Adaptation of BHK cells producing a recombinant protein to serum-free media and protein-free medium

Helder J. Cruz¹, José L. Moreira¹, Glyn Stacey², Elsa M. Dias¹, Katherine Hayes², Dennis Looby², Bryan Griffiths² & Manuel J. T. Carrondo^{1,3}

¹ IBET/ITQB – Instituto de Biologia Experimental e Tecnológica/Instituto de Tecnologia Química e Biológica, Apartado 12, P-2780 Oeiras, Portugal; ² CAMR – Centre for Applied Microbiology and Research, Porton Down, Salisbury, U.K.; ³ Fac. Ciências e Tecnologia/Univ. Nova de Lisboa, P-2825 Monte da Caparica, Portugal

Received 24 February 1997; accepted 3 June 1997

Key words: BHK cells, protein-free medium, recombinant human IgG, serum-free medium, soybean trypsin inhibitor

Abstract

In this work a recombinant BHK21 clone producing a fusion protein with potential application in tumour target therapy was adapted to five different serum-free media (SFM) and to a protein-free medium (PFM). Only the PFM did not require a gradual adaptation to cell growth in the absence of serum. All tested SFM required a gradual adaptation (up to 35 days). For the majority of the SFM tested, cell specific productivity was not affected by the decrease in serum concentration during adaptation; however, cell growth was significantly affected by the serum decrease. Both cell growth and productivity were increased when PFM SMIF6 was used instead of the control medium. Long term measurements (approximately 100 days) of cell specific productivity for PFM and the two best SFM showed that productivity was maintained. This indicates the media capability to be used in long term production processes.

Introduction

The fusion protein used in this work, produced by a recombinant BHK21 cell clone, is an anti-EGF receptor IgG1 antibody linked to a TNF- α cytokine tail and has been shown to be active with respect to antigen specificity and cytokine activity (Old, 1985; Tracey, 1994) and is a candidate for tumour target therapy.

Commonly used to support growth of animal cells in culture, serum provides a wide number of components such as hormones, growth factors and binding proteins (Hayashi and Sato, 1976; Barnes and Sato, 1980) but is also a potential source of bacterial, mycoplasmal and viral contamination, is the most expensive additive to cell culture media, has a high degree of batch variability and interferes with product recovery, rendering downstream processing more

difficult. For these reasons, current biotechnological approaches to cell culture avoid the use of serum. Although recent studies indicate that engineering the regulatory structure of the mammalian cell cycle is a direct alternative to culture adaptation as a means of removing serum from mammalian cell systems (Lee et al., 1996), proliferation of cells in medium free from serum is usually achieved by gradual adaptation. In this work cell adaptation to five SFM and one PFM was studied and compared.

Materials and methods

Cell lines and growth systems

BHK 21A cells genetically modified to produce a

fusion protein were obtained from Dr. Hansjörg Hauser (GBF, Braunschweig, Germany).

Cells were initially grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Glasgow, UK) containing 10% foetal bovine serum (FBS) (Sigma, St. Louis, USA), 4 mM glutamine and 1 g L⁻¹ glucose (Sigma) (all final concentrations).

Five different SFM were used: QBSF 56 (Sigma), CHO-S-SFM II (Life Technologies), Ex-cell 301 (JRH Biosciences, USA), CHO Master (AMS, UK) and DMEM/Ham's F12 (Life Technologies) derived medium previously used with BHK cells (Jager et al., 1988) as well as a PFM, Scharfenberg's modification 6 (SMIF6) (GBF, Braunschweig, Germany) (Scharfenberg and Wagner, 1995; Neermann and Wagner, 1996).

Studies were performed in 25 cm² static flasks at 37 °C (Greiner, Frickenhausen, Germany) containing 10 mL of medium and 2 × 10⁴ cells mL⁻¹ as inoculum. All media contained puromycin at 5 mg L⁻¹ (Sigma, St. Louis, USA). Cell subculture was carried out using trypsin and its inhibition by soybean trypsin inhibitor as reported in Cruz et al., 1997.

