Protection by a Recombinant *Mycobacterium bovis* Bacillus Calmette-Guérin Vaccine Expressing Shiga Toxin 2 B Subunit against Shiga Toxin-Producing Escherichia coli in Mice

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We have developed a novel vaccine against Shiga toxin (Stx)-producing *Escherichia coli* (STEC) infection using a recombinant *Mycobacterium bovis* BCG (rBCG) system. Two intraperitoneal vaccinations with rBCG expressing the Stx2 B subunit (Stx2B) resulted in an increase of protective serum IgG and mucosal IgA responses to Stx2B in BALB/c mice. When orally challenged with 10⁵ CFU of STEC strain B2F1 (O91: H21), the immunized mice survived statistically significantly longer than the nonvaccinated mice. We suggest that intraperitoneal immunization with rBCG expressing Stx2B would be a potential vaccine strategy for STEC.

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) or enterohemorrhagic *Escherichia coli* (EHEC) is a causative agent of hemorrhagic diarrhea (33), hemolytic uremic syndrome (HUS) (12), and neurological damage (36). In Sakai City, Japan, 7,892 schoolchildren and 74 teachers were affected in a large outbreak in 1996 (25). One hundred twenty-one individuals developed HUS, and three females died with complications of the central nervous system. A large outbreak of Shiga toxin 2-producing enteraggregative *E. coli* (EAEC) O104:H4 occurred in northern Germany in May 2011. In total, 16 countries in Europe and North America reported 3,222 infectious cases resulting in 810 (25%) cases of HUS and 50 deaths (7). In Japan, between late April and early May 2011, an outbreak of Stx2-producing EHEC O111 infection occurred in Toyama, Fukui prefecture, and Yokohama. A total of 169 people developed diarrhea, 30 (17.8%) developed HUS, and 50% lethality. As the cause is suspected (14), there is an increased toxicity after toxin activation by mouse intestinal mucus (15). Protective responses have been achieved with nucleotide 4324 of 28S RNA of the 60S ribosomal subunit (5), rendering ribosomes inactive for protein synthesis (30). Each B subunit binds with high affinity to the glycosphingolipid globo-triaosylceramide (Gb3), which is expressed on the surface of eu-karyotic cells (18).

Bosworth et al. reported that vaccination of pigs with a genetically modified nontoxic form of Stx2e (E167Q) produced antibodies to Stx2e (2). Pigs vaccinated with Stx2e (E167Q) had a reduced incidence of subclinical edema disease and never developed clinical edema disease. Acheson et al. reported that oral administration of an Stx1 B-subunit-expressing *Vibrio cholerae* vaccine strain [CVD 103-HgR(pDA60)] caused development of neutralizing serum antibodies to Stx1 in rabbits (1). Rabbits immunized with Stx2 toxoid were fully protected from the intravenous administration of 10 and 50% lethal doses (LD₅₀) of Stx1, and this correlated directly with protection from binding of this toxin to target organs (19).

Bacillus Calmette-Guérin (BCG) is a live attenuated strain of *Mycobacterium bovis* that offers potential advantages as a vector for mucosal delivery of antigens. Recombinant BCG (rBCG) elicits protective humoral immune responses to a variety of antigens. A single intranasal vaccination with rBCG expressing the outer surface protein A antigen from *Borrelia burgdorferi* resulted in a protective systemic IgG response and a highly sustained secretory IgA response, and, surprisingly, prolonged effects lasted more than 1 year (15). Protective responses have been achieved with...
rBCG in pneumococcal infection (16), leishmaniasis (4), and rodent malaria (23). Here, we have constructed an rBCG that expresses and secretes the Stx2 B subunit (Stx2B) to efficiently elicit host humoral immune responses against STEC infection. Our rBCG system expressed the Stx2B as a fusion protein in live BCG and caused humoral responses against Stx2B as serum IgG and mucosal IgA in mice. We have constructed a shuttle vector expressing Stx2B (pSOJK1-Stx2B) and transformed BCG with vector pSOJK1 or pSOJK1-Stx2B by electroporation. Finally, a streptomycin-treated mouse model infected with Stx2d-producing STEC strain B2F1, which is highly virulent in mice, was used to confirm the effects of rBCG in a challenge test.

