CHAPTER VI

CHARACTERIZATION OF HUMANIZED MONOCLONAL ANTIBODIES DEVELOPED USING DEIMMUNIZATION METHOD AND LOGICAL APPROACH METHOD: BINDING STUDIES *IN VITRO* USING CELL-BASED ELISA AND IMMUNOGENICITY STUDIES *IN VIVO* IN NON-HUMAN PRIMATES

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6.1 OBJECTIVE

The main objective of this chapter is to compare the efficiency of both deimmunization and logical approach methods to develop humanized anti-C2 mAbs having reduced immunogenicity and are still able to bind to C2-antigen expressed on colorectal cancer cells. The H2C2 mAb was developed using logical approach method with monocistronic humanized-pAH4602B and humanized-pAG4622B expression vectors generated from chapter 3 and co-transfected into NS0 mammalian cells.

The transfectomas producing high levels of H2C2 mAbs were isolated using ClonePix FL system and were purified using Äktaprime plus system as described in chapter 4 and 5, respectively. The binding of both HIC2 and H2C2 mAbs to C2-antigen were evaluated *in vitro* by cell-based ELISA using SW1116 colorectal carcinoma cells, while the immunogenicity of both humanized mAbs were evaluated *in vivo* using non-human primates *Macaca fascicularis*. Based on the analysis of the functionality and immunogenicity of H1C2 and H2C2 mAbs, the efficiency of deimmunization method and logical approach method for the development of humanized anti-C2 mAbs were compared.

6.2 INTRODUCTION

The use of monoclonal antibodies generated from hybridoma technology for diagnosis and treatment of human cancers has been the subject of intense research for many years (Zafir-Lavie et al. 2007). Unfortunately, since mouse splenocytes are used, the resulting mouse mAbs are likely to induce AAR, known as HAMA response when administered in humans (Hwang & Foote, 2005).

Although the use of chimeric mAbs may reduce the AAR, they still contain one third of antibody of mouse origin, and could still induce a significant AAR, known as HACA. On the other hand, humanized mAbs which are commonly developed by complementary-determining regions (CDR)-grafting method may have negligible AAR but are often non-functional due to the loss of antigen-binding function.

This is because some non-CDR mouse residues in the framework are still required for the effective binding of humanized mAbs and injudicious substitution of critical residues that maintain the CDR conformation required for specific binding to antigen may cause loss of binding ability (Mateo et al. 2000). Consequently, tedious and laborious back mutations are required to restore their functionality.

Therefore, in the process of humanization it would be desirable to substitute mouse amino acid residues in the variable region as judiciously as possible. A high degree of homology in the mouse and human framework will ensure that the CDR will have the greatest chance of retaining the binding properties. However, there will still be a certain degree of amino acid mismatch in these frameworks between human and mouse antibodies, which may give rise to AAR.

Thus careful consideration of strategic substitution of these mismatched residues is needed for the humanized mAb to maintain binding affinity and at the same time induce minimum AAR when being administered in humans.

6.3 MATERIALS AND METHODS

The H1C1 and H2C2 mAbs which were purified using Äktaprime plus system as described in chapter 5 were used. The H1C1 mAb was developed using deimmunization method while H2C2 mAb was by logical approach method.

6.3.1 Characterization of mAbs

6.3.1.1 SDS-PAGE

All purified mAbs were analyzed by SDS-PAGE under reducing and non-reducing conditions according to Laemmli (1970). The SDS-PAGE gel consisted of a 10% (v/v) separating gel topped by a 5% (v/v) stacking gel. Full range Rainbow marker (GE Healthcare, USA) was used as a reference for the estimation of the molecular weight of the protein bands.

6.3.1.2 *In vitro* binding studies

6.3.1.2.1 Immunofluorescence cell-based assay

Since it is critical 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). The SW1116 cell line (cat. no.: CCL-23, ATCC, USA) which expresses the C2-antigen on its cell surface was used. The cell line was grown at 37ºC using Leibovitz's L-15 medium (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (Biochrom, Germany).

For the preparation of fixed cell-based ELISA, 96 well plates were cultured with 200 µl of 10^4 SW1116 cells/well and incubated at 37ºC 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 is a mixture of acetone and methanol at equal volumes.

Wells were washed before they were blocked by the addition of 200 µl of blocking buffer (pH 7.4 phosphate buffer (1 M) containing 3% bovine serum albumin (BSA) and 0.1% tween-20) into each well and incubated at 37ºC for 90 minutes. Wells were then washed 3 times with washing buffer.

