Chapter 6

Construction and Characteristics of a Recombinant Single-Chain Antibody Fragment against Bacterial Type III Secretion

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Additional information is available at the end of the chapter

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Abstract

Pseudomonas aeruginosa, a Gram-negative pathogen, causes life-threatening infections. Lung injury and the development of sepsis depend largely on expression of the virulence genes associated with the type III secretion system of this bacterium. The type III secretion system functions as a molecular syringe to deliver type III secretory toxins directly into the cytosol of eukaryotic cells and also acts to inhibit innate immune mechanisms, thereby preventing bacterial clearance. Antibodies against PcrV, the cap structure in the translocational needle of type III secretory apparatus of *P. aeruginosa*, block toxin translocation of the type III secretion system. We have been investigating the therapeutic use of a recombinant anti-PcrV single-chain antibody. In this chapter, as a preliminary step toward an antibody-based immunotherapy against bacterial infections, we summarize our experience of constructing a recombinant single-chain antibody (called scFv166), in which the heavy (V_H) and light chain (V_L) variable regions of the anti-PcrV monoclonal IgG are joined by a flexible peptide linker. The practical methodologies used to make recombinant scFv166 against a bacterial protein component are described in detail.

Keywords: single-chain antibody, PcrV, *Pseudomonas aeruginosa*, type III secretion system

1. Introduction

Bacterial infections still frequently cause life-threatening diseases in humans. New pathogens have emerged, old pathogens have reemerged, and the prevalence of multidrug resistant microorganisms has increased despite the introduction of various new antibiotics since antibacterial agents were first developed in the early twentieth century. The difficulties

associated with treating infections in immunocompromised patients have increased the need for new adjunctive immunotherapies. During the last 20 years, major advances in the techniques used to generate human antibodies and humanize murine monoclonal antibodies have seen antibody-based therapies to arrive as potential candidates for adjuvant therapies for infectious diseases. However, today, antibody therapy for bacterial infections is still indicated in relatively few situations, although more attention should be focused on it because of the increased levels of bacterial drug resistance and higher numbers of immunocompromised patients.

We have been investigating the therapeutic use of recombinant antibodies against the Gram-negative pathogen, *P. aeruginosa*. *P. aeruginosa* is an opportunistic pathogen responsible for a variety of acute infections in immunocompromised patients, and chronic infections in those with cystic fibrosis [1, 2]. *P. aeruginosa* is also highly resistant to various antibiotics and causes nosocomial pneumonia with an associated high mortality rate despite aggressive treatment with antimicrobial drugs [3, 4]. We have been studying the pathogenesis of acute infections caused by *P. aeruginosa* to identify a therapeutic target in this pathogen, and have reported that its ability to cause epithelial injury, to disseminate into the circulation, and to avoid host innate immune responses is highly associated with its type III secretion system (TTSS) [5–9]. The TTSS of Gram-negative bacteria mediates the translocation of toxins from the bacterial cytoplasm directly into the cytosol of host eukaryotic cells [10, 11]. Once inside the eukaryotic cell, these translocated bacterial toxins interfere with signal transduction. TTSSs with homology to *P. aeruginosa* have been described in most Gram-negative bacterial pathogens (e.g., *Yersinia*, *Shigella*, *Salmonella*, and *Escherichia coli*), and all of them are associated with pathogenicity [12].

Here, as a preliminary step toward antibody-based immunotherapy against bacterial infections, we summarize our trial to block the TTSS-associated virulence of *P. aeruginosa* using recombinant antibody technologies. Especially, cloning of the variable domains of the light and heavy chain from a hybridoma cell and assembling the cloned V_H and V_L domains to recombinant single-chain antibody (scFv) to confirm the binding to the target antigen are required steps to humanize murine antibody. In addition to a brief explanation of the *P. aeruginosa* TTSS and the concept of a virulence blockade, the advantage of a recombinant single-chain antibody (scFv), the detailed methods to clone the variable domains from hybridoma cells and construction of scFv166, in which the heavy (V_H) and light chain (V_L) variable regions of the anti-PcrV monoclonal IgG molecule are joined by a flexible peptide linker, will be described.

