CHAPTER VI
DISCUSSION

1. Neutralization test of MAbPT6-2G6

Ability of specific antibody to protect against disease, *i.e.* diphtheria, was first discovered by von Behring and Kitasato in the early 1890s. Since then the antibody based-therapy, called serum therapy, was practiced for treatment of various infectious diseases in human. The discovery of sulfonamides and antibiotics in the mid 1930s, however, made a change in the treatment strategy of infectious diseases, *i.e.* the serum therapy was mostly replaced by the antimicrobial chemotherapy. Nevertheless, the antibody retains its therapeutic niche for many viral infections as well as for envenomation, *e.g.* snakebite, and bacterial intoxication, *e.g.* tetanus, botulinum and pertussis (Casadevall and Scharff, 1995; Dolby *et al*., 1975). For pertussis, even though effector mechanisms of protective immunity against the disease is not completely understood and may involve both humoral and cell mediated immune mechanisms (Buchwald and Pirofski, 2003), evidences in the literature have pointed out that specific antibodies to PT play important role in amelioration of the pertussis severe morbidity and in protection against the *B. pertussis* infection (Sato and Sato, 1990; Sato *et al*., 1984).

In 1994, Murine hybridoma secreting monoclonal antibody specific to PT was produced through a fusion of immune splenocytes of a BALB/c mouse immunized with PT and mouse myeloma P3-X63-Ag8.653 cell (Buddhirakkul, 1994). The specific hybridoma, named PT6-2G6, secreted IgG1 monoclonal immunoglobulin with antigenic specificity to S1 subunit of PT. In this study, the monoclonal antibody secreted from PT6-2G6 hybridoma (MAbPT6-2G6) was re-confirmed for its antigenic specificity by Western blot analysis. It was found that MAbPT6-2G6 bound specifically to S1 subunit of PT (**Figure 15**). MAbPT6-2G6 was determined the neutralizing activity against PT mediated-functions *in vitro*: hemagglutination (HA), *ex vivo*: Chinese hamster ovarian (CHO) cell clustering (CC) and *in vivo*: leukocytosis-promotion (LP).

In vitro*; hemagglutination (HA)*

Hemagglutination or erythrocyte agglutination of PT was established in 1976 by Arai and Sato. The hemagglutination of PT was mediated by S2-S4 and S3-S4 dimers of B oligomer (Tamura *et al*., 1982) by binding to their ligand on the cell membrane of erythrocytes, leading to formation of a red blood cell lattice and eventually agglutination of the cells. Hemagglutination of chicken red blood cells was usually used as a model to assess the PT binding inhibition effect of monoclonal anti-PT antibodies in several studies (Sato *et al.*, 1984; 1987; Schmidt and Schmidt, 1989). In this study, the finding that intact MAbPT6-2G6 could inhibit the *in vitro* effect of PT mediated-hemagglutination, which is normally mediated by the B oligomer of PT (Tamura *et al*., 1982), implies that the MAbPT6-2G6 specific to S1 subunit can stereologically block the binding of PT holotoxin to its receptor on the erythrocytes.

Ex vivo: *Chinese hamster ovarian (CHO) cell clustering (CC)*

The CHO cell cytotoxicity assay or CHO cell cultering (CC) which the shape of PT-treated CHO cells became rounding up and clustering was the first *ex vivo* assay for assessing neutralizing antibodies to pertussis toxin (Hewlett *et al*., 1983). They suggested that pertussis toxin-induced morphological changes of CHO cells were not due to alterations in adenylate cyclase since clustering occurred when basal cyclic-AMP levels were unaffected by the toxin. Moreover, the morphological change induced by pertussis toxin was strikingly different from that caused by cholera toxin, which alters cell morphology by increasing cyclic AMP levels (Hewlett *et al*., 1983). In spite of the absence of changes in cyclic AMP levels, CHO cell clustering appears to be due to toxin-catalyzed ADP-ribosylation of a 41-kilodalton G_i proteins that are $G_{i\alpha\text{-}2}$ and $G_{i\alpha\text{-}3}$ (Burns *et al*., 1984; 1987; Gerhardt and Neubig, 1991). It is believed that ADP-ribosylation of $G_{i\alpha}$-protein leads to the actin depolymerization, resulting in cell clustering and rounding up.