Next, 100 µl of 200 ng/ml purified H1C2 and H2C2 mAbs were added in triplicate to designated wells. For positive controls, wells were reacted with 100 µl of 200 ng/ml of mouse and chimeric anti-C2 mAbs. For negative controls, 100 µl of 200 ng/ml

immunopure Human IgG (cat. no.: 31154, Thermo Fisher Scientific, USA) and immunopure Mouse IgG (cat. no.: 31202, Thermo Fisher Scientific, USA) were added. All samples were diluted using pH 7.4 phosphate buffer (1 M) prior to use. Wells were incubated at 37ºC for 90 minutes and washed thrice with washing buffer, and binding of primary antibody was assessed using appropriate FITC-conjugated secondary antibodies. Anti-human secondary antibody was added to wells reacted with chimeric, humanized and human antibodies while anti-mouse secondary antibody was added to wells reacted with mouse antibodies.

The secondary antibodies used were diluted to a ratio of 1:64 in phosphate buffer before use. Then, 100 µl of diluted secondary antibodies conjugated to FITC: anti-human detection agent-FITC (cat. no.: K8200, Molecular Devices, USA) or anti-mouse detection agent-FITC (cat. no.: K8220, Molecular Devices, USA) were added.

The mixtures were incubated at 37ºC for 60 minutes and unbound secondary antibodies were removed by washing with washing buffer before 50 µl of sterile water were added. The cell binding was visualized under a fluorescence microscope (Nikon, USA).

6.3.1.2.2 Competitive cell-based ELISA

To evaluate the affinity of the humanized mAb to C2 antigen, a competitive cell-based ELISA was performed. The fixation of SW1116 cells, blocking and washing of the plates were similarly performed as described in immunofluorescence cell-based assay. Next, 50 µl of 0.1, 1.0 and 10.0 µg/ml of purified H1C2, H2C2 and QC2 mAbs were each mixed with 50 µl of 1.0 µg/ml of purified MC2 mAb. The mixtures were then added in triplicate to designated wells. For negative control, immunopure Human IgG

was used. The plates were incubated at 37°C for 90 min. Wells were washed 3 times with washing buffer and 100 µl of diluted secondary antibody: anti-mouse IgG conjugated to peroxidase enzyme (cat. no. A3673, Sigma-Aldrich) were added to each well. The secondary antibody used is specific to the γ-chain of mouse mAbs and was diluted to a ratio of 1:2500 in phosphate buffer before use. The mixtures were again incubated at 37°C for 60 min. Unbound secondary antibodies were removed by washing, and 100 µl ABTS substrate solution (cat. no. 11112422001 and 11112597001, Roche) were added. The mixture was incubated at room temperature in the absence of light for 30 min. Finally, the enzyme-substrate reaction was stopped by adding 50 µl of 0.5 M of sulfuric acid and absorbance was measured at 405 nm using a Synergy HT multi-mode microplate reader (BioTek, Winooski, Vermont, USA).

6.3.1.3 *In vivo* immunogenicity studies

To predict the immunogenicity of H1C2 and H2C2 mAbs in humans, non-human primates, macaque monkeys (*Macacca fascicularis)* were used. The use of monkeys for this study was approved by the Animal Care and Use Committee (ACUC), Laboratory Animal Science Centre, Faculty of Medicine, University of Malaya and granted ACUC ethics number: FIS/14/07/2010/SD (R) (Appendix G). All procedures were performed on anesthetized *M. fascicularis* under the supervision of a veterinarian.

6.3.1.4 Immunization and blood collection of *M. fascicularis*

Five captive-born monkeys between 2-4 years old were obtained from Laboratory Animal Science Centre, University of Malaya. Each monkey was randomly chosen to be immunized with either with H1C2, H2C2, QC2, MC2 mAbs or placebo. For the first

immunization, 0.2 mg mAb emulsified in 1 ml of complete Freund's adjuvant (CFA) (Sigma-Aldrich, USA) were injected into each monkey. For subsequent booster immunizations, CFA was replaced with incomplete Freund's adjuvant (IFA) (Sigma-Aldrich, USA). Placebo consisted of adjuvants emulsified in the absence of mAb. Antibodies or placebo were injected intradermically at 5 different spots with approximately 0.2 ml per spot on day 0, 14, 28 and 42.

Prior to each immunization, monkeys were anaesthetized with Zoletil (Virbac, USA) at a dose of 8 mg/kg via intramuscular injection. Then, the mass of each monkey was measured and blood was collected on day 0, 14, 28, 42 and 56 from the cephalic vein using needle and syringe. Blood was immediately transferred to EDTA-treated tubes, inverted several times and centrifuged at 2000 rpm for 5 minutes at 4ºC. The plasma samples were then aliquoted into small volumes into PCR tubes and stored at -20ºC.

