Production of anti TNF- α antibodies in eukaryotic cells using different combinations of vectors carrying heavy and light chains

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Abstract Tumor necrosis factor- α (TNF- α) plays a key role in rheumatoid arthritis and some other autoimmune diseases. Therapy with anti-TNF- α recombinant antibodies (Ab) appears to be highly effective. Production of new hyper-producing eukaryotic cell lines can decrease the treatment cost, which currently is very high. However, due to the complexity of protein transcription, translation, processing, and secretion in mammalian cells, the stages at which antibody expression is affected are still poorly determined. The aim of this work was to compare the productivity of two cell lines developed in CHO DG44 cells, deficient in dihydrofolate reductase, transfected with vectors carrying either heavy (H) or light (L) chains of chimeric antibody under different combinations of selective elements. Both H and L chains were cloned either in pOptiVEC or pcDNA3.3 vectors and different combinations were used to produce HL and LH cell lines. We have shown that Ab production has been low and

comparable between HL and LH cells until selection on methotrexate (MTX) when LH but not HL cells have responded with 3.5 times increased productivity. Flow cytometry analysis has demonstrated that intracellular concentration of full size Abs in LH cells was 5.6 times higher than in HL ones due to higher amount of H chain synthesis. No differences in viability between HL and LH cells have been found. We have concluded that the expression of H chain in the pOptiVEC vector, which is responsible for MTX resistance, has led to the suppression of H chain synthesis and limitation in full Ab assembly.

Keywords Tumor necrosis factor- α · CHO · DG44 · Antibody · DHFR

Introduction

Tumor necrosis factor (TNF- α) is a pro-inflammatory cytokine produced primarily by immune cells in a soluble form. Besides soluble TNF- α , the transmembrane form of TNF- α demonstrates some unique biologic functions, such as cytotoxic activity and polyclonal B cell activation (Utsumi et al. 1989). Antibodies (Abs) to soluble or membrane-associated forms of TNF- α have provided a new possibility to treat severe forms of rheumatoid and psoriatic arthritis, cutaneous Crohn's disease, psoriasis, and possibly other inflammatory skin diseases like Behcet's disease and pyoderma gangrenosum (Baldi et al. 2005; Khoo and Rubeai 2008; Kohler 1980; Schlatter et al. 2005).

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Several anti-TNF- α antibodies (Etanercept, Adalimumab, Infliximab, Golimumab, Certolizumab) that have the potential to neutralize the soluble form of TNF- α were approved by the U.S. Food and Drug Administration for clinical applications (Jayapal et al. 2005). However, the efficacy of different TNF- α antagonist can vary depending on the disease (Mounach et al. 2013; Sizova 2011). For instance, Etanercept is not clinically effective for the treatment of granulomatous diseases, in which the transmembrane form of TNF- α may play a major role (Li et al. 2005). Therefore, production of new Abs to TNF- α can represent a novel activity to trigger antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and apoptotic or other signaling mechanisms (Dwek 1995; Goochee and Monica 1990; Jenkins and Curling 1994; Li et al. 2005; Utsumi et al. 1989).

Mammalian cell lines are usually the system of choice for the production of recombinant Abs due to their similarity to human cells. Human proteins are not accurately reproduced by other common expression systems such as bacteria or yeasts (Gaillet et al. 2007). Eukaryotic Chinese hamster ovarian cells (CHO) DG44, GS-NS0, HEK293, HEK293-EBNA, and some others are widely used for stable production of recombinant Abs (Gaillet et al. 2007). Of these cells, CHO cells adapted to the growth in suspension in a serum-free condition are preferred by biopharmaceutical companies because of their high proliferation, high antibody production rates, and resistance to mechanical stress. Proteins produced by CHO are correctly glycosylated and folded (Trill et al. 1995), which is important for high protein solubility, activity, and extended blood circulation (Bianchi and McGrew 2003; Schutt et al. 1997; Trill et al. 1995). Stable expression of a targeted gene in eukaryotic cells eliminates the need for transient expression and provides the possibility to optimize the cultivation conditions for scale-up procedures. Production of recombinant Ab by stably transfected cell lines depends on the presence of effective genetic regulatory elements in vectors, selective agents, selection of parental cell line, and culture medium (Page and Sydenham 1991). For some Abs the Ab production can be as high as 2 g/L (Zhou et al. 1997). The highest production in HEK293 cells grown in a serum-free medium ranged from 20 to 1,000 mg/L (Barnes et al. 2001). In GS-NS0 cells, an Ab production level from 40 to 2,700 mg/L was reported (Barnes et al. 2001; Kunert et al. 2000; Zhou et al. 1997). There are several reasons