In this study, it was found that MAbPT6-2G6 at 6.25 μg ccould neutralize partially (~50%) the CC caused by CCD50 of PT (5.76 pg).

In vivo*; leukocytosis promotion (LP)*

In 1967, Morse and Riester showed that the lymphocytosis induced in mice by *Bordetella pertussis* is due to the presence in the circulation of an increased number of pre-existing lymphocytes and is an inhibition of lymphocyte emigration which is

primarily the consequence of an effect on the cell. Several studies confirmed that lymphocytes were inhibited in the migration from blood circulation to lymphoid tissue (Spangrude *et al*., 1985; Spangrude *et al*., 1984; Sugimoto *et al*., 1983). Hudnall and Molina showed that patients with pertussis were markedly increased in L-selectin– negative leukocytes (Hudnall and Molina, 2002). It was demonstrated that leukcytosis was the result of ADP-ribosylation of $G_{i\alpha}$-protein (Chaffin *et al*., 1990). But the extact mechanism is under investigation.

In this study, it was found that the MAbPT6-2G6 could cause significant decrease ($p< 0.05$) in LP mediated PT in a dose dependent manner (**Figure 19**).

As mentioned above, CC and LP of PT were caused by the ADP-ribosylation of $G_{i\alpha}$-protein. However, MAbPT6-2G6 that neutralized significantly the LP activity inhibited partially the CHO clustering mediated by PT. Neutralization of pertussis toxin with the CHO cell assay seems to be a poor predictor of protection from disease. Because, Sato *et al*. (1991) showed that monoclonal antibodies (especially those against S1 subunit) that protected mice from pertussis toxin-mediated lymphocytosis conferred protection from aerosol challenge, while monoclonal antibodies that neutralized pertussis toxin with the CHO cell assay did not mediate protection from aerosol challenge.

In this study, the mouse-protection test of MbPT6-2G6 against lethal *B. pertussis* infection was not performed due to the absence of such a virulent strain of *B. pertussis* in Thailand.

2. Mimotope analysis of MAbPT6-2G6

Several murine monoclonal antibodies specific to various PT subunits were tested for their protective activities in infant mice against the aerosol and intracerebral challenge with *B. pertussis* (Sato and Sato, 1990; Sato *et al*., 1991). It was found that while many of the monoclonal antibodies especially those directed against S1 were highly protective when passively administered to the mice, several other preparations directed against the same protein were less effective or did not confer any immunity at all (Sato and Sato, 1990; Sato et al., 1991). The finding indicates that only certain epitopes in the S1 protein are important in neutralizing the PT activities. In this study,

a neutralizing epitope of MAbPT6-2G6 on the S1 of PT was identified by using phage display peptide library as experimental tool.

A random peptide phage display library was used to identify a mimotope of the specific MAb to S1 subunit of PT. This random peptide phage display library has been successfully applied for identifying mimotopes of various monoclonal antibodies specific to various pathogens, such as bovine herpes virus (Lehmna *et al*., 2004) and *Neisseria meningitides* (Charalambous and Feavers, 2000). A random peptide phage library designated T7S constructed by Dr. Gunner Froman, Department of Microbiology, Uppsala University, was used previously for the determination and characterization of a mimotope that bound to monoclonal antibody to mouse polyomavirus large T-antigen (Houshmand *et al*., 1999), a mimotope of monoclonal specific to *Leptospira* spp. (Tungtrakanpoung *et al*., 2006) and a mimotope of monoclonal antibody to cockroach allergen (Sookrung *et al.,* 2006).

In general, during the bio-panning process for selecting a phage clone displaying peptide that binds to a monoclonal antibody, the diversity of phage clones was decreased after multiple rounds of bio-panning and with higher-stringency selection (Irving *et al*., 2001). Therefore, to increase the population of phage bound specifically by the MAbPT6-2G6, four rounds of bio-panning were done with the decreased duration of the binding step while the time of washing was increased. The bio-panning results revealed that there were increased in the titers of the phages through successive rounds of selection (Table 2), suggesting that the numbers of phages with high affinity binding were increased during the selection process.

