Isolation, characterization and recombinant protein expression in Veggie-CHO: A serum-free CHO host cell line

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Abstract

The dihydrofolate reductase-deficient Chinese hamster ovary cell line, DXB11-CHO, commonly used as a host cell for the production of recombinant proteins requires 7.5% serum-supplementation for optimal growth. Regulatory issues surrounding the use of serum in clinical production processes and the direct and indirect costs of using serum in large-scale production and recovery processes have triggered efforts to derive serum-independent host cell lines. We have successfully isolated a serum-free host that we named Veggie-CHO. Veggie-CHO was generated by adapting DXB11-CHO cells to growth in serum-free media in the absence of exogenous growth factors such as Transferrin and Insulin-like growth factor, which we have previously shown to be essential for growth and viability of DXB11-CHO cells. Veggie-CHO cells have been shown to maintain an average doubling time of 22 hr in continuous growth cultures over a period of three months and have retained the dihydrofolate reductase -deficient phenotype of their parental DXB11-CHO cells. These properties and the stability of its serum-free phenotype have allowed the use of Veggie-CHO as host cells for transfection and amplified expression of recombinant proteins. We describe the derivation a serum-free recombinant cell line with an average doubling time of 20 hr and specific productivity of 2.5 Units recombinant Flt-3L protein per 10⁶ cells per day.

Introduction

Chinese hamster ovary (CHO) cells are widely used for the production of several complex recombinant proteins, e.g. cytokines, clotting factors, antibodies (Brasel et al., 1996; Kaufman et al., 1988; McKinnon et al., 1991; Wood et al., 1990). The dihydrofolate reductase (DHFR)-deficient mutant cell lines (Urlaub and Chasin, 1980), DXB-11 and DG-44, are the CHO host cell lines of choice because the efficient DHFR selectable and amplifiable gene expression system allows high level recombinant protein expression in these cells (Kaufman, 1990). In addition, these cells are easy to manipulate as adherent or suspension cultures and exhibit relatively good genetic stability. CHO cells and recombinant proteins expressed in them have been extensively characterized and have

been approved for use in clinical manufacturing by regulatory agencies.

Serum is often used for the propagation of CHO cell lines, and there is increasing pressure to remove serum from the manufacturing process of therapeutics for several reasons. The expense of serum, variance between serum lots and serum quality, regulatory concerns regarding viral contamination in serum and the burden of removing serum proteins in downstream processing are some of the driving reasons to implement serum-free, cell-culture technology. Therefore, this technology has become an essential component of the production process and the ability to adapt adherent, serum-requiring recombinant CHO cell lines to grow as suspension cultures in serum-free media has been demonstrated (Adamson, 1994; Thomas and Fung, 1994). However, adaptation of recombinant cell lines to suspension and serum-free growth can be a time consuming step in process development

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Figure 1. Isolation of Veggie-CHO cells by adaptation of DXB11-CHO cells to growth in serum-free media. Figures 1A and B show the average viability and cell doubling time respectively of DXB11-CHO cells during their adaptation to the gradual removal of serum, followed by the removal of growth factors: IGF-1 and transferrin.

Figure 2. Growth and expression of a recombinant cytokine protein in Veggie-CHO and parental DXB11-CHO host cells. The doubling time, protein titers and specific productivity (qp) of the DXB11:FLT-3L and Veggie-CHO:FLT-3L cultures over 40 passages are shown.

with variable effects on recombinant protein expression and protein quality. Hence, a suspension and serum-free preadapted DHFR-deficient host cell line that could be used to generate recombinant cell lines expressing high levels of recombinant protein would greatly facilitate the process and enhance efficiency in the serum-free process development of recombinant proteins.

We used an adaptation strategy to derive the Veggie-CHO cells which grow in serum-free media in the absence of exogenous growth factors such as Transferrin and Insulin-like growth factor (IGF-1). This involved the gradual reduction of serum supplementation in the media and the replacement of serum with low levels of growth factors, IGF-1 and transferrin, in an enriched cell growth media. These serum-free cells were then weaned off these growth factors. The adaptation of cell lines to media with reduced or no serum is based on careful adjustment of medium components to provide cells with optimal nutrient levels and provide them with a mixture of growth factors essential for survival and growth in the absence of serum (Barnes and Sato, 1980; Evans et al., 1956; Hamilton and Ham, 1977). This approach has been demonstrated for several cell lines, including CHO cells (Gandor et al., 1995; Sinacore et al., 1996). The basal medium of a majority of serum-free formulations usually consist of Hams F-12, DMEM or a mixture of both. Over the past few years several serum-free, protein-free media and chemically defined media have become commercially available for use in development of serum-free cell lines. Some of these have been formulated for use with CHO cell lines and require an adaptation process to acquire optimal growth (Wyatt and Doak, 1995).

