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Full Length Research Paper

Engineering and evaluation of a mouse/human chimeric antibody against Shiga toxin 2

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Hemolytic-uremic syndrome (HUS), mainly caused by Shiga toxin (Stx) producing *Escherichia coli* (STEC) such as *E. coli* O157:H7 and STEC/EAggEC, is a serious complication predominantly leading to renal failure and even death. We have previously reported that a monoclonal antibody effectively neutralizes Stx2 *in vitro* and *in vivo* toxicity models. As a therapeutic agent against HUS, the mouse origin of this antibody can trigger human anti-murine antibody (HAMA) reactions thereby restricting its clinical application. In order to reduce its immunogenicity for use in humans, in this study, a mouse/human chimeric antibody designated rS2C4-IgG1 was developed in baculovirus/insect cell expression system. Analysis of antigen-binding and competitive binding revealed that rS2C4-IgG1 possessed specificity and affinity similar to that of S2C4. Results from cytotoxicity assays and mouse toxicity model analysis showed that rS2C4-IgG1offers neutralizing activity comparable to its parent MAb *in vitro* and *in vivo*. Therefore, the chimeric rS2C4-IgG1 had great potential for use in the treatment of STEC infection.

Key words: Shiga toxin 2, HUS, STEC, chimeric rS2C4-IgG1.

INTRODUCTION

Infection with Shiga toxin (Stx) producing *Escherichia coli* (STEC) is a global serious problem (Phillip et al., 2005). Following ingestion of pathogen-contaminated food and several days of incubation, patients usually develop watery, and bloody diarrhea (Akiyoshi et al., 2010). More seriously, some of the patients, especially children and elderly, even develop hemolytic uremic syndrome (HUS) which is characterized by acute renal failure, thrombocytopenia, micro-angiopathic hemolytic anemia, and even death (Scheiring et al., 2008). The dominant serotype etiologically associated with HUS is generally believed to be O157:H7, such as what was observed in the STEC outbreak in the fall of 2006 in USA which was induced by contaminated spinach, and in the large-scale epidemic that happened in China in 1999-2000 (Seto et al., 2007). However, an unusual *E. coli* serotype O104:H4 was identified to be responsible for German STEC outbreak in 2011, which caused thousands of infection and HUS cases, demonstrating horizontal gene exchange event during enterobacteria natural evolution (Bezuidt et al., 2011). Of the several pathogenic factors, two antigenically distinct types, Stx1 and Stx2 primarily mediate STEC virulence (Donohue et al., 1989).

Stx consists of an A-subunit monomer and a B-subunit pentamer. A-subunit contains enzymatic RNA N-glycosidase activity that hydrolyzes the N-glycoside bond of adenosine of the 28S rRNA of 60S ribosomes and

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hence inhibits protein synthesis, while B is involved in receptor binding (Furutani et al., 1990). Stx2 is closely related to a family of Stx2 variants or alleles (Stx2c, Stx2d, Stx2e, and Stx2f). These respective Stx2 variants have 99.7, 94.9, 94.0, and 63.4% nucleotide sequence identity in their A subunits and 95.2, 86.6, 79.0, and 75.4% nucleotide sequence identity in their B subunits to the corresponding subunits of the Stx2-encoding gene.

Although the mechanisms of action of Stxs are thought to be the same, Stx2 is more clinically significant than Stx1 and frequently associated with the development of HUS (Tesh et al., 1993).

There are no preventive measures or specific therapeutics for HUS. Certain antibiotics, particularly the quinolones, trimethoprim, and furazolidone, which work as bacterial SOS inducers, can activate the lytic cycle of Stx-encoding bacteriophages, resulting in increased toxin gene copy number, transcription and Stx production *in vivo* (Neely Friedman, 1998). Thus, the use of these antibiotics can exacerbate patients' infection and should be avoided in clinical practice. Passively administered toxin-specific antibodies have been shown to be highly effective at preventing toxin-mediated diseases as little endogenous serum antibody against Stxs is induced following STEC infection (Bitzan et al., 1993). Several Stx2-specific monoclonal antibodies (MAb) with murine or human origins have been developed, and many have been shown to neutralize the activity of Stx2 *in vitro* and/or *in vivo* (Sheoran et al., 2003; Yamagami et al., 2001). We have previously reported the generation and characterization of a murine MAb designated S2C4 which neutralizes the cytotoxicity of Stx2 and its variants (Jiao et al., 2009).