Thirty phage clones bound by MAbPT6-2G6 were randomly selected from 4^{th} round. DNA segments of the T7 phage encoding the 10B capsid protein were amplified from individual phage clones using PCR technique and subjected to DNA sequencing. It was found that DNA of only 12 clones, *i.e.* T7PT6-1 to 12, could be sequenced and the deduced amino acid sequences of these clones were divided into 6 mimotope groups: M1; LTPCRN (1 phage clone), M2; LTPCRTK (2 phage clones), M3; LSRIFIAN (5 phage clones), M4; LLTVRRA (2 phage clones), M5; VISIVGV (1 phage clone) and M6; SCVNSLL (1 phage clone).

The screening of phage-displayed random peptide library typically contains a small population of phage clones individually expressing peptides that bind selectively

to the antigen-binding sites of the particular antibodies. These phages are "target-specific binders", which can be further divided into high-affinity binder and the much more abundant low-affinity binders. Phage-displayed random peptides library also contains population of "target-unrelated peptides" (TUP), which may react with constant antibody regions or other components of the screening system, such as the beads, plates, or the capturing molecule (streptavidin, protein A, *etc*). In this study, all of the six mimotopes were compared with previously reported TUP (Menedez and Scott, 2005; Adey *et al.,* 1995) and also compared with vector sequences in GenBank using Vecscreen software. It was found that all mimotope were not similar to TUP sequence and vector sequences.

When all of six mimotope peptide sequences were aligned with the amino acid sequences deposited in GenBank database using BLASTP software, all mimotopes were not found to matched with S1 (data not shown). The possible explanation was that the GenBank is a big genome database of organism so that short mimotope sequences would have less chance to match with S1 but have more chance to match with other proteins deposited in database.

Thus, the six mimotope sequences were aligned with amino acid sequence of S1 using SIM software. It was found that all of them matched to the amino acids at positions 213-225 (TSRRSVASIVGTL) on S1 subunit (**Figure 21**) and when locating amino acids 213-225 (TSRRSVASIVGTL) on the 3D structure of S1 using PyMOL 0.99rev8 software, it was revealed that the amino acids 213-225 (TSRRSVASIVGTL) were located on the surface of the S1 and at juxtaposition of the S2-S4 dimer of PT **(Figure 22)**. Kirsi *et al.* (1992) showed that PT must at least be bivalent for acting as a hemagglutinin. Thus, blocking one of the two binding sites (S2-S4 and S2-S4 heterodimers) results in abolition of the hemagglutination activity of PT. Moreover, the S2 subunit, especially amino acids at positions 37-52, was responsible for binding B oligomer to the ligand on membrane of RBC.

All together, the finding implied that the peptide sequence, amino acids 213-225 (TSRRSVASIVGTL), is the mimotope of MAbPT6-2G6 and the MAbPT6-2G6 neutralized the PT-mediated hemagglutination by following proposed mechanism: the specific binding of MAbPT6-2G6 to S1 subunit at amino acid positions 213-225 (TSRRSVASIVGTL) juxtaposed to S2-S4 heterodimer of the B subunit lead to

stereologically hindering the binding region of the dimer, resulting in abolishing the hemagglutination of PT.

Although the molecular events involved in the cellular entry of PT is not well understood, evidence suggested that PT may enter the cell by traversing across the plasma membrane instead of using the receptor mediated endocytotic pathway (Kaslow and Burns, 1992). It is also known that intact antibody molecule, because of its large size and molecular hydrophilicity, cannot normally penetrate the hydrophobic plasma membrane of the living cells and thus is inaccessible to its intracellular target. The findings that previous incubation of the PT with the MAb PT6-2G6 which is specific to S1 subunit could inhibit the CHO cell clustering and leukocyte-promotion activities are interesting because these PT functions are mediated by the ADP-ribosyltransferase activity of the intracellular S1 (Castro *et al*., 2001). Neutralization of the PT by the intact MAb should not be mediated by interfering the enzymatic function of the S1 *per se* but rather preventing; 1) the binding of PT to target cell, and 2) the intracellular entry of the toxin by forming PT-antibody complexes that are unable to traverse the plasma membrane.