We demonstrate the convenience and advantage of using Veggie-CHO cells to generate recombinant CHO lines by using Veggie-CHO as host cells for the methotrexate (MTX)-amplified expression of the FLT-3L cytokine molecule. The FLT-3L molecule expressed in Veggie-CHO is active and is expressed at levels similar to those obtained using the conventional DXB11-CHO host cells.

Materials and methods

Materials

DMEM-F12, Nucleoside-free DMEM-F12 (–) and custom enriched formulations of these media, IMX

2.3 and IMX 1.2 respectively, were purchased from JRH Biosciences. Dialyzed and non-dialyzed fetal bovine serum (FBS) was obtained from Hyclone Laboratories. Tissue culture flasks were obtained from Corning and spinner flasks were obtained from Bellco. Recombinant Insulin-like growth factor (Long R3-IGF-1) (McKinnon et al., 1991) was purchased from GroPep and bovine were purchased from JRH and plant soy hydrolysate (NZ soy) was purchased from Quest. Pluronic F68 was purchased from BASF, and Trace elements A,B,C, were purchased from Cellgro. MTX was from Immunex.

Cell culture methods

DXB11 and Veggie CHO host cells

DXB11-CHO monolayers were maintained in DMEM-F12 media supplemented with 7.5% dialyzed FBS. Monolayers were subcultured three times weekly by trypsinization. Suspension CHO cultures were maintained in the DMEM-F12 media supplemented with decreasing amounts of serum as indicated in Figure 1. Serum-free IMX media (SF-IMX) consisted of the custom IMX2.3 base media containing pluronic and supplemented with trace elements, soy hydrolysates, intralipids, trace element mixes A, B, C, (to provide essential selenium and ferrous sulphate to supplement for iron carriers). Growth factors, IGF-1 and transferrin, were added at indicated concentrations. Glycine (2.52 mM), hypoxanthine (174 μ M), thymidine (174 μ M) were added to the DMEM F-12 and IMX2.3 media for the derivation and maintenance of Veggie-CHO cells in order to enrich for these components and minimize selection and outgrowth of any DHFR revertants. In addition, the peptone hydrolysates also provide additional amino acids.

Suspension adapted DXB11-CHO cell cultures were maintained as 100 mL cultures in 250 mL spinner flasks for the derivation of the Veggie-CHO cells. Cells were passaged three times weekly by centrifugation and 100% media replacement. Cells were seeded at a density of 2×10^5 cells mL^{-1} into enriched media (SF-IMX) with decreasing levels of serum or growth factor

Cell counts were determined by haemocytometer, and cell viability was determined by propidium iodide or trypan blue exclusion assay. DNA dyes, propidium iodide and Hoechst 33342 were used in flow cytometry analysis to monitor viability and apoptosis of cell cultures (Dive et al., 1992; Sun et al., 1992). Cell doubling time (Td) was calculated using the following

Figure 3a–b. Stability of recombinant FLT-3L expression in Veggie-CHO cells. A. Veggie -CHO:FLT-3L Cells were passaged in select media containing 150 nM MTX or in DHFR non-select media. FLT-3L protein levels in the culture media were determined at each passage using ELISA assays and used to calculate the specific productivity (qp). B. Chromosomal DNA was isolated from the cell cultures at various passages and FLT-3L gene copy number was determined using quantitative southern blot analysis (Sambrook et al., 1989). DNA was treated with restriction enzymes to release a single fragment of the FLT-3L coding sequence and probed with radiolabelled FLT-3L cDNA sequence. The signal was normalized for genomic DNA loading in each lane. DNA was loaded as follows: Lanes 1 and 5 control untransfected DXB-11, Lanes 2–4 Veggie-CHO:FLT-3L; Lane 2, 4 are DNAs from the +MTX culture at passage 37 and 63 respectively. Lane 3 is DNA from passage 63 no MTX. Lanes 6–10 are DNAs from DXB-11:FLT-3L. Lanes 6, 8, 9 and 10 are from +MTX DXB11 cultures at passage 66, 39, 62 and 82 respectively and lanes 7 is DNA from the nonselect (-MTX) DXB11 cultures at passage 66.