2. Antibody-based blockade of *P. aeruginosa* type III secretion

P. aeruginosa translocates its virulent TTS toxins (ExoS, ExoT, ExoU, and ExoY) directly into eukaryotic cells to disrupt their normal cellular processes [12, 13]. The translocation of ExoS and ExoT proteins, which both have ADP-ribosyltransferase and GTPase activities, inhibits

the phagocytic activities of macrophages [14, 15]. The translocation of ExoU, which has patatin-like phospholipase A_2 activity, is correlated with acute cytotoxicity *in vitro* and lung damage, sepsis, and mortality in animal models [7, 16–20]. The translocation of ExoY, which possesses adenylate cyclase activity, causes an increase of cytosolic cyclic AMP in eukaryotic cells and affects cell morphology [21]. In our past clinical study, we discovered an association between patients infected with TTSS-expressing *P. aeruginosa* strains and mortality [22], and other reports from various countries have supported the association of TTSS with severe clinical outcomes in patients infected with this bacterium [19].

In the TTSS, the translocated toxins are not exposed extracellularly and evade direct recognition by the host immune system. Therefore, targeting the protein factors involved in the "*secretion*" or the "*translocation*" process of the TTSS seems a rational approach for blocking TTS virulence. To target the TTSS of *P. aeruginosa*, we have been developing neutralizing antibodies capable of blocking the translocation process of the TTSS [23]. An obvious candidate for a protective antigen was PcrV as it shares relatively high homology with the protective antigen from *Yersinia* sp., LcrV [6, 24-33]. Using genetic analyses, we demonstrated that PopD and PcrV were required for the delivery of *P. aeruginosa*-encoded TTS toxins [23]. In addition, recombinant TTS proteins, such as ExoU, PcrV, and PopD, were produced, purified, and tested for their protective capacities in a model of acute lung infection in mice. Only PcrV was protective in these experiments. Antibodies to PcrV protected against type III intoxication as shown by the inhibition of translocation of ExoY and by the inhibition of macrophage cytotoxicity mediated by ExoU. Passive protection with anti-PcrV reduced the inflammatory response, minimized bacteremia, and prevented septic shock [23]. Moreover, the protective capacity of the antibody was Fc-independent because $F(ab')_2$ fragments of polyclonal anti-PcrV were also effective [34–36].

In our previous study, the Mab166 murine monoclonal antibody, which has neutralizing effects on virulence of the *P. aeruginosa* TTSS, was developed [35]. Also, the Fab fragments of the Mab166 had comparable therapeutic effects to the whole IgG of Mab166 in preventing *P. aeruginosa*-induced acute lung injury, and the Fc-dependent opsonization of the bacteria does not seem critical for the efficacy of the Mab166 [36]. These results implicate that the blockade of type III secretion-associated virulence can be attained by the effective Fab fragment of IgG molecules. Because the Fc-portion of IgG may induce unfavorable inflammatory responses such as complement fixation, activation of macrophages, the administration of the whole IgG may cause some inflammatory side effects. If the Fab fragment had the same therapeutic potency as the whole IgG, the therapeutic administration of either Fab fragments or scFv might overcome the disadvantages of the intratracheal administration of whole IgG. Therefore, the *E. coli*-derived recombinant scFv against PcrV is attractive to be an effective therapeutic agent against *P. aeruginosa* pneumonia.

In the next chapter, we describe the methods used to clone the variable antibody domains V_H and V_L from hybridoma cells and assembly of a single-chain antibody as an *E. coli*-derived recombinant protein. Previously, the engineered recombinant Fab fragment against PcrV was humanized to allow it to be considered for adjunctive therapy in patients [37–39].

3. Methods for construction of a single-chain antibody

3.1. Cloning the variable V_H and V_L domains from hybridoma cells

3.1.1. Poly A⁺ RNA extraction

The anti-PcrV IgG Mab166 hybridoma cell line [35] was cultured in a standard culture medium. After the cells had reached confluence in a 75 cm^2 flask, they were harvested by centrifugation at 600 rpm for 5 min. The cell pellet was homogenized in 2 mL of TRIzol™ reagent (Thermo Fisher Scientific, Waltham, MA, USA), and total RNA extracted after chloroform fractionation, isopropanol precipitation, and washing with 70% ethanol. Poly A^+ RNA was extracted with an oligotex mRNA spin-column (Qiagen, Valencia, CA).

