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Generation of a Human Monoclonal Antibody to Cross-Reactive Material 197 (CRM_{197}) and Development of a Sandwich ELISA for CRM_{197} Conjugate Vaccines

Dain Kim[1], Hyeseon Yoon[2], Sangkyu Kim[1], Jimin Wi[3], Heesu Chae[1], Gyunghee Jo[1], Jun-Yeol Yoon[1], Heeyoun Kim[2], Chankyu Lee[2], Se-Ho Kim[1], and Hyo Jeong Hong[1,3]*

[1]*Department of Systems Immunology, College of Biomedical Science, Kangwon National University, Chuncheon 24341, Republic of Korea*
[2]*Eubiologics Co., Ltd., Chuncheon 24232, Republic of Korea*
[3]*Scripps Korea Antibody Institute, Chuncheon 24341, Republic of Korea*

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**Corresponding author*
Phone: +82-33-250-8381;
Fax: +82-33-259-5643;
E-mail: hjhong@kangwon.ac.kr,
hjhong@skai.or.kr

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Cross-reactive material 197 (CRM_{197}) is a non-toxic mutant of diphtheria toxin containing a single amino acid substitution of glycine 52 with glutamic acid. CRM_{197} has been used as a carrier protein for poorly immunogenic polysaccharide antigens to improve immune responses. In this study, to develop a sandwich ELISA that can detect CRM_{197} and CRM_{197} conjugate vaccines, we generated a human anti-CRM_{197} monoclonal antibody (mAb) 3F9 using a phage-displayed human synthetic Fab library and produced mouse anti-CRM_{197} polyclonal antibody. The affinity (K_D) of 3F9 for CRM_{197} was 3.55 nM, based on Bio-Layer interferometry, and it bound specifically to the B fragment of CRM_{197}. The sandwich ELISA was carried out using 3F9 as a capture antibody and the mouse polyclonal antibody as a detection antibody. The detection limit of the sandwich ELISA was <1 ng/ml CRM_{197}. In addition, the 3F9 antibody bound to the CRM_{197}-polysaccharide conjugates tested in a dose-dependent manner. This ELISA system will be useful for the quantification and characterization of CRM_{197} and CRM_{197} conjugate vaccines. To our knowledge, this study is the first to generate a human monoclonal antibody against CRM_{197} and to develop a sandwich ELISA for CRM_{197} conjugate vaccines.

Keywords: Cross-reactive material 197, vaccines, human monoclonal antibody, phage display, sandwich ELISA

Introduction

Cross-reactive material 197 (CRM_{197}) is a non-toxic mutant of diphtheria toxin, which is produced in *Corynebacterium diphtheriae* and commonly causes respiratory disease among children [1]. Diphtheria toxin is a single-chain 58 kDa protein that consists of two fragments (A and B), which are linked by disulfide bridges and separated after mild trypsin treatment and reduction in vitro [2–4]. The fragment A (21 kDa) is the catalytic domain that inhibits protein synthesis, and fragment B (37 kDa) binds to the host cell receptor, heparin binding EGF-like growth factor [5, 6]. CRM_{197} contains the single amino acid substitution from glycine to glutamic acid at position 52 in the catalytic domain [7]. This substitution affects the conformations of both fragments A and B because of their tight interaction, resulting in conformational differences between CRM_{197} and native diphtheria toxin [8]. Biogio *et al*. observed that among the four monoclonal antibodies (mAbs) raised against CRM_{197}, all bound to CRM_{197} but only one bound to the native diphtheria toxin [8].

It was found that CRM_{197} was an ideal carrier for conjugate vaccines against encapsulated pathogenic bacteria such as *Haemophilus influenzae* type b (Hib), *Streptococcus pneumonia*, and *Neisseria meningitides* serogroups A, C, W-135, and Y [9–14]. The use of carrier protein greatly enhances the

immunogenicity of polysaccharide or oligosaccharide antigens, which are poorly immunogenic in the immature immune systems of infants and young children [13, 15–19]. The large-scale collection of data from clinical trials indicates very favorable immunogenicity, safety, and tolerability profiles [20]. For over a decade, licensed CRM_{197}-based vaccines against invasive pneumococcal, meningococcal, and Hib diseases have been used worldwide and the safety profiles have been well-accepted in all age groups [20].

