CHAPTER VI
DISCUSSION

Vaccination is the primary strategy for prevention and control of influenza while anti-virus drug together with supportive measures are used for treatment of severe influenza clinical manifestations. The prophylaxis and therapy are facing several obstacles. Current influenza virus vaccines, *i.e*., the inactivated whole virus, split product, or subunit vaccines for intramuscular injection (Kanra *et al*., 2004) and the live attenuated virus vaccine for intranasal route (FluMist) (Treanor *et al*., 2000), elicit relatively short duration of immunity which are clade and/or subtype specific (Cox *et al.*, 2004). They only protect against the disease caused by the same strains, clade, or to the most, the same subtype to the influenza virus that contained in the vaccine (Baras *et al.*, 2008). Thus, the vaccine strains of influenza viruses must be adapted annually such that the vaccine contains the strains matched with the circulating virus. Moreover, influenza has short incubation period which the immunological memory elicited by the vaccination is unlikely to be recalled in time. Therefore, frequent vaccination, *e.g.*, annually, is required to keep a sustained protective serum level of the virus neutralizing antibody which is principally anti-hemagglutinin that block the virus entry into cells. The protective rates of the vaccines vary among groups of the vaccinees, with low protection observed among the elderly (Kang *et al.*, 2004). The presently available vaccines are, thus, unlikely to confer protection to humans against the new influenza virus that may emerge as a result of the genetic reassortment of the existing 16 H and 9 N influenza A subtypes, or the highly pathogenic avian influenza H5N1 subtype that might be adapted to more efficient human-to-human spread. There is a perceived necessity of a broad spectrum influenza vaccine that protects across clades/subtypes for both human and veterinary uses. However, there are milestones to the success in developing such vaccine even though several world renowned scientists in many prestigious laboratories are devoting for it. The influenza virus exposed individuals especially those with severe influenza symptoms requires anti-influenza drug. Unfortunately, there is only one family of the drugs that still effective against influenza, *i.e.,* oseltamivir (Tamiflu) which is a neuraminidase inhibitor (Colman,

1989). Nevertheless, the drug must be given to the patients as early as possible after the infection in order to be effective (Regoes and Bonheffer, 2006). Besides, the medicine is unlikely to be adequately supplied, if a large epidemic or pandemic ever broke out, and drug resistant influenza virus have already emerged (Regoes and Bonheffer, 2006). Thus, new anti-virus drugs or their alternatives should be sought for large influenza outbreak or pandemic.

While the available anti-influenza virus drugs are aiming at blocking the activities of M2 ion channel protein and neuraminidase, no drugs are using the virus RNA polymerase as the targets. RNA-dependent RNA polymerase of influenza A virus plays pivotal role in virus transcription and replication. Thus, in this research, a hypothesis was set: antibodies that specifically bound to the functionally active sites on the virus polymerase subunits (PB1, PB2 and PA) in the infecting host cells would interfere with the virus infectious cycle which should bring about a termination of the infection. Therefore, the human monoclonal antibodies to N-terminal portion of individual polymerase subunits were produced by using human antibody phage display library constructed in our laboratory from 60 blood donors, which is a library with the largest antibody repertoire (2.6×10^8 diversity) (Kulkeaw, 2008).

The N-terminal portion of the polymerase subunit proteins were chosen as the antibody targets for several reasons. The N-terminal PA subunit has two nuclear localization signals located at the amino acid residues 124-139 and 186-247 in the molecule (Detjen *et a*l., 1987). The N-terminal PB1 binds to 3' and 5' of vRNA at amino acid positions 1-83, binds to PA in the polymerase complex at amino acid residues 1-25, and has also two nuclear translocation signals at amino acid residues 180-195 and 202-252, and the N-terminal PB2 contains viral nucleoprotein binding site at amino acids 1-269, PB1 binding site at amino acids 206-258, and cap binding site at amino acids 242-282. Interfering with the biological functions of these polymerase proteins by the antibodies would be inhibitory for the virus replication. Besides, the production of full length of functional recombinant polymerase proteins of the influenza virus is definitely not an easy task. To my knowledge, no evidence in the literature report successful production of full length recombinant polymerase proteins of the influenza virus by using the prokaryotic expression system. Only some

attempts have been made to amplify the protein coding sequences (Hoffmann *et al*., 2001; Chan *et al.*, 2006) but not the recombinant protein expression

1. Cloning of DNA sequences encoding N-terminal halves of the PA, PB1 and PB2-polymerases

The total RNA from influenza A virus subtype H5N1 [Influenza A/duck/144/Thailand/2005 (H5N1)] was extracted and used for complementary DNA synthesis by RT-PCR. In the RT-PCR, the ThermoScript™ Reverse Transcriptase was used. This transcriptase enzyme was chosen because it can function in synthesizing a long cDNA, *i.e.*, from 100 bp to >12 kb at high temperature. It is also good for RT-PCR for exceptionally difficult templates like the cDNA of the virus RNA polymerase sequences. Chan *et al.* (2006) compared the efficiency of reverse transcriptases between ThermoScript™ Reverse Transcriptase and M-MLV reverse transcriptase for amplification of all eight gene segments of influenza A virus H1N1 and H3N2 subtypes. They found that full length of PA, PB1 and PB2 coding DNA sequences could be amplified from the cDNA only when the ThermoScript™ Reverse Transcriptase was used. In this study, the Uni12 oligonucleotides was used as a specific primer for the first stand cDNA synthesis. The Uni12 primer has been previously used with success for influenza A virus cDNA synthesis by Gorman *et al*. (1990), Kawaoka *et al*. (1990), and Hoffmans *et al*., 2001. The sequences bound by the primer are found on the 3' end of all gene segments of the influenza virus.