A study on immunogenicity of these PT6-2G6 mimotopes in eliciting antibodies that can neutralize PT activity should be performed. Positive results will imply that the mimotopes may be used as either peptide analogue of PT for pertussis therapy or the phages carrying the mimotopes (phagotopes) may be used as a vaccine in inducing protectve immunity to PT.

3. Production of humanized-PT6-2G6-ScFv (PT6-2G6-HuScFv)

PT-neutralizing MAbPT6-2G6 has potential as non-drug therapeutic applications in patient with pertussis especially at the catarrhal and paroxymal stages. However, murine antibodies are foreign proteins to the human immune system and therefore elicit an immune response in the form of human anti-murine antibodies (HAMA). Since the original study of antibody humanization by Jones *et al*. (1986), in which complementarity determining regions (CDRs) of a murine Ab that are responsible for antigen binding were grafted onto a homologous human VL and VH frameworks, this strategy, termed “CDR grafting” has been frequently performed to decrease the degree of foreignness of the murine antibodies in human (Riechmann *et al*., 1988; Jones *et al*.,

1986; Jones *et al*., 1986). Humanized-antibodies overcome the main limitation of murine MAbs, which is their immunogenicity in humans (Carter, 2001). In this study, *PT6-2G6-huscFv* DNA was constructed and soluble PT6-2G6-HuScFv was produced by genetic engineering.

Construction of PT6-2G6-huscFv

The murine CDRs of MAbPT6-2G6 VL and VH were determined by using IMGT/V-QUEST (http://imgt.cines.fr) which is an integrated alignment tool for the immunoglobulin (IG) and T cell receptor (TR) nucleotide sequences.

The most crucial step in the humanization of antibody by CDR grafting is to choose the most appropriate human frameworks for using as templates for preserving the conformation of the murine CDR loops of HuScFv (Banfield *et al.,* 1997). The most appropriate human frameworks can be selected based on strategies that 1) relies on the known X-ray structures of human frameworks (Riechmann *et al*., 1988; Jones *et al*., 1986) and the searching the Protein Data Bank (PDB) database to identify the human antibody structure that shows the closest overall identity to the antibody to be humanized, and 2) relies on the similarity of the sequence of human frameworks and target antibodies. The most appropriate human frameworks can be selected by homology searches of immunoglobulin sequences from nucleotide or protein databases like GenBank (http://www.ncbi.nlm.nih.gov), EMBL (http://www.ebi.ac.uk/embl), SwissProt (http://www.expasy.ch/sprot) and PIR (http://pir.georgetown.edu) using programs such as Fasta. In this study, most matched human frameworks were selected based on the similarity of the sequences of the human frameworks to VL and VH of MAbPT6-2G6. By BLAST homology search tool in GenBank, the human VL (accession no. bac01708.1) and VH (accession no caa78175.1) with 78% and 60% homology to the murine regions were selected and used as canonical frameworks. The murine CDRs derived from MAbPT6-2G6 were grafted onto the selected of VL and VH frameworks of human immunoglobulin frameworks by using splice overlapped extension-polymerase chain reaction (SOE-PCR) technique originally described by Ho *et al*.(1989). Human frameworks and HuVL and HuVH of PT6-2G6 were linked together with $(Gly_4Ser)_3$ also by SOE-PCR