3.1.2. RNA oligo-capping

To clone the variable V_H and V_L domains from the total RNA, the oligo-capping method reported by Maruyama and Sugano [40] using a GeneRacer™ kit (Thermo Fisher Scientific) was used. mRNA (250 ng) was incubated with calf intestinal phosphatase at 50 °C for 1 h to dephosphorylate non-mRNA or truncated mRNA species. After the reaction, phenol-chloroform extraction and ethanol precipitation were performed, and the dephosphorylated RNA was incubated with tobacco acid pyrophosphatase at 37°C for 1 h to remove the 5′-cap structure from the full-length mRNA. After phenol-chloroform extraction and ethanol precipitation, the synthetic RNA oligo (GeneRacer™ RNA Oligo, Thermo Fisher Scientific) was ligated to the decapped RNA with T4 RNA ligase at 37°C for 1 h. After phenol-chloroform extraction and ethanol precipitation, the RNA was suspended in diethylpyrocarbonate-treated water.

3.1.3. Reverse transcription of mRNA

The RNA-oligo ligated, full-length mRNA was reverse transcribed using a 54 base-pair primer containing an 18 nucleotide dT tail (GeneRacer™ Oligo-dT, Thermo Fisher Scientific) and avian myeloblastosis virus reverse transcriptase at 42°C for 1 h. After the reaction, the sample was diluted four times with sterile water.

3.1.4. Construction of a single-chain antibody gene

The cDNAs encoding V regions of the heavy and light (kappa) chains were PCR-amplified using a set of primers (V_H forward: 5'-TGA GGA GAC GGT GAC TGA GGT TCC-3', V_H reverse : 5'-CAG GTG CAG CTG AAG CAG TCA GG-3', V_{k2} forward: 5'-CCG TTT TAT TTC CAG CTT GGT CCC-3', V_k reverse : 5'-GAC ATC CAG ATG ACT CAG TCT CCA-3'). PCRs were run over 30 cycles (94°C for 30 sec, 60°C for 40 sec, and 72°C for 40 sec). V_H and V_L fragment-amplified PCR products were purified separately by agarose gel electrophoresis. The PCR products derived from the murine immunoglobulin V_H and V_L domain of Mab166 were subcloned into a pCR2.1 vector (TOPO cloning™, Thermo Fisher Scientific) and submitted to

a DNA sequencing service for DNA sequence acquisition and analysis. Sequencing of the immunoglobulin variable genes for Mab166 was analyzed by The International imMunoGeneTics Database IMGT (http://www.imgt.org).

The purified V_H and V_L cDNAs were each assembled into a single gene using a DNA linker fragment-encoding a glycine-serine $(Gly_4Ser)_3$ linker peptide, thereby connecting the two cDNAs in the correct reading frame. Assembly PCR was run with a set of primers to multiply V_H-linker-V_L. The assembled fragment was amplified using two oligonucleotide primers with either an *Nco*I or *Xba*I restriction enzyme site at the 5′ end to facilitate cloning of the PCR product into a pBAD/gene III plasmid (Thermo Fisher Scientific) (**Figure 1**). The ligation mixture was used to transform *E. coli* TOP10 cells (Thermo Fisher Scientific), and subsequently to transform *E. coli* LMG194.

3.2. Expression and purification of recombinant single-chain antibody fragments

3.2.1. Expression and purification of scFv166

scFv166 protein expression was induced in the *E. coli* plasmid-harboring transformants by adding L-arabinose to a final concentration of 0.004%. After 24 h culture at 26°C with agitation at 200 rpm, the cells were collected by centrifugation at 5000× g for 20 min and then incubated in phosphate-buffered saline (PBS) with 1 mM ethylenediaminetetraacetic acid for 10 min on ice to obtain the periplasmic fraction. The osmotically shocked lysate was centrifuged at 15,000× g for 20 min, passed through a 0.4-μm-pore-size filter and dialyzed overnight against

