AsPac J. Mol. Biol. Biotechnol. 2011
Vol. 19 (2) : 63-71

Application of cytotechnology techniques: A case study for the production, purification and characterization of humanized antibody secreted by NS0 transfectoma

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Received March 2011 / Accepted June 2011

Abstract. Humanized monoclonal antibodies are widely used for diagnosis and treatment of cancer due to their reduced immunogenicity and high specificity. These valuable biopharmaceuticals are produced using mammalian cells which often require serum when cultured *in vitro*. Since the use of serum involves many ethical, safety and scientific complications, a high-producing NS0 transfectoma which was isolated using Clone Pix FL system, was directly adapted to serum-free growth media (SFGM) in T75 flasks. This transfectoma has been engineered to stably secrete humanized anti-C2 monoclonal antibody (hum-C2 mab) which is highly specific for a colorectal tumor associated antigen. However, the serum-independent NS0 transfectoma had low cell viability when cultured in spinner flasks. Therefore, triple flasks were used instead. The growth characteristics and also hum-C2 mab productivity were comparable between cells cultured in serum-free and serum-supplemented media. Besides that, an automated liquid chromatography system, Äktaprime Plus, was used to purify hum-C2 mab from filtered and concentrated cell culture supernatant since the conventional method of antibody purification is time-consuming, laborious and prone to errors. The high-throughput nature of Äktaprime Plus enabled the purification hum-C2 mab in less than 30 minutes. In addition, the real-time monitoring and the automated fraction collection of Äktaprime Plus eliminated the need for downstream analysis and decreased the risk of spillage or misplacing of fractions containing precious hum-C2 mab. To evaluate the functionality of hum-C2, a conventional antigen-based ELISA could not be used due to the lack of commercially-available purified C2 antigen. As an alternative, a cell-based ELISA was performed using SW1116 cells and even after humanization, the purified hum-C2 mab was still able to bind to the C2 antigen expressed on the surface of SW1116 cells. In summary, the production of NS0 transfectoma in SFGM using triple flasks and the convenient one-step affinity chromatography using Äktaprime Plus resulted in hum-C2 mab being free from exogenous protein contamination especially from bovine polyclonal antibodies found in serum. From the cell-based ELISA, cell binding was observed which confirms the functionality of purified hum-C2 mab after humanization.

Keywords: Automated antibody purification, Cell-based ELISA, Humanized monoclonal antibodies, Serum-free adaptation, Small-scale antibody production.

Abbreviations: 99mTc-mC2: mouse anti-C2 monoclonal antibodies labeled with Technetium-99m; **ATCC:** American type culture collection; **bIgGs:** bovine polyclonal IgG antibodies; **cat. no.:** catalog number; **DMEM:** Dulbecco's modified eagle media; **ELISA:** enzyme-linked immunosorbent assay; **Fc:** fragment-crystallizable; **hum-C2 mab:** humanized anti-C2 monoclonal antibodies; **IgG1:** immunoglobulin isotype G subclass 1; **KCl:** potassium chloride; **K_2HPO_4:** dipotassium hydrogen orthophosphate; **KH_2HPO_4:** potassium dihydrogen orthophosphate; **kDa:** kilo Dalton; **NaCl:** sodium chloride; **M:** molar; **MWCO:** molecular weight cut-off; **$(NH_4)_2SO_4$:** ammonium sulfate; **nm:** nanometer; **NS0:** myeloma cell line; **NS0-TFA33:** monoclonal NS0-A33 transfectoma; **rcf:** relative centrifugal force; **rpm:** revolutions per minute; **SFM:** serum-free media; **SFGM:** serum-free growth media; **T75:** cell culture flask with 75 cm^2 surface area; **TFF:** tangential flow filtration; **UP:** ultra-pure; **UV:** ultraviolet; **v/v:** volume/volume; **w/v:** weight/volume.

INTRODUCTION

In a recent publication (Dharshanan *et al.*, 2011), we have reported the isolation of monoclonal NS0 transfectomas secreting humanized anti-C2 monoclonal antibodies (hum-C2 mabs) using the Clone Pix FL system. The hum-C2 mabs had been engineered to have lower immunogenicity compared to their precursors which were developed in the mouse through hybridoma technology (Dharshanan *et al.*, 2011; Mateo *et al.*, 2000; Yazaki *et al.*, 2004). The hum-C2 mabs are also of IgG1 isotype and highly specific for a colorectal tumor associated antigen, C2, a novel 145-190 kDa glycoprotein preferentially expressed on the surface of malignant colorectal cells (Iznaga-Escobar *et al.*, 2004).

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When mouse anti-C2 mabs labeled with Technetium-99m (^{99m}Tc-mC2) were used for the diagnosis of ovarian cancer, ^{99m}Tc-mC2 has been confirmed to have very limited binding with normal adult tissues (Solano *et al.*, 2006). It has also been shown to be able to recognize colorectal tumors with 94% sensitivity and 100% specificity (Tejuca *et al.*, 2004).