A major disadvantage of the murine MAbs is that they are recognized as heterogenous proteins in the patients when used for therapeutics (Jones et al., 1986). The human anti-mouse antibody (HAMA) response and other pharmacodynamic side effects following treatment with mouse MAbs have hampered their efficacy in human studies (Shawler et al., 1985). In this study, as a first step towards antibody humanization, a mouse-human chimeric antibody of S2C4 has been constructed and expressed in baculovirus insect cell expression system, and the recombinant protein efficacy was evaluated *in vitro* and *in vivo*.

MATERIALS AND METHODS

Construction of vector expressing chimeric S2C4-IgG1

Total RNA was extracted from S2C4 hybridoma cell line (1×10^7) using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's instructions, and first-strand cDNA was synthesized from total RNA by using a First-Strand cDNA Synthesis Kit (Invitrogen, New York, USA). Variable region of heavy (VH) and light (VL) chain encoding genes were amplified by PCR with a set of primers complementary to the 5′-ends of the mouse constant regions (MSCG1ab-B for VH; MSCVK 10 for VL) and to the 5′-ends of the leader sequence upstream of the variable region (MSCVH13 for VH; MSCJK12-BL for VL) (Williams et al., 2010). The sequences of these primers are as follows: MSCG1ab-B 5' CCT GGC CGG CCT GGC CAC TAG TGA CAC ATG GGG CTG TCG TTT TGG C 3'; MSCVK 10 5' GGG CCC AGG CGG CCG AGC TCG ACA TTG AGC TCA CCC AAT C 3'; MSCVH13 5' GGT GGT TCC TCT AGA TCT TCC CTC GAG GTG CAG CTT GTT GAG TC 3'; MSCJK12-BL 5' GGA AGA TCT AGA GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC GCC ACC AGA GGA TTT TAT TTC CAG TTT GGT CCC 3'. Using standard PCR technology, reactions were set up using EX Taq (Takara, Dalian, Liaoning, China). The PCR program used for all reactions was 94°C for 3 min and 30 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 1min, followed by a final extension at 72°C for 10 min. The PCR products were cloned into TA-vector (Takara, Dalian, Liaoning, China) for sequencing. A second set of primers were designed to amplify VH and VL encoding genes of S2C4 which was determined by sequencing results described above. The VL and VH genes were separately cloned into pAc-K-CH3 baculovirus vector (Liang et al., 1997) for chimeric IgG expression via *SacI/HindIII* and *XhoI/NheI* sites, respectively. The resulting chimeric IgG1 was referred to as rS2C4-IgG1.

Generation of rS2C4-IgG1 in baculovirus expression system

A recombinant baculovirus, AcNPV- rS2C4, was generated by co-transfection with rS2C4-IgG1 plasmid and linearized AcNPV baculovirus DNA (Pharmingen, San Diego, USA) into Sf9 insect cells according to manufacturer's instructions. A single recombinant baculovirus was selected by plaque assay, and amplified in Sf9 cells. The recombinant virus stock was used to re-infect insect cells at a multiplicity of infection (MOI) of 5 for rS2C4-IgG1 production. The expressed rS2C4-IgG1 was affinity purified by Protein A (GE Healthcare, New Jersey, USA), and kept in -20°C for later use.