for such wide variability, including insufficient aeration, lack of free amino acids in the culture medium, and limitations in glucose, glutamine, sodium pyruvate, or growth factors concentration. However, in some cases the efficacy of protein transcription and translation can be predetermined by the selection of the plasmid and the cell line used, as well as by specific features of the protein itself.

The aim of this study was to produce and characterize a pair of new recombinant chimeric anti-human TNF- α Abs developed in CHO DG44 cells deficient in dihydrofolate reductase. For this we used different combinations of vectors pOptiVEC and pcDNA3.3 carrying either heavy or light chains of chimeric Ab. Vector pOptiVEC is responsible for MTX resistance and dihydrofolate reductase activity while pcDNA3.3 carries antibiotic genetecin G418 resistance. Incubation of cells with MTX and G418 provides dual resistance and facilitates Ab heavy and light chains production in the same cell. Antibody folding and assembly pass several checkpoints in the cell to prevent secretion of mis-folded antibodies (Schroder and Kaufman 2005). Due to the complexity of protein transcription, translation, and processing, the stages at which antibody expression is affected are still poorly determined. The selection process in CHO DG44 cells is asymmetrical and includes incubation in the selective medium to restore dihydrofolate reductase activity due to pOptiVEC presence followed by G418 selection mediated by pcDNA3.3; and finally, by MTX selection dependent on pOptiVEC elements. This paper has demonstrated that cloning of light chain in pOptiVEC vector and heavy chain in pcDNA3.3 resulted in cells producing 6–10 times more Ab than in the opposite combination. Cloning by cell sorting and feeding cells with different supplements have additional effect on the productivity of LH cells. By demonstrating the suppressive effect of MTX on the production of heavy chain cloned in pOptiVEC, we hope to better understand how CHO cells regulate exogenous antibody expression.

Materials and methods

Construction of vectors

Variable domains of the heavy and light chains of murine monoclonal F10 Ab directed to human TNF- α

Table 1 Construction of chimeric genes

	Vector	Selective agent	Gene	Construct	Name
1	pcDNA3.3-PL	G418	Light chain	pcDNA 3.3-LC	DNA-L
2	pcDNA3.3-PL	G418	Heavy chain	pcDNA 3.3-HC	DNA-H
3	OptiVEC-PL	MTX	Light chain	pOptiVEC-LC	Opti-L
4	OptiVEC-PL	MTX	Heavy chain	pOptiVEC-HC	Opti-H

were characterized earlier (Radko et al. 2002). To construct chimeric Ab expression plasmids, we used vectors pcDNA3.3-TOPO TA and pOptiVEC-TOPO TA (Invitrogen, Carlsbad, CA, USA) modified by polylinker (PL) to introduce NheI and XhoI restriction sites. PLs were introduced using a synthetic oligonucleotide duplex. New vectors were designated pcDNA3.3-PL and pOptiVEC-PL, respectively. The coding sequence of the light chain variable region was fused with the constant human kappa domain using the splicing by overlapping extension-polymerase chain reaction (SOE-PCR) method (Ho et al. 1989). Restriction sites for NheI and XhoI were introduced, respectively, into 5'- and 3'-termini using specific primers. To obtain a chimeric heavy chain, restriction sites for NheI and ApaI were introduced into the 5'- and 3'-termini of the variable region of the heavy chain, while the XhoI site was incorporated into the 3'-terminus of the constant C_H1-domain of human immunoglobulin G (IgG). For the fusion of variable and constant domains, the endogenous site for ApaI located at the 5'-terminus of the C_H1-domain was used. The genes of the chimeric light and heavy chains were cloned into pcDNA3.3-PL and pOptiVEC-PL using the NheI and XhoI sites. Four constructs containing Ab heavy and light chain genes were prepared (Table 1).