Initially, these hum-C2 mab producing NS0 transfectoma cells were cultured and maintained in cell culture media containing fetal bovine serum; however the use of serum involves ethical, scientific and safety complications (Even *et al.*, 2006). Therefore, it is crucial to adapt and maintain the transfectoma cells in serum-free growth media. Unfortunately, the versatile function of serum makes its removal from cell culture media a challenging task especially in non-static conditions such as in spinner flasks. Besides that, the purification of mab is usually done using the conventional affinity chromatography method, owing to its low cost. However, this method has two main disadvantages. First, being non-automated in nature, it is labor intensive, time consuming and increases the risk of errors, such as spillage or misplacing of tubes. Second, the lack of real-time monitoring in the traditional method often requires subsequent downstream analysis to determine the exact fractions which contain the purified antibodies. Also, the antigen-based ELISA which is commonly used to evaluate the functionality of humanized antibodies could not be applied for hum-C2 mab due to the absence of a commercially-available purified form of C2 antigen.

Hence, in this case study, we report on the application of cytotechnology techniques to circumvent the disadvantages and limitations associated with the use of serum, spinner flasks and a conventional antibody purification method. First, the use of serum and its attendant complications was eliminated by directly adapting the serum-dependent NS0 transfectoma cells to a commercial serum-free media for hybridoma cells containing a synthetic cholesterol supplement. Then, the low viability of the serum-independent NS0 transfectomas during the production in spinner flasks was overcome by using triple flasks instead. Third, an automated liquid chromatography system, Äktaprime Plus, was used to purify antibodies, replacing the conventional method which is time consuming, laborious and prone to errors. Finally, as an alternative to antigen-based ELISA, a cell-based ELISA was used to evaluate the functionality of hum-C2 mab.

METHODS AND MATERIALS

Isolation of monoclonal NS0 transfectoma secreting hum-C2 mab NS0 transfectomas secreting humanized anti-C2 mab were developed using methods described by Dharshanan *et al.* (2011) and Roque-Navarro *et al.* (2003). Briefly, linearized plasmids coding for humanized heavy and light anti-C2 antibody chains were diluted in sterile water to a final concentration of 2 µg/100 µl. Transfection complexes were formed by adding 16 µl of FuGENE HD transfection reagent (Roche, Germany) and 100 µl of each of the diluted plasmids in a 1.5 ml centrifuge tube. The mixture was then vortexed vigorously for 5 seconds and incubated at room temperature (15-20°C) for 15 minutes. Transfection complexes were added to NS0 cells drop-wise and then incubated for 48 hours. The growth of non-transfected cells was inhibited by the addition L-histidinol dihydrochloride (Sigma-Aldrich, USA).

Transfectomas were then seeded into semi-solid media (Genetix, UK) and incubated for 7 days. In order to identify high-producing transfectomas, the visible clones were imaged using Clone Pix FL (Genetix) and the individual transfectomas with high fluorescent intensity were aspirated with micro-pins controlled by the Clone Pix FL system. The selected transfectomas were dispersed automatically in a 96 well plate containing growth media which consisted of DMEM (Biochrom, Germany), 1% (v/v) of glutamax (Life Technologies, USA), 1% (v/v) of antibiotic/antimycotic (Life Technologies) and 5% (v/v) of serum (Biochrom). The transfectomas were then incubated at 37°C, 5% CO_2 with high humidity and subsequently expanded to T75 flasks (Nunc, USA).

Adaptation of NS0 transfectoma in serum-free media For subsequent downstream applications, monoclonal NS0 A33-transfectoma (NS0-TFA33) was chosen due to its high hum-C2 mab productivity compared with other monoclonal transfectoma clones. To circumvent the problems associated with the use of serum, NS0-TFA33 cells were directly adapted into serum-free media, SGFM. Briefly, NS0-TFA33 cells growing at the exponential phase in serum-supplemented media were dislodged from the surface of a T75 flask by tapping and transferred to a 50 ml centrifuge tube. The cells were pelleted by centrifugation at 1000 rpm for 5 minutes at 25°C. The supernatant was discarded and the cells were re-suspended in a sterile pH 7.4 phosphate buffer (containing a mixture of 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of K_2HPO_4 and 0.24 g of KH_2PO_4 dissolved in 1 L of ultra-pure (UP) water) and centrifuged again at 1000 rpm for 5 minutes at 25°C. The washing step was repeated at least twice to ensure that all previous media which contained serum were completely removed. After that, approximately 2×10^6 of NS0-TFA33 cells were inoculated into new T75 flasks containing 20 ml of SFGM which was made up of hybridoma-SFM (Cat. no. 12045, Life Technologies) and 1% (v/v) synthechol (Cat. no. S5442, Sigma-Aldrich). The transfectomas were then incubated at 37°C with 5% CO_2 to allow the adaptation and growth in a serum-free environment. The viable cell concentration and percentage cell viability in SFGM were determined every 24 hours by Trypan blue dye exclusion assay. In this assay, 10 µl of serum-independent NS0-TFA33 cells were taken and transferred to a 0.6 ml centrifuge tube containing 90 µl of Trypan blue dye (Biochrom). After the mixture was briefly vortexed, 10 µl of the mixture were then loaded onto a haemocytometer and using an inverted microscope, the number of viable and the non-viable cells were determined. Once the serum-independent