6.3.1.5 Measurement of monkeys' anti-antibody response

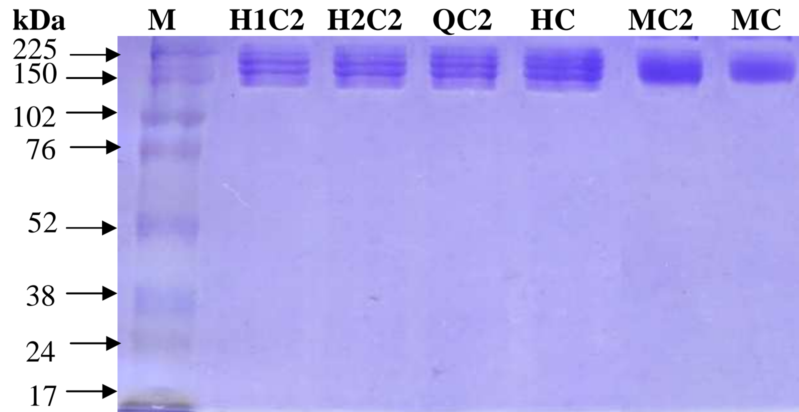
The AAR responses were determined by the measurement of blood IgM and IgG levels employing ELISA methods described in chapter 4 (section 4.3.5) with modifications. Briefly, purified MC2 mAb (1 µg/ml) or purified QC2 mAb (1 µg/ml) were used as capture antibodies, anti-monkey IgM (cat. no.: 617-103-007, Rockland Immunochemicals, USA) and anti-monkey IgG (cat. no.: A2054, Sigma-Aldrich, USA) were used as the secondary antibodies to detect IgM and IgG levels, respectively. Both secondary antibodies are conjugated to peroxidase and were diluted to a ratio of 1:2500 in phosphate buffer before use. Monkey plasma samples were diluted to a ratio of 1:2500 and 1:5000 in phosphate buffer.

6.4 RESULTS

6.4.1 SDS-PAGE

From the SDS-PAGE analysis, bands of approximately 150 kDa were stained for all mAbs under non-reducing conditions (Figure 6.1a). Under reducing conditions (Figure 6.1b), two bands of approximately 55 and 25 kDa, corresponding to VH and VL, respectively, were identified for all mAbs. No other additional bands were detected under both non-reducing (Figure 6.1a) and reducing conditions (Figure 6.1b), indicating that all the purified mAbs were not contaminated with significant quantities of endogenous or exogenous proteins. Since mAbs were produced in serum-free environment as described in chapter 5, all the purified mAbs were free from bovine polyclonal IgG antibody contamination (Dharshanan et al. 2011b).

a)



b)

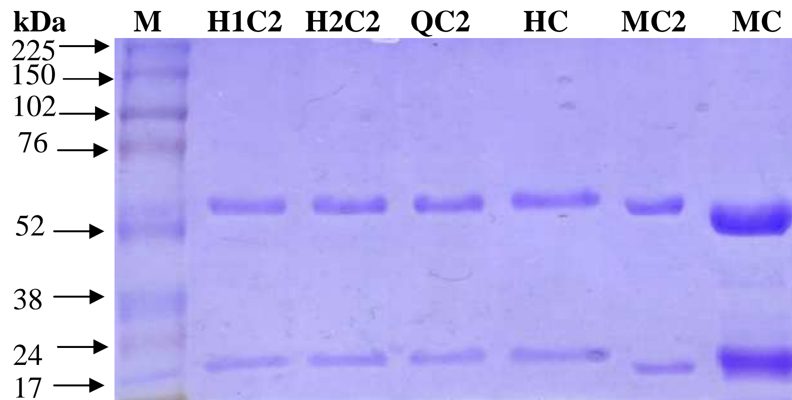


Figure 6.1: *In vitro* characterization of purified mAbs using SDS-PAGE under non-reducing (a) and reducing conditions (b). From the SDS-PAGE analysis, bands of approximately 150 kDa were stained for all mAbs under non-reducing conditions. Under reducing conditions, two bands of approximately 55 and 25 kDa, corresponding to the VH and VL, were identified for all mAbs. No additional bands were detected under both conditions.

6.4.2 *In vitro* binding studies

6.4.2.1 Immunofluorescence cell-based assay

A critical issue concerning humanized antibodies is the possible loss of binding ability after modifications are made to reduce their 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 cell which is colorectal adenocarcinoma cell-line expressing C2-antigen on its surface as the capture antigen in what is otherwise a conventional antigen-based ELISA.

Since SW1116 cells are adherent cells, 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 examination revealed that significant loss of non-fixed live SW1116 cells occurred during washing. In contrast, the use of fixation solution might have anchored cells firmly in the wells, but still preserved the structure of C2-antigen and therefore decreased the experimental variation for consistent and reproducible cell-binding ELISA results (Yang et al. 2003).

