum specific growth rate of a hybridoma culture and Takagi et al. (2001) demonstrated that ammonium concentration higher than 4 mM reduces markedly the growth of CHO cells.

Glucose consumption was similar in all cultures, the lowest concentration being attained with the microcarrier Cytodex 1, Figure 5a. Lactate production may also be considered similar in the various experiments, Figure 5b, a higher level of production being observed with Cytodex 1 as consequence of the higher glucose consumption in this culture. The lactate concentrations obtained in these experiments (up to 63 mM) probably had no toxic effect on CHOZMD cells, since the second best performance with regard to cell density was obtained with Cytodex 1, which produced the most lactate. Similarly, Fassnacht and Pörtner (1999) found that the influence of lactate concentration on hybridoma growth was rarely of concern, since inhibitory

concentrations were not usually reached in normal culture conditions. Lao and Toth (1997) demonstrated that when approximately 60 mM lactate was added, CHO cell growth was reduced by 45%, although the viability remained high ($\geq 97\%$).

Disintegrin detection

In order to overcome the problem caused by protein interference, a serum-free medium (CHO-SFM-II) was tested for CHOZMD cell culture. Combining this medium with the Pronectin F microcarrier, which showed high yields of cells, a culture was carried out by replacing gradually the 10% v/v serum DMEM by the serum-free medium. Even though a lower cell density was attained with this medium at 192 hrs, 1.8×10^6 cells/mL, as can be observed in Figure 6, against 2.8×10^6 cells/mL at 168 hrs with DMEM plus 10% v/v of serum (in Figure 3), the dot blot assay allowed a clearer demonstration of the presence of disintegrin in the medium CHO-SFM-II (Figure 7). The use of the serum-free medium is also interesting to facilitate the stage of purification of the disintegrin in a large-scale development of the bioprocess.

Performance of the microcarriers

The diversified performance shown by the microcarriers can be ascribed to the variety and relevance of the main features built into each one. The best results for culturing the CHOZMD cells were obtained with the microcarrier Pronectin F. Even though the adhesion process on this microcarrier was slow, it allowed the highest specific growth rate (μ_{\max}) and the highest cell density achieved. Based on these results, since disintegrin production should be proportional to the cell number, Pronectin F is expected to be the most promising choice for larger-scale cultures.

CONCLUDING REMARKS

With regard to cell attachment, the microcarrier Cultispher S showed the lowest adhesion time-constant $K_a = 0.278$ hrs

Table 3. Maximum specific growth rates (μ_{\max}) of CHOZMD cells in spinner flasks for the four microcarriers, calculated by linear regression of equation 2 on data in the exponential growth phase.

Microcarrier	Maximum specific growth rate $\mu_{\max}(\text{d}^{-1})$ Value \pm SE*	Correlation coefficient
Cytodex 1	0.45 \pm 0.02	0.998
Cultispher S	0.34 \pm 0.01	0.998
Pronectin F	0.67 \pm 0.05	0.995
Immobasil FS	0.52 \pm 0.12	0.949

SE: Standard error.

(or the highest adhesion rate), followed, in increasing order, by the microcarriers Immobasil FS (0.846 hrs), Cytodex 1 (0.909 hrs) and Pronectin F (5.125 hrs).

Comparing the growth, the highest value for specific maximum growth rate, μ_{max} , was obtained with the Pronectin F microcarrier with $0.67d^{-1}$, followed, in decreasing order, by Immobasil FS with $0.52d^{-1}$, Cytodex 1 with $0.45d^{-1}$ and Cultispher S with $0.34d^{-1}$. The highest value of final cell density was obtained with the microcarrier Pronectin F (2.76×10^6 cells.mL⁻¹), followed by Cytodex 1 (1.47×10^6 cells.mL⁻¹), Cultispher S (1.34×10^6 cells.mL⁻¹) and Immobasil FS (3.84×10^5 cells.mL⁻¹).

The specific consumption and production of the amino acids (in grams x (10⁶ adhered cells)⁻¹) were higher in the CHOZMD cell cultures with the microcarriers coated with adhesive molecules, Pronectin F and Cultispher S, than in the cultures with uncoated microcarriers, Immobasil FS and Cytodex 1. The glucose consumption and lactate production did not differ significantly. In the case of ammonia production, the trends were clearly different when the cell was cultivated with the coated and uncoated microcarriers; it seems that the uncoated microcarriers having an abiotic adhesion surface, dissimilar to that found *in vivo*, stimulated the production of higher quantities of ammonia.

The results obtained in this work suggest that the use of the microcarrier Pronectin F for large-scale production of the recombinant disintegrin with the CHOZMD cell is very promising. A better and quantitative assay of the amount of the recombinant protein, such as an enzyme-linked immunoassay (ELISA), is under development and will allow the process to be followed more consistently.

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