CHAPTER VI
DISCUSSION

Passive serum/antibody therapy has been practiced since the early 1900s by Emil von Behring (Casadevall and Scharff, 1994). It was since applied for the treatment and intervention of various human diseases including infections, intoxications as well as non-communicable diseases such as cancers and autoimmune conditions. Antibody therapeutics may derive either from homologous or heterologous species. Human derived antibody preparations are relatively safe with minimum untoward reactions in the recipients but the supply is limited and ethical issues have been of concern. Animal (*e.g*., horse, ovine) derived-polyclonal antibodies and murine monoclonal antibodies have been used as alternatives to human polyclonal antibodies (Blake *et al*., 1976; Tacket *et al*., 1984; Aulitzky *et al*., 1991; Shapiro *et al*., 1998 and Casadevall, 1999). However, because of the heterologous nature of these preparations anti-isotype responses do occur in recipients which cause side effects, e.g. immediate and immune complex mediated-hypersensitivities (Black and Gunn, 1980; Carter, 2001). Several attempts have been made to reduce/abrogate such undesirable effects by minimizing the foreignness of the animal protein in humans by using (in falling order of immunogenicity) human-mouse chimeric monoclonal antibodies (in which the Fc portion of the mouse antibody was replaced by a human one), murine Fab fragments, humanized-murine preparations, and fully human monoclonal antibody which were derived from genetically engineered sources (Morrison and Schlom, 1990; Zhang *et al*., 2007).

For human leptospirosis, delayed case recognition and late treatment often result in severe clinical manifestations especially pulmonary hemorrhage and renal failure which are the main reasons for fatality (Faine, 1982; Magaldi *et al*., 1992; Magill, 1998; Trevejo *et al.,* 1998 and Yersin *et al*., 2000). Intravenous antibiotic therapy, together with other supportive measures, is currently the mainstay practice for acutely ill and complicated cases. A fraction of patients develop drug hypersensitivity and a Jarisch-Herxheimer reaction (JHR) caused by the massive release of bacterial

products, which aggravate the pathological condition. As such, therapeutic antibodies should be an appropriate alternative to antibiotics for such patients.

1. Characterization of murine monoclonal antibody clone LPF1

The murine hybridoma clone LPF1 secreted monoclonal antibody specific to all strains of pathogenic *Leptospira* was established (Sakolveree *et al*., 2007). This clone was selected to study because it secretes a monoclonal antibody (MAb) that is specific to an epitope found in several pivotal proteins involved in the metabolism and survival of pathogenic *Leptospira* as summarized in **Chapter V**.

The *in vitro* hemolysis of red blood cells (RBC) by different serovars of pathogenic strains of *Leptospira* has been studied by a number of workers (Hathaway and Marshall, 1980). The mechanism was not described, however, porin formation (Elkins and Sparling, 1990; Lee *et al*., 2002) at the surface of RBC or other mammalian cells in order to gain the enriched nutrient such as iron and fatty acid inside the erythrocyte and resulting in hemolysis might be possible. The different strains of *Leptospira* spp. can mediate vary degree of hemolysis. In this study, the MAb LPF1 was found to inhibit *Leptospira* mediated RBC hemolysis *in vitro*. The neutralizing mechanism underline of MAb LPF1 may bind to the outer membrane of leptospires especially the LipL32 (hemolysis associated protein 1) (Sakolvaree *et al*., 2007), inhibit the pore formation and the MAb LPF1 might bind to the flagellin protein and interfere the motility of the leptospire. The original MAbLPF1, muscFv and huscFv at 2 μg could inhibit 56.8, 76.6 and 67.2%, and 30.37, 58.74 and 57.07% of hemolysis mediated by 1.4 HD50 and 2.8 HD50, respectively, of the pathogenic *Leptospira*.

The MAb LPF1 was characterized it therapeutic efficacy in experimental animal. A Syrian hamsters are very susceptible to leptospiral infection, they have often been used to determine the virulence of leptospiral organisms. This infection is also used as a model to study acute experimental leptospirosis (Miller and Wilson, 1966). MAb LPF1 gave 100% therapeutic efficacy against 5 LD50 of *Leptospira interrogans* serovar Pomona in experimental leptospirosis hamster model. Although the mechanism of MAb inside the body has not been characterized yet, it may be involved in the binding of the MAb LPF1 and pivotal and virulent proteins that play important

role in the pathogenicity of the disease. The motility of the leptospires was inhibited by the antibody, subsequently enhanced the elimination of the bacteria by the process of phagocytosis. The MAb might inhibit the hemolysis due to the outer membrane protein, LipL32 were malfunctioned.

The methodology to detect and identify infecting *Leptospira* serovars in clinical and post-mortem samples is by culture (Faine *et al*., 1999). However, this is a fastidious and time-consuming method (Boqvist *et al*., 2003). Even the persistence of live leptospires still occurred in the survived hamster, however, the degree was decreased when compared to the positive leptospirosis control and live leptospires may shed in the urine for months (Faine, 1982; Van Crevel *et al*., 1994 and Kelley, 1998).