The characterization and validation of CRM_{197} conjugate vaccines requires precise measurement of the vaccine concentration in vitro and in vivo. A sandwich enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies (mAbs) or polyclonal antibodies is a common tool for antigen quantitation. The mAbs are currently generated by hybridoma and in vitro display technologies [21–25]. Phage display of combinatorial antibody libraries is a powerful tool to generate mAbs and has become the most frequently used display technology [26, 27]. Here, we present the generation of a human anti-CRM_{197} mAb from a phage-displayed human synthetic Fab library and mouse anti-CRM_{197} polyclonal antibodies and their application in a sandwich ELISA for the quantitation and characterization of CRM_{197} and CRM_{197} conjugate vaccines.

Materials and Methods

Production of CRM_{197}

CRM_{197} was produced by fermentation of the *C. diphtheriae* C7 (β 197) tox^- strain [28]. The proteins secreted into the culture supernatant were recovered by centrifugation, filtration, and concentration. Protein purification was performed by anion exchange chromatography and hydroxylapatite chromatography followed by concentration. The purity of the isolated CRM_{197} was >95%.

Preparation of CRM_{197}–Polysaccharide Conjugates

Pneumococcal polysaccharide (PnPS) was produced by fermentation of *Streptococcus pneumoniae* isolates (serotypes 9V, 11A, 18C, 19A, and 19F) from the Culture Collection of University of Goteborg, as described previously [29]. The polysaccharides were further purified by hydroxylapatite chromatography and ultrafiltration. The purity of the isolated PnPS was >90%. The PnPS was reduced to smaller sizes by microfluidization and conjugated to CRM_{197} using an adipic acid dihydrazide (ADH) linker and an 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) catalyst, as described previously [30, 31]. The PnPS-CRM_{197} conjugate was recovered by filtration and concentration using an ultrafiltration/diafiltration (UF/DF) system. The Vi capsular polysaccharide (Vi) was produced by fermentation of *Salmonella typhi* Ty2 from the American Type Culture Collection, as described previously [32]. The purity of the isolated Vi was >95%. The Vi was conjugated to CRM_{197} using ADH and EDAC, as described previously [33]. The Vi-CRM_{197} conjugate was recovered by filtration and concentration using a UF/DF system.

Biopanning of Phage-Displayed Human Synthetic Fab Library

Biopanning was performed using the phage-displayed human synthetic Fab library (2×10^9 diversity) constructed in our laboratory against CRM_{197}, as described previously [34]. The input and output phage titers were measured for each round.

Indirect and Quantitative ELISAs of Phage-Displayed Fabs Selected after Panning

After three rounds of panning, infected TG1 cells from each colony were inoculated at OD_{600} = 0.1 and cultured in 2 × YT/carbenicillin/glucose broth until reaching OD_{600} = 0.5. The cells were infected with KM13 helper phage at 20 MOI without stirring at 37°C for 30 min and then with stirring for 30 min. The infected cells were centrifuged at 2,900 ×*g* for 10 min, and the cell pellet was resuspended in 2 × YT/carbenicillin/kanamycin broth and cultured at 30°C with stirring for 12 h. After centrifugation, the supernatant containing Fab phages was analyzed by ELISA. For the indirect ELISA, the phage Fabs were incubated with CRM_{197} or BSA (1 μg/ml) coated on each well at 37°C for 1 h. After washing, the bound phage Fabs were incubated with anti-M13 antibody-HRP (1:5,000 v/v, GE Healthcare). For the quantitative ELISA, the phage Fabs were incubated with anti-human kappa antibody (100 ng/well, Thermo Fisher Scientific) coated on the wells. After washing, the bound phages were incubated with the anti-M13 antibody-HRP (1:5,000 v/v, GE Healthcare). Finally, TMB substrate reagent [31] was added and incubated for 6 min. The reaction was stopped with 2.5 M H_2SO_4.

Screening for CRM_{197}-Specific Fab Clones

To confirm the enrichment of positive clones against CRM_{197}, screening was performed as previously described [35]. The culture supernatant in each well, containing phages, was analyzed by indirect ELISA and quantitative ELISA, as described above.