Characterization of rS2C4-IgG1 binding specificity

The binding specificity of rS2C4-IgG1 compared to its parent MAb was assessed by competitive ELISA, and Western Blot. For ELISA, costar 96-well EIA/RIA Strip well immunoplates were coated with Stx2 protein diluted to a concentration of 2 µg/ml by carbonate-bicarbonate buffer, pH 9.6 (50 µl/well), and incubated overnight at 4°C. After washing with washing buffer consisting of PBS, pH 7.2, and 0.05% Tween 20, the plates were blocked with 300 µl/well of 5% fat-free milk powder in PBS and incubated overnight at 4°C. They were then washed as described above. Fifty microlitres of purified rS2C4-IgG1 that was serially diluted twofold were mixed with same volume of S2C4 MAb and then added to the plate. The enterovirus 71 specific human IgG was used as an irrelevant control. After incubation in a moist chamber for 30 min at 37°C, plates were washed 5 times with washing buffer, and 100 µl per well of the goat anti-mouse antibody-HRP conjugate was added (1:2000, Sigma Aldrich, St. Louis, USA). Plates were incubated for 30 min at 37°C and washed 5 times, and 100 µl per well of tetramethylbenzidine (TMB) substrate (Thermo Fisher, Illinois, USA) was used for detection. Plates were incubated at room temperature for 10 min, and 100 µl per well of 1M H_2SO_4 was added to stop the reaction. Absorbance was read at 450 nm. For Western Blot, the purified Stx2 was electrophoresed by sodium dodecyacyl sulphate-polyacrylamide gel electrophresis (SDS-PAGE) and transferred to nitrocellulose membrane (GE Healthcare, New Jersey, USA). Membranes were separately incubated with diluted rS2C4-IgG1 and S2C4 MAb. After washing, diluted HRP-anti-human IgG (for rS2C4-IgG1, 1:2000, Sigma Aldrich, St. Louis, USA) or HRP-anti-mouse IgG (for S2C4 MAb, 1:2000, Sigma Aldrich, St. Louis, USA)) conjugate was added. Blot was developed with ECL plus Western blotting detection system kit (GE Healthcare, New Jersey, USA).

Vero cell cytotoxicity assay

The cytotoxin-neutralizing ability of chimeric rS2C4-IgG1 was assayed on Vero cells. Firstly, the 50% cytotoxic dose (CD_{50}) of the Stx2 preparation was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, USA) reduction conversion assay which determines the concentration of Stx2 that results in approximately 50% killing of the Vero cells. Briefly, Vero cells were plated at 3.0×10^4/well on 96-well plate in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Invitrogen, New York, USA) and incubated overnight at 37°C and 5% CO_2. Purified Stx2 was serially diluted, added to each well and incubated for 3 days. Then, 20 µl of MTT at 5 µg/L was added to each well, and incubation was continued for 2 h. The formazan crystals resulting from mitochondrial enzymatic activity on MTT substrate were solubilized with 100 µl of 20% (wt/vol) SDS/50% (vol/vol) N,N-dimethylformamide (pH 4.7), and incubated overnight. Absorbance was measured at 590 nm by using a microplate reader. Result was expressed as percent of viability compared with control culture viability from assays performed in the absence of Stx2. For assessment of rS2C4-IgG1 neutralizing potency, a 50 µl volume of toxin solution in DMEM containing 5 times the CD_{50} of the toxin was pre-incubated with 50 µl of diluted rS2C4-IgG1, for 1 h at 37°C, and the remaining unbound toxin cytotoxicity was measured by cytotoxicity assay as described above. The S2C4 MAb was used as a control. Results were expressed as percent of viability compared with control culture viability from assays performed with anti-Stx2 mouse sera without Stx2 (100% viability) and with only Stx2 (0% viability).

Mouse toxicity model

A murine Stx2 toxicity model was used to examine the ability of chimeric rS2C4-IgG1 to neutralize the effects of Stx2 *in vivo*. About 16 h after intraperitoneal (ip) injection of 5 ng Stx2 per mouse (dose equal to 100% lethality), rS2C4-IgG1 and MAb S2C4 were serially diluted from 30 to 0.5 µg/mouse in 200µl of PBS or PBS alone (control) and administered intravenously (iv) to each group of 10 6-week-old female BALB/c mice (Slac Laboratory Animal Co., Shanghai, China). Mice were observed twice daily for survival and experiments was terminated at day 12.

RESULTS

Cloning of S2C4 MAb variable region genes and expression of chimeric rS2C4-IgG1

After nucleic acid amplification and sequencing, S2C4 MAb variable region encoding genes, with sizes of 378 bp for VL, and 396 bp for VH, respectively, were obtained (Figure 1A). By aligning them with immunoglobulin sequences in GenBank database, VL was assigned to VK iii subfamily, whereas VH was belonged to IgG1 class VH iii subgroup. For chimeric full antibody expression, VL and VH were inserted into pAc-k-CH3 vector under the two very late baculovirus expression promoters of p10 and polyhedrin, respectively (Figure 1B). The expressed rS2C4-IgG1 was purified to homogeneity from culture supernatant by affinity chromatography scheme. Heavy and light chains with expected sizes of 55 and 30 kDa were observed under reducing condition of SDS-PAGE (Figure 1C).