From the cell-based ELISA (Figure 6.2) with H1C2, H2C2, QC2 and MC2 mAbs, high fluorescence images were obtained compared to wells where unspecific human and mouse antibodies were used. Intense fluorescence were seen in wells incubated with H1C1 and H2C2 mAbs, as with QC2 and MC2 mAbs.

This shows that both H1C2 and H2C2 mAbs were still able to bind to C2-antigen expressed on SW1116 cells. This is to be expected because no humanization was done on mouse residues in the CDR or Vernier zone which are both important for antigen recognition (Mateo et al. 2000).

6.4.2.2 Competitive cell-based ELISA

Figure 4 compares the immunoreactivity of H1C2, H2C2 and QC2 mAbs to C2 antigen expressed on SW1116 colorectal carcinoma. Competitive ELISA showed that MC2 mAb was inhibited in a dose-dependent manner by increasing concentrations of chimeric and humanized anti-C2 mAbs. Half displacement concentrations of H1C2, H2C2 and QC2 mAbs were 1.6, 1.7 and 0.85 g/ml, respectively, (Figure 4). Thus, the mutations introduced in the variable region of humanized anti-C2 mAbs provoked approximately 50% reduction in binding affinity for both H1C2 and H2C2 mAbs.

To evaluate 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.

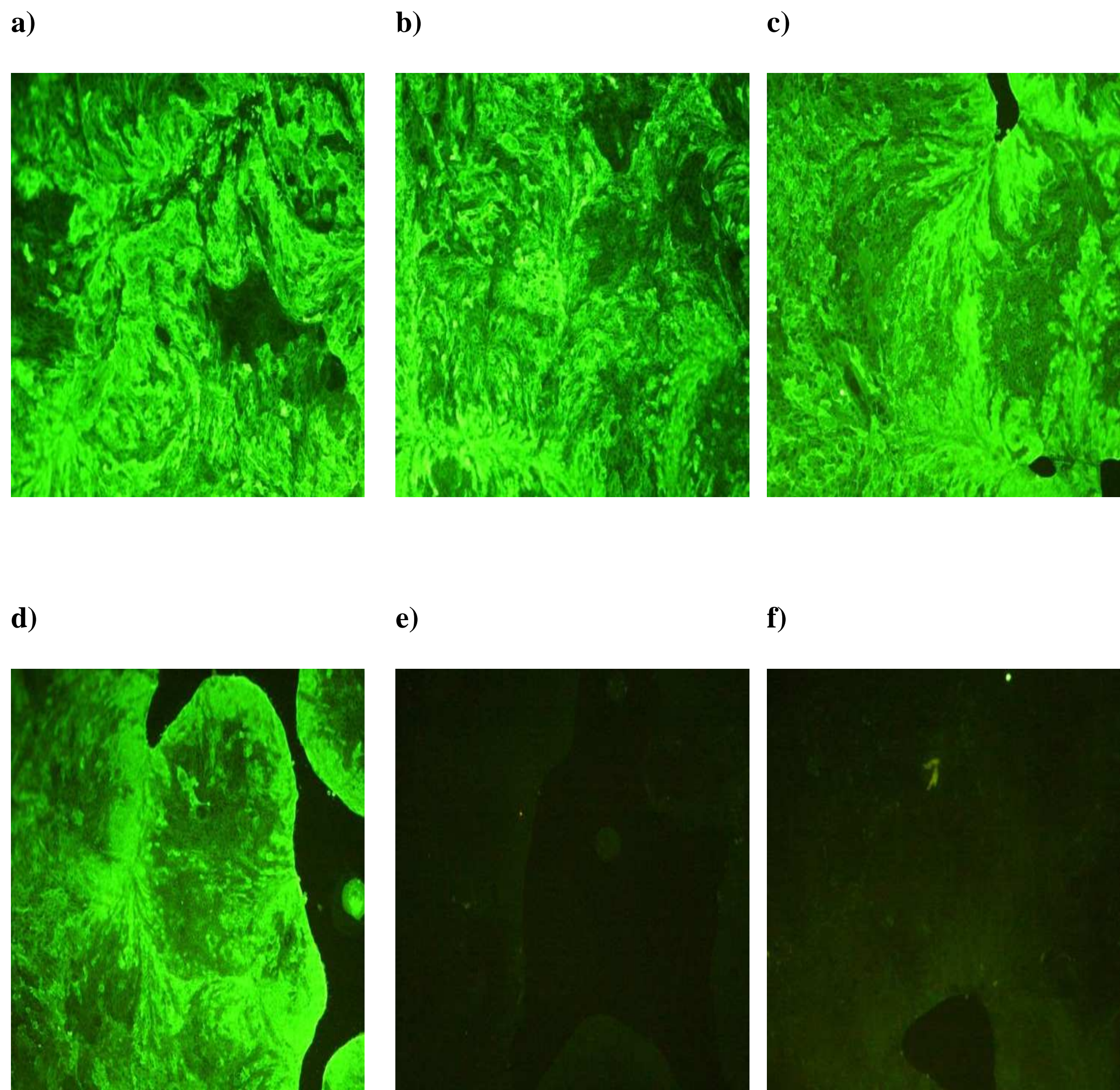


