Tohoku J. Exp. Med., 2004, **203**, 295-303

Characterization of a Shiga Toxin 1-Neutralizing Recombinant Fab Fragment Isolated by Phage Display System

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INOUE, K., ITOH, K., NAKAO, H. TAKEDA, T. and SUZUKI, T. *Characterization of a Shiga Toxin-1-Neutralizing Recombinant Fab Fragment Isolated by Phage Display System.* Tohoku J. Exp. Med., 2004, **203** (4), 295-303 —— Shiga toxin (Stx)-producing *Escherichia coli* (STEC) causes bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome. Molecular structural analysis of Stx-neutralizing monoclonal antibody (mAb) will be helpful for development of therapeutics and prophylactics for STEC infection. In this study, we cloned the genes of Stx 1-neutralizing mAb, termed 5-5B from hybridoma cells by phage display system and characterized its recombinant Fab (rFab) fragment. 5-5B rFab fragment reacted with Stx1, but not with Stx2 and bovine serum albumin (BSA). It also showed the neutralizing activity against the cytotoxicity of Stx1. These results imply that 5-5B rFab fragment is functionally identical to parent mAb. The variable heavy (V_H) and light domains were found to be highly homologous with the derived germ line sequences. As for V_H domain, the complementarity determining regions (CDRs) showed higher replacement/substitution mutation ratio than that in the frame work regions. Among the regions, CDR2 showed the most frequent nucleotide and amino acid substitutions. These results suggest that heavy chain CDR2 may mainly be associated with the 5-5B function, that is neutralizing cytotoxicity of Stx1. ——— phage display; recombinant fab; shiga toxin 1; monoclonal antibody

Received March 24, 2004; revision accepted for publication June 2, 2004.
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Footnotes: The nucleotide sequence data reported in this paper are available from DDBJ/EMBL/GenBank nucleotide sequence databases under accession numbers, AB116255-6.
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Shiga toxin (Stx)-producing *Escherichia coli* (STEC) has been recognized as an emerging food borne pathogen, found mainly in industrialized countries, that causes bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (Griffin and Tauxe 1991). A large outbreak of STEC affecting nearly 10 000 patients occurred in Japan in 1996. The major virulence factor is Stx, which binds globotriaosylceramide (Gb3) on the surface of eukaryotic cells, internalized, inhibits protein synthesis, and causes cell death (Lingwood et al. 1987). Stx has two major types, Stx1 and Stx2, both of which inhibit protein synthesis of eukaryotic cells (Melton-Celsa and O'Brien 1998).

Although antibodies that neutralize Stxs are most likely to prevent disease, a minority of patients develop rising levels of Stx-neutralizing antibodies following STEC infection. Thus a specific therapeutic approach is needed for the prevention of severe disease caused by STEC infection. Although one of the promising candidates is Stx-neutralizing monoclonal antibody (mAb), a major drawback with the use of mAbs in the treatment of human diseases is their immunogenicity and the consequent elicitation of human anti-mouse antibodies following their repeated injection into patients. Small fragments with the antigen binding activity, such as $F(ab')_2$, Fab, and recombinant Fab (rFab) fragment would be preferable for clinical use because of their reduced immunogenicity and better pharmacokinetic properties (Yokota et al. 1992) compared with whole antibody. Small peptides including antibody complementarity determining region (CDR) motif, termed CDR peptides (Park et al. 2000), would also be promising as therapeutics of human diseases.

Phage display technology provides the successful selection of recombinant antibody fragments specific for the antigen of interest by displaying the functional antigen binding sites on the surface of M13 filamentous phage (Burton and Barbas 1994; Winter et al. 1994). rFab fragments against viral pathogens (Burton et al. 1991; Barbas et al. 1992; Williamson et al. 1993; Itoh et al. 1999a), autoantigens (Barbas et al. 1995; Eggena et al. 1996; Graus et al. 1997; Ditzel et al. 2000), and tumor-associated antigens (Itoh et al. 1999b, 2001a, 2003; Nakayashiki et al. 2000; Hansen et al. 2001) have been successfully isolated from the antibody gene library of human- or murine-origin by phage display system.