NS0-TFA33 cells in T75 flasks had reached a concentration of 3×10^6 cells/ml with ≥90% viability in SFGM even after five passages, the cells were harvested and cryopreserved. For cryopreservation, approximately 3×10^7 of the serum-independent NS0-TFA33 cells were pelleted at 1000 rpm for 5 minutes at 4°C and then dissolved in a serum-free cell freezing media containing DMSO (Cat. no. C6295, Sigma-Aldrich). The mixture was cooled gradually at -80°C using CoolCell (Biocision, USA) and then transferred to liquid nitrogen for long term storage.

Production of NS0 transfectoma in serum-free media Initially, spinner flasks were used for the production of 1 L of the serum-independent NS0-TFA33 cells but despite repeated trials, the viability of the transfectoma decreased rapidly after 48 hours. As a result, 5 triple flasks (Nunc), with a working volume of 200 ml each were used instead. A total of 2×10^7 serum-independent NS0-TFA33 cells were suspended in 200 ml of SFGM and then transferred to a triple flask. The triple flasks were then incubated at 37°C with 5% CO_2 to allow cell multiplication and hum-C2 mab secretion for seven days. The viable cell concentration, percentage cell viability and hum-C2 mab concentration in SFGM were determined every 24 hours using Trypan blue dye exclusion assay and quantitative ELISA (Dharshanan *et al*., 2011). At the end of seven days, all the supernatants were pooled.

Purification of humanized anti-C2 mab The mab in the pooled supernatant was purified by chromatography using the Äktaprime Plus (GE Healthcare, USA) system. First, the cell debris was removed by centrifuging the supernatant in 50 ml centrifuge tubes at 6000 rcf at 4°C for 10 minutes. The clarified supernatant was subjected to tangential flow filtration (TFF) using Vivascience Vivaflow 200 (Sartorius, Germany). During TFF, the supernatant was filtered through a membrane with a molecular weight cut-off (MWCO) value of 50 kDa and also was simultaneously concentrated from 1 L to approximately 50 ml. Protein in the concentrated supernatant was precipitated with ammonium sulfate ($(NH_4)_2SO_4$). Fifty ml of 80% (w/v) concentrated $(NH_4)_2SO_4$ was added in a drop-wise manner to 50 ml of filtered and concentrated cell culture supernatant. The mixture was then incubated at 4°C for 60 minutes with constant stirring.

After 60 minutes, the mixture was centrifuged at 20,000 rcf at 4°C for 15 minutes and the supernatant was carefully discarded. The pellet was dissolved in 3 ml of pH 7.4 phosphate buffer. The $(NH_4)_2SO_4$ was removed by transferring the mixture to dialysis tubing with MWCO value of 50 kDa and was dialyzed against 3 changes of 2 L of UP water. Water was changed at 3 hour intervals. Later, the hum-C2 mab was conditioned to pH 7.4 phosphate buffer by substituting the UP water with 3 L of the phosphate buffer and dialysis was continued at 4°C against 6 changes of buffer, with 3 hours between each change.

Prior to the purification of hum-C2 mab, the HiTrap Protein A HP 1 ml column (GE Healthcare) and Äktaprime Plus system were flushed and calibrated according to the manufacturer's instructions. The hum-C2 mab was then purified through a pre-programmed protocol in Äktaprime Plus system using pH 7.4 phosphate buffer as the binding buffer, UP water as the washing buffer and a commercial IgG Elution Buffer with a pH of 2.8 (Cat. no. 21004, Thermo Fisher Scientific, USA) as the elution buffer. Finally, purified hum-C2 mab was immediately conditioned back to pH 7.4 phosphate buffer by dialysis and stored at -20°C.

Characterization of humanized anti-C2 mab Since it is important to evaluate the functionality of hum-C2 mabs after humanization, a cell-based ELISA was performed using a method described with modifications (Hong *et al*., 2001). SW1116 cells (cat. no. CCL-23, ATCC, USA) were grown at 37°C using Leibovitz's L-15 medium supplemented with 10% fetal bovine serum. For cell-based ELISA, 96 well plates were cultured with 200 µl of 10^4 SW1116 cells/well and was incubated at 37°C for 72 hours. Plates were then washed three times with 200 µl of washing buffer (sterile water containing 0.09% sodium chloride and 0.05% Tween-20) to remove previous media. The cells were then fixed using 200 µl of fixation solution and incubated at room temperature for 5 minutes. The fixation solution was a mixture of acetone and methanol at equal volumes. Wells were washed before being blocked by the addition of 200 µl of blocking buffer (phosphate buffer containing 3% bovine serum albumin (BSA) and 0.1% Tween-20) into each well and incubated at 37°C for 90 minutes. Wells were then washed 3 times with washing buffer.