E. coli XL-1 blue cells from Stratagene (La Jolla, CA, USA) were transformed with constructs listed in Table 1 using the Ca²⁺ precipitation method as described (Sambrook et al. 2001). After transfection, cells were grown for 16 h at 37 °C in 200 mL of LB medium (consisting of 10 g/L Trypton (Becton Dickinson, Franklin Lakes, NJ, USA), 5 g/L Yeast Extract (Becton Dickinson) and 10 g/L Sodium Chloride (Helicon, Moscow, Russian Federation) in the presence of ampicillin (JSC Synthesis, Kurgan, Russian Federation). Plasmid DNA was purified using midiPrep columns (Invitrogen). The structure of chimeric Ab genes was verified using DNA sequencing.

Table 2 Development of CHO DG44 cells expressing full-length Abs

	Gene 1	Gene 2	Combination	Name
1	Opti-H	DNA-L	Heavy + light	HL
2	DNA-H	Opti-L	Light + heavy	LH

The abbreviations Opti-H, Opti-L, DNA-H, DNA-L are described in the Table 1

Transfection of CHO DG44 cells

CHO DG44 cells were cultured in 30 mL of CD DG44 medium (Invitrogen, USA) in 125 mL glass Erlenmeyer flasks shaking at 135 rpm on an orbital shaker (ELMI S-3L, Riga, Latvia) in a CO₂-incubator, at 37 °C, using 8 % CO₂. 48 h before transfection the cells were seeded at 3 × 10⁵ cells/mL. This seeding was repeated 24 h before transfection to synchronize the cells. Transfection was performed by Amaxa Nucleofector II (Amaxa, Cologne, Germany) according to manufacturer's protocol (Lonza Cologne Protocol 2009). We used 5 µg of each plasmid to transfect 5 × 10⁶ cells. Different combinations of plasmid constructs carrying heavy and light chain Ab genes were used for the development of stable cell lines (Table 2).

Enzyme-linked immunosorbent assay (ELISA)

The concentration of Abs in conditioned media from transiently transfected cells was determined by ELISA. Flat-bottom, 96-well plates (Costar-Corning, Corning, NY, USA) were coated overnight with 10 µg/mL of human recombinant TNF-α (Shingarova et al. 2010) in phosphate-buffered saline (PBS). All incubations were performed in the presence of 1 % bovine serum albumin BSA (Amresco, Solon, OH, USA) in PBS (B-PBS) at room temperature. Plates were washed between incubations in 0.05 % Tween-20 in PBS (T-PBS). Different dilutions of conditioned medium were incubated for 2 h in B-PBS. After washing, anti-human IgG-horseradish

peroxidase (HRP) (Sorbent Ltd., Moscow, Russian Federation) was added at a 1:25,000 dilution and incubated for 1 h. The color was developed with TMB One (NVO Immunotech, Moscow, Russian Federation). Optical density was determined using the microplate reader Model 680 (Bio-Rad, Hercules, CA, USA). An Ab to TNF- α with a known concentration was used as the reference standard.