Immunoreactivity of the chimeric antibody

To determine whether the chimeric rS2C4-IgG1 could maintain its parent MAb S2C4's binding specificity, we performed antigen-binding assay by competitive enzyme linked immunosorbent assay (ELISA) and Western blot. As shown in Figure 2A, rS2C4-IgG1 could effectively compete with its parental mouse antibody S2C4, in a dose-dependent manner for binding to Stx2, while no antagonist effect was induced by control IgG. In western blot, rS2C4-IgG1 specifically bound to the A subunit of Stx2 at a size of 26KDa, similar to its parent MAb S2C4 (Figure 2B). These results demonstrate that the murine derived variable regions possess specificity similar to that of the original murine antibody.

Neutralization of Stx2 in vero cell cytotoxicity

The ability of rS2C4-IgG1 to neutralize the cytotoxic effects of Stx2 was analyzed on Vero cells. As shown in Figure 3, rS2C4-IgG1 induced an increased cell survival rate (3% to 99%) at doses ranging from 39 to 2500 µg/L in the presence of fixed amount of Stx2 (5 times of CD_{50}, 207 fg/L), demonstrating that the chimeric antibody, like its parent MAb, neutralizes Stx2 efficiently and in turn inhibits its toxicity to Vero cells, while an irrelevant control antibody (EV71 specific) lacked this activity.

Neutralization of Stx2 *in vivo*

Chimeric rS2C4-IgG1 was further evaluated in the mouse toxicity model to determine if it can significantly prolong survival and have the potential to work as therapeutic agent. Both rS2C4-IgG1 and MAb S2C4 showed neutralizing potency at all 7 doses tested compared to that for the PBS control group (Figure 4). Mice were given either rS2C4-IgG1 or S2C4 MAb iv 16 h after Stx2 administration. Both antibodies with doses from 1.9 to 30 µg/mouse resulted in the highest percentage of survival (90-100%) within 12-day interval, while 0.5 and 0.9 µg-dose groups the lowest (20 and 60%, respectively), indicating a similar *in vivo* protection profiles for the two antibodies. However, in some time points of low dose groups, the percent survival with rS2C4-IgG1 was higher than that of MAb S2C4 (for instance, in 0.9µg group, at day 6 the survival was 80% for rS2C4-IgG1 vs 60% for MAb S2C4).

DISCUSSION

Mouse monoclonal antibodies have been shown to have more limited use as therapeutic agents than anticipated