It has been characterized that the MAb LPF1 recognized the pathogenic strains of *Leptospira*, protect against leptospirosis both *in vitro* and *in vivo*, however, murine antibodies, as foreign proteins, may elicit immune reactions that reduce or eliminate its therapeutic efficacy and/or evoke allergenic or hypersensitivity reactions in human (Schroff *et al*., 1985).

2. Construction of murine scFv (muscFv) from hybridoma clone LPF1

The hybridoma clone LPF1 was chosen as a template for producing the single chain variable fragment due to the excellent therapeutic efficacy of the antibody as discussed in **Section 1**.

Amplification of heavy and light genes of the immunoglobulin from hybridomas using the polymerase chain reaction and the development of prokaryotic expression vectors for secretion of functional antibodies in the periplasmic space of *E. coli* (Skerra and PIuckthun, 1988) and on the surface of phage (McCafferty *et al*., 1990) has advanced the field of antibody engineering.

The production of recombinant antibodies such as scFvs is dependent on the ability to isolate the variable regions of antibodies (Kabat and Wu, 1991). The design of universail pnmers for the amplification of any light or heavy chain variable region cDNAs from B cells or hybridomas was critical in the initiation of antibody engineering. The amplification of irrelevant V genes that do not encode the hybridoma specificity has been reported to be a widespread problem.

Another potential problem in the cloning of immunoglobuiin genes from hybridomas lies in the choice of primers, particuladry those that amplify from the framework regions. Although these regions are highly wnserved, universai degenerate primers may not be ideal for amplifying these variable genes. They may discriminate against variable genes that have undergone somatic mutation within the 5' or 3' sequences (Orlandi *et al*., 1989; Johnson and Bird, 1991). Additionally, PCR can tolerate mismatches which, when using degenerate primers, can lead to the substitution of non-native amino acids (Orlandi *et al.*, 1989; Ward *et al*., 1989). Changes in the N-terminus may alter the structure and therefore the binding properties of these antibody fragments (Owens and Roberts, 1994).

In my research, the heavy (*VH*) and light (*VL*) chain variable regions of the LPF1 immunoglobulin were amplified by the specific primers designed by Cheng *et al* (2003). The degenerate primers were designed for amplification of universally gene family of the immunoglobulin. To construct the murine single chain variable fragment, the glycine and serine residue was used as linker. Hydrophillic properties of serine allow hydrogen bonding to the solvent and glycine provides the necessary flexibility (Argos, 1990). The repeat of glycine and serine amino acid sequences were commonly use because of the simplicity and its can retain the flexibility of the molecules (Argos, 1990). A variety of linkers with different lengths and sequences have been used (Huston *et al*., 1991). The lengths and sequences of the linker peptide could significantly affect the properties of the molecule. The twenty amino acid of the $(Gly_4Ser)_4$ linker is the intermediate length of the linker which used in this study. The murine *VH* and *VL* DNA sequences were linked together *via* linker and splice overlap extension PCR (SOE-PCR). This RCR technique was first described by (Ho *et al*., 1989). The advantages of the SOE-based method of scFv gene assembly over conventional cloning are two-fold. Firstly, it abrogates the necessity for including a restriction enzyme recognition site in the linker between *VH* and *VL*. The second advantage of the SOE is that it facilitates the cloning of the scFv gene, in that a simple ligation involving just two fragments is required (Nicholls *et al*., 1993).

The *muscFv* DNA sequence was introduced into the phagemid vector in order to generate the phage displayed muscFv molecule at the filament of the M13 phage as a fusion protein with g3p. The pCANTAB5E phagemid vector was used in this study.

There are several evidence showed the successfully used of the pCANTAB5E vector (Yamamoto *et al*., 1999; Cheng *et al*., 2003). The complete phages expressing scFv was established in the *E. coli*. The recombinant scFv molecule expressing from each phage was different in term of completely and conformationaly (Skerra and Plucktun, 1998). In this study, the process called biopanning and phage ELISA were used for enriching and selecting the specific scFv against *Leptospira* antigen.

The *E. coli* strain HB2151 was used to express soluble scFv because in this strain the amber stop codon interposed between the E-tag sequence and g3p gene prevented the expression of a scFv-g3p fusion protein. The soluble scFv is secreted into the periplasm *via* the g3p leader sequence, where it is folded in its native form. In this study, dot blot and Western blot analysis were verified that soluble scFv was concentrated in periplasmic fraction as the same to the study of He *et al*. (2002).

The recombinant muscFv antibody conferred both *in vitro* and *in vivo* neutralization of pathological manifestations mediated by heterologous *Leptospira* spp., at a similar magnitude to its original MAb LPF1. However, some parts of the muscFv molecule are foreign to the human especially the murine immunoglobulin framework regions. This region can elicit the human anti-murine antibody response (HAMA) (Ref) after repeated administration to the human leading to the undesirable side effects as well as accelerated clearance of the antibodies from the circulation.