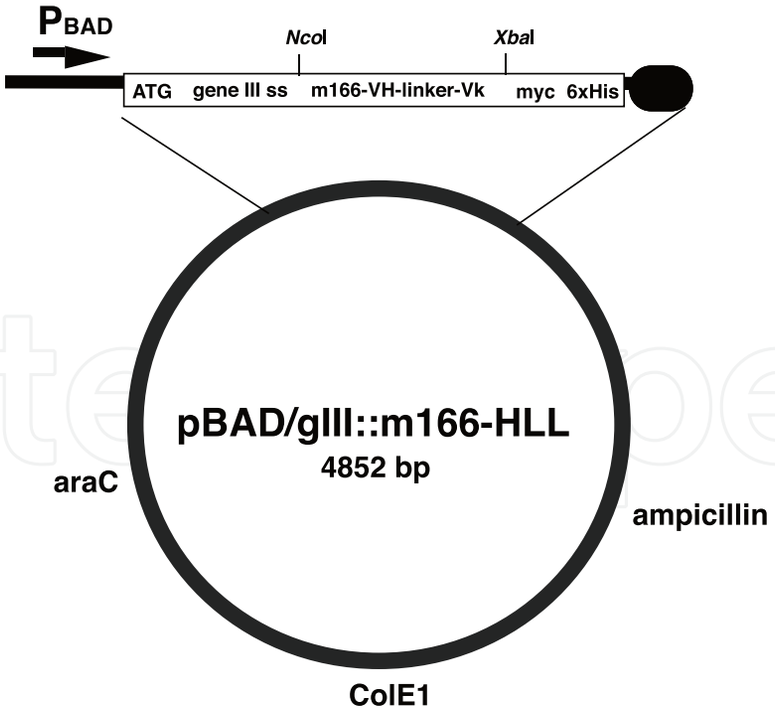

Figure 1. Expression vector pBAD/gIII::m166-HLL. The assembled scFv166 gene was subcloned into the pBAD/Gene III *E. coli* expression vector, downstream of, and in frame with, the gene III secretory leader sequence using *NcoI* and *XbaI* restriction sites.

lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 0.05% Tween 20, pH 8.0). The lysate was mixed with nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) for 30 min at 4°C with gentle shaking. After the Ni-NTA agarose was collected by centrifugation (4000× g), it was resuspended in lysis buffer and packed onto the chromatography column.

The column was washed twice with washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 0.05% Tween 20, pH 8.0), and the bound scFv166 antibodies were eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 0.05% Tween 20, pH 8.0). The eluate was dialyzed against PBS overnight and applied to an endotoxin removal column (Detoxi-Gel, Thermo Fisher Scientific) to get rid of the contaminating endotoxin. The purified antibodies were stored at −80°C until use.

In this study, we assembled the variable regions of the heavy and light chains of the anti-PcrV monoclonal IgG together with a glycine-serine linker in a single-chain antibody format. First, we assembled scFv166 in two different formats: one with V_H-linker-V_k positioned between the two variable segments (**Figure 2**), the other with V_k-linker-V_H positioned between two variable segments. The assembled scFv166 gene was subcloned into the *E. coli* pBAD/gene III expression vector, downstream of, and in frame with, the gene III secretory leader sequence. Expression of recombinant scFv166 was induced in *E. coli* by arabinose, after which it was purified via its C-terminal hexahistidine tag using Ni-NTA resin and conventional affinity column techniques. However, scFv-V_L-linker-V_H was highly insoluble, despite the expressed protein being detected in the whole lysates from *E. coli* cells after arabinose induction. Because scFv166 with its associated V_H-linker-V_L fragment was easier to purify as a soluble protein, we decided to focus on purifying it in that format. The purified scFv166 recognized the PcrV antigen in ELISAs and western immunoblots, as described in the next section.