Next, 100 µl of 200 ng/ml purified hum-C2 mab were added in triplicate to designated wells. Similarly, 100 µl of diluted cell culture supernatants containing 200 ng/ml of mouse-, chimeric-, and humanized anti-C2 mabs which were produced using serum-supplemented media were also used as positive controls. For negative controls, 100 µl of 200 ng/ml immunopure Human IgG (cat. no. 31154, Thermo Fisher Scientific) and immunopure Mouse IgG (cat. no. 31202, Thermo Fisher Scientific) were used. All samples were diluted using pH 7.4 phosphate buffer prior to use. Wells were incubated at 37°C for 90 minutes and washed thrice with washing buffer. Then, 100 µl of diluted secondary antibodies conjugated to peroxidase enzyme: anti-human IgG (cat. no. A6029, Sigma-Aldrich) or anti-mouse IgG (cat. no. A3673, Sigma-Aldrich) were added to the designated well. The anti-human secondary antibody was added to wells with chimeric, humanized and human antibodies, while the anti-mouse secondary antibody was added to wells with mouse antibodies. Both secondary antibodies were diluted to a ratio of 1:2,500 in phosphate buffer before use. The mixtures were again incubated at 37°C for 60 minutes. Unbound secondary antibodies were removed by washing and 100 µl 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) substrate solution were added. The mixture was incubated at room temperature in the absence of light for 30 minutes. Finally, the enzyme-substrate reaction was stopped by adding 50 µl of 0.5 normality (N) of

sulfuric acid and absorbance was measured at 405 nanometer (nm) using a Synergy HT multi-mode microplate reader.

RESULTS AND DISCUSSION

Adaptation of NS0 transfectoma in serum-free media Fetal bovine serum is a complex mixture containing a large number of components such as growth factors, hormones, transport proteins, detoxifying agents, attachments factors and protease inhibitors. It also provides nutrients, purines, pyrimidines, vitamins, trace elements, inorganic and shear protective compounds which are all essential for the growth and support of NS0 cells (Keenan *et al.*, 2006). However, the use of serum is unfavorable for a number of reasons. First, the collection of serum involves animal welfare issues as it is obtained from the unborn calf (Valk *et al.*, 2004). Second, the qualitative and quantitative variation of the serum composition results in batch-to-batch variations, which explains the lack of reproducibility of experiments (Kannan *et al.*, 2009). Third, being a chemically undefined mixture, serum poses a potential source of contaminants and pathogens such as bacterial endotoxins, fungi, mycoplasma, prions and viruses (Even *et al.*, 2006; Falkner *et al.*, 2006), thus rendering any mabs produced unsuitable for human use. In fact, Wessman and Levings (1999) reported that between 20-50% of commercial serum were virus-positive. Fourth, although only 5-10% (v/v) of serum is usually used for mammalian cell culture, it is still very costly as serum accounts for up to 70% of the cost of overall media formulation.

Another major drawback with the use of serum for the production of hum-C2 mab is the high protein content especially from bovine polyclonal IgG antibodies (bIgGs) (Rasmussen *et al.*, 2005). These contaminating bIgGs and other exogenous proteins will subsequently be introduced into the pool of expressed hum-C2 mab and may exceed the concentration of the hum-C2 mab product by five- to several thousand-fold (Even *et al.*, 2007). Using protein A affinity chromatography (Swinnen *et al.*, 2007), it is possible to purify the hum-C2 mab from most contaminating exogenous proteins except for the bIgGs. Protein A has a high affinity to the Fc-region of hum-C2 mab but also a low undesired affinity to the Fc-region of bIgG (Hober *et al.*, 2007; Huse *et al.*, 2002). Thus, even after protein A affinity chromatography, the use of serum during NS0-TFA33 production will result in hum-C2 mab still being contaminated with bIgGs.

Several methods could be employed to circumvent this problem. One method is to perform a two-step chromatography, with protein A as the capture step and ion-exchange (Jiskoot *et al.*, 1991) or hydrophobic interaction (Grunfeld and Moore, 1997) as the second step. Although it is possible to obtain hum-C2 mab with reduced bIgGs contamination by this method (Aybay and Imir, 2000), the increase in the number of purification steps will subsequently lower the yield of hum-C2 mab. Another approach is to employ a single-step chromatography procedure using protein L. This method has been used to purify hum-C2 mab from NS0-TFA33 cells cultured in growth media containing 5% (v/v) serum. Unlike protein A, protein L only binds to the kappa light chains of human antibodies and does not bind to the Fc-region of either human or bovine antibodies. As a result, even with the use of serum-supplemented media, it should be possible to obtain pure hum-C2 mab without the contamination of exogenous proteins or even bIgGs. Unfortunately, although hum-C2 mab consists of two kappa light chains, we were unable to purify hum-C2 mab using protein L affinity chromatography. This may be due to the fact that protein L only recognizes certain subclasses of kappa light chains and not that of hum-C2 mab. The next possible option was to culture the serum-dependent NS0 transfectomas using commercial serum with depleted bIgGs, however this option was unfavorable because serum with depleted bIgGs is costly and issues are still present concerning contamination with hazardous substances associated with the use of serum. In order to overcome these concerns it would be most advantageous to adapt the NS0-TFA33 cells to serum-free culture conditions.