Stx1 is known to be identical to the Stx of *Shigella dysenterie* type1 or differs by only one amino acid in the A subunit (Kozolov et al. 1988; Takao et al. 1988). Stx2 seems to be more important epidemiologically in the development of helmolytic uremic syndrome (HUS) than Stx1 (Boerlin et al. 1999). Moreover, the binding property of Stx1 and Stx2 against Gb3 are different from each other (Kiarash et al. 1994; Itoh et al. 2001b). These observations suggest that the molecular structural and functional characterization of Stxs-neutralizing mAbs would be helpful for development of prophylactic and therapeutic reagents for STEC infection. Nakao et al. (2002) isolated both a Stx1-neutralizing mAb, 5-5B and a Stx2-neutralizing mAb, VTm1.1 (Nakao et al. 1999). In this study, we focused on the investigation of a structure-function relationship of Stx1-neutralizing mAb, 5-5B. We performed the molecular cloning of 5-5B genes by phage display system and determined the binding property of cloned genes-derived rFab fragment. We then characterized the variable heavy (V_H)- and variable light (V_L)- chain sequences in relation to the Stx1-neutralizing activity. The findings indicate that the cloned gene encodes the Fab part of 5-5B, and that the heavy chain (HC) CDR (HCDR) 2 may play an important role in expression of neutralizing activity.

Materials and Methods

Library construction and phage selection

Fab phage display combinatorial library was constructed from 5-5B-producing hybridoma cells (provided by Dr. C. Kataoka at the Kansai Research Institute, Kyoto) as described previously (Itoh et al. 1999b, 2001a). Briefly, total RNA of

5-5B hybridoma cells (1×10^7) was extracted with RNeasy Mini Kit (Qiagen, Tokyo). First-strand cDNA was then synthesized with a primer of oligo $(dT)_{15}$ using an avian myeloblastosis virus (AMV) reverse transcriptase first-strand cDNA synthesis kit (Life Sciences, St. Petersburg, FL, USA). Polymerase chain reaction (PCR) amplification of the HC Fd region and whole κ light chain (LC) were performed with the GeneAmp 2400 PCR system (Perkin-Elmer, Norwalk, CN, USA) using the family-specific variable region and isotype-specific constant region primers (Itoh et al. 2001a) designed based on the Kabat database (Kabat et al. 1991).

Gel-purified PCR products were double digested with restriction enzymes (S*pe*I/X*ho*I for HC and S*ac*I/X*ba*I for LC) respectively. All enzymes were purchased from Roche Diagnostics, Tokyo), and then ligated sequentially into the phage display vector *pComb3* (provide by Dr. Dennis R. Burton, The Scripps Research Institute, La Jolla, CA, USA) (Barbas et al. 1991). The constructed library was electroporated into *E.coli* XL1-Blue cells (Stratagene, La Jolla, CA, USA) by *E.coli* pulser (Bio-Rad, Richmond, CA, USA), then streaked onto LB agar plates containing 100 μg/ml of carbenicillin and 100 mM glucose. Single colonies were cultured in Super broth (SB) with 50 μg/ml of carbenicillin, and Fab-displayed phage (Fab-phage) clones were rescued by infection with VCSM13 helper phage (Stratagene). The amplified Fab-phage clones were tested for the binding to Stx1 by a phage enzyme-linked immunosorbent assay (ELISA) as described below. The identity of the positive clones was determined by the *Bst*NI fingerprinting (Eggena et al. 1996).

Nucleic acid sequencing

Nucleic acid sequencing was carried out using a PRISM 310 genetic analyzer (ABI, Foster City, CA, USA) with a dye terminator cycle sequencing FS ready reaction kit (ABI). The primers SeqT3 (5´-ATT AAC CCT CAC TAA AG-3´) and KEF (5´-GAA TTC TAA ACT AGC TAG TTC G-3´), both hybridizing to the (-)-strand, were used for sequencing V_H and V_L domains, respectively. Comparison to the reported immunoglobulin germline sequences from Genbank/EMBL/DDBJ was performed by the NCBI's Ig BLAST analysis and by the DNAPLOT analysis.