formula where t = days in culture, N_t is the final cell density and N_0 is the initial seeding density.

$$Td = \ln 2 / \mu$$

$$\mu = \ln(N_t/N_0) \times 1/t$$

Derivation and analysis of recombinant cell lines

The cDNA for the FLT-3L protein was subcloned into a bicistronic CHO expression vector with a CMV promoter and contained the selectable, amplifiable DHFR cDNA (Kaufman et al., 1991; Morris et al., 1997). Genomic DNA was isolated from recombinant cell lines and southern blot analysis was performed as described in the manual by Sambrook, Fritsch and

Maniatis (1989). Recombinant protein expression in cell conditioned media was monitored using the Flt3L specific ELISA (Mckenna et al., 1995).

Effect of butyrate, IGF-1 and temperature on the recombinant Veggie-CHO and DXB11 cell lines was studied using 20 mL suspension batch cultures in 125 mL agitated shake flasks. Cells were seeded at 1×10^6 to 2×10^6 cells mL^{-1} in the presence or absence of 1 mM butyrate and in the presence or absence of IGF1 (100 ng mL^{-1}) in serum free media (non selective SF-IMX). The lids of the shake flasks were slightly vented and cultures were incubated on a shaking platform at 31 or 37 °C for the period of the experiment. Cell counts, cell viability and protein expression were monitored as described above.

Figure 4. Effect of butyrate and temperature on growth and recombinant protein expression in Veggie-CHO cells. The cell density, protein titers and qp values of high density batch cultures of DXB-11:A and Veggie-CHO:FLT-3L in the presence of butyrate or absence of butyrate at 37 or 31 °C are shown.

Isolation of Veggie-CHO recombinants

Approximately 20×10^6 Veggie-CHO cells in mid-log phase of growth were plated in T75 tissue culture flasks on the day of transfection in serum-free media. Cells were left in a CO₂ incubator at 37 °C for one hour to allow the cells to adhere to the plates. Cells were then transfected with 50 μ g of the FLT-3L cDNA expression vector in serum-free media using Lipofectamine reagent from Life Technologies following the general recommendations of the manufacturer. Transfection efficiency of Veggie-CHO cells with lipofectamine was several fold lower than that of DXB11 cells when monitored by transient expression of Lac Z protein. The transfected Veggie-CHO cells have a semi-adherent phenotype and were allowed to recover for 3 to 4 days and then gently trypsinized and selected for the selectable, amplifiable DHFR gene in the nucleoside-free, Selective SF-IMX1.2 media. The surviving cells or 'transfectant pools' were then selected in increasing concentrations of MTX (50 to

150 nM) in the Selective SF-IMX media to amplify recombinant protein expression. The amplified transfectant pool in 150 nM methotrexate expressed the highest level of recombinant FLT-3L molecule. This amplified transfectant pool was subcloned by limiting dilution in 96-well dishes using 3% dialyzed serum-supplemented, nucleoside-free DMEM-F12 (DHFR selective) media. The wells were visually screened for individual colonies and cells from these wells were transferred to 24-well dishes in serum-free media. Individual clonal colonies arising in the 96 wells are semi-adherent phenotype. Isolates were successively transferred to suspension growth in agitated shake flasks and then to spinner flasks in 10 to 12 passages and maintained as suspension cultures in Selective SF-IMX media. Clones were screened for recombinant FLT-3L expression using ELISA assay to select the final Veggie-CHO:FLT-3L recombinant clone. Although most of the clones assumed their serum-free and suspension growth phenotype, a small number

(20%) were terminated because of their long doubling times or low culture viabilities when passaged into serum-free and suspension growth

Isolation of DXB11 recombinants

Monolayers of adherent DXB11-CHO host cells were transfected using Lipofectamine reagent and plated as adherent cultures in nucleoside-free DHFR selection growth media (7.5% dialysed serum, DMEM-F12). Individual transfectant colonies were screened for FLT-3L expression using the ELISA assay. Colonies expressing the highest cytokine levels were further amplified in increasing amounts of methotrexate, up to a final concentration of 250 nM methotrexate. The 250 nM MTX pool was subcloned using the 96-well limiting dilution technique and the resulting colonies were screened for expression. The final DXB11:FLT-3L recombinant clone was identified as the highest expressing isolate. These cells were suspension adapted by passaging cells over several generations in serum-containing selective growth media in a spinner flask (100 mL culture in a 250 mL spinner flask)