In this study, we have successfully adapted NS0-TFA33 directly from serum-supplemented media into serum-free media, SFGM, which consists of a serum-free media (SFM) for hybridoma cells with the addition of 1% (v/v) synthechol. The cell growth and viability in both original serum supplemented medium as well as SFGM were similar. The NS0-TFA33 initially inoculated at 0.1×10^6 cells/ml, was able to reach to a concentration of 3.25×10^6 cells/ml in just 5 days even after several passages. The fact the serum-dependent NS0-TFA33 could be directly switched from a serum-supplemented medium to a serum-free medium clearly demonstrates the analogy in nutrient components and composition in serum-supplemented media and SFGM required for the growth and support of NS0-TFA33 cells. Synthethol, which is synthetic cholesterol, was added to SFGM because unlike hybridoma cells, NS0 cells are cholesterol-auxotrophic due to the demethylation of lanosterol to C-29 sterols (Seth *et al.*, 2006). While antibiotics had been used previously in serum-supplemented media, it was not added in SFGM because the use of antibiotics may result in a decrease in cell viability of serum-independent NS0-TFA33 cells, most likely due to the absence of certain protective components available in serum (Valk *et al.*, 2010). Furthermore, the addition of antibiotics in SFGM was not necessary because compared to serum-supplemented media SFGM is less susceptible to microbial contamination.

Production of NS0 transfectoma in serum-free media For small-scale production, initially, spinner flasks were employed. Three spinner flasks containing 50 ml of SFGM and one spinner flask containing 50 ml of serum-supplemented media were inoculated with 0.1×10^6 cells/ml of serum-independent and serum-dependent NS0-TFA33 cells respectively. After 48 hours, the cell viability in all three flasks containing SFGM had decreased to approximately 30%, but the cells cultured in serum-supplemented media had 94% of