Conversion of Fabs into IgG and Analysis of Antigen-Binding Activity

To convert the selected Fabs into IgG format, the cloning and expression of IgG were performed as described previously [34]. The IgG was purified from the culture supernatant by affinity chromatography on Protein A-agarose beads, and the protein concentration was determined using a NANO-DROP 2000 (Thermo Fisher Scientific). The integrity and purity of the purified antibody were assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The purified IgG was analyzed by indirect ELISA and sandwich ELISA using anti-human IgG Fc-HRP (1:8,000 v/v, Thermo Fisher Scientific).

Production of Mouse Anti-CRM_{197} Polyclonal Antibody

Mouse polyclonal antibody was obtained by immunizing three mice (BALB/C, AbFrontier) with CRM_{197} for 6 weeks. After primary injection, the second and third boosters were given 7 days after each immunization. Final sera containing anti-CRM_{197} polyclonal antibodies were obtained 7 days after the third immunization. The anti-CRM_{197} mouse sera obtained was used for the sandwich ELISA.

Sandwich ELISA

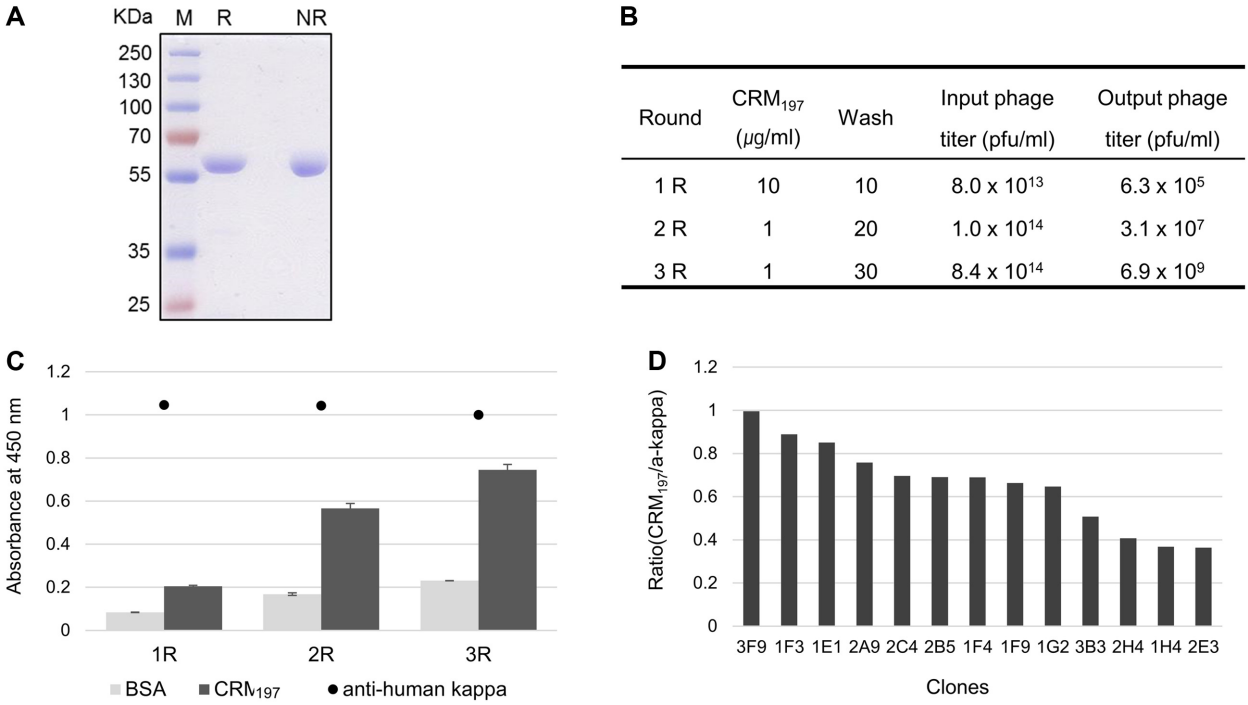
The 96-well microplates (Nunc) were coated with 2.5 μg/ml anti-CRM_{197} mAb in coating buffer (Na_2CO_3, $NaHCO_3$, pH 9.6) overnight at 4°C. The next day, microplates were blocked with blocking solution (2% skim milk in 0.05% phosphate buffered saline with Tween 20 [PBST]). After washing twice with 0.05% PBST, diluted CRM_{197} (0–20 ng/well) in PBA (0.1% BSA in phosphate buffered saline [PBS]) was added to each well then incubated with mouse anti-CRM_{197} polyclonal antibody in PBS (1:10,000 v/v). After washing four times, 100 μl of anti-mouse Ig Fc-HRP (1:5,000 v/v, Thermo Fisher Scientific) was added to each well. All incubations were performed at 37°C for 1 h. Finally, TMB substrate reagent [31] was added and incubated for 5 min. The reaction was stopped with 2.5 M H_2SO_4.