MATERIALS AND METHODS

Bacterial culture. BCG Tokyo was used as a host for plasmid pSOJK1 (22) and its derivatives. BCG Tokyo and its transformants were grown in Middlebrook 7H9 broth (BD Diagnostic Systems, Sparks, MD) supplemented with 10% albumin-dextrose-catalase (ADC) enrichment (BD Diagnostic Systems, Sparks, MD) and 0.05% Tween 80 (7H9 ADC medium). For immunization of mice, rBCG-Stx2B or rBCG with shuttle vector (rBCG-SV) was grown with shaking (100 rpm) at 37°C in 7H9 ADC. After 3 days of incubation with shaking, rBCG was pelleted at 4,000 × g and washed twice in phosphate-buffered saline (PBS). The optical density at 600 nm (OD600) was measured, where 1.0 OD600 unit was estimated to be 10⁸ CFU/ml of rBCG. Also, to confirm the bacterial count obtained using the OD600, rBCG was counted by the colony assay method on Middlebrook 7H10 agar plates.

EHEC O91:H21 strain B2F1 producing Stx2d (provided by Alison O’Brien) was grown in Luria broth (L broth) (Bacto tryptone, 10 g; Bacto yeast extract, 5 g; NaCl, 5 g; glucose, 1 g; H₂O, 1 liter) at 37°C overnight, after which the organism was washed once with PBS by centrifugation (6,000 × g for 20 min). The bacterial suspension of B2F1 producing Stx2d was adjusted to approximately 10³ CFU/ml in PBS.

Purification of Stx2. Stx2 used in this study was purified as described previously (37) and was determined to be free of detectable lipopolysaccharide by the amebocyte lysate test. Purity was also confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and cytotoxic potency in a Vero cell assay.

Construction of an Escherichia coli-mycobacterium shuttle vector that expresses the Stx2 B subunit. The gene encoding E. coli Stx2B was designed from the B subunit of bacteriophage 933W (accession no. X07865; positions 1210 to 1476) and amplified from E. coli 933W by PCR using designed primers. The nucleotide sequences of the oligonucleotides were as follows: forward primer, 5’-CCCTCGAGTGCGGATTGTGCTAAAG-3’, and reverse primer, 5’-CCCTCGAGGGTCATTATTAAACTGCA-3’ (restriction sites for XhoI are underlined). These primers contained the sequences for XhoI restriction sites to facilitate ligation into pSOJK1. The amplified DNA was cloned in pGEM-T Easy vector (Promega Co, Madison, WI). The DNA sequence of the Stx2B gene and addi-
tion. XhoI sites were confirmed by DNA sequencing with an ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA) to be matched with the Stx2 B-subunit gene under accession no. X07865 (see Fig. S1 in the supplemental material). The Stx2B gene fragment was digested with XhoI and was cloned into the XhoI site of the Mycobacterium kansasii K-alpha (antigen 85B) gene in pSOJK1 (22). The resulting plasmid, pSOJK1-Stx2B or pSOJK1, was transformed into BCG by electroporation as described previously (23) (Fig. 1A). rBCG-Stx2B was selected on Middlebrook 7H10-oleic acid-albumin-dextrose-catalase (OADC) agar (BD Diagnostic Systems, Sparks, MD) containing 20 μg/ml kanamycin (Meiji Seika Co. Ltd., Tokyo, Japan) and by culturing at 37°C for 4 weeks. Kanamycin-resistant colonies were then transferred to Sauton medium as described by Gheorghiu et al. (9) and grown at 37°C for 3 weeks. Culture supernatants were then obtained by filtration of the culture medium of rBCG through a membrane filter with a pore size of 0.45 μm (Nihon Millipore K.K., Tokyo, Japan) and then concentrated by ammonium sulfate precipitation. Twenty micrograms of secreted protein was examined by SDS-PAGE and by immunoblotting as described previously (24). The membrane was then reacted with polyclonal anti-K-alpha antibody or anti-monoclonal Stx2B antibody (ViroStat Inc. Portland, ME). K-alpha was purified from the supernatant of rBCG-SV that was transformed with only pSOJK1 as described previously (28). Polyclonal anti-K-alpha sera were obtained from female rabbits (Japan SLC, Shizuoka, Japan) after multiple injections of K-alpha emulsified with incomplete Freund’s adjuvant (Sigma, St. Louis, MO).

i.p. immunization. Ten-week-old female BALB/c mice were purchased from JAX Mice and Services (Bar Harbor, ME). Mice were immunized by intraperitoneal i.p. injection of 10^8 or 10^7 CFU of rBCG-Stx2B or 10^7 CFU of rBCG-SV followed by one booster 2 weeks later. This experiment was approved by the Animal Care and Use Committee, Kyushu University.