Figure 6.2: *In vitro* characterization of purified mAbs by cell-based ELISA using colorectal carcinoma expressing C2-antigen. From the cell-based studies, cell-binding were observed as indicated by the high fluorescence in images obtained when H1C2 (a), H2C2 (b), QC2 (c) and MC2 (d) mAbs were used, compared to the lack of fluorescence in images using unspecific human (e) and mouse (f) control antibodies. This demonstrates that even after humanization both H1C2 and H2C2 mAbs developed using deimmunization and logical approach methods, respectively, were still functional.

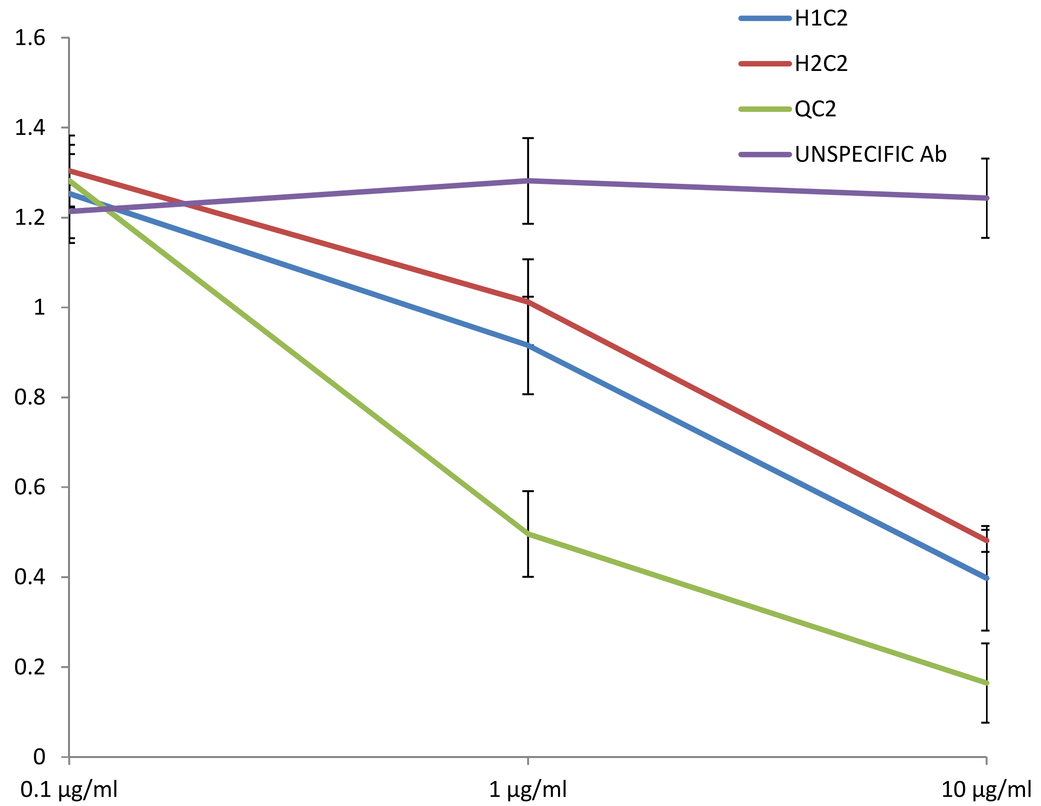


Figure 6.3: *In vitro* reactivity of purified mAbs measured by competitive cell-based ELISA. Displacement of MC2 mAb binding to C2 antigen, a membrane glycoprotein expressed in SW1116 human colorectal cell line-coated plates, by H1C2 (blue line), H2C2 (red line), and QC2 (green line) mAbs. An unspecific human mAb (purple line) was included as negative control.

6.4.3 *In vivo* immunogenicity studies

The next critical issue was whether the humanized mAbs developed by deimmunization and logical approach method would have a decreased immunogenicity when they were administered in humans. To address this issue, *M. fascicularis* were used because of the similarity of monkeys' immune system to that of humans', thus providing a reliable indicator of potential human AAR. Although *M. fascicularis* are only listed in appendix II (currently not threatened with extinction) by Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), in Malaysia, however, it is classified as a Protected Animal, under schedule two of the Protection of Wild Life Act 76, 1972.