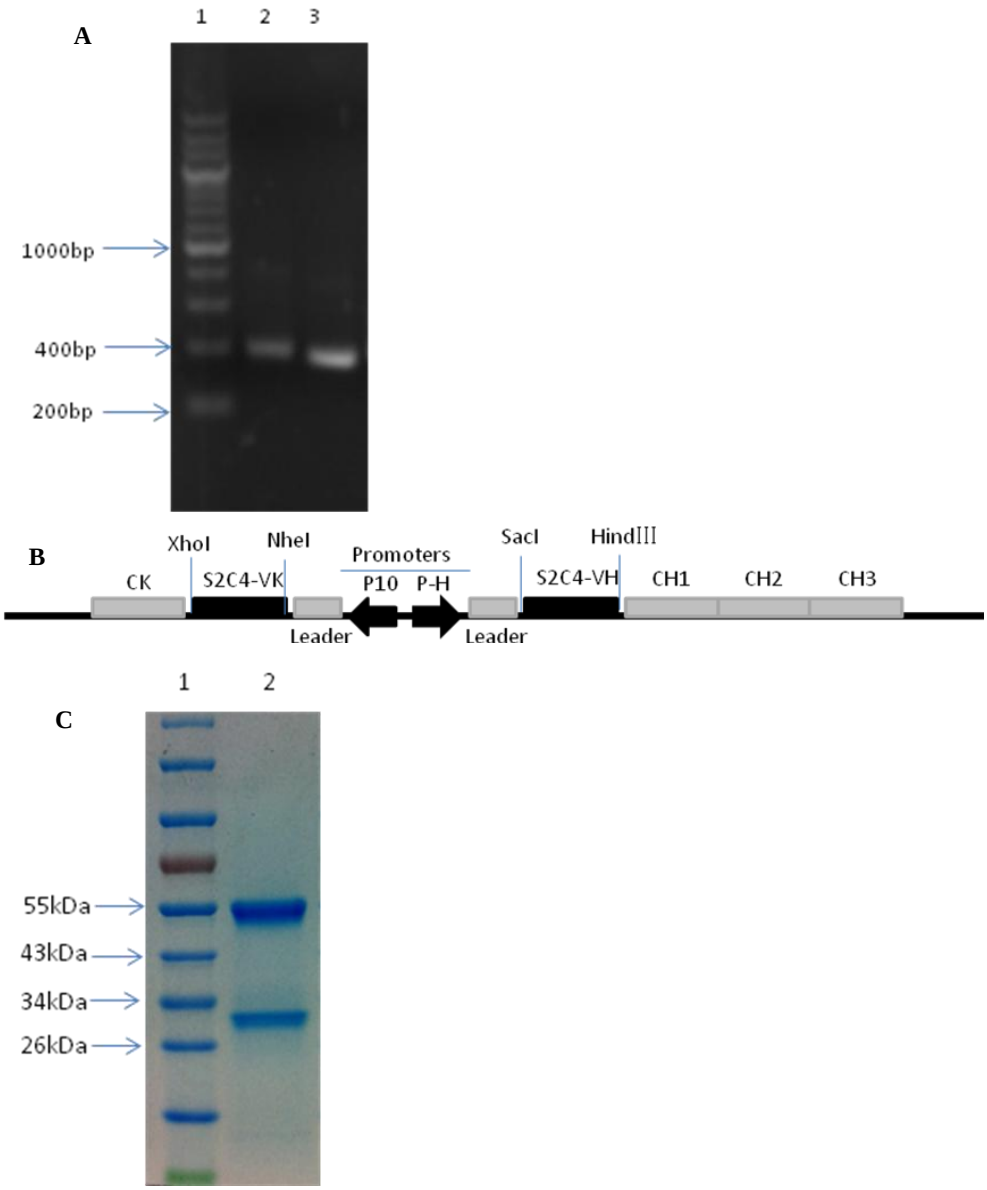

Figure 1. Cloning and expression of Chimeric rS2C4-IgG1 (A) S2C4 MAb variable region encoding genes were amplified with two pairs of specific primers by PCR. Lane: 1, DNA ladder; lane 2, amplified VH gene with a size of 396bp; lane 3, amplified VL gene with a size of 378 bp. (B) The antibody coding regions of baculovirus vector for chimeric antibody expression. (C) SDS-PAGE of purified rS2C4-IgG1 protein. Lane: 1, Protein marker; lane 2, rS2C4-IgG1.

because of their short serum half-life, their inability to trigger human effector functions, and the induction of a human anti-mouse antibody response (Shawler et al., 1985). To overcome or alleviate these deficiencies, a variety of antibody constructs, including antibody fragments, chimeric, and humanized antibodies, etc, via recombinant DNA methodologies have been developed (Waldmann, 2003). Antibody fragments, including ScFv and Fab which correctly align variable light and heavy chains, can retain the antigen binding specificity of the parent antibody, and can be produced as functional proteins in bacterial expression systems (Casey et al., 1995). However, they may be quickly cleared by the kidneys due to their smaller size (the predicted sizes of the Fab and ScFv are about 50kDa and 30kDa, below the 60kDa threshold of the glomerular filter), which results in a short vascular half-life (Zola, 2000). Another issue is that antibody fragments, which lack an Fc region, do not activate complement or elicit cytotoxicity (Vandevyver et al., 1993). Considering the important roles of variable and Fc regions of antibodies in mediating biological effects, and to further reduce immunogenicity in humans, in this