The first protocol for the immune response and Stx2 i.p. challenge test is shown in Fig. 2. Ten-week-old BALB/c mice were immunized with 10^6 or 10^7 CFU rBCG-Stx2B or with PBS. The second protocol, to test a lasting immune response, is shown in Fig. 3. Ten-week-old BALB/c mice were immunized with 10^6 or 10^7 CFU rBCG-Stx2B or PBS i.p.

Oral challenge with strain B2F1 in streptomyacin-treated mice. Immunized mice were given water containing streptomyacin (5 g/liter) (Meiji Seika Co. Ltd., Tokyo, Japan) ad libitum for 3 days to reduce the level of normal intestinal flora before oral inoculation with B2F1 (27). On day 3 of streptomyacin treatment, the mice were starved for one-half day, giving water containing streptomyacin, and then orally inoculated with 0.5 ml of B2F1 suspension (10^7 CFU). After the inoculation, food was resumed.

B2F1 is the most virulent of all EHEC strains after oral infection of streptomyacin-treated mice (LD_{50} of 10 CFU after oral infection) (17). The mice were observed for 2 weeks after oral inoculation of the B2F1 strain.

Challenge of mice with purified Stx2. Ten-week-old female BALB/c mice were immunized by i.p. injection of 10^6 or 10^7 CFU of rBCG-Stx2B or 10^7 CFU of rBCG-SV followed by one booster 2 weeks later. The numbers of mice per test group for mice inoculated with 10^6 or 10^7 CFU rBCG-Stx2B or 10^7 CFU rBCG-SV were 7, 6, and 5, respectively. Two weeks after vaccine administration, a dose of 2.9 ng/mouse (approximately 2 LD_{50}) of purified Stx2 was injected i.p., and the mice were then observed for 2 weeks.

ELISA of serum IgG and mucosal IgA anti-Stx2B antibodies. Antibody responses in serum and stool were determined by enzyme linked immunoassay (ELISA) under conditions similar to those previously described (10). Briefly, 96-well ELISA plates (Costar, Cambridge, MA) were coated overnight at room temperature with 1.5 mg/ml of purified Stx2 in PBS. Nonspecific sites were blocked by incubation with 1.0% bovine serum albumin (Sigma, St. Louis, MO). After washing, the plates were incubated at room temperature for 2 h with 1:500-diluted serum samples. The bound Stx2-specific IgG was determined with sheep anti-mouse IgG horseradish peroxidase-conjugated antibody (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) and subsequently developed with 3,3′,5,5′-tetramethylenbenzidine substrate solution (Thermo Fisher Scientific Inc., Rockford, IL). After addition of stop solution (2 N H2SO4), the wells were read at 450 nm. IgG concentrations were determined by plotting the sample values against a standard curve.

Analysis of mucosal IgA was performed as described previously (10). Briefly, stool was collected from animals in each group and rehydrated in PBS containing 3% bovine serum albumin and 10 mM phenylmethylsulfonyl fluoride (PMSE) (Sigma, St. Louis, MO) overnight. Samples were centrifuged at 10,000 × g, and the supernatant stored at −20°C until required. For IgA analysis, the wells for the test samples were coated with 2.0 mg/ml of purified Stx2 in PBS. For the standard curves, the wells were coated with goat anti-mouse IgA affinity-purified antibody (Bethyl Laboratories, TX). ELISA was performed as described above. The bound Stx2-specific IgA was determined with goat anti-mouse IgA horseradish peroxidase-conjugated antibody (Bethyl Laboratories, TX) diluted 1:5,000, added to all of the wells, and incubated at room temperature for 2 h. The concentration of immunoglobulin was calculated from the optical density at 450 nm with standard curves. ELISA was performed on the three test groups (i.e., 10^6 or 10^7 CFU of rBCG-Stx2B or 10^7 CFU of rBCG-SV), with serum or stool samples from PBS-injected mice as controls for each ELISA run. The ELISA results for each group were divided by the result for normal intestinal flora before oral inoculation of the B2F1 strain.