Production and purification of rFab fragment

Reconstruction of a phagemid DNA and production of rFab fragment from *E.coli* was performed as described previously (Barbas et al. 1991; Burton et al. 1991). In brief, single *E.coli* colonies bearing the phagemid DNA reconstructed for rFab production were inoculated into 100 ml of SB with 50 μg/ml of carbenicillin and 20 mM $MgCl_2$ and cultured for 6 h at 37˚C with vigorous shaking. Protein expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside (Roche Diagnostics) and cells incubated overnight at 30˚C. rFab fragment was purified from supernatant of bacterial lysates by Ni-chelate chromatography using a Ni-NTA spin kit (Qiagen) as described previously (Itoh et al. 2001a).

ELISA

Binding of Fab-phage or rFab fragment against Stx was determined by a direct ELISA. Each 50 μl of Stxs (5 μg/ml) was fixed to the wells of ELISA plate (3690 EIA/RIA plate, Costar, Cambridge, MA, USA) by incubation for overnight at 4ºC. The wells were then filled with 100 μl of phosphate-buffered saline (PBS) containing 1% (w/v) BSA (BSA-PBS) and incubated for 1 hour at 37ºC. Fifty μl of Fab-phage or rFab fragment was added to each well and incubated for 1 hour at 37ºC. The wells were washed 5 times with PBS containing 0.05% (v/v) Tween20 (T-PBS) and filled with 50 μl of a 1/500 of alkaline phosphatase (ALP) labeled rabbit anti-mouse IgG $F(ab')_2$ (Jackson Immunoresearch, West Grove, PA, USA) followed by an incubation for 1 hour at 37ºC. After 5 times washing with T-PBS, 50 μl of *p*-nitrophenyl phosphate (Sigma104 phosphatase substrate 5 mg tablet, Sigma, St. Louis, MO, USA) dissolved in 1M diethanolamine buffer (pH 9.8) was added

and incubated for 30 minutes at 37ºC. Color development was stopped by addition of 2M sodium hydroxide, then the absorbance of resultant *p*-nitrophenol in each well was determined at 405 nm by a microplate reader (Model 550, Bio-Rad).

Cytotoxicity test

ACHN (human renal adenocarcinoma; ATCCRL1611) cells were grown in Dulbeccos' modified Eagle's medium supplemented with 10% fetal calf serum (Gibco BRL, Rockville, MD, USA). Cytotoxicity was assayed in wells of a microtiter plate (Iwaki Glass, Chiba). Approximately 7×10^4 cells in 90 μl of growth medium were seeded into each well. A 10-μl volume of test sample was added to each well and incubated under 5% CO_2 in air at 37ºC for 3 days. Cytotoxic effect was visualized by neutral red vital staining. After incubation at 37ºC for 75 minutes with 100 μl of 0.014% neutral red per well, the supernatant was removed and cells were rinsed twice with PBS. Addition of an equal volume of 0.5N HCl-35% ethanol released the dye from the lysosomes of viable cells. The absorbance of the dye at 540 nm was measured with a microplate reader and was directly related to the number of viable cells in each well. Results were expressed as percent viability compared with control culture viability (100%) from assay performed in the absence of Stxs.

Cytotoxin neutralization assay

The Cytotoxin-neutralizing ability of 5-5B rFab fragment and isotype-matched rFab fragment against CD98 (Itoh et al. 2001a) was assayed on ACHN cells. A 10-μl volume of toxin solution containing 5 times higher titer of the 50% cytotoxic dose (CD_{50}) of toxin was preincubated with 25 μl of filtrated and diluted rFab fragment solution for 1 hour, and then remaining unbound toxin cytotoxicity was measured by cytotoxicity assay as described above. Results were expressed as percent viability compared with control culture viability from assays performed with rFab fragments without Stxs (100% viability) and with only Stx (0% viability).