The PA-5', PB1-5', and PB2-5' sequences were amplified from the cDNA by using three pairs of oligonucleotide primers designed from DNA sequences of the influenza A virus subtype H5N1 deposited in the database. The PA-5' sequence encodes N-terminal 1-240 amino acids of the PA protein which contain two nuclear localization signals (NLS), one is located at amino acid residues 124-139, and another is located at the amino acid residues 186-240 (Detjen *et al*., 1987). The PB1-5' sequence encodes N-terminal PB1 protein (1-378 amino acids) containing the PA binding site at amino acid residues 1-25, RNA binding site at amino acid residues 1-83 and 249-256, and 2 NLS at amino acid residues 180-195 and 202-252 (Detjen *et al*., 1987). The PB2-5' sequence encodes N-terminus of PB1 (404 amino acids) containing the PB1 binding site at amino acid residues 206-259. This portion of the

PB2 molecule could bind the host cap at the amino acid residues 242-282 and contains NP binding site at amino acid residues 1-269 (Detjen *et al*., 1987). All of the mentioned components located at the N-terminal portions of the polymerase molecules are important in viral transcription and replication processes. Thus, blocking of these components might interfere the viral life cycle and inhibit the viral pathogenesis. The PA-5', PB1-5', and PB2-5' encoding DNA sequences were successfully amplified from the cDNA. Their amplicons were seen as bandsof about 720, 1,134, and 1,212 bp, respectively. Sequence analysis of the amplified PB1-5', PB2-5' and PA-5' sequences showed that there were 99-100% identity of nucleotide sequences to those of the sequences deposited in the database. It has been previously reported that there was 98.8-100% identity of the PB1, PB2 and PA nucleotide sequences among the H5N1 virus strains isolated from Thailand during 2004-2005. The nucleotide sequences of 97.2-97.7% homology of were found for the PB1, PB2 and PA coding sequences of the H5N1 strains isolated in Thailand in 2005 and China in 2005 (Amonsin *et al*., 2006).

The respective gene sequences were also successfully cloned into *E. coli* expression hosts. In the prokaryotic expression system, the pET-20b(+), pQE30 and pQE31 protein expression vectors were commonly used. The reason to use these vectors is that they are especially constructed for the purpose of producing a fusion protein in which 6x His tag is added spontaneously to the protein sequence. The 6x His tag is relatively small and does not interfere with the structure of function of the purified protein. The advantage of using this tag is the ability of the tag to bind to the metal-chelating surface; therefore the recombinant fusion protein can be simply purified and detected by using nickel-affinity column and anti-His antibody. The pET-20b(+) vector contains the 6x His tag at the C-terminal of the inserted fragment. The advantage of this vector is to ensure that only full-length of the target proteins are purified and detected by anti-His antibody. The pQE expression vectors, *i.e.*, the pQE30 and pQE31, contain the 6x His tag at the N-terminal of the inserted fragment. The recombinant proteins could be also purified by using the nickle column. Both pET-20b(+) and pQE protein expression vectors contains strong promoter, bacteriophage T7 and T5 promoters, respectively. Although the pET-20b(+) vectors can be used for expression of all three recombinant polymerase proteins, we have

explored the possibility of using the pQE expression vectors for the recombinant PB2 and PA polymerase protein expression with success.

2. Production of recombinant N-terminal PA, PB1 and PB2-polymerases proteins

PB1-5' gene segments were subcloned into pET-20b(+) protein expression vector and the *E. coli* BL21 (DE3) pLysS cell was used as an expression host. The PA-5' and PB2-5' gene segments were subcloned into pQE31 and pQE30 protein expression vectors, respectively and the M15 *E.coli* cells were used as a expression host. Recombinant N-terminal PA, PB1 and PB2 proteins have approximate *Mr* of 26, 43 and 41, respectively. The recombinant N-terminal PA contains amino acid 1 to 240 of the native PA subunit; the recombinant N-terminal PB1 contains amino acid 1 to 378 of the native PB1 subunit; and the recombinant N-terminal PB2 contains amino acid 1 to 404 of the native PB2 subunit. The recombinant N-terminal PB1 and PB2-5' were detected in the insoluble cytoplasmic fractions while the recombinant N-terminal PA protein was found in both soluble and insoluble cytoplasmic fractions but the amount in the latter was more. To obtain the soluble target proteins, the conditions of protein expression were modified by decreasing the concentration of IPTG (0.5-0.2 mM) and the bacterial culture temperature (30^{o}C). The amount of soluble recombinant PA protein was slightly increased (data not shown). However, the recombinant PB1 and PB2 proteins were still found in the insoluble cytoplasmic fractions. Nevertheless, all of the recombinant proteins in such expressed conformations were used for selecting phage clone displaying HuScFv to the epitopes on the molecules with the awareness that the important epitopes may not be exposed on the surface and could not be recognized by the HuScFv on the phage particles.