Results

Isolation of Veggie-CHO Cells by adaptation of DXB11-CHO cells to growth in serum-free media and protein-free media

Since removal of serum leads to removal of cell attachment factors, we first adapted the adherent DXB11-CHO host cell line to grow in suspension in serum-supplemented DMEM-Hams F-12 media in stirred spinner flasks. These suspension adapted DXB11-CHO cell cultures were passaged every 2 to 3 days (by 100% media replacement) into decreasing levels of serum (Figure 1). We used a basal media (SF-IMX) consisting of a mix of Hams F-12 and DMEM enriched with amino acids and vitamins and supplemented with growth factors IGF-1 ($100 \mu\text{g L}^{-1}$) and transferrin (10 mg L^{-1}) to replace serum components. We have found that unlike insulin, IGF-1 acts as a growth and viability factor at physiological levels in DXB-11 cells (Thomas and Fung, 1994), and we used it initially, along with bovine transferrin, to replace serum.

Adaptation of the cells to the stepwise removal of serum from their growth media required approximately 100 passages (over approximately 160 cumulative cell doublings). These cells, designated SF-CHO, maintained stable growth (25 hour doubling

time) and high cell viability (over 90%) in serum free media over 6 passages. This is indicative that the cells are completely adapted to grow in SF-IMX containing growth factors (IGF-1 and transferrin). Removal of transferrin from the serum-free media of SF-CHO could be accomplished in a few passages, without a significant drop in viability (below 90%) or growth (data not shown). Therefore, serum-free adaptation of the SF-CHO cells has also made these cells independent of transferrin as a growth factor. However, the SF-CHO cells continue to require IGF-1 for growth and viability. Sudden dramatic decrease in levels or removal of IGF-1 from the SF-CHO cell line lead to loss in culture viability and lack of growth. We chose to keep the transferrin in the SF-CHO media, to stabilize IGF-1, while we adapted these cells to lower concentrations of IGF-1. After the cells were adapted to be completely independent of IGF-1 for growth by passage 170 (approximately 383 cumulative cell doublings) the bovine transferrin was eliminated from the media. Cells rapidly adjusted to the removal of transferrin (Figure 1) to grow in protein-free media and were then designated as Veggie-CHO. Addition of iron-carriers, i.e. tropolone, to substitute for transferrin, did not enhance cell growth or viability.

Stepwise reduction of the serum or IGF-1 supplementation in the growth media often led to a transient decrease in cell viability at some of reduction steps, which was often accompanied by a transient increase in doubling time (see Figure 1). More dramatic reductions in the levels of serum and IGF-1 than those indicated in Figure 1 were attempted but led to loss of culture viability to below 50% (data not shown) and these cultures were not maintained further. The adherent DXB11-CHO cultures have been reported to grow with an average doubling time of 18 hr (Urlaub and Chasin, 1980). We found that our suspension and serum-free adapted cultures grew with an average doubling time of 28.6 hr. Removal of IGF-1 from this media led to cultures with an average doubling time of 25.7 hr and the Veggie-CHO cells have an average doubling time of 22.5 hr.

The serum-free adapted cultures and the parental DXB11 cells were analyzed for reversion of the DHFR phenotype using fluorescent methotrexate. FACS analysis of cells were incubated for 20 hr with fluorescent methotrexate showed similar profiles for both cell types with a single nonstaining population. In addition, cells were taken from different stages of the serum and growth factor weaning and tested for growth in serum-free and serum-containing DHFR-selective

growth media. Lack of growth and the rapid loss of cell viability in the DHFR-selective media along with the fluorescent methotrexate analysis demonstrate the DHFR-deficient phenotype of the serum-free cell host.

Veggie-CHO and SF-CHO cells and the recombinant transfectant pools and clones generated from these cells (see below) can be cryopreserved in serum-free, protein-free media containing 10% DMSO. Cells were shown to recover and maintain growth and viability after 6 months of storage in liquid nitrogen.

