CHAPTER VIII

OVERALL CONCLUSION

8.1 SUMMARY

In this project, we aimed to develop procedures to produce, with high efficiency in terms of continuity of production, lowered cost and increased speed, humanized mAbs against C2-antigen (hum-C2 mAbs), a marker specifically expressed in colorectal and ovarian cancer, from mAb of mouse origin. The resulting hum-C2 mAbs must still retain their original specificity and functionality, but possess reduced immunogenicity. Two methods of antibody humanization were used to identify potential immunogenic mouse amino acids in the variable region: a deimmunization method and a logical approach method.

The deimmunization method (H1C2 mAb) uses both IgBLAST software and a computer algorithm, AMPHI, while the logical approach method (H2C2 mAb) uses only IgBLAST software to predict the potential immunogenic amino acids in mouse variable regions and then judicious replacements of those few amino acids residues by the corresponding residues from the highest homologous human sequences.

The respective mouse amino acids were then replaced with their corresponding human residues by overlapping-PCR mutagenesis. The DNA sequences coding the humanized variable regions from both humanization methods were then cloned into monocistronic pAH4602 and pAG4622 expression vectors and transfected into NS0 cells.

Although limiting dilution method is routinely used to isolate monoclonal NS0 cells secreting high levels of mAbs, however it was not used in this research. This is due to the fact that the limiting dilution method is time-consuming, has low probability of monoclonality and is significantly limited by the number of clones that can be feasibly screened. In order to have a reasonable probability of obtaining high producer clones which are extremely rare, a high number of transfected clones are required for screening, which will not be possible using the low-throughput limiting dilution method.

Therefore to minimize the duration and to increase the probability of obtaining high-producing clones with high degree of monoclonality, an automated colony picker, ClonePix FL system was used to replace limiting dilution method. Using ClonePix FL system, 1×10^5 clones were screened and the producer clones with different exterior fluorescent intensities were automatically isolated in just 7 days. Rare high-producers (> 3000 FU) with frequency of as low as 0.003% of the population were able to be isolated.

These rare producers require serum when cultured *in vitro* and since the use of serum involves many ethical, safety and scientific complications, high-producing NS0 transfectomas which were isolated using ClonePix FL system, were directly adapted to serum-free growth media (SFGM) in T75 flasks.

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 mAbs 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 of hum-C2 mAbs in less than 30 minutes.

In addition, the real-time monitoring and the automated fraction collection procedure of Äktaprime Plus eliminated the need for downstream analysis and decreased the risk of spillage or misplacing of fractions containing precious hum-C2 mAbs. Thus, the production of NS0 transfectoma in SFGM using triple flasks and the convenient one-step affinity chromatography using Äktaprime Plus, resulted in the purified hum-C2 mAbs being free from contamination with exogenous proteins especially from bovine polyclonal antibodies found in serum.

To evaluate the functionality of H1C2 and H2C2 mAbs, 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 the observation of cell-binding to the C2-antigen expressed on the surface of SW1116 cells confirmed the functionality of purified H1C2 and H2C2 mAbs developed using both the deimmunization and the logical approach method respectively.

In terms of immunogenicity, hum-C2 mAbs developed from both humanization methods also have reduced immunogenicity compared to mouse anti-C2 mAb in *Macaca fascicularis*. However, only H1C2 mAb which was developed by

deimmunization method had lower immunogenicity in *Macaca fascicularis* compared to chimeric anti-C2 mAb and humanized mAb (H2C2 mAb) produced by the logical approach method. This clearly indicated the superiority of deimmunization method and demonstrated the fact that it could not be substituted by the logical approach method.

Nevertheless, the use of overlapping-PCR mutagenesis to humanize mouse residues in the deimmunization method frequently results in undesired mutations which causes the method to be time-consuming and thus is not cost-effective. Therefore, we substituted the conventional protocol of the deimmunization method with a method using synthetic DNA coding for the H1C2 mAb variable regions which were then transfected into NS0 and CHO mammalian cells using pFUSE and UCOE expression vectors. The productivity of H1C2 mAb in NS0 and CHO cells using the two different expression vectors were then compared.