Three-step selection process of cells producing Abs to TNF- α

Selection using OptiCHO selective medium

After electroporation the cells were cultivated in the CD DG44 medium for 2 days and then transferred into OptiCHO selective medium (Invitrogen) supplemented with 8 mM of L-glutamine and 0.18 % Pluronic F-68. Cells seeded at 3×10^5 cells/mL in 30 mL of medium in 125 mL Erlenmeyer flasks were cultivated at 8 % CO₂ in a CO₂-incubator under constant shaking. The cell concentration was adjusted to 3×10^5 cells/mL every 72 h. After 2 weeks of cultivation the doubling time of the cell line was around 24 h and cell viability was ~95 %.

Selection using geneticin G418

After the first cycle of cultivation in OptiCHO medium during 2 weeks, cells were subjected to antibiotic selection by the addition of 500 μ g/mL of geneticin G418 (Invitrogen) to the medium. The cells were cultivated as described above.

Selection using MTX

After 2 weeks of cultivation, when cell viability became ~95 %, they were subjected to the last cycle of selection using MTX (UfaVita, Ufa, Russian Federation). The cells were sub-cultivated in the same way as described above every 72 h steadily increasing MTX from 50 to 500 nM as indicated. Some cultures were also tried with 1 μ M MTX.

Enrichment of recombinant cells by cell sorting

To enrich the culture with high Ab producers after the full protocol of selection, the cells were subjected to

cell sorting. To do this, the original protocol (DeMaria et al. 2007) was used with changes. Exponentially growing cultures were collected, washed twice in fresh cold PBS (+4 °C), and stained with anti-human IgG-fluorescein isothiocyanate (FITC) (Santa Cruz, Santa Cruz, CA, USA) for 1 h. Sample analysis and cell sorting were performed on a DiVa digital upgraded FACSVantage SE (Becton–Dickinson, Franklin Lakes, NJ, USA) systematically equipped with a 488 nm laser and a 530/30 nm filter. The sorting procedure was set up with a pressure of 27 psi and a 100- μ m nozzle. The cells with maximal green fluorescence (5 %) were sorted and subsequently seeded in OptiCHO medium at 0.3×10^6 cells/mL with 8 mM of L-Gln, pen/strep (10,000 units/mL of penicillin, 10,000 μ g/mL of streptomycin), and amphotericin (25 μ g/mL of Fungizone) (Sigma, St. Louis, MO, USA). The unstained CHO DG44 cells were used as a control.

Flow cytometry analysis of intracellular H and L chain accumulation

To determine intracellular synthesis of H and L chains in LH and HL cell lines, cells were fixed with 4 % paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37 °C, centrifuged and permeabilized with 0.1 % Triton X-100 (Sigma-Aldrich) in PBS for 30 min. Aliquots were stained with mouse monoclonal anti-human kappa IgG (Polygnost, Saint-Petersburg, Russian Federation) followed by secondary anti-mouse IgG-FITC (SantaCruz) and polyclonal rabbit anti-human Fc-PE (SantaCruz). Unstained or stained with secondary Ab cells were used as controls. Cells were analyzed by FACScan (Becton–Dickinson) equipped with CELLQuest soft. Aliquots of living cells were stained with mouse monoclonal anti-human kappa IgG (Polygnost, Saint-Petersburg, Russian Federation) followed by secondary anti-mouse IgG-FITC (Santa Cruz) and polyclonal rabbit anti-human Fc-PE (Santa Cruz). Unstained or stained living cells with secondary Ab cells were used as controls. Total 5,000 events were collected and analyzed by WinMDI 2.8 free soft. Aliquots of living cells were stained with mouse monoclonal anti-human kappa IgG (Polygnost) followed by secondary anti-mouse IgG-FITC (Santa Cruz) and polyclonal rabbit anti-human Fc-PE (Santa Cruz). Unstained or stained living cells with secondary Ab cells were used as controls. For the comparison live

cells were also labeled by the same pair of antibodies to check the membrane expression of Ab chains.

Feeding additives

To further increase the Ab production rate, yeast extract (5 g/L), D-glucose (4 g/L), or combinations thereof were added to the exponentially growing cultures. The cells were incubated in 8 mM of L-Gln, 0.18 % Pluronic F-68, 500 µg/mL of G418, and 1 µM of MTX in 30 mL of the culture medium in 125-mL Erlenmeyer flasks at 8 % CO₂ in a CO₂-incubator under constant shaking.