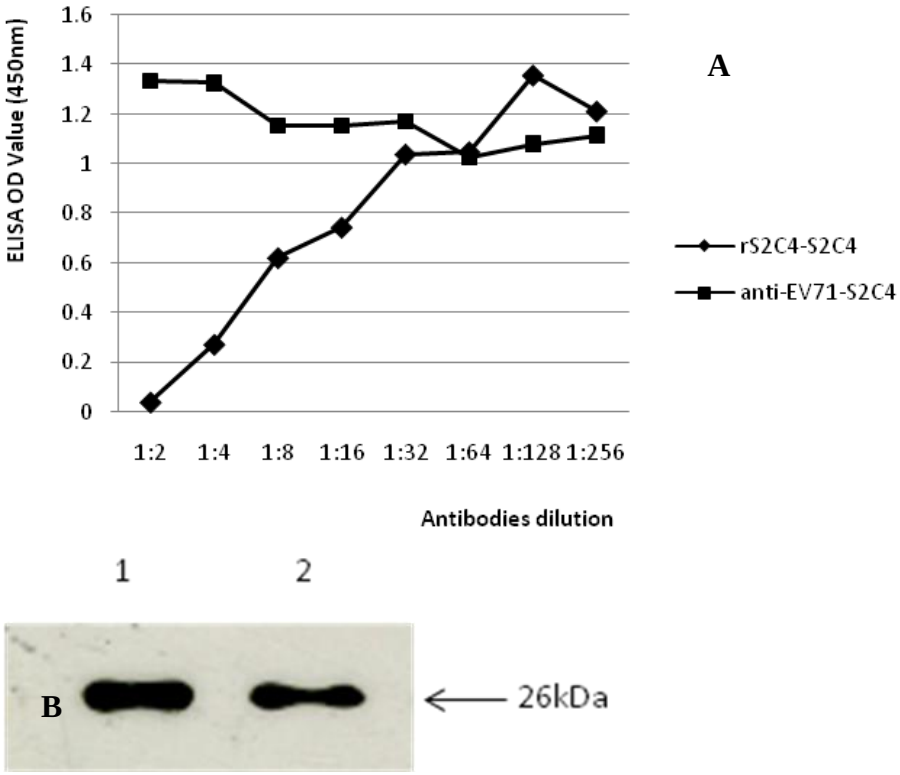

Figure 2. Evaluating the immunoreactivity of the chimeric rS2C4-IgG1. (A) rS2C4-IgG1competed with its parent MAb to bind target antigen, A subunit of Stx2 in ELISA format. Non-specific human IgG was used as control IgG. (B) A Western blot analysis of rS2C4-IgG1 binding activity as described in Materials and methods. 1, rS2C4-IgG1; 2, S2C4 MAb.

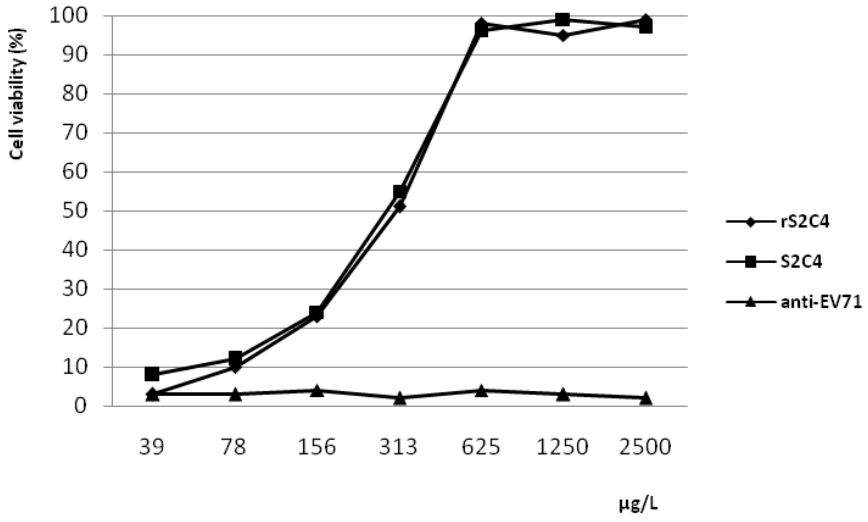

Figure 3. Neutralizing activity of chimeric rS2C4-IgG1 against Stx2 in cytotoxicity assay. Purified Stx2 with a cytotoxicity of 5 times of CD_{50} was incubated with diluted rS2C4-IgG1 and S2C4 MAb at 37°C for 1 h, and the remaining unbound toxin cytotoxicity was measured by cytotoxicity assay as described in materials and methods. Cell viabilities were calculated according to the following formula: (A_{590} of sample -A_{590} obtained with only Stx2)/(A_{590} with only S2C4 MAb -A_{590} obtained with only Stx2)×100% (Nakao et al.,1999).