Affinity Determination of 3F9 Antibody Using Octet Red

The affinity of the antibody was determined by bio-layer interferometry (BLI) using Octet RED (ForteBio, USA) as described previously [34]. The CRM_{197} antigen was prepared using 2-fold serial dilutions (50, 25, 12.5, 6.25, and 3.13 nM in 0.1% PBA) and the association step was 10 min.

Epitope Mapping of the 3F9 Antibody

The CRM_{197} protein (10 μg) was treated with 0.1 μg trypsin at 37°C for 5 min and analyzed by 10% SDS-PAGE under reduced and non-reduced conditions. The protein bands were transferred to a nitrocellulose membrane (GE Healthcare, France). Then the membrane was incubated with 3F9 mAb (1 μg/ml) or mouse anti-CRM_{197} polyclonal antibody (1:2,000 v/v) diluted in blocking solution (5% skim milk in 0.05% TBST), followed by anti-human IgG Fc-HRP conjugate (1:6,000 v/v, Thermo Fisher Scientific) or anti-mouse IgG Fc-HRP (1:5,000 v/v, Thermo Fisher Scientific), respectively. Finally, the bands were visualized using a chem-iluminescent substrate (WEST-ZOL plus, iNtRON BioTechnology, Korea).



Round	CRM_{197} (μg/ml)	Wash	Input phage titer (pfu/ml)	Output phage titer (pfu/ml)
1 R	10	10	8.0×10^{13}	6.3×10^{5}
2 R	1	20	1.0×10^{14}	3.1×10^{7}
3 R	1	30	8.4×10^{14}	6.9×10^{9}

Fig. 1. Isolation of anti-CRM_{197} Fab clones from a human synthetic Fab library.

(**A**) 10% SDS-PAGE of purified CRM_{197} (58 kDa). M, molecular weight standards. R, reduced sample. NR, non-reduced sample. (**B**) Input and output phage titers of each panning round; pfu, plaque-forming unit. (**C**) Indirect ELISA of polyclonal phage Fabs using CRM_{197} or BSA as a negative control and quantitative ELISA using anti-human kappa antibody coated on each well. The bound phages were detected by anti-M13-HRP conjugate. Values were obtained from duplicate wells and are expressed as the mean ± SEM. (**D**) The antigen binding activities of unique Fab clones were ranked by indirect ELISA and quantitative ELISA.