Recombinant cytokine protein expression in Veggie-CHO

The Veggie-CHO serum-free host and the parental serum-requiring host DXB11-CHO were genetically engineered using the DHFR/MTX selection and gene amplification method to secrete high levels of recombinant FLT-3L (McKenna et al., 1995). The Veggie-CHO transfectant pools were selected for growth in 150 nM MTX to amplify recombinant protein expression. Selection of the Veggie-CHO transfectant pool in MTX levels higher than 150 nM concentration did not result in significant increase in the expression of this cytokine molecule. The DXB11 transfectant pools (i.e. colonies) were step amplified to a final 250 nM MTX concentration as described in the methods section.

Clonal FLT-3L expressing recombinant cell lines (Veggie-CHO:FLT-3L and DXB11:FLT-3L) were isolated from these final amplified pools using the 96-well limiting dilution technique. Growth and recombinant protein expression of the cultures were monitored and are shown in Figure 2. The Veggie-CHO:FLT-3L cells exhibit better growth; they have a shorter doubling time and reach higher cell densities than the DXB11:FLT-3L cells as illustrated in figure Figure 2 and Table I (also see Figure 4 below). These two cell lines produce similar final titers of recombinant protein.

Genetic stability of recombinant Veggie-CHO:FLT-3L cells

Genetic stability of the DXB11 and Veggie-CHO recombinant cell lines were evaluated by monitoring recombinant FLT-3L protein expression and integrated gene copy number of FLT-3L over 87 passages in cells grown in DHFR-selective or non-selective growth media. This corresponded to 133 cumulative cell doublings (CCD) in selective media and 124 CCD in non selective growth media. Protein expression and integrated gene copy number of FLT-3L were monitored

Figure 5. Effect of butyrate and IGF-1 on cell viability and apoptosis in batch cultures of Veggie-CHO. The viability and apoptosis in high density batch cultures of DXB-11:A and Veggie-CHO:FLT-3L in the presence of 1mM sodium butyrate or absence of butyrate with 100 $\mu\text{g mL}^{-1}$ IGF-1 or absence of IGF-1 are shown.

Table 1. Specific protein productivity (qp) and growth rates (doubling time) of the two FLT-3L recombinant cell lines, Veggie-CHO:FLT-3L and DXB11:FLT-3L

Cell type	qp (Units 10e6-cells ⁻¹ .day ⁻¹)	Doubling time (hr)
DXB11:FLT-3L	5.58±2.39	42.1±11.8
Veggie-CHO:FLT-3L	2.27±2.94	21.2±2.9

Figure 6. SDS-PAGE analysis of recombinant protein expressed in Veggie CHO and DXB11 cells. Cytokine protein purified from conditioned media derived from 10 day production cultures was analyzed on reducing 16% SDS PAGE gel electrophoresis. Positions of the three different glycoforms of the FLT-3L are indicated by solid arrows, position of the clipped FLT-3L is indicated by dashed arrow. Sample order is as follows: lane 1, molecular weight standards; lane 2, Veggie-CHO:FLT-3L (5 µg); lanes 3 and 4, DXB11:FLT-3L (5 and 10 µg, respectively).

using ELISA assays and southern blot analysis respectively (Figure 3). Results of these studies show that integrated copy number of FLT-3L in Veggie-CHO:FLT-3L cells was maintained at 8 copies/cell over the 87 passages in both, the presence or absence of selective pressure for the DHFR gene function (Figure 3, lanes 2–4).

The data in Figure 2 seem to suggest that the DXB11:FLT-3L recombinant cell line seems to be losing recombinant FLT-3L protein expression over 40 passages in selective growth media. We found however, that inoculum cultures derived from later passages (passage 38 and passage 80) of this culture do not show decreased protein expression when treated with sodium butyrate. We have also found that the integrated gene copy number of the FLT-3L cDNA stays stable at 12 copies and does not decrease in the later passages (Figure 3, lanes 8–10). It is possible that the apparent decrease in recombinant protein expression in the DXB11:FLT-3L may be a result of some reversible mechanism of gene down-regulation (i.e. histone acetylation, Chen et al., 1997), the effect of which

seems to be reversed by sodium butyrate. We have not observed this butyrate-reversible decrease in protein expression in other DXB11 or Veggie-CHO cells recombinant cell lines. We also observed that both protein expression and integrated gene copy number of FLT-3L decreased over 40 passages in DXB11:FLT-3L cultures grown in DHFR-nonselect media (Figure 3, lane 7). This instability could be a function of the integration site in this cell line.