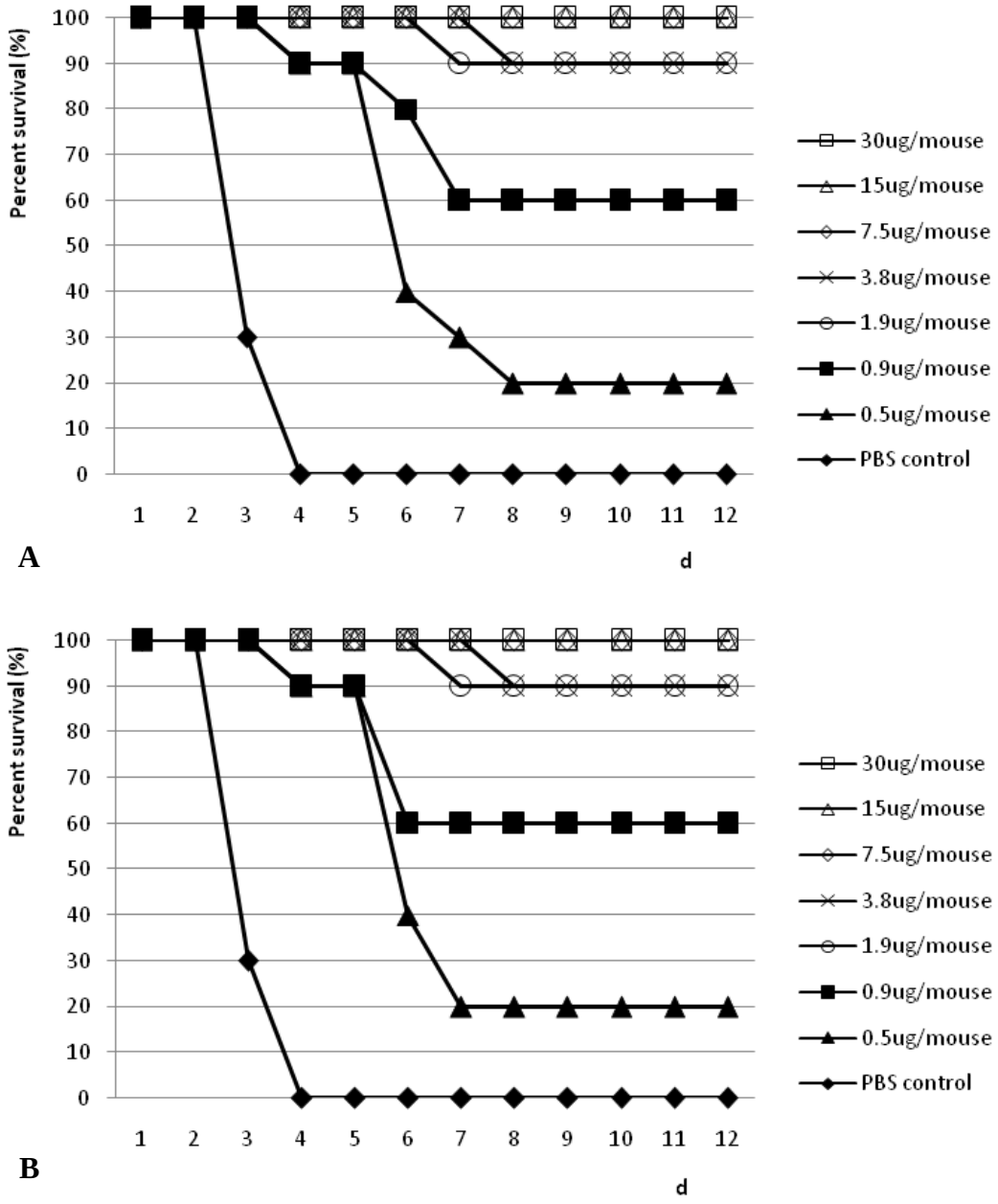

Figure 4. Neutralization activity of the chimeric rS2C4-IgG1 and S2C4 MAb against Stx2 in the mouse toxicity model. (A) Number of surviving mice administered with 30, 15, 7.5, 3.8, 1.9, 0.9, 0.5 µg of rS2C4-IgG1 or PBS control in 200 µl volume at 16 h after ip administration of a 100% lethal dose of the Stx2. rS2C4-IgG1 protected 90% of the mice at the doses ranging from 1.9 to 30 µg/mouse compared to PBS control, but prolonged survival was not observed at the two lower doses of 0.9 and 0.5 µg/mouse. Experiments were terminated on day 12. Mice that survived through day 12 were euthanized and were given a survival score of 12 days. (B) S2C4 MAb was used as a positive control, and experimental methodology for S2C4 MAb was the same as rS2C4-IgG1 described above.

study, a complete mouse/human chimeric antibody format was generated in the baculovirus/insect cell expression system for a well-characterized murine-derived antibody against Stx2, and its efficacy *in vitro* and *in vivo* was also evaluated.

The chimeric antibody, designated rS2C4-IgG1 was made by joining cDNA sequences encoding the variable regions, with their respective leader sequences, of the light and heavy chain of mouse MAb S2C4 to cDNA sequences encoding the human κ chain and γ1 constant region, respectively. The recombinant vector was transfected into SF9 insect cells with the help of

linearized AcNPV baculovirus DNA, and a complete immunoglobulin molecule of chimeric rS2C4-IgG1 was detected in the culture supernatant. Because the variable regions that constitute the antigen-binding site are not altered, chimeric antibody is expected to bind as well as the parental mouse antibody (Williams et al., 2010). Our previous report demonstrated that S2C4 MAb's activity was mainly mediated by an antagonist effect, in which S2C4 MAb blocks Stx2 binding to its receptor on the target cell. In this study, the rS2C4-IgG1 showed a similar neutralizing profile to that of the parental mouse antibody *in vitro* and *in vivo* assays.