Due to the stringent laws and regulations in Malaysia, only five captive-born *M. fascicularis* were permitted to be used for this study. For immunization, the intradermal route was chosen because of the high population of matured dendritic cells in the dermis layer of the skin (Cui et al. 2003). During the study, no evident changes in behavior, food consumption and body mass were observed in all *M. fascicularis*.

Compared to *M. fascicularis* immunized with humanized and chimeric anti-C2 mAbs, the *M. fascicularis* immunized with MC2 mAb had the highest monkey IgG and IgM AAR irrespective whether MC2 mAb (Figure 6.3) or QC2 mAb (Figure 6.4) was used as capture antibody. Decreased but significant AAR responses were obtained when *M. fascicularis* were immunized with QC2 and H2C2 mAbs. However it can be seen that when H1C2 mAb was used as immunogen, the monkey's AAR was similar to that of those immunized with placebo (Figures 6.3 and 6.4).

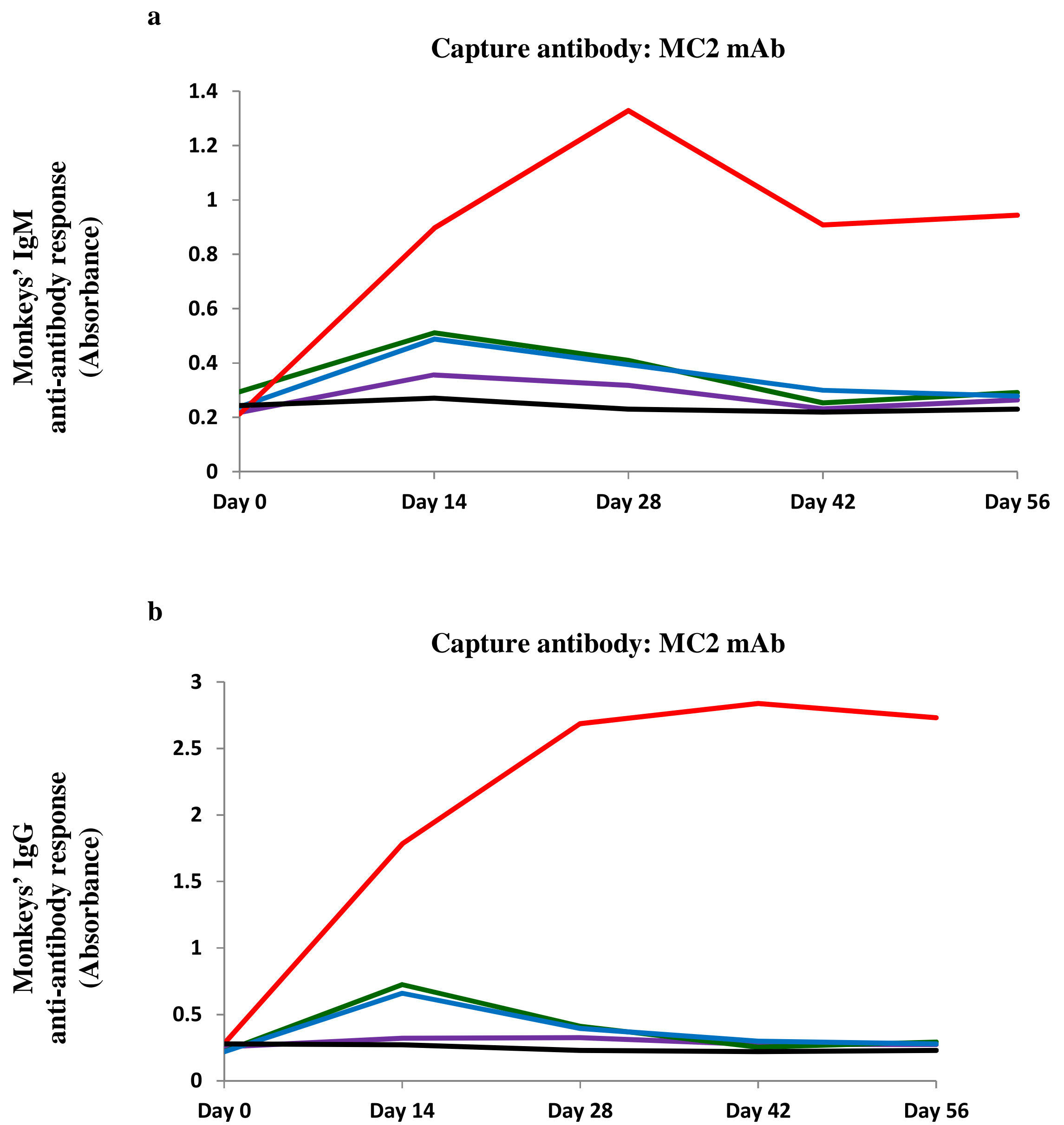


Figure 6.4: Monkeys' anti-antibody responses to mouse-, chimeric- and humanized anti-C2 antibodies using MC2 mAb as capture antibody. These graphs show the AAR of monkeys to MC2 (red), QC2 (blue), H1C2 (purple), and H2C2 (green) mAbs as measured by titers of IgM (a) and IgG (b). MC2 mAbs were used as capture antibody. For negative control, *M. fascicularis* was immunized with placebo (black).

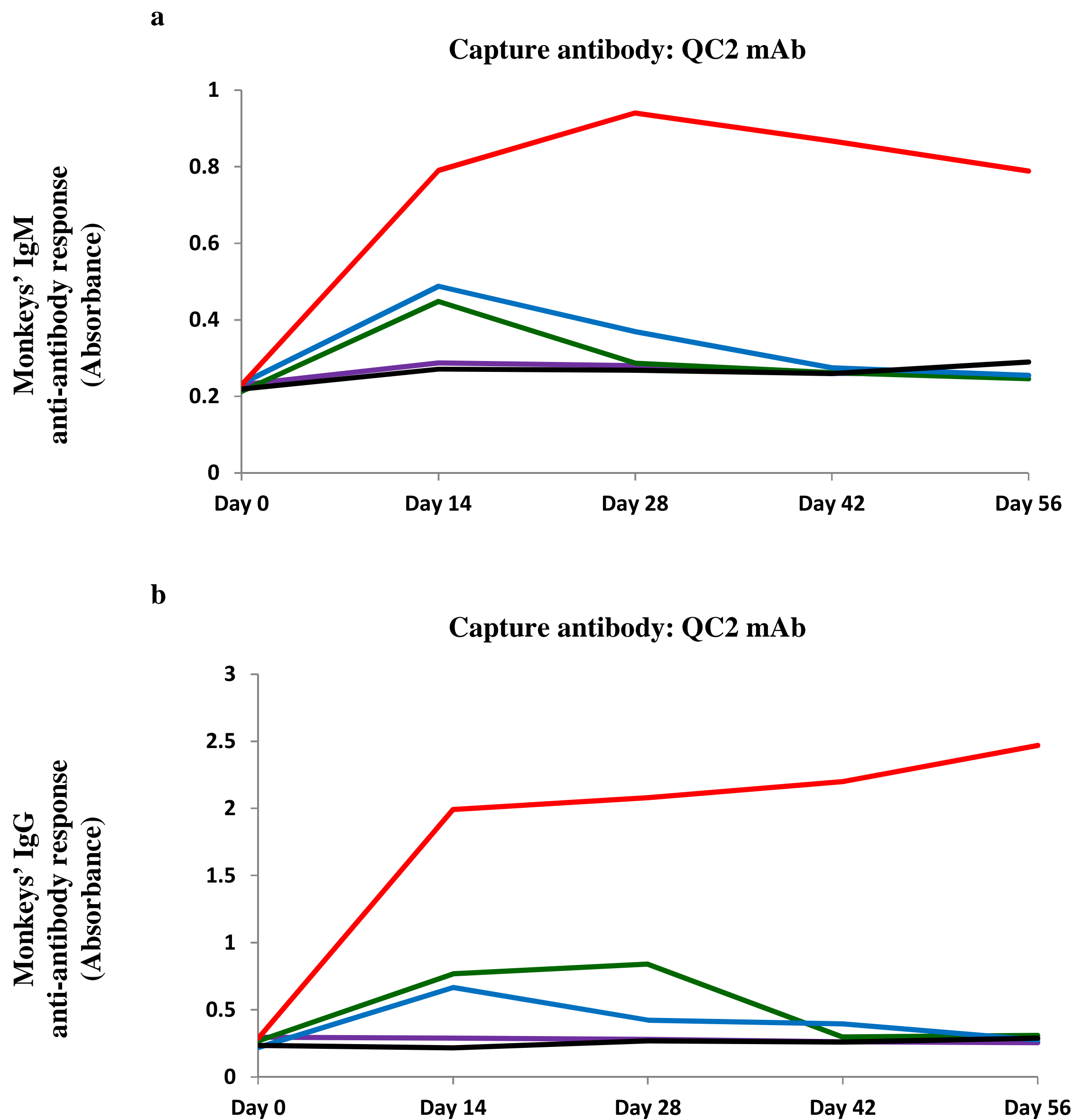


Figure 6.5: Monkeys' anti-antibody responses to mouse-, chimeric- and humanized anti-C2 antibodies using QC2 mAb as capture antibody. These graphs show the AAR of monkeys to MC2 (red), QC2 (blue), H1C2 (purple), and H2C2 (green) mAbs as measured by titers of IgM (a) and IgG (b). QC2 mAbs were used as capture antibody. For negative control, *M. fascicularis* was immunized with placebo (black).