3.3. Protein gels and immunoblot analyses

The purity of scFv166 was evaluated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Blue staining (**Figure 3**). Briefly, samples of *E. coli* lysate were loaded onto a 4–15% Tris-HCl gel (BioRad Laboratories Inc., Hercules, CA, USA) and, after electrophoresis, the gel was stained with Coomassie Blue. For the immunoblot analysis, after SDS-PAGE, the protein was transferred to a nitrocellulose membrane and immunostained with a horseradish peroxidase-conjugated anti-c-myc IgG antibody, after which the blot was developed with a chemiluminescent substrate (ECL, GE Healthcare Bioscience, Piscataway, NJ). Immunoblots of scFv166 and precipitated *P. aeruginosa* proteins were also performed (**Figure 4**). *P. aeruginosa* PA103 was cultured in tryptic soy broth deferrated with nitrilotriacetic acid for 24 h at 31°C and, after centrifugation at 5000× g for 20 min, the supernatant was harvested. Saturated ammonium sulfate solution was added (final concentration, 55%), and the solution was incubated on ice for 1 h, and then centrifuged (20,000 × g, 30 min). The precipitated proteins were resuspended in 100 μL of PBS. After adding 100 μL of SDS-PAGE sample buffer and boiling for 5 min, the sample was analyzed by SDS-PAGE. After electrophoresis, the proteins were blotted onto a nitrocellulose membrane, and then immunostained with scFv166 and a horseradish peroxidase-conjugated anti-c-myc IgG secondary antibody, and the blot was developed with ECL.

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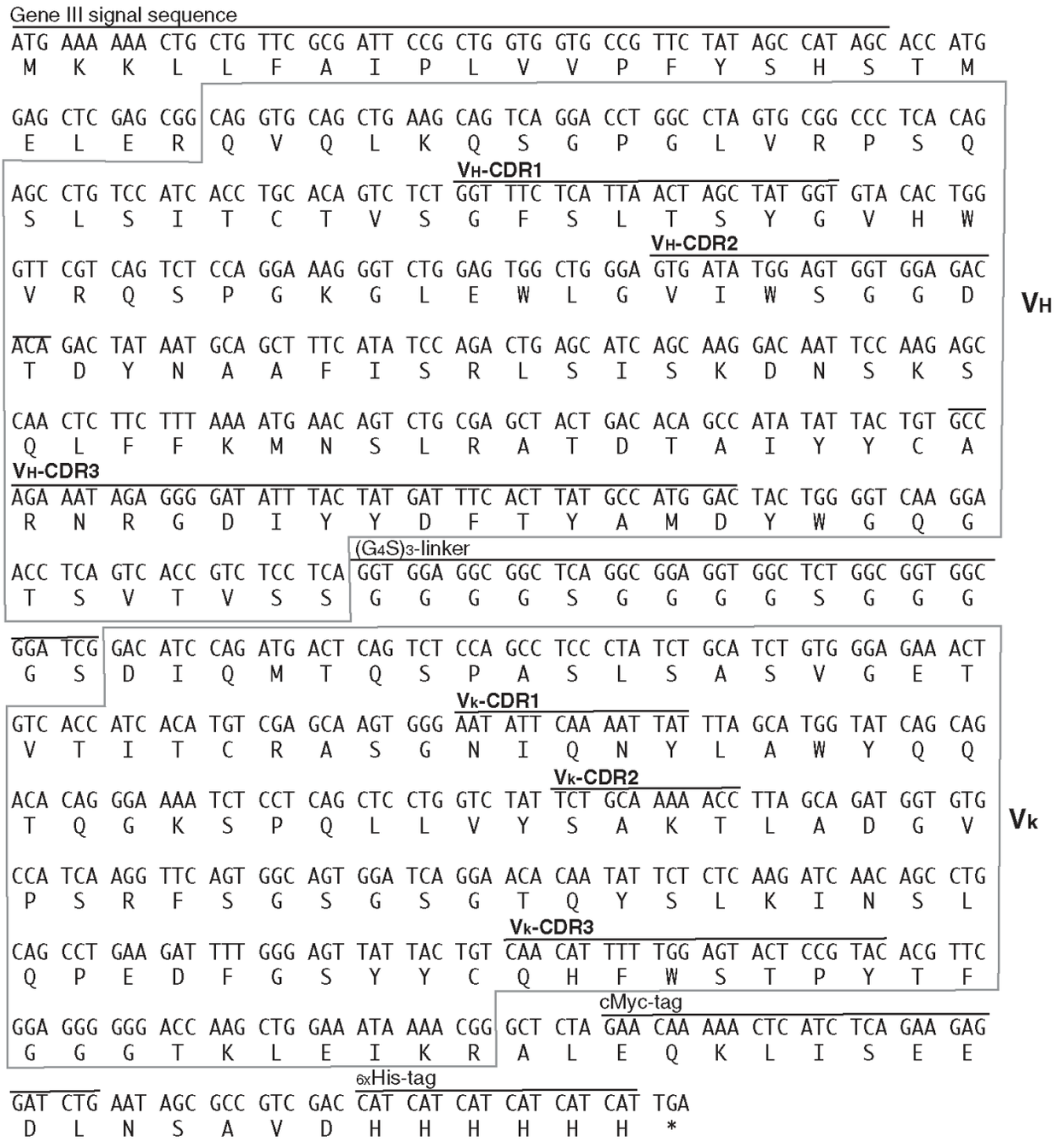