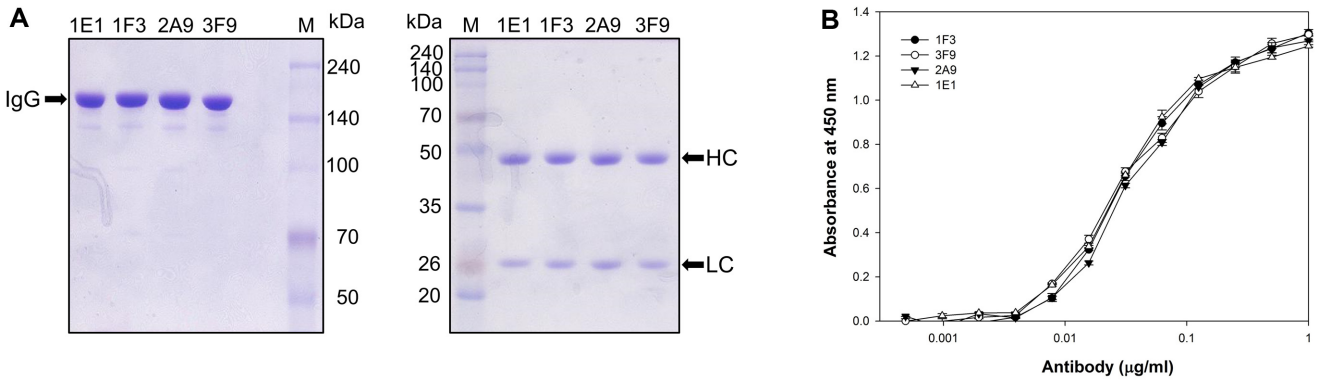


Fig. 2. Analysis of antigen-binding activities of anti-CRM_{197} mAbs.
(**A**) 6% SDS-PAGE of four purified mAbs under non-reducing conditions (left panel) and 10% SDS-PAGE under reducing conditions (right panel). (**B**) Analysis of antigen-binding activities of the four purified mAbs by indirect ELISA. Values were obtained from duplicate wells and are expressed as the mean ± SEM.

Results and Discussion

Bio-Panning of Phage-Displayed Human Synthetic Fab Library against CRM_{197}

Three rounds of bio-panning against the purified CRM_{197} (Fig. 1A) were performed for enrichment of anti-CRM_{197} phage Fabs. The output phage titers largely increased after the third round of panning (Fig. 1B), and anti-CRM_{197} phage Fabs were enriched, when assessed by ELISAs using polyclonal phage Fabs (Fig. 1C).

Selection of Anti-CRM_{197} Fabs

To analyze individual anti-CRM_{197} Fab clones, 285 colonies were randomly selected and phage Fabs were screened by indirect ELISA and quantitative ELISA using BSA. Forty clones with the highest antigen-binding activities were selected and 13 unique clones were obtained after DNA sequencing (Fig. 1D). Finally, four unique Fab clones (3F9, 1E1, 1F3, and 2A9) with the highest antigen-binding activities were selected for further study. The HCDR3 of 3F9 has 13 amino acid residues, while that of the other clones has 8 residues, which are different from each other.

Conversion of Fabs into IgG and Analysis of Antigen-Binding Activities by Indirect ELISA

The four Fab clones were converted into IgG and expressed transiently in HEK293F cells. The secreted IgG in culture supernatants was purified using affinity chromatography with Protein A agarose beads, and the purity and integrity of the purified antibody was confirmed by SDS-PAGE (Fig. 2A). The purified anti-CRM_{197} mAbs were analyzed by indirect ELISA to compare their antigen-binding activities. The four mAbs (3F9, 1E1, 1F3, and 2A9) showed similar binding activities (Fig. 2B).

Analysis of the Antigen-Binding Activities of anti-CRM_{197} mAbs by Sandwich ELISA

To develop a sandwich ELISA for detection of CRM_{197} in biological samples, mouse anti-CRM_{197} polyclonal antibodies were produced by immunizing three mice with the purified CRM_{197}, and each antiserum (#1-#3) was serially diluted (1:5,000–1:100,000 v/v) to perform an indirect ELISA. As shown in Fig. 3A, the #2 mouse antiserum showed the highest binding activity. Next, to measure the antigen-binding activity of the mAbs by sandwich ELISA, the 3F9 antibody was coated on each well and incubated with CRM_{197} at different concentrations in 0.1% PBA, then the #2 antiserum (1:10,000 v/v) was added followed by anti-mouse IgG(Fc)-HRP and the plate was incubated (Fig. 3B). The 3F9 antibody showed high antigen-binding activity, and the detection limit of the sandwich ELISA was <1 ng/ml CRM_{197} (Fig. 3C). In addition, the reactivity of the 3F9 antibody to the CRM_{197} in human normal sera was similar to that in PBA (data not shown).

To examine whether 3F9 can bind to CRM_{197} conjugate vaccines, one type of Vi-CRM_{197} and five types of PnPS-CRM_{197} conjugates (serotypes 9V, 11A, 18C, 19A, and 19F) were prepared and purified (Fig. 4), as described in the Materials and Methods, and their reactivity to 3F9 was measured by sandwich ELISA. As shown in Fig. 3D, the 3F9 antibody bound to the CRM_{197}–polysaccharide conjugates in a dose-dependent fashion.