Sampling

Cells were counted using a Fuchs-Rosenthal haemocytometer (Brand, Wertheim, Germany). Viable cells were determined using the trypan blue dye exclusion method. Adherent cells were determined after cell release from the static surface by digestion with trypsin.

Product quantification was performed using an ELISA protocol obtained from Dr. Erwin Rieke (Merck KGaA, Darmstadt, Germany).

Results and discussion

Single step adaptation to serum-free medium

As shown in Table 1, none of the SFM tested supported cell growth after direct switch from serum-containing to serum-free medium. Reduction from 10 to 5% serum did not lead to any change in cell culture, the attached cells still showing normal fibroblast-like morphology. In most cases (excepting CHO Master) reduction to 2.5% serum did still allow cell growth, although at a lower rate; thus, decreased cell growth is obtained below 5% serum content. In most cases the proportion of suspended cells increased and adherent aggregates appeared with decreasing serum concentration.

Multi-step adaptation to serum-free medium

All SFM tested required a gradual adaptation from DMEM with 10% FBS to the SFM under test. As can be seen in Table 2 cell growth during adaptation (expressed by days to confluency) did decrease with decreasing serum content. Some cultures did not survive at low serum content (CHO Master at 1% serum and DMEM/F12 at 0% serum). Also cell attachment to the surface was lower (the proportion of suspended cells increased with decreasing serum content) and in most cases led to cell aggregation, similar to that reported for BHK21 pSVII cells producing interleukin-2 (Jager et al., 1988). This is also in agreement with the reported fact that both BHK21 and CHO cells formed spheroids of up to 500 µm in diameter after transfer to serum-free culture conditions (Ackermann et al., 1995).

In an adaptation process it is critically important to screen the resultant cell population to ensure that the selection events do not result in loss of target cell function as, in this case, expression of the recombinant fusion protein. From the results depicted in Figure 1 it can be seen that CHO-S-SFM II and QBSF 56 media supported high productivity down to low serum levels (i.e. 0.25 and 0.5%, respectively). DMEM/F12 showed maintenance of cell productivity, but cell growth was very low (Table 2). CHO Master showed an overall low productivity and, furthermore, it showed a decrease in productivity with decreasing serum content. A marked decrease in productivity was also observed for Ex-cell 301.

In conclusion, it can be said that the gradual change from 10% serum containing medium to reduced amounts of serum did not lead to changes in cell specific productivity in most of the serum-free media examined. However, for all media tested, cell growth was reduced with serum concentrations below 5%. This is in agreement with the reported decrease in BHK cell growth at serum concentrations below 5% (Moreira et al., 1995).

From the cell growth (Table 2) and productivity values (Figure 1) obtained, the best SFM was identified as being CHO-S-SFM II followed by QBSF56, both supplemented with 0.5% FBS.

Single step adaptation to protein-free medium

The change from 10 to 0% serum containing medium in a single step has only been achieved with protein-free medium SMIF6. These cells showed a lag phase of

Table 1. Results obtained from single step adaptation to serum-free medium

Media	Serum (%)	Days to confluency	Attached cells	Suspended cells
CHO-S-SFM II	5	3	Normal morphology	Few
	2.5	4	Some aggregates	Some
	0	nr	Aggregates	Many
Ex-cell 301	5	3	Normal morphology	Few
	2.5	4	Normal morphology	Few
	0	nr	Normal morphology	Few
CHO Master	5	4	Normal morphology	Few
	2.5	nr	Few	Many
	0	nr	Few aggregates	Many
QBSF 56	5	3	Normal morphology	Few
	2.5	4	Some aggregates	Few
	0	nr	Aggregates	Few
DMEM/F12	5	4	Normal morphology	Few
	2.5	5	Some aggregates	Few
	0	nr	Aggregates	Some

nr = Not reached.

Figure 1. Multi step adaptation in static cultures for five different serum-free media from 10 to 0% serum content.