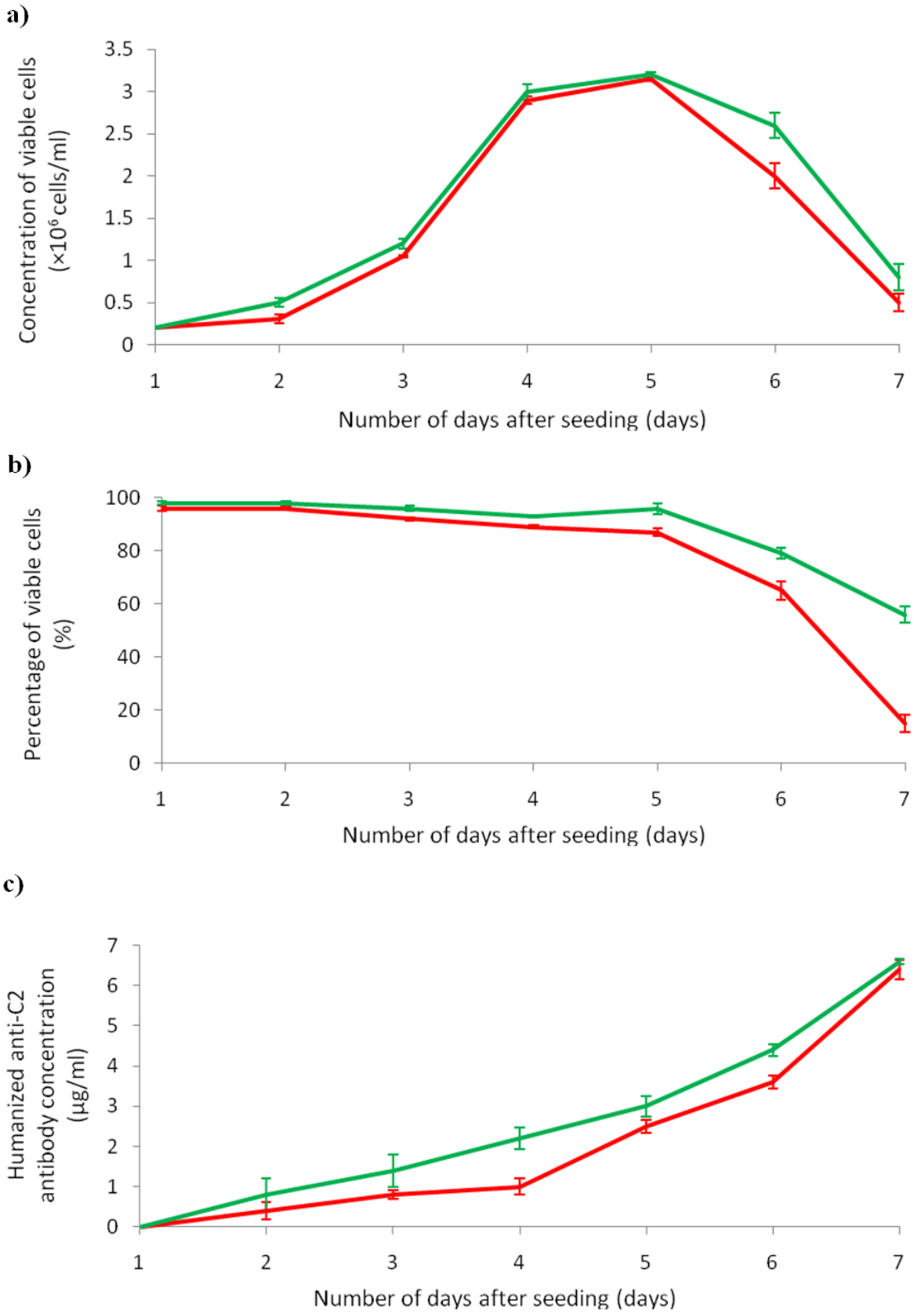

Figure 1. Comparison of growth characteristics and productivity of NS0 A33-transfectomas cultured in the absence (red line) or presence of serum (green line) using triple flasks. The viable cell concentrations (a), percentage of viable cells (b) and concentration of humanized anti-C2 monoclonal antibody in cell culture supernatant (c) of NS0 A33-transfectomas cultured in SFGM were all slightly lower compared to that of cells cultured in serum-supplemented media.

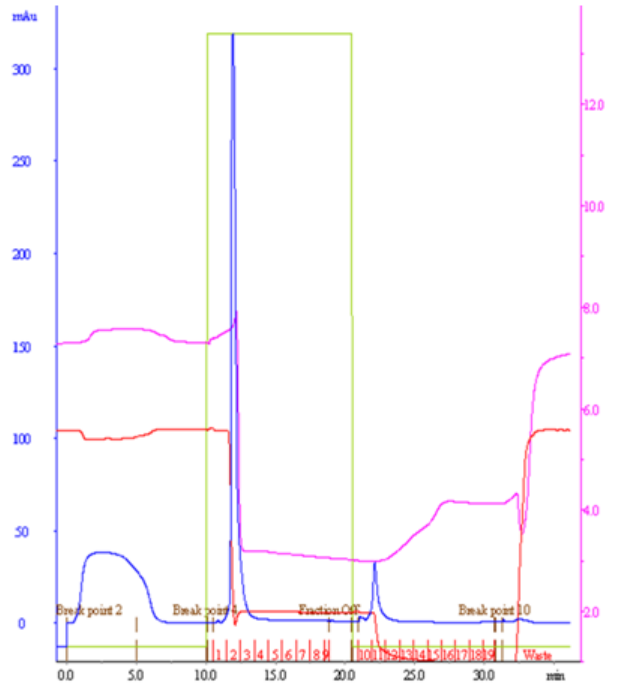

Figure 2. Real-time chromatograms obtained during purification of humanized anti-C2 monoclonal antibodies using Äktaprime Plus. Various parameters such as pH (pink line), conductivity (red line) and UV absorbance at 280 nm (blue line) were displayed by using PrimeView software. The green line depicts the start to end of elution process and together with the built-in pump and fraction collector; the humanized antibodies were purified in just 30 minutes. From the real-time monitoring, the increase in UV absorbance shows that the humanized anti-C2 antibodies are eluted in fraction 2 and 3.

Since all other parameters were constant, SFGM obviously lacked the shear protective compounds present in serum. It was also observed that the use of spinner flasks often resulted in the undesired increase of the temperature in the CO_2 incubator which could be caused by the frictional-heat effect during magnetic agitation.

Therefore, triple flasks which are disposable cell culture vessels each having three growth surfaces totaling 500 cm^2 each, were used instead. Since triple flasks are static cell culture vessels similar to T75 flasks, the shear effect and undesired temperature increase associated with the use of spinner flasks were no longer problems. As expected, the serum-independent NS0-TFA33 cells had only slightly lower viable cell concentration, percentage of viable cells and hum-C2 mab productivity compared to their serum-dependent counterparts when both were cultured in triple flasks (Figure 1). After 7 days, the supernatant from serum-independent NS0-TFA33 were collected because the viable cell concentration and percentage of viable cells had decreased to 0.4 × 10^6 cells/ml and 14% (Figure 1a and 1b). It was felt that further incubation would not significantly increase the concentration of hum-C2 mab in the cell culture supernatants. Although the use of triple flasks might have overcome the shear effect present with the use of spinner flasks, triple flasks are more suitable for small scale production, and for medium and large scale production, stirrer-based bioreactors might still have to be used. Therefore, for medium and large scale production, pluronic, a shear protective compound, might be used to combat the shear effect of spinner flasks on NS0 transfectomas (Whitford, 2003).