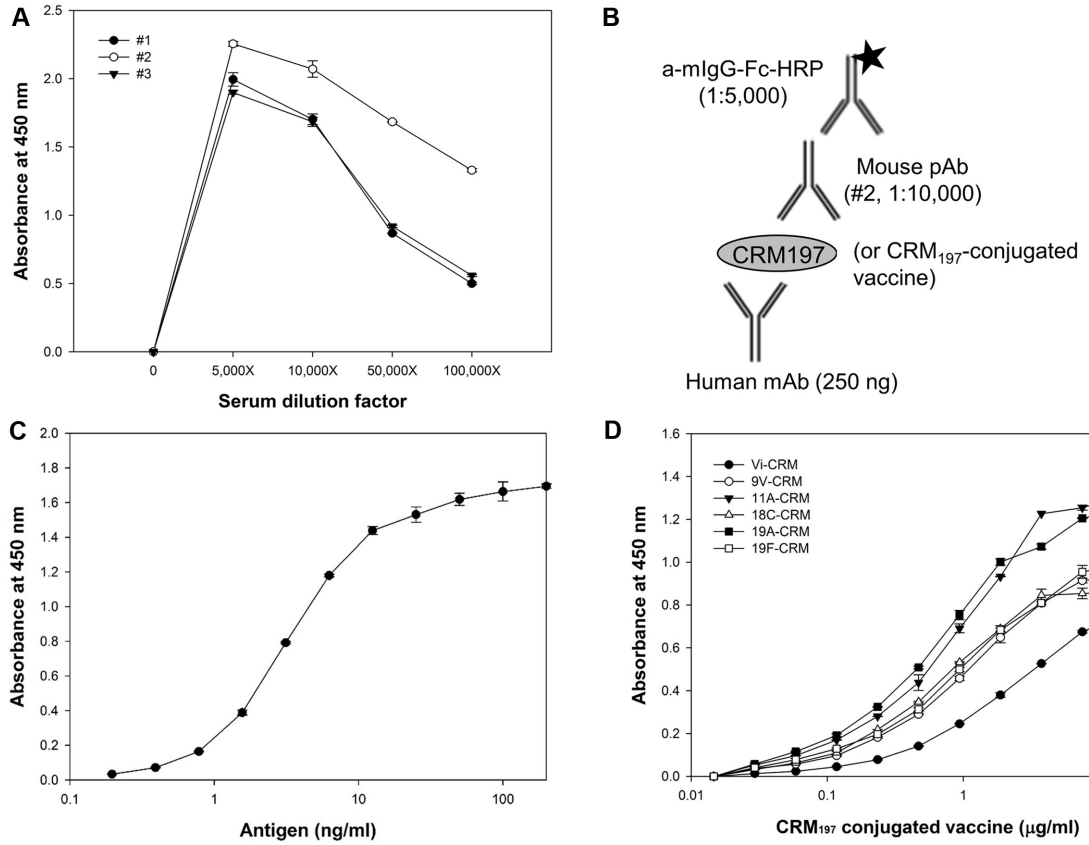


Fig. 3. Sandwich ELISA of an anti-CRM_{197} mAb.

(**A**) Indirect ELISA of anti-CRM_{197} polyclonal antibodies obtained from three mice (#1-#3) immunized with CRM_{197}. Mouse serum was diluted at various concentrations (5,000-fold, 10,000-fold, 50,000-fold, and 100,000-fold) and incubated with CRM_{197} coated on each well followed by anti-mouse IgG(Fc)-HRP conjugate diluted 1:5,000. (**B**) Schematic representation of sandwich ELISA. (**C**) Sandwich ELISA of 3F9 using CRM_{197} prepared as a 2-fold serial dilution. (**D**) Sandwich ELISA of 3F9 antibody using CRM_{197}–polysaccharide conjugates diluted in 0.1% PBA and mouse anti-CRM_{197} polyclonal antibody (1:1,000 v/v). All values were obtained from duplicate wells and are expressed as the mean ± SEM.