FIG 2 Two immunizations of rBCG-Stx2B, serum IgG antitoxin response, and challenge test with purified Stx2. (A) On day 0 and day 14, BALB/c mice were immunized i.p. with rBCG-Stx2B; each group received 10^6 (n = 7) or 10^7 (n = 8) CFU of rBCG-Stx2B or PBS (n = 6). On day 28, anti-Stx2B serum IgG antibody was measured using ELISA. *, P < 0.05. (B) On day 28, the challenge test with purified Stx2 was performed. The survival rates are shown. *, P < 0.05.
the control. Since the IgG or IgA concentration for PBS-injected control mice was different for each ELISA plate of the three groups, the results shown are a ratio of IgG or IgA concentration in serum and stool samples from PBS-injected mice compared to that in the three groups.

Statistical analysis. Statistical analysis involved analysis of variance, which was followed by unpaired Student t, Bonferroni, and log rank (Mantel-Cox) tests with SPSS 19. Statistical differences were considered to be significant at a P value of $< 0.001$ (**) or $< 0.05$ (*).

RESULTS

Antigen production by rBCG-Stx2B. rBCG harboring pSOJK1-Stx2B expressed the K-alpha–Stx2B fusion protein (38.7 kDa) in culture supernatants (Fig. 1B). The expression of fusion protein was observed only in the supernatant of rBCG-Stx2B. The fusion protein reacted with both anti-K-alpha serum and anti-Stx2B monoclonal antibody. There were also additional bands of 22 and 14 kDa that were recognized by anti-K-alpha rabbit sera (Fig. 1B).

Immune response of mice to Stx2B and challenge with Stx2. On days 0 and 14, 10-week-old female BALB/c mice were immunized i.p. with either PBS ($n = 9$), $10^6$ CFU of rBCG-SV ($n = 6$), or $10^7$ CFU of rBCG-Stx2B ($n = 6$). (A and B) The IgG (A) and IgA (B) ratios were obtained as the sample OD$_{450}$/control OD$_{450}$ with PBS-treated mice. *, $P < 0.05$. (C) On day 0 and day 14, BALB/c mice were immunized i.p. with rBCG-Stx2B, and each group received $10^6$ ($n = 7$) or $10^7$ ($n = 8$) CFU of rBCG-Stx2B or $10^7$ ($n = 6$) CFU of rBCG-SV. The challenge test of oral inoculation of $10^5$ CFU of B2F1 was performed on day 28. The survival rates are shown. **, $P < 0.001$; *, $P < 0.05$.

FIG 3 Immune response to Stx2B and oral challenge with strain B2F1 in streptomycin-treated mice. On day 0 and day 14, BALB/c mice were immunized i.p. with either PBS ($n = 9$), $10^6$ CFU of rBCG-SV ($n = 6$), or $10^7$ CFU of rBCG-Stx2B ($n = 6$). (A and B) The IgG (A) and IgA (B) ratios were obtained as the sample OD$_{450}$/control OD$_{450}$ with PBS-treated mice. *, $P < 0.05$. (C) On day 0 and day 14, BALB/c mice were immunized i.p. with rBCG-Stx2B, and each group received $10^6$ ($n = 7$) or $10^7$ ($n = 8$) CFU of rBCG-Stx2B or $10^7$ ($n = 6$) CFU of rBCG-SV. The challenge test of oral inoculation of $10^5$ CFU of B2F1 was performed on day 28. The survival rates are shown. **, $P < 0.001$; *, $P < 0.05$. 

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not last for 2 months. IgA antibody responses lasted for 2 months in mice immunized with 10^6 or 10^7 rBCG (Fig. 3B).

For the challenge test, we used a streptomycin-treated mouse model of infection with the most virulent strain of Stx2d-producing E. coli, strain B2F1. Forty-two days after the first of two immunizations, mice were challenged with 10^3 CFU of B2F1 for protection against STEC infection. The 14-day survival rates for the 10^6 and 10^7 CFU rBCG-Stx2B inoculation groups were 57% and 63%, respectively. In addition, the group inoculated with 10^0 or 10^2 CFU of rBCG-Stx2B survived statistically significantly longer than control mice inoculated with rBCG-SV (P = 0.04 [*] or P < 0.001 [**], respectively, by the log rank test) (Fig. 3C).