Purification of humanized anti-C2 antibody Ammonium sulfate precipitation was done prior to the purification of hum-C2 mab using protein A affinity chromatography. To reduce the total $(NH_4)_2SO_4$ needed the supernatant was concentrated to 50 ml from the original 1 L using TFF. Thus the quantity of $(NH_4)_2SO_4$ required was reduced from 800 g to 40 g.

Äktaprime Plus system was used to purify the hum-C2 mab. Using this system and a HiTrap Protein A HP column, hum-C2 mab in 10 ml of phosphate buffer was purified in less than 30 minutes (Figure 2), rather than around 6 hours if the conventional method were used instead. The significant reduction in the duration was possible because of the use of a built-in pump in Äktaprime Plus that contributed to the high flow-rate, whereas the absence of a pump in the conventional method meant that the flow-rate was entirely dependent on gravitational force. The built-in fraction collector in Äktaprime Plus also allowed the automated collection of fractions during the elution process which minimized the risk of spillage or misplaced tubes which are common during conventional method of antibody purification.

The PrimeView software of Äktaprime Plus system allowed real-time monitoring of the hum-C2 mab as well as providing vital information such as the pH, temperature, conductivity and pressure in the column during the purification process. During purification of hum-C2 mab, the increase in UV absorbance at 280 nm (Figure 2), directly confirmed that hum-C2 mab was eluted in fractions 2 and 3 only: therefore a downstream analysis to determine the antibody containing fractions was not required as in the conventional method.

Characterization of humanized anti-C2 antibody A critical issue concerning humanized antibodies is the possible loss of binding ability after modifications made to reduce its immunogenicity. Therefore, the functionality of the humanized antibodies is usually evaluated using conventional ELISA which requires antigens in purified form. Given the lack of commercially-available purified form of C2 antigen, a cell-based ELISA was used instead. This assay uses SW1116 cells which are colorectal adenocarcinoma cells expressing the C2 antigen on its surface as the capture antigen in what is otherwise a conventional antigen-based ELISA.

Since SW1116 cells are also adherent cell type, live SW1116 cells were used initially to avoid potential alteration of the epitope on the C2-antigen caused by drying or fixation (Hong *et al.*, 2001). Although cell binding was observed, the variation in binding of the triplicate wells with live cells was significantly higher compared to the situation where fixed SW1116 cells were used. Microscopic examina-