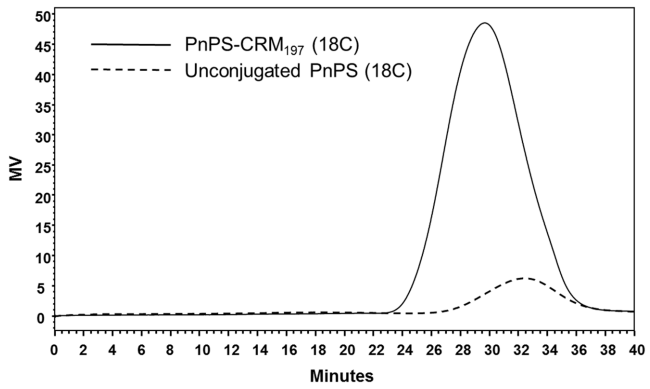
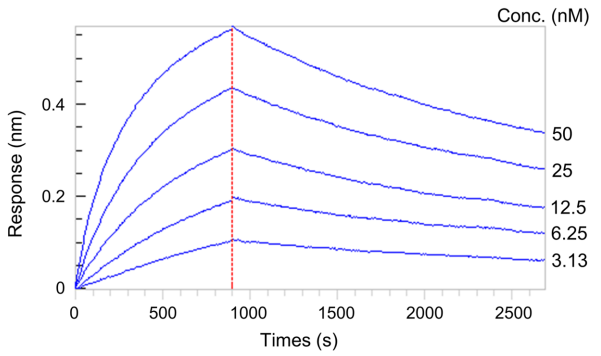


Fig. 4. HPLC-SEC of PnPS-CRM_{197} conjugates.

HPLC-SEC profiles (refractive-index detection) of unconjugated PnPS 18C and PnPS-CRM_{197} conjugate after elution (flow 0.5 ml/min) from a TSKgel 6000–5000 PWXL column equilibrated with 150 mM NaCl, 10 mM sodium phosphate, pH 7.2.



Antibody name	K_D (M)	k_{on}(1/Ms)	k_{off}(1/s)	Full R^2
3F9	4.450×10^{-9}	6.701×10^{4}	2.982×10^{-4}	0.9976

Fig. 5. Affinity determination of 3F9 using Octet RED.

CRM_{197} was prepared using 2-fold serial dilutions (50, 25, 12.5, 6.25, and 3.13 nM). The calculated association and dissociation rates and K_D value are presented.