Antibody purification

Recombinant Abs from the conditioned medium were purified using the Protein A agarose column (Gibco BRL, Grand Island, NY, USA) according to the manufacturer's instructions (self loaded, 0.5 mL of resin). Briefly, the culture medium was loaded on a pre-equilibrated column, washed with 5 volumes of 50 mM Tris-HCl, pH 7.0 binding buffer, eluted with 700 µL of 100 mM glycine, pH 3.0. The proteins were concentrated 20 times by filtration on Amicon Ultracel centrifuge filters 100 K (Millipore, Billerica, MA, USA).

Gel electrophoresis

Purified Ab were denatured in sodium dodecyl sulfate (SDS)-sample buffer with or without 2-mercaptoethanol (2-ME) and subjected to 10 % SDS-polyacrylamide gel electrophoresis. The gels were stained with PageBlue (Fermentas, Vilnius, Lithuania).

Cell line production quantification

When the doubling time of HL and LH cell lines, treated with different doses of MTX, reached around 24 h with ~95 % cell viability, the cells were used to study maximal production of Ab. For this, cells were seeded at 0.3×10^6 cells/mL in OptiCHO medium and incubated in 8 mM L-Gln, 0.18 % Pluronic F-68, 500 µg/mL of G418 in 30 mL of medium in 125 mL Erlenmeyer flasks, 8 % CO₂ incubator at constant shaking for not <14 days. Supernatants were collected for quantification.

Statistical analysis

Statistical analysis was performed using Student's *t* test. Comparison values of $p < 0.05$ were considered statistically significant.

Results

Double transfection of CHO DG44 with heavy and light chains of F10 Ab against human TNF-α

Before transfection, CHO DG44 cells were grown for several passages in a logarithmic phase to synchronize the culture. Characteristics of transfectants are given in Tables 1 and 2. 48 h after transfection, supernatants of HL and LH cultures (Table 2) were collected for the analysis of recombinant Ab production. Both cultures produced comparable levels of Ab ranging from 1.5 to 2.0 µg/mL, as estimated by ELISA. When the viability of cells reached around 95 %, both cultures were transferred into a hypoxanthine- and thymidine-deficient OptiCHO selective medium and passaged every 4 days. The dynamics of cell selection is shown in Fig. 1a, b. Both HL and LH cultures completed first round of selection (more than 90 % cell viability) at passage 4 (Fig. 1a, b, time point "B") and were subjected to geneticin G418, a selective antibiotic for the pcDNA 3.3 plasmid, selection. The second cycle of selection, completed at passage 13 for both cultures (Fig. 1a, b, time point "C"), was followed by the addition of 50 nM of MTX. Before MTX addition and every time before MTX dose increase a portion of cells was seeded for maximal Ab production as described in Materials and Methods section. The resultant Ab concentrations are shown in Fig. 1c, where time points correspond to C, D, E, F passages and the last one to passage 41 shown in Fig. 1a, b. Production of Ab by the HL and LH cultures was comparable (from 1.5 to 2.0 µg/mL) until the beginning of MTX selection. Both HL and LH cultures increased 10 times Ab production at 50 and 100 nM MTX. Starting from 250 nM of MTX only LH but not HL culture continued to increase Ab production ($p = 0.01$ LH vs HL) (Fig. 1c). Gel electrophoresis of purified recombinant proteins showed the presence of IgG in both cultures (Fig. 1d). As a control, an isolated human Ab pool was used.