Construction and screening of phage-displayed PT6-2G6-HuScFv antibody library

One of the most powerful applications of phage display has been the isolation of recombinant antibodies with a unique specificity (Griffiths, 1993; Winter *et al*., 1994; Barbas *et al*., 1991; Neri *et al*., 1995; Hoogenboom *et al*., 1998). The antibodies can be fused with g3p fusion either by direct cloning to the phage genome (Parmley and Smith, 1988; Cwirla *et al*., 1990; McCafferty *et al*., 1990, 1991; Clackson *et al*., 1991; Swimmer *et al*., 1992) or by cloning into gene 3 present within a phagemid plasmid (Bass *et al*., 1990; Barbas *et al*., 1991; Hoogenboom *et al*., 1991; Griffiths *et al*., 1994). In this study, the pCANTAB5E phagemid was selected. Then *PT6-2G6-huscFv* was cloned into pCANTAB5E plasmid vector. Recombinant phages must be rescued with a helper phage (M13KO7 helper phage). A rescued recombinant phages display g3p protein from the wild-type helper phage and fusion g3p with *PT6-2G6-huscFv* from resident phagemids. That is contrast to display of g3p fusion encoded within the phage genome itself, where all g3p molecules are originally present as fusion. But, such fusion causes a decrease in infectivity of recombinant phages (Smith, 1985).

After the transformation to TG1 *E. coli* cells, at first only 200 clones were present (data not shown). This was a relatively low number compared with some other libraries. The main reason could be the transformation method. Generally, the heat shock method which efficiency is much lower than that of electroporation was employed. But, in this study, one kind *huscFv* was done. So, in principle, thee was a high chance that one recombinant antibody with one antigenic specificity could be generated. Although the heat shock method yields low efficiency, it was still good enough to obtain the positive phage clones. But in the case of other sources of material and other purposes (for example, creating a naive repertoire antibody library), it would be preferable to use electroporation method.

In TG1 *E. coli* cells, the amber stop codon (TAG) located between *huscFv–E tag* sequence and *pIII* gene can be read through. In the presence of helper phage M13KO7 or phage rescue, the E-tag fused-HuScFv was displayed with g3p on the surface of the recombinant phage, allowing for bio-panning and affinity screening by phage ELISA. In this study, the phage rescue is done directly from transformed or re-infected TG1 *E. coli* cells without the step of culturing the *E. coli* cells on plates. The direct phage

rescue is faster (saving one day), but it has the risk of losing candidate antibody clones. The reason is that differently transformed or re-infected cells may grow at different rates. Some antibody clones may grow very slowly, have a very low number of bacteria and produce only low amount of phages which can never be enriched by the bio-panning. For simple libraries such as the one in this study or library that the ScFv gene from hybridoma cells were cloned, hence not so many candidates to be selected, the direct phage rescue is useful. For a complex library which many candidate antibodies (for example, the naive repertoire antibody library which has about 10^{10} candidates) are required, one additional day of culture on plates is recommended.

Screening of phages displaying PT6-2G6-HuScFv by phage-ELISA

Screening is always needed because non-specific phages can never be removed by selection. In this study, even when the ELISA signal was highest after 4th round of bio-panning, only 5 clones that gave positive result by phage ELISA were obtained from 50 clones (**Figure 42**). This means that non-specific phages contribute ~90% of the whole phage population. The percentage of the specific binders in the whole phage population could be vary. Griffiths *et al.* (1993) showed that specific binders could be up to 45% after three rounds of the enriched selection.

Expression and purification of the PT6-2G6 HuScFv antibody

Since the HB2151 *E. coli* is transfected quite poorly, the transformed HB2151 *E. coli* cells were plated on the 2× YT-AG plate containing nalidixic acid (2× YT-ANG)in order to ensures that the resulting clones are truly nalidixic acid-resistant HB2151 bacteria and not due to a carryover of the infected TG1 *E. coli* cells that are susceptible to nalidixic acid.

By small scale expression of soluble PT6-2G6-HuScFv, it was found that HB2151 *E. coli* clones no. 3, 5, 12, and 23 that showed the binding activity to PT by phage-ELISA could not produce the soluble PT6-2G6-HuScFv. It may be caused from non-specific binding of phages to PT or they were clones that contained a stop codon which may not interfere with the production of phage-displayed-HuScFv in the TG1 *E. coli* but may interfere with the production of soluble HuScFv in the non-suppressor strain HB2151 *E. coli*.