Effect of butyrate and temperature on growth, cell viability and recombinant protein expression in Veggie-CHO cells

Temperature reduction is a common manipulation of cell cultures which has variable effects on cell viability and protein expression (Bloemkolk and Gray, 1992; Weidemann et al., 1994). We have observed increased viability and decreased growth of the Veggie-CHO and DXB11-CHO batch cultures at lower temperatures (Figure 4). Reduction in temperature to 31 °C also leads to a small increase in specific protein expression (qp) in Veggie-CHO cultures but not in DXB11-CHO cultures as shown in Figure 4. We have found that the response in protein expression to changes in temperature varies for different proteins expressed in Veggie-CHO.

Sodium butyrate has been reported to have several different effects on cells; it has been shown to effect gene expression, cell cycle distribution of cells in culture and induce cell differentiation (Chen et al., 1997; Kruh, 1982). Both cell lines, DXB11-CHO and Veggie-CHO, respond similarly to sodium butyrate addition (Figure 4). Addition of sodium butyrate results in decreased growth and a significant increase in specific recombinant protein expression at 37 °C but not at 31 °C. The recombinant DXB11 cells however grow less than the Veggie-CHO:FLT-3L cells at 37 °C even in the absence of butyrate in these experiments. This is because the DXB11 cells have a long doubling time (40 hr) in serum and these cells are not adapted to

grow in serum-free media used for these batch culture experiments (see Section Methods).

Butyrate was also found to have an adverse effect on viability of the cells (Figure 5). FACS analysis of the cells from these serum-free batch cultures to examine apoptosis revealed that 1mM sodium butyrate induces apoptosis in Veggie-CHO cultures (Figure 5) and DXB11 cultures (data not shown). Since we have previously found that IGF-1 suppresses butyrate induced apoptosis in DXB11-CHO cells we examined the effect of IGF-1 addition on cell viability and apoptosis in butyrate-treated Veggie-CHO cultures. Results of these experiments, shown in Figure 5, illustrate that addition of IGF-1 to the Veggie CHO batch cultures did not significantly enhance cell viability nor suppress butyrate-induced apoptosis in these cultures.

FLT-3L protein analysis

FLT-3L protein was purified from serum-free butyrate induced cultures of DXB11:FLT-3L and Veggie-CHO:FLT-3L with similar process parameters. Comparison of SDS PAGE analysis of the purified FLT3L protein show that the protein from both sources has multiple banding pattern, with three FLT-3L protein species (Figure 6). Western blot and glycoform analysis demonstrates that the differently migrating species represent various glycoforms of the FLT-3L molecule. The FLT-3L molecule has two N-glycosylated sites. The slowest migrating form represents most of the double glycosylated species and the middle band represents most of the single glycosylated species and the fastest migrating band represents the non-N-glycosylated species (C. Jochheim and L. Novick, personal communications). Although all three species are expressed in both cell types, the higher glycosylated species are more prevalent in the Veggie-CHO (Figure 6, lane 2) samples and least prevalent in the DXB11 samples (Figure 6, lanes 3 and 4). In addition the DXB11 sample has a fourth band (indicated with a dotted arrow in Figure 6) that represents a clipped version of FLT-3L. This fourth band is sometimes also seen in Veggie-CHO FLT-3L samples and can not be attributed to any one single culture parameter. Assay of the purified protein in '*in vitro*' bioassays show that the protein from both cell lines has similar biological activities. More detailed biochemical analysis aimed at better understanding of the post-translational modifications on this molecule and the effect of culture and purification conditions on these modifications are underway.

Discussion and conclusions

Veggie-CHO cells derived from DXB11-CHO cells maintain vigorous growth and high viability as well as their DHFR-deficient phenotype in serum-free, essentially protein-free media. We have demonstrated their utility as a host cell line for the stable, high level expression of active recombinant cytokine protein. Generation of serum-free recombinant cell lines directly from the preadapted Veggie-CHO host is both time and labor efficient since we have eliminated multiple rounds of adaptation, cloning and characterization that would be involved in isolating DXB11-CHO recombinants and then adapting them to growth in serum-free media. In addition the elimination of expensive growth factors and serum (except in 96-well cloning) during the generation of the recombinant Veggie-CHO lines and the recombinant protein production provided significant cost savings.