First, it was found that the use of synthetic DNA coding the variable regions of hum-C2 mAbs provides a convenient and fast route to develop H1C2 mAbs which were still functional. Second, irrespective of the vector DNA size, the linearization of vectors increases the transfection efficiency; however, the restriction endonuclease must be chosen with careful consideration. Third, pFUSE expression vectors work well in NS0 cells and UCOE expression vectors work well in CHO cells. Fourth, it is advisable to use two different selection antibiotics for co-transfection using two monocistronic vectors, in order to minimize the probability of transfectomas expressing only the light chain of the antibody. Finally, the use of UCOE vectors in CHO cells results in more high-producing and stable clones compared to the use of pFUSE vectors in NS0 cells.

It was also found that the level of expression of H1C2 mAbs in CHO cells using UCOE vectors was 100 times higher than NS0 cells transfected with pFUSE vectors. Since the majority of the cells are also stable high-producers, therefore using UCOE vectors it is much easier and faster to find high-yielding clones with the productivity and stability required for manufacturing, as opposed to the 'needle-in-a-haystack' approach using conventional vectors. Hence, it was concluded that the best approach to produce humanized anti-C2 mAbs is by using synthetic DNA coding the variable regions which were then cloned into UCOE expression vectors and transfected in CHO cells. In addition, to our knowledge, this is the first time humanized anti-C2 mAb was expressed in CHO cells.

8.2 CONCLUSION

In conclusion, this research has met all the general and specific objectives. Procedures to produce humanized monoclonal antibodies against C2-antigen from monoclonal antibody of mouse origin with high efficiency in terms continuity of production, lowered cost and increased speed had been successfully developed. Humanized anti-C2 mAbs had been successfully generated using two humanization methods. Knowledge and expertise of using an automated and high-throughput selection system, ClonePix FL for the selection of mammalian transfectoma cells secreting high quantity of humanized anti-C2 mAbs had also been acquired.

Cytotechnology techniques were also successfully applied for the serum-free adaptation of transfectoma cells, and also for the production, purification and characterization of humanized anti-C2 mAb developed by both deimmunization and logical approach

methods. It was found that for the development of humanized anti-C2 mAbs with reduced immunogenicity and retained functionality, the deimmunization method is superior compared to the logical approach method.

Although all initial objectives had been fulfilled, another objective was added for the improvement of the current procedures. Experiments were carried out to improve on the productivity of H1C2 mAbs and also to overcome the limitation of the deimmunization method by using the deimmunization method but employing synthetic DNAs instead which is less time-consuming and more convenient compared to overlapping-PCR mutagenesis method used previously. To improve the productivity of H1C2 mAbs, these synthetic genes were then cloned into new expression vectors, pFUSE and UCOE expression vectors and expressed in NS0 and CHO mammalian cells.

The productivity of H1C2 mAb expressed using UCOE vectors in CHO cells, which is the preferred cell line for large-scale production of therapeutic proteins, was at least 100 times higher compared to that of using the earlier procedures. The use of ClonePix FL and Äktaprime Plus systems also allows the isolation of high-producing transfectomas and purification of humanized mAbs to be performed efficiently and quickly. Hence, H1C2 mAbs and various other recombinant proteins could now be conveniently and rapidly produced at large-scale at a reduced cost and duration.

8.3 FUTURE RESEARCH

The immediate next course of action is to deliver the CHO transfectoma secreting the high level of H1C2 mAbs to Process Science Development, Inno Biologics, Malaysia,

for the downstream process optimization to increase the viability and growth of the cells in bioreactors. Once the optimizations are completed, using the state-of-the-art and current Good Manufacturing Practises (cGMP) expertise in Inno Biologics which also comply with European Medical Medical Agency and United States Food and Drug Administration regulatory requirements, a large-scale production and purification of H1C2 mAbs will be carried out. To evaluate and confirm the specificity of H1C2 and H2C2 mAbs towards C2 antigen expressed on SW1116 colorectal carcinoma, other various cell lines: breast carcinoma, melanoma, lung carcinoma, B-cell lymphoma, T-cell leukemia and even peripheral mononuclear cells could be used as controls. The H1C2 mAbs will then be sent for preclinical studies, followed by clinical phase 1, phase 2 and phase 3, and if proven to be effective and safe, it will be used for the diagnosis and/or treatment of colorectal and ovarian cancer patients.