In the chimeric antibody construct, the murine Fc was replaced by its human counterpart, which resulted in approximately 75% humanization of mouse antibody molecule (Morrison et al., 1984), and could therefore induce a significantly decreased HAMA response in human recipients. However, the remaining 25% mouse component in this chimera is still immunogenic, and human anti-chimeric antibody response (HACA) have been observed (Aarden et al., 2008). However, considering the particular nature of STEC infection in humans, where only a few times of antibody administration can effectively eliminate the toxin from the body, the severe side effect associated with cancer patients due to long term and large dosage use, would not happen in this situation. On the other hand, effector functions of antibodies such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) are mainly mediated by the Fc region (Vandevyver et al., 1993). Of the several isotype of human IgGs, IgG1 is believed to have superior activities than IgG2, IgG3, and IgG4 (Steplewski et al., 1988). In this study, we developed the chimeric antibody with human IgG1 isotope, and this molecule was shown to be more potent *in vivo* than its mouse counterpart only in some time-points of low dose groups (shown in results). However, whether the observed activity of recombinant chimeric antibody was contributed by Fc region and its underlying mechanism of action need to be further addressed.

The baculovirus expression system has been used for the expression of a wide variety of heterologous genes, and Ig is the first protein expressed in this system (Hasemann and Capra, 1990). Compared to other eukaryotic expression system, a primary advantage of baculovirus protein expression is its higher expression level, with an ordinary 10 mg secreted human IgG per liter yield from the supernatant of infected SF9 cells cultured in flasks. In this study, the deduced rS2C4-IgG1level is about 24 mg per liter (data not shown), with a good balance of heavy/light chains expression (Figure 1C). The insect cell has a different post translational glycoprotein pattern from mammalian hosts (Wilson et al., 2001). However, many studies have reported that these differences do not affect IgG activities (Boyd et al., 1995), consistent with our results that rS2C4-IgG1 exerted *in vitro* and *in vivo* functions similar to its parent antibody.

In conclusion, the chimeric rS2C4-IgG1 generated in baculovirus/insect cell expression system might be a promising candidate molecule for immunotherapy against HUS.

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