The location of expressed HuScFv antibody in the *E. coli* cells is important. The active antibody requires an intramolecular disulfide bridge (Harlow and Lane, 1988;

Alberts *et al*., 1989). Inside HB2151 cells, there is a reducing environment, where it is difficult to form a disulfide bridge, therefore the HuScFv antibody inside the cells are not active. By contrast, in the periplasmic space, the disulfide bridge between VL and VH could be formed. Therefore, HuScFv located in peripalsmic fraction and that secreted into the medium are the active ScFv. However, the concentration of secreted HuScFv in the supernatant could be very low that was not worthy of further handling. So, HuScFv antibody with the substantial amount contained in periplasm are suitable for purify and used in further experiment. In this study, under IPTG induction at 37°C, it was that the PT6-2G6-HuScFv was accumulated in the cytoplasmic fraction (data not showed). When the temperature of HuScFv expression was decreased from 37°C to 30°C, PT6-2G6 was secreted out into periplasmic space. So, temperature of HuScFv expression at 30°C was used in large scale expression.

PT6-2G6-HuScFv in periplasmic fraction was rapidly and easily purified by HiTrap anti-E tag affinity column, and its molecular weight was about 35 kDa (**Figure 44**). The disadvantage of this purify method is that, first, the HiTrap anti-E tag affinity column is very expensive. Second, since the E-tag binds tightly to the column, it is hard to completely recover HuScFv antibodies, consequently, if the same column is used to purify other ScFv antibody, cross-contamination may occur.

4. Characterization of PT6-2G6-HuScFv

PT6-2G6-HuScFV was successfully produced. By Western blot analysis, the antigenic specificity of PT6-2G6 was determined. The result showed that PT6-2G6-HuScFv bound specifically to S1 subunit (**Figure 51**). It implied that MAbPT-2G6 and PT6-2G6-HuScFv possessed the same antigenic specificity to PT by recognizing S1 subunit of PT.

For hemagglutination inhibition test, it was found that the PT6-2G6-HuScFv was retaining the hemagglutination inhibition activity of original MAb by blocking the binding of toxin to the cell receptor. Based on the number of the Fv portion of both antibody preparations, the hemagglutination inhibition efficacy of PT6-2G6-HuScFv was slightly decreased. MAbPT6-2G6 at 116 Fv portions caused 100% hemagglutination inhibition while PT6-2G6-HuScFv with 248 Fv molecules mediated 50% hemagglutination inhibition mediated by 125 ng PT. An obvious reason that may

be to explain th results. The less inhibitory effect of the PT6-2G6-HuScFv might be due to its has smaller size that can mediate less stereological hinderance the hemagglutination sites of the B pentamer. The leukocytosis-protmotion inhibition test of PT6-2G6-HuScFv would be tested in further experiment.

In 2006, Layer and Maynard showed that HuScFv derived from murine MAb that bound specifically to S1and neutralized the activity of PT was produced (http://aiche.confex.com/aiche/2006/techprogram/P73855.HTM. Accessed on 23/4/ 2007). However, the antigen affinity of that HuScFv to S1 was completely lost. Thus, in this study, it was the first time that HuScFv specific to S1 subunit of PT was successfully produced and demonstrated that it still contain the target binding properties of original MAb.

By the homology search of PT6-2G6-HuScFv using BLAST software, it revealed that HuVL and HuVH of PT6-2G6-HuScFv have 90% and 81% homologies with the closet human VL and VH molecules, respectively (**Figures 48 and 49**). The CDR of PT6-2G6-HuScFv showed the difference in amino acid sequence to the human CDR, especially CDR3 of VH. Because, CDR3 of VH falls in the region of the V-D-J joining, in which several mechanisms contribute to generation of its diversity, including selection of *V*, *D* and *J* gene segments, and alternative splicing patterns. (Kabat *et al*., 1991; Rock *et al*., 1994). It is possible that the CDRs of PT6-2G6-HuScFv, especially CDR3 of HuVH, may evoke anti-variable region including anti-idiotypic response in human who ever received this HuScFv in large amount. Therefore, to minimize that response, the further humanization that graft only the specificity determining residues (SDRs) within the CDRs onto the human antibody frameworks should be performed and the advantage and disadvantage of the more humanized-(SDR-grafted) product should be demonstrated. Presently, the PT6-2G6-HuScFv has the potential to use as the non-drug therapeutic agent in patient with serious pertussis.