Figure 2. scFv166 nucleotide sequence. After the gene III signal sequence (18 aa) and the short joint region (6 aa), the V_H region (123 aa) is followed by the glycine-serine linker (15 aa), the V_L region (108 aa), a cMyc-tag, and a hexahistidine-tag.

3.4. Affinity determination of scFv166

The affinity of scFv166 for its cognate antigen was determined by competition ELISA, and the result was compared with that of the hybridoma-derived parental Mab166, as described previously [41], **Figure 5**. Briefly, in the first step, the total antibody concentration range in which the absorbance correlates proportionately with the free antibody concentration was

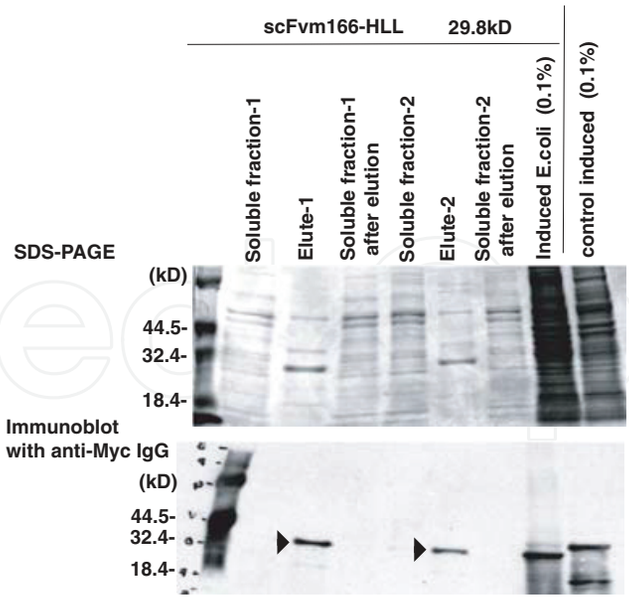

Figure 3. Expression and purification of scFv166. *E. coli* lysates were loaded onto a 4–15% gradient Tris-HCl gel and, after electrophoresis, the gel was stained with Coomassie Blue. For the immunoblot analysis, after polyacrylamide gel electrophoresis, the protein was blotted onto a nitrocellulose membrane and immunostained with a horseradish peroxidase-conjugated anti-c-Myc IgG antibody, and the blot was developed with a chemiluminescent substrate. The secreted scFv166 (298 amino acids) was detected as a 29.7 kD-band in the elute-1 and elute-2, designated by arrows. Soluble fraction: the osmotically shocked lysate; elute: the eluted solution from an Ni-NTA agarose column; soluble fraction after elution: the solution passed through an Ni-NTA agarose column (two sets of the lysate and the column elute were analyzed and labeled "–1" and "–2", respectively).