Fig. 1 Selection (a, b) and characterization (c, d) of HL and LH cultures. **a, b** Selection of CHO DG44 cell lines transfected with pOptiVEC-HC and pcDNA 3.3-LC (a, HL culture) or pOptiVEC-LC and pcDNA 3.3-HC (b, LH culture) expression constructs (Tables 1–2). Selection protocol: A CD OptiCHO, B CD OptiCHO + G418, C CD OptiCHO + G418 + 50 nM MTX, D CD OptiCHO + G418 + 100 nM MTX, E CD

OptiCHO + G418 + 250 nM MTX, F CD OptiCHO + G418 + 500 nM MTX. **c, d** Antibody production by (c) and PAAG electrophoresis of purified Ab (c) from HL and LH cultures. 10 % SDS-PAGE was run with or without 2-mercaptoethanol (2-ME). Significant differences ($p < 0.05$) are marked with asterisks

Enrichment with high-producing cells by cell sorting

It is known that many cells can express the proteins they produce on the cell surface. We stained live HL and LH cells with anti-human IgG-FITC and showed that both cultures exposed human IgG on their surfaces. Around 5 % of analyzed cells expressed Ab on their surfaces at very high levels (Fig. 2a). We hypothesized that hyper-expressing cells also secreted more IgG into the culture medium. To verify this, we sorted 12 % of the brightly stained cells and estimated the expression of IgG on cell surfaces. It appeared that both before and after sorting LH cultures expressed higher levels of human IgG [mean fluorescence intensity (MFI) was 3.7 and 15.2, accordingly] than HL cultures (2.8 and 9.2) (Fig. 2c, d). Sorting of LH cells resulted in 30 % increase in Ab production while HL production did not change significantly (Fig. 2b).

Negatively selected cells produced less recombinant protein than parental cells (data not shown), indicating that the surface IgG display is proportional to the level of protein production by the cells.

To understand the nature of lower Ab production by HL cells we followed in detail the dynamics of culture growth, cell viability, and the level of Ab production. Both cultures grew in a comparable manner with 95 % of viable cells however HL productivity was much lower in comparison with LH cell cultures (Fig. 3a, b). Thus, we concluded that decreased Ab production in HL cultures was its intrinsic property not associated with the cell death.

Effect of increased MTX and nutrients on cell growth

MTX appeared to have the most significant effect on the productivity of cell lines, so we attempted to

Fig. 2 Cell sorting of HL and LH cell lines. **a** Cells were stained with anti-human IgG-FITC and sorted in P3 and P4 fractions. **b** Antibody production of HL and LH cells before and after first round sorting. **c, d** Histograms of unlabeled (control),

labeled before (blue) and after (yellow) sorting (P3 quadrant) cells from HL (**c**) and LH (**d**) cultures. Significant difference ($p < 0.01$) is marked with *asterisk*

increase MTX concentration up to 1,000 nM. The increase to 1,000 nM of MTX inhibited the growth of HL cultures, both unsorted and sorted (data not shown) while LH cells selected by sorting increased Ab production to 138 % in comparison with 500 nM MTX (Fig. 3c). It is interesting to note that the unsorted LH cells responded to 1,000 nM MTX with slight but significant decrease in protein production (Fig 3c). We hypothesized that this could be a result of variation between cells in multiplicity of recombinant gene copies as we did not observe the decrease in the viability of cells in this culture. The cells with low numbers of copies could be more susceptible to suppressing effect of high dose MTX.

We also tried to increase the yield of Ab by the addition of supplements such as yeast extract, D-glucose, or combinations thereof. Supplementation with yeast extract but not glucose resulted in a 140 % increase in Ab production by LH cells (Fig. 3d).