His-tagged recombinant N-terminal PA, PB1, and PB2 proteins were purified by one-step purification process using nickel column. His-tagged protein was individually captured by the column while other *E. coli* contaminants were washed out by using a wash buffer. Bound recombinant proteins were then eluted out by an appropriate eluting buffer. The technique is convenient and the amounts of the target proteins are adequate for the experimental use. Nevertheless, for larger quantity, other expression system such as yeast or insect cell expression systems might have to be sought.

3. Production of HuScFv specific to recombinant N-terminal PA, PB1 and PB2 proteins

The recombinant proteins were individually used as antigens in the phage bio-panning for selection of the phage clones displaying HuScFv to the protein epitopes. Single round of the bio-panning was used instead of the repeated rounds of the process commonly used by other investigators (Claudio *et al.*, 2003, Mei-yum *et al.*, 2005, Takayuki *et al.*, 2007). The repeated bio-panning (~ 2-4 cycles) although eliminates the non-specific phage clones but tends to generate complete and partial deletions of the *scFv* gene insert from the phage genome rendering unproductive clones. From the single round bio-panning in this study, ~ 63.3-83.3% of HB2151 *E. coli* clones transformed with individual HuScFv-phage preparation contained the *huscFv* gene insert (**Figure 32-34**). Among them, 63.2-80% of the representative clones that were screened for the HuScFv expression were positive (**Figure 35-37**) which indicates further that some *huscFv*-phagemid carrying *E. coli* could not expressed the HuScFv under the condition used in this study. The reason that can be given to explain the finding is that the HuScFv might contain a stop codon within the nucleotide sequence. Nevertheless, the human monoclonal antibodies in the form of ScFv to the N-terminal PA, N-terminal PB1, and N-terminal PB2 polymerase proteins of influenza A virus H5N1 subtype were successfully produced using the human antibody phage display library.

To determine the *huscFv* sequences of the selected phage clones were determined by observing the multiplicity of the DNA banding patterns by RFLP. A RFLP pattern multiplicity of 80-100% was found for the *huscFv* of *E. coli* clones that could produce the HuScFv (**Figure 44-46**). This implies that the epitope diversity of the HuScFv might also be high, *i.e.*, the HuScFv produced from different *E. coli* clone may be bind to different epitope on recombinant N-terminal polymerase protein.

4. Binding specificity of the HuScFv to the recombinant N-terminal polymerase proteins

The expressed HuScFv were found to bind specifically to their homologous proteins when tested by indirect ELISA and Western blot analysis and/or dot ELISA *in vitro* (**Figures 39-43**). Experiments should be done to test the inhibitory activities of these specific HuScFv on the native polymerase functions and to identify their epitope specificity.

5. Some biological activity of the N-terminal PA

It has been reported that during the influenza infection the individually expressed PB1 and PA subunits were found in both the cytoplasm and the nucleus of the influenza infected cells, whereas the PB2 subunit tends to accumulate in the nucleus (Fodor *et al*., 2004). The N-terminal portion of the PA polymerase subunit contains two independent nuclear localization signals (NLS), one in the region I (amino acids 124 to 139) and another in the region II (amino acids 186-247), both of which contribute to nuclear import of the vRNP (Nieto *et al*., 1994). Deletion of either the region I or the region II did not prevent nuclear localization of the mutant PA. In this study, the recombinant N-terminal PA was produced and it contains nuclear localization signals, region I (amino acid 124 to 139) and a part of the region II (amino acids 186-240). In this study an assay was designed for testing the nuclear import activity of the so-produced recombinant N-terminal PA protein. The assay used the digitonin-permeablilized Vero cells that had been grown on glass cover slips in tissue culture medium. The digitonin permeabilizes the plasma membrane of cells, but retains the integrity of the nuclear envelope (Adan *et al*., 1990). It has been reported that the 70 kDa dextran labeled with Taxas Red could enter the plasma membrane but was almost totally excluded from the nucleus. This indicates that plasma membrane was permeabilized by the digitonin, while the integrity of the nuclear envelope was maintained (Wu *et al*., 2007). In this study, recombinant N-terminal PA (~26 kDa) was found in the cytoplasm and efficiently enter and accumulate in the nucleus of the digitonin-permeabilized Vero cells (**Figure 47**). Thus, this model of nuclear localization was used for testing the HuScFv inhibitory of the recombinant N-terminal PA. The HuScFv inhibition of PA nuclear import resulted in decreased nuclear

acculution of recombinant PA in Vero cells. From these results, it appears that HuScFv specific to recombinant N-terminal PA may be an effective means of interrupting a critical stage in the influenza A virus life cycle.