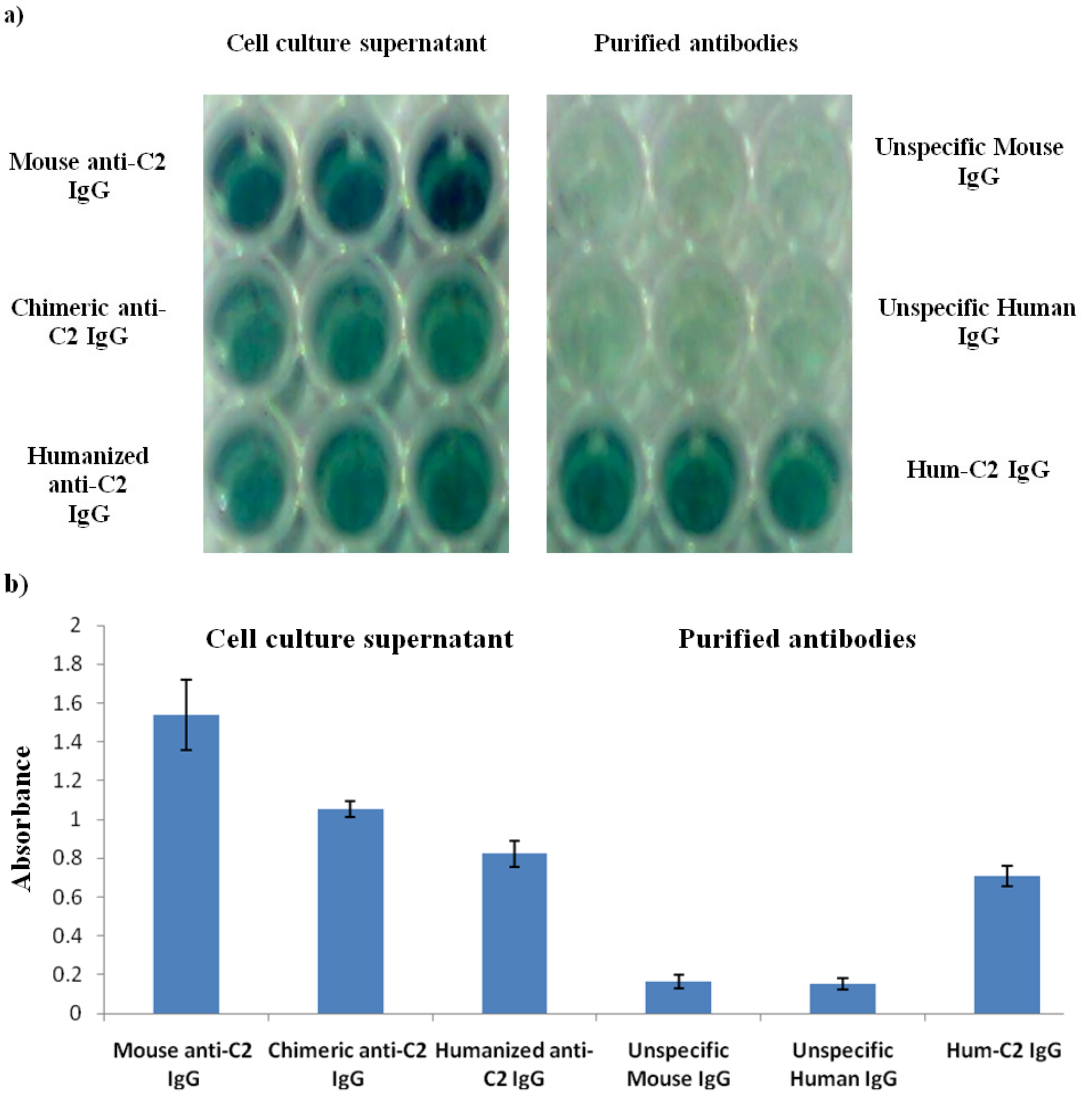

Figure 3. Cell-based ELISA using SW1116 cells to evaluate the functionality of purified humanized anti-C2 antibodies. The color intensity (a) and the absorbance value (b) of the enzyme-substrate reaction were used as indicators for the functionality of the antibodies. Cell culture supernatant containing mouse- chimeric- and humanized anti-C2 were used as positive controls. Unspecific human and mouse antibodies were use as negative controls. The purified (Hum-C2 IgG) and unpurified (Humanized anti-C2 IgG) form of hum-C2 mabs were still able to bind to C2 antigen expressed on SW1116 cells. The mouse anti-C2 antibody has the highest affinity for C2 antigen followed by chimeric anti-C2 antibody, while both unspecific antibodies do not bind to C2-antigen.

tion revealed that significant loss of non-fixed live SW1116 cells occurred during washing. In contrast, the use of fixation solution may have anchored cells firmly in the wells, but still preserved the antigenicity of C2 antigen and therefore decrease the experimental deviation for consistent and reproducible cell-binding ELISA results (Yang *et al.*, 2003).

From the cell-based ELISA (Figure 3), the absorbance value and color intensities of wells with both hum-C2 mabs in culture supernatant and in purified form were higher compared to wells containing antibodies used as negative controls. Nevertheless, in comparison to wells with mouse anti-C2 mabs, both forms of hum-C2 mabs had lower absorbance values. Similarly, chimeric anti-C2 mabs also had lower absorbance values than mouse anti-C2 mabs.

This could have been caused by a reduced affinity of hum-C2 mab and chimeric anti-C2 mab after modification (Roque-Navarro *et al.*, 2003), or the fact that the anti-mouse secondary antibody may have higher affinity to the γ-chain of mouse Fc region compared to that of the anti-human secondary antibody to the γ-chain of human Fc region found in chimeric or humanized anti-C2 mabs. However, the results show that the hum-C2 mabs generated in serum-free growth media by the procedures described still retains considerable functionality and will be useful for consideration for clinical use.

CONCLUSION

In this study, we have demonstrated that it is possible to directly adapt a serum-dependent mab secreting NS0 cells

into serum-free environment using SFGM and that the mabs produced by these cells still retained functionality. Although the serum-independent NS0 transfectomas were able to grow reasonably well in SFGM using T75 flasks, they failed to grow in spinner flasks. Therefore, for small-scale production of hum-C2 mabs, the serum-independent NS0 transfectomas were cultured using triple flasks. The use of Äktaprime Plus system also enabled the purification of hum-C2 mab to be carried out smoothly, effectively and in much shorter time compared to the conventional method of antibody purification. Since the lack of a commercially-available purified C2 antigen prelude the use of conventional antigen-based ELISA for functional studies of hum-C2 mabs, a cell-based ELISA was used instead. Using SW1116 cells which express C2 antigen on its surface, cell binding was observed; this confirms that the purified hum-C2 mab is still functional even after humanization.

ACKNOWLEDGEMENTS

We would like to thank Mr. Junaid Muneer Raja, Mr. Nur Halis Isa, Mrs. Ong Sheau Ching and Ms. Nor Shahida Mat Su for their valuable suggestions and comments. This work was fully funded by Ministry of Science, Technology and Innovation (MOSTI), Malaysia.

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