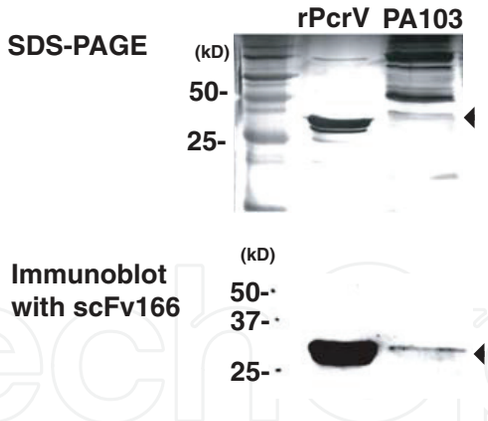

Figure 4. Immunoblot of *P. aeruginosa* proteins reacted with scFv166. Precipitated *P. aeruginosa* PA103 proteins were resuspended in 100 μL of PBS. After adding 100 μL of SDS-PAGE sample buffer and boiling for 5 min, the proteins in the sample were separated by SDS-PAGE with *E. coli*-derived recombinant PcrV (rPcrV) as a reference. After electrophoresis, the proteins were transferred to a nitrocellulose membrane, and immunostained with scFv166 and a horseradish peroxidase-conjugated secondary anti-c-Myc IgG antibody and the blot was developed with a chemiluminescent substrate. The bindings of scFv166 to both native PA103 PcrV (294 amino aicds, 32.4 kD) and recombinant PcrV (rPcrV, 306 amino acids, 33.8 kD) were detected as shown in arrows.

measured by indirect ELISA with the PcrV antigen coated at 1 μg/mL. In the second step, K_d, the dissociation constant, was measured using binding equilibrium studies (competition ELISA) to determine the concentration that gives 50% inhibition of maximum binding.

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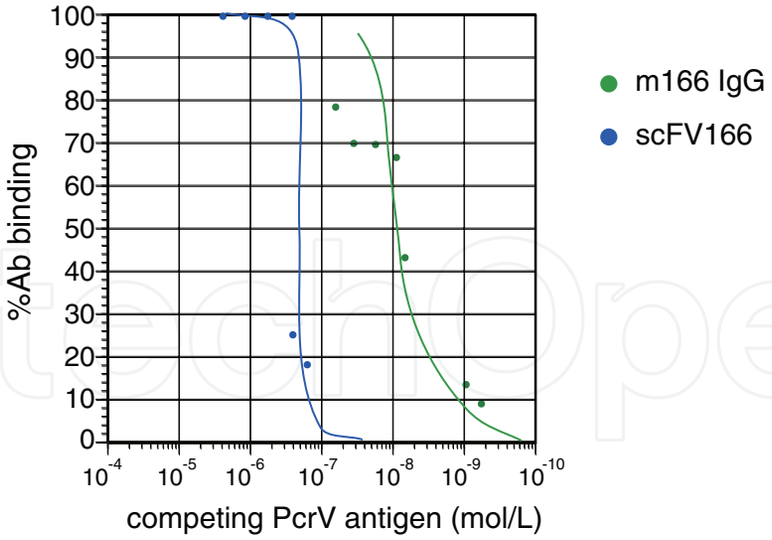

Figure 5. Binding affinities of Mab166 and scFv166 in competition ELISA. Binding affinities (K_d) of Mab166 and scFv166 to immobilized PcrV were evaluated in a competition ELISA.

4. Results

4.1. Aminoacid sequence of V_H, V_L of scFv166

The sequence of the Mab166 heavy chain region is shown in **Figure 2**. The DNA sequence of the 5′-untranslational region and a V-region segment in the heavy chain-containing complementarity determining regions (CDRs) 1 and 2 is identical (except two amino acids in the frame 3 region) to germline Musmus IGHV2S2 (IGHV subgroup 2, V_H#101, Accession #J00502). The V-region sequence also shows the same level of homology as that reported for pseudogene, IGHV2S5 (Accession #M21165). Transcription starts 24 nucleotides downstream of the TATA box of germline IGHV2S2. Nucleotides differ from the germline sequence at 10 positions, and these cause the following amino acid changes: position #61 in CDR2 S->D, #87 in FR3 V->L, #95 Q->R, and #96 S->A, #97 N->T. The first 15 nucleotides in the D-region encode the first 5 unique amino acids in CDR3, and the region consists of 16 amino acids in total. The J-region DNA sequence is identical to the IGHJ4 germline sequence (Accession #V00770). The unique CDR3 sequence includes the Arg-Gly-Asp (RGD) sequence, which functions as a recognition sequence for adhesion receptors in many adhesive proteins including fibrinogen, fibronectin, von Willebrand factor, and vitronectin.