1–2 days, after which they began to proliferate at a high rate. Cell growth, viability and adherent fraction were maintained at high levels, similar to those obtained in

serum-containing cultures; the cell morphology shown was healthy, fibroblast like shape.

Table 2. Results obtained from multi step adaptation to serum-free medium

Media	Serum (%)	Days to confluency	Attached cells	Suspended cells
CHO-S-SFM II	5	3	Normal morphology	Few
	2.5	5	Normal morphology	Some
	1	6	Some aggregates	Many
	0.5	7	Aggregates	Many
	0	9	Aggregates	Many
Ex-cell 301	5	3	Normal morphology	Few
	2.5	4	Normal morphology	Few
	1	5	Normal morphology	Few
	0.5	6	Normal morphology	Few
	0	7	Normal morphology	Few
CHO Master	5	3	Normal morphology	Some
	2.5	7	Few	Many
	1	cd	Few	Some
QBSF 56	5	3	Normal morphology	Few
	2.5	4	Normal morphology	Few
	1	5	Normal morphology	Few
	0.5	6	Normal morphology	Some
	0	nr	Normal morphology	Some
DMEM/F12	5	3	Normal morphology	Few
	2.5	4	Normal morphology	Some
	1	5	Normal morphology	Some
	0.5	11	Aggregates	Some
	0	cd	Aggregates	Some

nr = Not reached. cd = Cells died.

Serum-free media and protein-free medium comparison

The best serum-free media (CHO-S-SFM II and QBSF56 containing 0.5% FBS) were compared with the protein-free medium (SMIF6). As can be observed in Table 3, cell growth in SFM (0.015 to 0.019 h^{-1}) was lower than in the control with 10% FBS (0.024 h^{-1}).

The cell specific productivity obtained with PFM (SMIF6) was higher than that in the control and in the other media (188% of control); the same was also observed for cell growth (0.039 h^{-1}). Average viability was also higher for this PFM, being very similar to the control whereas both SFM showed significantly lower values. It can also be observed that cell specific productivity is maintained when comparing QBSF56 and CHO-S-SFM II with the control.

From this comparison it can be concluded that the PFM (SMIF6) has several advantages over the SFM used in this study, namely no need for a time prolonged multi-step adaptation, higher productivity and all the referred advantages of a protein-free medium. Thus, the PFM was chosen for the development of future work. A second choice would be CHO-S-SFM II, considering maximum cell concentration, specific growth rate and productivity obtained in this medium (Table 3).

Production stability

The maintenance of productivity with time is important to maintain process consistency and also high and constant production rates. Therefore it was necessary to test the stability of production in the best performant

Table 3. Summary of the static culture comparison for the different serum-free media

Medium	FBS (%)	Maximum cell concentration (10^6 cell mL^{-1})	Specific growth rate (h^{-1})	Cell specific productivity (% of control)	Average viability (%)	Average adherent fraction (%)
DMEM	10	0.81	0.024	100 ± 16	90	94
QBSF56	0.5	0.69	0.015	100 ± 12	76	78
CHO-S-SFM II	0.5	0.90	0.019	97 ± 16	79	81
SMIF6	0	0.93	0.039	188 ± 14	87	93

Figure 2. Stability studies for cell specific productivities with cells growing in SFM CHO-S-SFM II; SFM QBSF 56 and PFM SMIF6.

SFM and PFM. As can be seen from Figure 2, CHO-S-SFM II medium showed slightly lower, QBSF56 slightly higher and SMIF6 significantly higher average productivity than the control. All this data is compatible with the results obtained in culture studies, summarised in Table 3, confirming the best performance of CHO-S-SFM II when compared to QBSF56.

CHO-S-SFM II and QBSF56 media showed stability in product expression for approximately 100 days (respectively 90 and 50 generations). As for SMIF6 medium, a highly consistent cell specific productivity over 190 days (180 generations) was also obtained, indicating its capability to be used in long term production processes and also long term subculturing.

Conclusions

We have compared the relative performance of five different serum-free media and a protein-free medium with a view to the production of a complex recombinant fusion protein for therapeutic use from BHK cells.