Results and Discussion

The antibody HC Fd and LC genes were amplified by PCR with the family-specific variable region and isotype-specific constant region primers using cDNA synthesized from total RNA of 5-5B-producing hybridoma cells. Amplified products were then pooled, gel-purified, and digested with restriction enzymes corresponding to the cloning sites. The IgG_1, κ antibody library constructed by successive insertion of each LC and HC fragment into the phage display vector *pComb3* contained 1.4×10^6 transformants. A total of 40 single colonies from the library were separately cultured, then the helper phage-rescued Fab-phage clones were tested for binding to Stx1 by a direct ELISA. Finally, eight clones that show high reactivity to Stx1 with low background against BSA were selected as positive clones. B*st*NI fingerprinting revealed that all positive clones showed the identical digestion pattern (data not shown). Nucleotide sequencing confirmed that all positive clones were identical.

5-5B rFab fragment was recovered by freeze-thawing from the periplasm of IPTG-induced packed cells bearing the cloned DNA reengineered for soluble Fab expression. 5-5B rFab fragment was affinity-purified by Ni-chelate chromatography from the crude lysates since expressed rFab fragment has a hexa histidine tag next to the C terminal end of the HC fragment. A single band of approximately 50-kDa under non-reducing condition was confirmed by SDS-PAGE after Coomassie Brilliant Blue staining (data not shown).

The antigen binding activity and specificity of 5-5B rFab fragment was evaluated. 5-5B rFab fragment reacted with Stx1 in a dose-dependent manner though it showed no reactivity to Stx2 and BSA in the same concentration range as determined by a direct ELISA (Fig. 1). 5-5B rFab fragment neutralized the cytotoxic activity of Stx1 against ACHN cells in virto (Fig. 2). Toxin neutralization was 5-5B rFab-specific manner

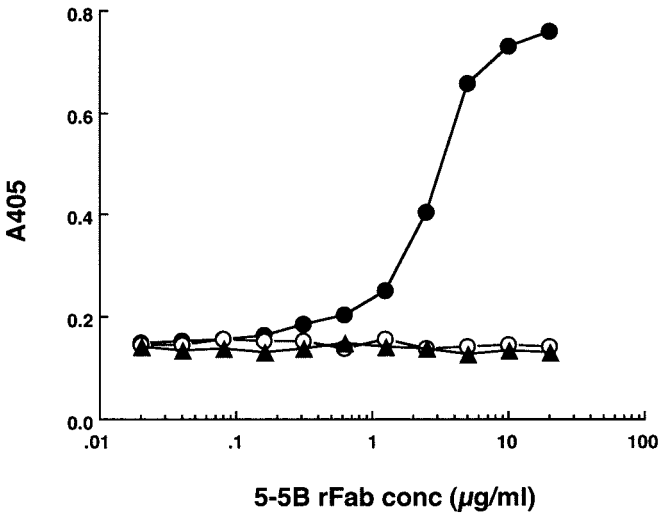

Fig. 1. Reactivity of 5-5B rFab fragment against Stx1, Stx2 and BSA. Serially diluted 5-5B rFab fragment was reacted with plate-coated Stx1 (5 μg/ml), Stx2 (5 μg/ml), and BSA (100 μg/ml). Ag-bound rFab fragment was detected by ALP-labeled anti-Fab secondary antibody. ●, Stx1; ○, Stx2; ▲, BSA .