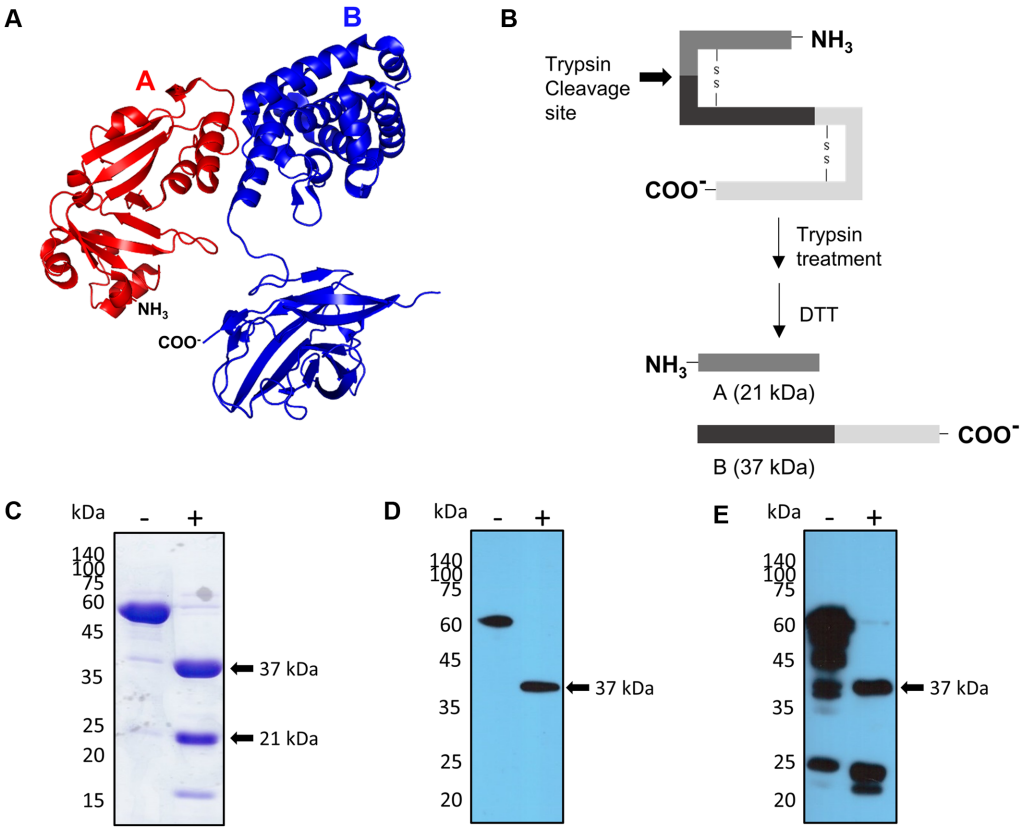


Fig. 6. Epitope mapping of the 3F9 antibody.
(**A**) Structure of CRM_{197} (PDB ID code 4AE0). Fragments A and B are colored as red and blue, respectively. (**B**) Schematic representation of CRM_{197} cleavage by trypsin. (**C**) 10% SDS-PAGE of CRM_{197} treated with mock (–) or trypsin (+) under reducing conditions. (**D** and **E**) The protein bands were analyzed by western blot analysis using 3F9 antibody and anti-human IgG (Fc)-HRP (**D**) or using mouse anti-CRM_{197} polyclonal antibody and anti-mouse IgG (Fc)-HRP (**E**).

Affinity Determination and Epitope Mapping of 3F9

The affinity (K_D) of the 3F9 antibody for CRM_{197} was measured using Octet Red and was 4.45 nM (Fig. 5). Next, to identify the epitope of 3F9, the CRM_{197} was treated with trypsin and DTT and analyzed by SDS-PAGE under reducing conditions and western blot analysis (Fig. 6). As expected, CRM_{197} (58 kDa) was cleaved into two fragments (37 kDa and 21 kDa) after treatment with trypsin and DTT (Figs. 6A–6C). In western blot analysis, 3F9 specifically bound to the B fragment (37 kDa) of CRM_{197} (Fig. 6D); while the mouse polyclonal antibody bound to both the A and B fragments (Fig. 6E). Taken together, the results indicate that 3F9 antibody binds specifically to a linear epitope in the B fragment of CRM_{197} with a high affinity. Considering the facts that CRM_{197} contains the single amino acid substitution from glycine to glutamic acid at position 52 in the A fragment and that 3F9 binds to a linear epitope in the B fragment, 3F9 may bind to the B fragment of native diphtheria toxin. It remains to be elucidated whether this antibody may have neutralizing activity against the toxin.

In conclusion, we generated a human mAb (3F9) that binds specifically to the B fragment of CRM_{197} with a high affinity and developed a sandwich ELISA using the human mAb as the capture antibody and mouse anti-CRM_{197} polyclonal antibody as the detection antibody. The 3F9 bound to CRM_{197} and CRM_{197}-polysaccharide antigen conjugates tested. To our knowledge, this study is the first to show the generation of human mAb to CRM_{197} and its application in a sandwich ELISA for CRM_{197} conjugate vaccines. This ELISA system will be useful for quantification and characterization of CRM_{197} and CRM_{197} conjugate vaccines.

Acknowledgments

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Industry & Energy (MOTIE), and the Korea Institute for Advancement of Technology (KIAT) through the Encouragement Program for The Industries of Economic Cooperation Region.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

Abbreviations

ADH, adipic acid dihydrazide; BLI, bio-layer interferometry; CRM_{197}, cross-reactive material 197; EDAC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; ELISA, enzyme-linked immunosorbent assay; Hib, *Haemophilus influenzae* type b; mAb, monoclonal antibody; PBS, phosphate buffered saline; PBST, phosphate buffered saline with Tween 20; PnPS, Pneumococcal polysaccharide; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; UF/DF, ultrafiltration/diafiltration; Vi, Vi capsular polysaccharide

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