Intracellular expression of heavy and light chains in HL and LH cultures

Finally we were interested to study the accumulation of light and heavy chains inside HL and LH cells to understand the difference between cultures. For this, stable HL and LH cultures developed after sorting, were permeabilized and stained with Ab specific to light kappa chain and Fc fragment. It appeared that HL cells produced 5.6 times less (9.5 vs 53.4 %, accordingly) amount of heavy chain than LH ones (Fig. 4a, b). The viability and density of both HL and LH cultures were comparable (see Fig. 3a, b). Almost all (96 %) heavy chain molecules were paired with the light chain (double stained) meaning that the availability of a heavy chain is a limiting parameter for Ab synthesis. We also demonstrated that around 99 % of cells in both cultures produced light chain of recombinant Ab (Fig. 4a, b), however the amount of light chain per cell in LH was significantly higher in

Fig. 3 Comparison of quantitative parameters between HL and LH cells. **a, b** Dynamics of cell density and antibody production in LH (**a**) and HL (**b**) cultures. **c** Effect of high dose methotrexate

(MTX) on antibody production in unsorted and sorted LH culture at different stage of MTX selection. **d** Effect of glucose, yeast extract or their combination on Ab production by LH culture

comparison with HL culture (Fig. 4c, d, MFI 1,020 and 150 accordingly).

It is of note that LH cells contained four different populations of cells producing high (6 %, MFI 2,150), medium (21 %, MFI = 430), low (16 %, MFI = 75) and very low (16 %, MFI = 15, negative control: 32 %, MFI = 4) numbers of heavy chain copies (Fig. 4b, circles). It can be hypothesized that this effect corresponds the number of gene copies per cell. A completely different situation was found in HL cells, where only two definitive subpopulations were found: producing high (2 %, MFI = 2,320) and very low (42 %, MFI = 13) (Fig. 4a, circles) number of heavy chain copies. It is evident that there is a regulatory restriction in HL cells preventing gene multiplication when Ab heavy chain is cloned under MTX selective element.

We finally checked the expression of light and heavy Ab chains on the surface of live cells and found

out high light chain expression on LH cells but not HL ones (Fig. 4e, f). Recognition of the heavy chain was minimal and equal in both cell lines.

Discussion

We have shown that the yield of recombinant Ab production in CHO DG44 cells depends on the cis-configuration of the MTX regulatory element inserted in the plasmids coding for H and L chains of Ab. Of the two plasmids used for the construction of full-length Ab to TNF- α , pcDNA 3.3 contains the gene, responsible for the resistance to geneticin G418, while pOptiVEC provides MTX resistance. Unexpectedly and for the first time it was shown that high concentrations of MTX in HL cultures, which contained H chain gene under MTX control, suppressed

Fig. 4 Accumulation of heavy and light chains in HL and LH cultures after MTX selection. **a, b** Dot plots showing intracellular accumulation of human IgG light and heavy chains in HL (**a**) and LH (**b**) permeabilized CHO cells. **c, d** Overlay of histograms of light (kappa-FITC, **c**) and heavy (Fc-PE, **d**) chain expression in HL (grey solid) and LH (dotted line) versus secondary antibody control (solid line). **e, f** Expression of IgG, its heavy and light chains on the surface of HL (**e**) and LH (**f**) live CHO cells

multiplication of H chain gene resulting in low number of recombinant molecules ready to couple with the light chain. Antibodies are heterotetramers consisting of an equimolar ratio of H and L chains. Earlier it was shown that for recombinant Ab production in CHO cells equimolar ratio is not optimal and 2–5 times excess of L chain in stably transfected cells represents a balance between H chain abundance limiting

productivity and the requirement for excess of L chain to render Ab folding and assembly more efficient (Schlatter et al. 2005). Our experiments supported these results by demonstrating that low H chain polypeptide synthesis limits Ab production. Several other publications have studied some effects of H and L chain imbalance on Ab folding and protein production (Gomez et al. 2012; Li et al. 2005; Schlatter et al.