3. Production of humanized-murine *scFv* (*huscFv*) DNA sequence

The humanization process is the method to decrease the foreign cponterpart whereas retains the antigen binding site at the complementarity determining regions (CDRs) of both VH and VL. The nucleotide sequences of the *VH* and *VL* chain of the MAb LPF1 were sequenced. To select the high most matched human immunoglobulin template for grafting the murine CDRs onto human frameworks, the murine amino acids sequences were subjected to align against the immunoglobulin database. The IMGT program was chosen as immunoglobulin database (Ref).

There are several strategies to produce the humanized-murine scFv which contain human frameworks and murine CDR as summarized in **part II, section 3 of CHAPTER I**. In this case, we found that the immunoglobulin frameworks (FRs) of our murine monoclonal antibody shared only 66.3% and 63.3% homologies with the

closest human VH and VL structures, respectively; thus, in the humanization process we chose to transfer the the whole of murine *CDRs* to human scaffold templates to create a humanized-murine *scFv* sequence (Hudson, 1998; Kashmiri *et al*., 2005; Vinetz, 2001) instead of using site directed nucleotide mutagenesis (Delagrave *et al.*, 1999) or other strategies. This strategy was the simplest humanization methodology as first described by Jone *et al*. (1986). This method may diminishes antibody affinity because certain framework residues can interact directly to the antigen, affect the conformation of the CDR loops and influence the packing interactions between the β-sheet strands (Chotia *et al*., 1985). However, the constructed muscFv and huscFv still retain the specificity against *Leptospira* antigen by Western blot analysis. The primers induced mutagenesis and splice overlap extension PCR (SOE-PCR) were used to create the gene encoding human frameworks and murine CDR. The flexible linker $(Gly_4Ser)_4$ still used in the construct to link between humanized VH and humanized VL together.

4. Characterization of huscFv

In this study, a humanized-mouse monoclonal antibody was successfully produced using gene sequences encoding variable heavy and light chain segments of a. The MAb was also previously found to neutralize *Leptospira* mediated-pathological reactions both *in vitro* and *in vivo*. However, The humanized-murine scFv protein purified from a transformed *E. coli* clone conferred both *in vitro* and *in vivo* neutralization of pathological manifestations mediated by heterologous *Leptospira* spp., at a similar magnitude to its original murine scFv counterpart, indicating the preservation of the primary epitope binding specificity and affinity of the humanized-molecules. This was confirmed by the results of the Western blot analysis which shows that all preparations reacted similarly to *Leptospira* proteins at ~36 kDa. The original MAb LPF1, muscFv and huscFv at 2 μg could inhibit 56.8, 76.6 and 67.2%, and 30.37, 58.74 and 57.07% of haemolysis mediated by 1.4 HD50 and 2.8 HD50, respectively, of the pathogenic *Leptospira*. The significantly lower magnitude of percent haemolysis inhibition exerted by the intact MAb compared to its two derivatives on the same weight basis (2 μg) as seen in the test using 2.8 HD50 of *Leptospira* spp. might be due to the lower number of antigen binding sites in the

original MAb preparation. The MAb contains not only epitope binding sites, but also, in large proportion, constant parts, *i.e*., the Fc and CH1 domains of the heavy chain and the CL domain of the light chain, while the ScFv-preparations contained solely the epitope binding domains. The constant portions as well as the bivalent antigen binding nature of the intact antibody are important in certain immune mediated functions, *e.*g., bacterial agglutination, phagocytosis and complement mediated lysis. However, the MAb LPF1 has been shown to bind specifically to *Leptospira* proteins involved in bacterial protein synthesis, metabolism, motility and cellular integrity. Therefore, merely binding to the target epitopes by the antigen binding sites in the scFv preparations should be enough to interfere with the ultimate functions of the target proteins and consequently to neutralize the *Leptospira*-mediated pathology as demonstrated by the *in vitro* and *in vivo* experiments of this study. The finding that the humanized-scFv has a comparative degree of *Leptospira* neutralization efficacy to its murine scFv counterpart indicates that CDR transferation to the human canonical fragments did not alter any of the important structural features of the antigen combining sites, *i.e*., the association of individual CDRs on both VH and VL and the rest of the molecule that were crucial for the antibody affinity to the epitope.

In this study, the whole murine CDRs were grafted onto the human immunoglobulin frameworks to create a humanized-molecule. There is a possibility that the preparation might still be immunogenic to human recipients. In order to further reduce/abrogate the remaining degree of foreignness, only the specificity determining residues (SDRs) within the CDRs should be used instead of the whole CDRs in the humanization process. However, such extensive molecular modification might alter the conformation of the antigen binding site and result in alterations of the epitope binding affinity and specificity. Experiments are needed to demonstrate the advantage and disadvantage of the more humanized-(SDR-grafted) product. For the time being, our humanized-murine preparation has a high potential as a therapeutic alternative for human leptospirosis in patients with antibiotic hypersensitivity.