6.5 DISCUSSION

In general, although both H1C2 and H2C2 mAbs had reduced immunogenicity compared to MC2 mAb, only the H1C2 mAb developed using deimmunization method had its immunogenicity virtually eliminated. On the other hand, the immunogenicity of H2C2 mAb (developed using logical approach method), was still similar to that of QC2 mAb.

The reduced immunogenicity of H1C2 mAbs compared to H2C2 mAbs may be due to the fact that H1C2 mAbs have less amphipathic regions compared to that of H2C2 mAbs. In VH of H1C2 mAb, the humanized residues 10 and 17 are located in the first amphipathic region, whereas the remaining humanized residues 44 and 45 are located in the second amphipathic region (Figure 3.5). Similarly, in VL of H1C2 mAb, the humanized residue 15 is located in the first amphipathic region and residue 50 is located in the second amphipathic region. Therefore, both chains of H1C2 mAb had only 1 remaining amphipathic region; VH (third amphipathic region) and VL (third amphipathic region).

On the other hand, the H2C2 mAb developed using logical approach method had one humanized residue 10 in VH which is in the first amphipathic region and 2 humanized residues 68 and 81 which are in the second amphipathic region in VL. Thus, H2C2 mAb have two remaining amphipathic regions in each VH (second and third amphipathic regions) and in VL (first and third amphipathic regions). The additional amphipathic regions may have caused the immunogenicity of H2C2 mAb to be higher that of H1C2 mAb.

The deimmunization method combines veneering (based on Padlan's approach) (Padlan, 1991) to effectively humanize surface residues (thus removing B-cell epitopes) with the identification and removal of potential helper T-cell epitopes from engineered antibodies. Helper T-cell epitopes are short peptide sequences within proteins that bind to MHC class II molecules. The peptide-MHC class II complexes are recognized by T-cells and trigger the activation and differentiation of helper T-cells, thus stimulating a cellular immune response. Helper T-cells initiate and maintain immunogenicity by interacting with B-cells, resulting in the production of antibodies that bind specifically to the administered antibody. In deimmunization method, helper T-cell epitopes are identified within the primary sequence of the antibody using prediction software and these sequences are altered by amino acid substitution to avoid recognition by T-cells.

Thus, the nature of the protein surface is important for its recognition by the monkey or human immune system because the protein internalization and processing by antigen-presenting cells and the presentation of processed peptides to T-helper cells in the context of class II major histocompatibilty complex molecules are also essential events for the development of an immune response against administered mAbs (Mateo et al. 2000). Therefore, the removal of most linear epitopes that may have been presented to T-cells had considerably reduced the immunogenicity of H1C2 mAb. In contrast, the presence of additional potential T-cell epitopes of H2C2 mAb even after humanization may have contributed to its higher immunogenicity.

Hence the deimmunization method is a superior method for the development of functional humanized mAbs with reduced immunogenicity. The fact that, deimmunization method had also been applied previously on ior-egf/r3, a mouse mAb which blocks the epidermal growth factor receptor (EFGR) and the resulting humanized

antibody retained its antigen-binding affinity and was less immunogenic in monkeys than either their mouse or chimeric predecessors, further proves the superiority of deimmunization method compared to logical approach method (Mateo et al. 2000).

6.6 CONCLUSION

In conclusion, although the small number of monkeys permitted for this study prelude statistical analysis of the data, it is noteworthy that *M. fascicularis* immunized with H1C2 mAb had reduced immunogenicity compared to *M. fascicularis* immunized with QC2 and MC2 mAbs. While both H1C2 and H2C2 mAbs developed by deimmunization method and logical approach method, respectively, are functional even after humanization, the reduced immunogenicity using logical approach method however is not guaranteed as indicated by H2C2 mAb. Therefore, for the development of functional humanized mAbs with reduced AAR, it is recommended that amphipathic mouse residues be targeted for humanization, while those located in the CDR or Vernier zone be left alone. Therefore, it is concluded that the deimmunization method should be used to humanize mouse monoclonal antibodies. The retention of functionality and induction of minimal AAR make humanized H1C2 mAbs ideal for use in human therapeutics.