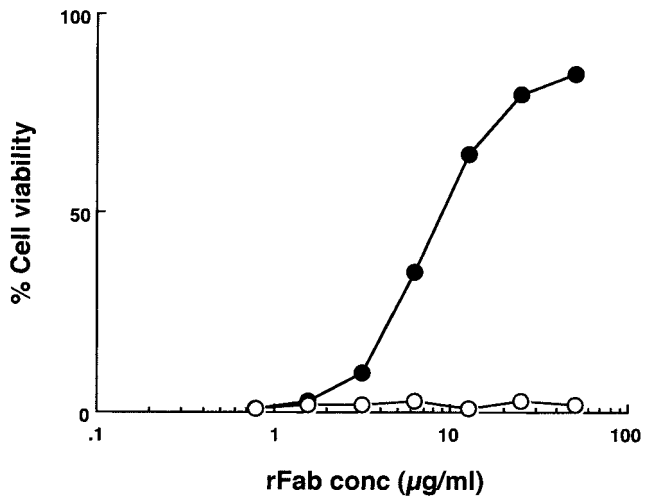

Fig. 2. Neutralizing activity of 5-5B rFab fragment against Stx1. Stx1 with a cytotoxicity of 5 times higher titer of the CD_{50} was preincubated with serially diluted 5-5B rFab fragment or isotype-matched rFab fragment against CD98 for 1 hour, and the remaining unbound toxin cytotoxicity against ACHN cells was then measured by cytotoxicity assay.
●, 5-5B rFab fragment; ○, rFab fragment against CD98.

since isotype-matched rFab fragment against CD98 showed no neutralization activity at any concentration. These results imply that cloned gene surely encode the Fab part of 5-5B mAb.

We demonstrated that cloning of post-reararanged antibody genes from antibody-producing hybridoma cells can be easily performed by antibody phage display. We used the *pComb3* system first reported by Barbas et al. (1991). This system is optimized for the expression of antibody genes as the Fab form and for targeting recombinant proteins to the periplasmic space of host cells by the pelB leader sequence. The HC Fd and LC fragments are thought to associate through the formation of a disulfide-bond in the periplasmic space under reducing conditions. rFab fragments that accumulate into the periplasmic space can easily be recovered by sonication or freeze-thawing the host cells with a minimum loss of antigen binding activity. A commercially available secondary antibody directed to IgG $F(ab')_2$ can be used to detect antigen-bound rFab fragments. The *pComb3* system used in this study is, therefore, better suited to the cloning and expression of mAb genes than the other systems.

To investigate the structural characteristics of V_H and V_L domains of 5-5B in relation to the Stx1-neutralizing activity, we sequenced and analyzed the cloned gene. The deduced amino acid sequences together with the derived germline genes are shown in Fig. 3. 5-5B V_H was found to be a member of the mouse VH5 family, and derived from *VH37.1* germline with 96% homology in nucleotide level and 92% homology in amino acid level (Table 1), while V_L belongs to the mouse Vk23 group V subgroup group I and derived from *23-43* germline with 96% homology in nucleotide and 94% homology in amino acid.

The V_H domain of an antibody is known to play a major role in antigen recognition and binding (Jaton and Reisen 1976). Somatic mutations in V segments during B cell maturation are indispensable for rearranged antibodies to obtain high specificity and affinity (Kalinke et al. 1996; Manser et al. 1998). Based on these observations,

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A
          FR1                              CDR1   FR2             CDR2
VH37.1    -----ESGGGLVKPGGSLKLSCAASGFTFS   SYGMS  WVRQTPEKRLEWVA  TISGGGSYTYYPDSVKG
5-5BVH    QVKLL.........................   H....  ..............  A....PT......G.R.

          FR3                                CDR3        FR4
VH37.1    RFTISRDNAKNNLYLQMSSLRSEDTALYYCAR   ----------  ----------
5-5BVH    ..I............................   YSYANNYFDY  WGQGTTLTVSS

B
          FR1                        CDR1         FR2              CDR2
23-43     -----QSPATLSVTPGDSVSLSC    RASQSISNNLH  WYQQKSHESPRLLIK  YASQSIS
5-5BVL    QIVLS..................   ..T.I......  ...............  .......

          FR3                                CDR3       FR4
23-43     GIPSRFSGSGSGTDFTLSINSVETEDFGMYFC   QQSNSW---  ------------
5-5BVL    ............K....I..............   ..T...PHT  FGGGTKLEIKRA
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Fig. 3. Deduced amino acid sequences of the V_H and V_L domains of 5-5B with the closest germline. (A) V_H sequence was compared with the closest germline *VH37.1*. (B) V_L sequence was compared with the closest germline *23-43*. Dashed lines in the nearest germline sequence indicate no amino acid corresponding to 5-5B sequences. Dots in 5-5B sequences indicate the identity with the closest germline sequence.