Cell adaptation in a single step change from 10 to 0% serum containing medium has only been achieved with SMIF6, the protein-free medium used in this work. The gradual change from 10% serum containing medium to lower amounts of serum in the majority of the serum-free media tested did not lead to changes in cell specific productivity. However, cell growth was reduced when serum concentration decreased to below 5%. Two media (CHO Master and Ex-cell 301) showed

decreasing productivity with decreasing serum content; one of these (CHO Master) showed significantly lower production levels. Adherence also showed an overall decrease as serum concentration decreased, with cells tending to form aggregates as serum levels were decreased to very low amounts. Protein-free medium SMIF6 showed higher cell growth and productivity, this leading to higher titres than the control and the serum-free media tested. Productivity stability was unaffected by long periods of cultivation (up to 180 cell generations for the SMIF6 medium).

SMIF6 was chosen for further process development given its protein-free composition and also as it allowed better cell growth and productivity and did not need any adaptation process. Compared with the serum-free media tested, the protein-free medium also has the lowest cost and permits easier downstream processing. A second choice would be CHO-S-SFM II, considering that it showed to be the best performant of the several SFM tested.

Acknowledgements

The authors acknowledge the financial support received from the European Commission (PL933069) and Junta Nacional de Investigação Científica e Tecnológica, Portugal (PBICT/BIO/20333/95 and PRAXIS XXI/BD/2764/94). The authors also thank Drs. Klaus Scharfenberg and Roland Wagner from GBF, Germany, for providing the protein-free medium used in this work.

References

- Ackermann M, Marx U and Jager V (1995) Influence of cell- and media-derived factors on the integrity of a human monoclonal antibody after secretion into serum-free cell culture supernatants. *Biotechnol Bioeng* 45: 97–106.
- Barnes D and Sato G (1980) Methods for growth of cultured cells in serum-free medium. *Anal Biochem* 102: 255–270.
- Cruz HJ, Dias EM, Moreira JL and Carrondo MJT (1997) Cell dislodging methods under serum-free conditions. *Appl Microbiol Biotechnol* 47: 482–488.
- Hayashi I and Sato G (1976) Replacement of serum by hormones permits growth of cells in a defined medium. *Nature* 259: 132–134.
- Jager V, Lehmann J and Friedl P (1988) Serum-free growth medium for the cultivation of a wide spectrum of mammalian cells in stirred bioreactors. *Cytotechnol* 1: 319–329.
- Lee KH, Sburlati A, Renner WA and Bailey JE (1996) Deregulated expression of cloned transcription factor E2F-1 in chinese hamster ovary cells shifts protein patterns and activates growth in protein-free medium. *Biotechnol Bioeng* 50, 273–279.
- Moreira JL, Alves PM, Feliciano AS, Aunins JG and Carrondo MJT (1995) Serum-free and serum-containing media for growth of suspended BHK aggregates in stirred vessels. *Enz Microbiol Technol* 17: 437–444.
- Neermann J and Wagner R (1996) Comparative analysis of glucose and glutamine metabolism in transformed mammalian cell lines, insect and primary liver cells. *J Cell Physiol* 166: 152–169.
- Old LJ (1985) Tumor Necrosis Factor (TNF). *Science* 230: 630–632.
- Scharfenberg K and Wagner R (1995) 'A reliable strategy for the achievement of cell lines growing in protein-free medium', in Beuvery C, Griffiths JB and Zeijlemaker (eds.) *Animal Cell Technology: Developments Towards the 21st Century* (pp. 619–623) Kluwer Academic Publishers, Dordrecht.
- Tracey K (1994) 'Tumour Necrosis Factor-Alpha', in Angus Thomson (ed.) *The Cytokine Handbook* (pp. 289–297) Academic Press, London.

Address for correspondence: Manuel J. T. Carrondo, IBET/ITQB – Instituto de Biologia Experimental e Tecnológica/Instituto de Tecnologia Química e Biológica, Apartado 12, P-2780 Oeiras, Portugal.