DISCUSSION

Alpha antigen (antigen 85B) is the most potent immunodominant and secreted antigen of mycobacteria. Previously, our group had succeeded in expressing foreign antigens in BCG as a fusion protein with K-alpha. The rBCG system has advantages, and BCG is perhaps the safest vaccine that has been used in humans (31). In this study, we inserted the Stx2B gene into the K-alpha gene and constructed an rBCG strain expressing Stx2B. In the first protocol (Fig. 2A), we did not expect a longer survival time for the group inoculated with 10^6 CFU rBCG-Stx2B because the group did not have a good serum IgG antibody response to Stx2B following the immunization with rBCG-Stx2B. In the second protocol (Fig. 3A), the immunization with 10^6 CFU rBCG-Stx2B induced a high mucosal IgA response that lasted for 2 months. It was determined whether the mucosal IgA response induced by 10^6 CFU rBCG-Stx2B could neutralize purified Stx2d. The group inoculated with 10^0 CFU of rBCG-Stx2B survived statistically significantly longer than control mice inoculated with rBCG-SV (Fig. 3C). We conclude that an increase of serum IgA antibody against Stx2B required 10^7 and 10^8 CFU of rBCG-Stx2B and the mucosal IgA response induced by 10^6 CFU rBCG-Stx2B had an inhibitory effect, absorbing Stx2d produced by the B2F1 from mouse intestines. Importantly, at day 28, higher IgA levels were observed in the mice inoculated with 10^7 than in those inoculated with 10^2 CFU of rBCG-Stx2. Inoculation with 10^6 CFU rBCG-Stx2B did not result in a high serum IgG or IgA response, and this group did not survive longer following an oral challenge with B2F1 (data not shown). It appears that this high dose of rBCG-Stx2B may interfere with the production of functional IgA antibody to Stx2B at day 28. Additionally, a high dose of purified Stx2B (361 ng/mouse) with adjuvant had the same result in that the IgA mucosal response to Stx2 reached an 8.27-fold level but no IgG response was observed at 56 days (data not shown). The IgA, but not IgG, response may be easily induced by Stx2B. Langermann et al. reported that a single intranasal vaccination with rBCG expressing OspA, an outer surface protein of Borrelia burgdorferi, resulted in a prolonged protective systemic IgG response for more than 1 year (15). On the other hand, a single i.p. inoculation of rBCG expressing E. coli heat-labile enterotoxin induced IgA and IgG antibodies for 5 months (10). These studies show that effective routes of rBCG inoculation should be considered and selected. Kawahara et al. reported that oral inoculation with freeze-dried rBCG-pSV3J1 induced HIV-1-specific mucosal and systemic immune responses in guinea pigs (13). When Stx1B was used together with cholera toxin for the intranasal immunization of BALB/c mice, strong enhancement of the immune response was observed (11). Recently, Tsuji et al. reported that intranasal vaccination with His-tagged StxB plus a mutant heat-labile enterotoxin is effective for preventing Stx1 or Stx2 toxemia in mice (35). For intranasal or oral immunization with rBCG-Stx2B, further studies will be needed.

Recently, Western blotting and ELSA were used to detect antibody to Stx2B in plasma specimens from HUS patients (6). Cohen et al. reported that B lymphoid cells are susceptible to Stx1 and that the vast majority of Stx1-sensitive B cells belong to the IgG- and IgA-committed subset, whereas most IgM-producing cells are resistant to Stx (3). Susceptibility of mouse B lymphoid cells to Stx2 has yet to be reported. Not only Stx holotoxin but also extracellular recombinant StxB (20) and anti-CD77 monoclonal antibody (34) induced apoptosis in a Burkitt’s B-cell lymphoma cell line. It is possible that a very high production of IgG in response to Stx2B might make a murine immune system imbalanced, and as a result, the IgG response in the present study did not last for more than 2 months. In conclusion, intraperitoneal immunization with rBCG expressing Stx2B was protective in the streptomycin-treated mouse model of STEC infection with the virulent strain of Stx2d-producing E. coli, strain B2F1. The production of antibodies against Stx2B, serum IgG and mucosal IgA, was maintained for 2 months in BALB/c mice.

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