TABLE 1. *Molecular structural characterization of the V_H domain of 5-5B by comparing with the closest germline sequence*

VH family	Closest germline	Amino acid homology (%)	Nucleotide homology (%)	R/S ratio FRs	R/S ratio CDRs
VH5	VH37.1	92	96	1/1:1.0	6/2:3.0

The frequency of silent and replacement mutations and the percentage homologies, in terms of nucleotide and amino acid sequence as compared with the closest germline sequence, are shown.

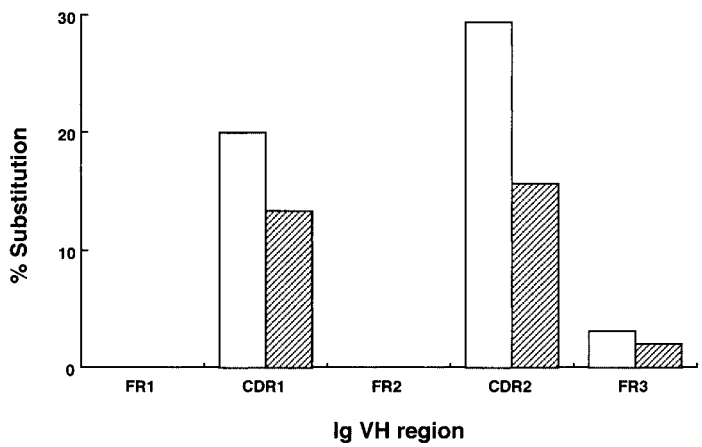

Fig. 4. Analysis of the distribution of nucleotide and amino acid changes within the V_H segment of 5-5B compared with the closest germline sequence. The percentage of mutation for individual regions (FR1, CDR1, FR2, CDR2, and FR3) was calculated as the number of changes divided by the total number of nucleotide for the region. Similarly, the average percentage of amino acid substitution for individual regions was calculated as the number of amino acid changes divided by the total number of amino acids for the region.
□, Amino acid level; ▨, Nucleotide level.

we further analyzed the V_H sequence of 5-5B. The frequency of silent and replacement mutations (R/S ratio) in frame works (FRs) and CDRs was compared with the *VH37.1* germline. As shown in Table 1, R/S ratio in CDRs (3.0) is higher than that in FRs (1.0). Next, we analyzed the distribution of nucleotide and amino acid changes within the V_H domain of 5-5B as compared with *VH37.1* germline. Frequent substitution was occurred in CDR2 and CDR1 in both nucleotide and amino acid levels, on the other hand, slight substitution was occurred in only FR3 (Fig. 4). Although the point mutation study should be done for confirmation, these results suggest that HCDR2 may be associated with the 5-5B function, which is the neutralization capacity to Stx1.

For clinical application of mAbs, human antibodies against Stxs would be the best, though it is very difficult to isolate them because of the low immunogenicity of Stxs in humans. Figini et al. (1998) reported that human antibody against the forate-binding protein (FBP), a cell surface antigen that is overexpressed in many human ovarian carcinomas, has been isolated using the genes of high affinity mouse mAb against FBP as a guides for selection, termed guided selection. The 5-5B mAb genes cloned in this study can be applicable as guides for selection of human antibody against Stx1 not only with binding activity but also with neutralizing activity. Construction of antibody combinatorial library from STEC-infected patient and isolation of anti-Stx1 human rFab fragment, which neutralizes cytotoxicity by guided selection is now in progress.

Acknowledgements

We are grateful to Dr. Chiwa Kataoka (The Kansai Research Institute) for gift of 5-5B producing hybridoma cells. We thank Dr. Dennis R. Burton (The Scripps Research Institute, USA) for the generous gift of *pComb3* vector. This work was supported in part by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (#13672379).

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