The Veggie-CHO cells that we derived by gradual reduction of serum and growth factors using an enriched mix of DMEM and HamsF-12 base media have retained their DHFR-deficient phenotype after extensive culture in serum-free conditions.

DHFR enzyme is involved in the synthesis of glycine, hypoxanthine and thymidine which are in turn required for DNA synthesis and cell division. Glycine (2.52 mM), hypoxanthine (174 μ M), thymidine (174 μ M) were added to the non-selective DMEM F-12 and SF-IMX medias used for the derivation and maintenance of Veggie-CHO cells in order to enrich for these components and minimize selection and outgrowth of any DHFR revertants. Although we tried more drastic reduction in serum and IGF-1 we choose to follow a very gradual removal of these in order to maintain the cell viability at over 60% in deriving the VeggieCHO cells. Two other groups (Gandor et al., 1995; Sinacore et al., 1996) reported the adaptation of DXB11-CHO cells to growth in serum-free media. The serum-free cells derived by these groups revert to a DHFR-positive phenotype during prolonged continuous culture in the serum-free media. Both these groups used more drastic removal of serum which resulted in drops in cell viability to 20% or lower. In addition the other investigators did not enrich their serum-free media with as high levels of nucleosides and glycine as we did in deriving the Veggie-CHO cells. Unlike the other groups, we also supplement our media with plant hydrolysates that also provide additional glycine and other nutrients. Any one or combination of these differences

could have helped decrease the selective pressure and help maintain the DHFR-deficient phenotype in the Veggie-CHO cells.

Other investigators have found that previously adapted serum-free cultures reverted to serum-dependent phenotype when cultured in serum-containing media (Yao et al., 1991). The serum-free and suspension growth phenotype of the Veggie-CHO cells is stable after these cells have been maintained for several generations as adherent cultures in serum-containing media. This is demonstrated here in the cloning of the recombinant cell lines in 3% serum-containing media in 96 well-dishes and the subsequent culturing of the clones in serum-free media. Majority of the clones did not require a prolonged adaptation period to growth in serum-free media.

The recombinant Veggie-CHO line expressing FLT-3L can grow and maintain high viabilities in two other plant derived peptones as well as in the complete absence of peptone supplementation in the SF-IMX growth media. Experiments to study protein expression in these other media are under way. The observation that growth and viability of these Veggie-CHO cells are maintained even in the absence of peptone supplement suggests that these cells are not dependent on peptides present in the plant hydrolysates, which could function as growth factors.

To further evaluate their advantage and utility, the Veggie-CHO and the SF-CHO cells have been used as host cells for the stable expression of several other soluble proteins (receptors and ligands). Expression levels (titers) and functional activity of some of these proteins expressed in these serum-free hosts were comparable to those from suspension adapted recombinant lineages generated using the conventional, serum-requiring DXB11-CHO cells. In general, most of the recombinant serum-free CHO transfectant exhibit faster growth and attain higher cell densities when compared to the conventional DXB11-CHO derived, suspension adapted, recombinant cells expressing the same protein. The DXB11:FLT-3L cell line described here however exhibits an atypically long doubling time compared to other DXB11 cell lines. In addition, three other recombinant cell lines derived from the serum-free hosts and expressing different recombinant proteins, have been shown to retain stable recombinant protein expression over 30 passages.

It seems likely that the selection and amplification procedures used to derive clonal Veggie-CHO recombinant lines, i.e. selection and amplification in suspension pool cultures of the transfectants followed by

clone isolation, allow for the selection and out-growth of the fastest growing transfectants. These fastest growing populations may not have the highest levels of specific recombinant protein expression. Hence the best expressing transfectants may be eliminated from the amplified pool before isolation or screening of final clones. The conventional DXB11-CHO cell lines were generated by isolating and screening individual colonies at most selection and amplification steps, thereby allowing transfectants with the best specific production, and often slower growth rates, to be screened and chosen. Cloning transfected Veggie-CHO pools at early stages of the selection and amplification steps may eliminate this bias toward the faster growing transfectants. Examining clones at earlier stages may lead to isolation of Veggie-CHO recombinants with a wider range in their growth and specific protein production phenotypes than we currently observe among the Veggie-CHO isolates derived from amplified pools. This may lead to the isolation of serum-free recombinant cell lines with higher specific production.

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