2005; Sung et al. 2004). Li et al. (2005) have demonstrated using bi-cistronic constructs of the encephalo myocarditis virus internal ribosome entry site, a significant increase in Ab production in HEK293T cells when the expression of H chains exceeded that of L ones. The authors stated that this was not true for CHO cells, where the intracellular excess of heavy chain production led to decreased yields of Ab in the supernatant. On the contrary, it was demonstrated (Zhou et al. 1997) that commercial stably selected Ab-producing DHFR-deficient CHO cells (Biogen Idec, USA) produced 1.4–2.0 times more H chains than L ones. In our work, we found that the synthesis of H chain was a limiting factor in Ab production. More important we showed that expression of H chain in the pOptiVEC vector, which contains the MTX regulatory element, severely suppressed gene multiplication. The importance of disproportional H and L chain production for optimal Ab assembly was studied by Davies et al. (2011). The authors used dual or single open reading frame expression vectors to drive H and L chains expression independently or jointly. The analysis of stable transfectants showed that those using the joint expression system significantly reduced Ab production. These results demonstrate that for each cell line preliminary pilot work has to be done to estimate the optimal ratio between H and L chains for the development of a high-producing stable line.

The LH cell line, in which the production of the Ab light chain was controlled by the MTX element, was used to study the effect of high MTX concentrations. The increase to 1 μ M MTX concentration led to 1.4 fold increase in Ab production. These results are in agreement with Jiang et al. (2006), where an increase in the MTX concentration from 50 nM in parental cultures to 3 μ M in daughter cultures corresponded to a 2.2–5.5-time increase in Ab production. In our hands the increase to 1 μ M MTX did increase the Ab production by LH cells, however higher MTX concentrations induced culture death. Our data demonstrated heterogeneity between cells in the production of H polypeptide. It can be hypothesized that further selection of cells with multiple insertions of H chain gene would produce a population more responsive to higher MTX concentration giving higher yield of Ab. To do this high-yield cells expressing recombinant Ab should be isolated by screening multiple individual clones using limiting dilution techniques.

As an alternative to the limiting dilution method, we applied the fluorescence-activated cell sorting (FACS) technology for the selection of the pool of hyper-producing cells. Earlier, Zuberbühler et al. (2009) found that recombinant CHO cells producing Ab can be directly stained using anti-human IgG. The authors explained that “antibodies transiting the membrane of stably transfected cells” are labeled, which indeed is possible. Our experiments supported the data obtained by Zuberbühler et al. both in the number of positive cells and by showing that cells expressing higher amount of IgG on their surfaces later produced higher amounts of recombinant proteins. However, we showed that the enrichment of culture by cell sorting has a limited capacity due to the expression of mostly L chain on the surface of cells while the expression of H chain is more important for the productivity of cells. Our data on the effect of cell sorting showed that second and third cycle of bulk selection did not further increase the yield of Ab. The same results were published by Pichler et al. (2011) where CHO-K1 and CHO-S host cells transiently transfected with human Ab were sorted in bulk cultures by three rounds followed by one round of a single-cell cloning. The productivity of sorted cultures was comparable to that of the parental cells until single-cell cloning was performed. This last cloning resulted in 3 out of 16 clones that produced 2–3 times more Ab.

It is well known that the production of recombinant proteins in serum-free conditions is both desirable and less effective (Kim et al. 2006). To keep the advantages of safe protein production in animal protein free media one can use low-cost hydrolysates such as yeast, soy, wheat gluten, or rice hydrolysates as medium additives (Sung et al. 2004). Of the positive effects of these hydrolysates, those of yeast hydrolysate on human thrombopoietin production in CHO cells were the most significant. Other growth-supporting factors for small-scale cultures include supplementation with glucose and glutamine (Hu et al. 2011). In our case, the addition of yeast extract significantly increased Ab production, while feeding with extra glucose had no effect.

Development of high-yield cells for the production of Ab in serum-free conditions is an important but very time-, labor-, and money-consuming technique. We have demonstrated that a wise selection of cloning vectors for the expression of antibody chains can increase the chances to obtain stable cell lines that produce high quantity of Ab. In spite of the discrepancy

in the opinion of what ratio between the intracellular H and L chains is optimal for protein production, our results showed that L chain is expressed abundantly by all cells while H chain synthesis is a limiting factor. Further attempts to clone LH cells by limiting dilution method will provide more information on H and L chain balance in CHO cells.

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