The nucleotide sequence of the variable region of the kappa light chain, along with its predicted amino acid sequence, is shown in **Figure 2**. The CDRs are underlined, and the amino acids are numbered according to a convention. This kappa variable chain is a class II mouse kappa variable region. Although its sequence is not identical to any germline variable regions present in the data bank (The International ImMunoGeneTics Database IMGT), the DNA sequence of the 5′-untranslational region, and V-region of the kappa light chain shows the highest

homology to germline Musumus IGKV12-41*01F (IGKV subgroup 12, Accession #AJ235953). Transcription starts from nine nucleotides downstream of the TATA box. Nucleotides differ from the germline sequence at four positions (+#192, C->A), (+#218, A->C), (+#250, A->T, +#251, A->C), and they cause amino acid changes at the following positions: #30 in CDR1 H->Q, #45 in FR2 K->T, and #56 in CDR2 N->S. The DNA sequence in the J-region is identical to germline IGKJ2 (Accession #V00777).

4.2. Evaluation of the expressed scFv166

Immunoblot to the anti-cMyc tag visualized the secreted scFv166 (298 amino acids) as a predicted 29.8 kD-band in the eluted solution from Ni-NTA agarose as shown in **Figure 3**. The bindings of scFv166 to both native PA103 PcrV (294 amino acids, 32.4 kD) and recombinant PcrV(rPcrV, 306 amino acids, 33.8 kD) were confirmed as shown in **Figure 4**. The binding affinity of Mab166 was 1×10^{-8} M, while that of scFv166 was 5×10^{-6} M (**Figure 5**).

4.3. Humanization and affinity maturation

The next step, for human use, after testing the binding affinity of scFv166 to a target molecule, together with the affinity maturation steps, is the elimination of the human-specific antigenic mouse amino acid sequence. In fact, Mab166 has already been humanized by antibody affinity engineering by serial epitope-guided complementarity replacement (SECR) which is a licensed humanization/affinity maturation technique of KaloBios Pharmaceutical Inc (Brisbane, California, USA) [33, 37] (**Figure 6**). In brief, SECR provides for a method for obtaining human idiologs for any nonhuman antibody to any target by epitope-guided replacement of variable regions using competitive cell-based methods in which the competitor can be either the reference antibody or a ligand that binds to the same epitope on the target as the reference antibody [37]. Fab 1A8 of humanized Mab166 by SECR bound to PcrV with approximately a twofold-higher affinity than the original murine Mab166 Fab [37]. Therefore, a further modification of scFv166 can be done by referring to the existing information available for the modified amino acid sequences in Fab 1A8 [33].

heavy chain IgG2b	CDR3	murine (Mab166) humanized (1A8)	NRGDIYYDFTYAMDY NRGDIYYDFTYAXDZ (X D M/F, Z D I/S/Q)
light chain κ	CDR3	murine (Mab166) humanized (1A8)	QHFWSTPYT QQFWXTPYT (X D S/G)
	FR4	murine (Mab166) humanized (1A8)	FGGGTKLEIKR FGGGTKLTVLR

Figure 6. Amino acid sequence differences between murine Mab166 and humanized Fab 1A8. CDR3 in the heavy chain, CDR3 and FR4 in the light chain (*k*) of humanized Fab 1A8 have sequence modifications following humanization and affinity maturation compared to corresponding sequences of Mab166.

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5. Conclusion

We have shown in an *in vivo* study that instillation of a single dose of Fab into the lungs of mice protected them against a lethal pulmonary challenge with *P. aeruginosa* [36]. The ability to use a recombinant Fab fragment for the treatment of *P. aeruginosa* infection in patients with ventilator-associated pneumonia or chronically infected cystic fibrosis patients has potential to minimize acute lung injury and mortality associated with TTS virulence of *P. aeruginosa*. Further optimization, such as the affinity maturation and PEGylation, will be the next step to achieve clinical application in humans. An engineered single-chain antibody that binds to the *P. aeruginosa* PcrV protein with high affinity has strong potential to be an effective new therapeutic reagent for infections caused by *P. aeruginosa*.

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Abbreviations

CDR	complementarity determining region
P. aeruginosa	*Pseudomonas aeruginosa*
SECR	serial epitope-guided complementarity replacement
TTS	type III secretory
TTSS	type III secretion system

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