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# Protection against Shiga-Toxigenic *Escherichia coli* by Non-Genetically Modified Organism Receptor Mimic Bacterial Ghosts

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**Shiga-toxigenic *Escherichia coli* (STEC) causes severe gastrointestinal infections in humans that may lead to life-threatening systemic sequelae, such as the hemolytic uremic syndrome (HUS). Rapid diagnosis of STEC infection early in the course of disease opens a window of opportunity for therapeutic intervention, for example, by administration of agents that neutralize Shiga toxin (Stx) in the gut lumen. We previously developed a recombinant bacterium that expresses a mimic of the Stx receptor globotriaosyl ceramide (Gb3) on its surface through modification of the lipopolysaccharide (A. W. Paton, R. Morona, and J. C. Paton, Nat Med 6:265–270, 2000, http://dx.doi.org/10.1038/73111). This construct was highly efficacious *in vivo*, protecting mice from otherwise fatal STEC disease, but the fact that it is a genetically modified organism (GMO) has been a barrier to clinical development. In the present study, we have overcome this issue by development of Gb3 receptor mimic bacterial ghosts (BGs) that are not classified as GMOs. Gb3-BGs neutralized Stx1 and Stx2 *in vitro* with high efficiency, whereas alternative Gb3-expressing non-GMO subbacterial particles (minicells and outer membrane blebs) were ineffective. Gb3-BGs were highly efficacious in a murine model of STEC disease. All mice (10/10) treated with Gb3-BGs survived challenge with a highly virulent O113:H21 STEC strain and showed no pathological signs of renal injury. In contrast, 6/10 mice treated with control BGs succumbed to STEC challenge, and survivors exhibited significant weight loss, neutrophilia, and histopathological evidence of renal damage. Thus, Gb3-BGs offer a non-GMO approach to treatment of STEC infection in humans, particularly in an outbreak setting.**

Shiga-toxigenic *Escherichia coli* (STEC) causes diarrhea and hemorrhagic colitis in humans, which can be complicated by life-threatening systemic sequelae, such as the hemolytic uremic syndrome (HUS) (1–4). Infection is principally food-borne, and modern large-scale food production and distribution practices leave the community vulnerable to massive outbreaks of STEC disease, such as that which occurred in Europe (principally northern Germany) in 2011 (5). Both the severe gastrointestinal symptoms and the systemic complications associated with STEC infections are caused principally by Shiga toxin (Stx), which is a *sine qua non* of virulence. During infections, STEC colonizes the gut and releases Stx into the gut lumen; the STEC does not invade the gut mucosa to any significant extent, but toxin is absorbed into the circulation and targets tissues, such as the microvasculature of the gut, kidneys, and brain, which display the appropriate glycolipid receptor (4, 6).

Development of rapid and sensitive methods for early diagnosis of STEC infection has created a window of opportunity for therapeutic intervention. Indeed, STEC infection may be detected up to a week before onset of HUS (4, 7). Furthermore, increased awareness during major outbreaks will result in more patients presenting during the prodromal stage. Contacts of persons with proven or suspected STEC infection also could be treated. Unfortunately, antibiotic therapy is contraindicated for STEC infection, because it increases free Stx in the gut lumen, probably by inducing toxin gene expression (4, 8). Thus, adsorption or neutralization of Stx in the gut is a potentially important alternative therapeutic strategy. STEC strains associated with human disease produce one or more of the recognized types of Stx (Stx1, Stx2, Stx2c, and Stx2d). Although they differ in amino acid sequence, all of these Stx types recognize the same receptor, globotriaosyl ceramide ($Gb_3$), which has the structure Galα[1→4]Galβ[1→4]Glc-ceramide (6). We have previously exploited this specificity to develop a recombinant bacterium expressing a mimic of the $Gb_3$ oligosaccharide on its surface (9). This involved insertion of a plasmid carrying two *Neisseria* galactosyl transferase genes, *lgtC* and *lgtE* (10), in a derivative of *E. coli* R1 (CWG308), which has a *waaO* mutation in the outer core lipopolysaccharide (LPS) biosynthesis locus such that a truncated LPS core terminating in glucose (Glc) is produced (11). Expression of *lgtC* and *lgtE* resulted in the linkage of Galα[1→4]Galβ[1→4] onto the terminal Glc, generating a chimeric LPS terminating in an exact mimic of the Stx receptor $Gb_3$ (9). This presents a high-density array of receptor mimics on the bacterial surface, each capable of lateral diffusion in the fluid outer membrane to optimize docking with the Stx B subunit pentamer. This bacterium adsorbed and neutralized Stx1, Stx2, Stx2c, and Stx2d with very high efficiency *in vitro* (1 mg dry weight could neutralize over 100 μg of purified toxin). Moreover, oral administration of the bacterium was 100% protective in a mouse model of STEC-induced renal damage (9). Oral adminis-

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**TABLE 1** Bacterial strains and plasmids

| Strain/plasmid | Description | Source or reference |
|---|---|---|
| Plasmids | | |
| pK184 | Cloning vector | 29 |
| pGb3 | pK184 carrying cloned *Neisseria lgtC* and *lgtE* genes | 9 |
| pAWJ | Carries lysis gene E from bacteriophage PhiX174 | 17 |
| *E. coli* strains | | |
| CWG308 | *waaO* mutant of *E. coli* R1 | 11 |
| 97MW1$^{SR}$ | Streptomycin-resistant derivative of O113:H21 STEC expressing Stx2 | 9 |
| CB168 | O111:H− STEC producing Stx1 | 18 |
| C600Δ*waaOB* | *E. coli* C600 with *waaOB* deleted | 13 |
| C600Δ*waaOB*Δ*lpp* | C600Δ*waaOB* with *lpp* deleted | This study |
| C600Δ*waaOB*Δ*ompA* | C600Δ*waaOB* with *ompA* deleted | This study |
| C600Δ*waaOB*Δ*lpp*Δ*msbB* | C600Δ*waaOB* with *lpp* and *msbB* deleted | This study |
| C600Δ*waaOB*Δ*lpp*Δ*msbB att*λ::$P_{araB}$-*lpp* Δ*ompA* | C600Δ*waaOB* with *lpp*, *msbB*, and *ompA* deleted and *lpp* under the control of arabinose promoter | This study |
| C600Δ*waaOB*Δ*lpp*Δ*msbB att*λ::$P_{araB}$-*lpp* Δ*araBAD*Δ*ompA* | C600Δ*waaOB* with *lpp*, *msbB*, *araBAD*, and *ompA* deleted and *lpp* under the control of arabinose promoter (conditional blebbing strain) | This study |
| C600Δ*waaOB*Δ*lpp*Δ*msbB att*λ::$P_{araB}$-*lpp* Δ*araBAD*Δ*ompA*/pGb3 | Conditional blebbing strain expressing the Stx receptor mimic Gb3 | This study |
| C600Δ*waaOB*Δ*minB*Δ*msbB* | C600Δ*waaOB* with the *minB* operon and *msbB* deleted, minicell-producing strain | This study |
| C600Δ*waaOB*Δ*minB*Δ*msbB*/pGb3 | Minicell-producing strain expressing the Stx receptor mimic Gb3 | This study |

tration of this novel agent to individuals diagnosed with, or at risk of, STEC infection has the potential to adsorb and neutralize free Stx in the gut lumen, thereby preventing absorption of toxin and the concomitant life-threatening systemic sequelae associated with STEC disease.

Notwithstanding the therapeutic potential of the live receptor mimic bacterium, the fact that it is a genetically modified organism (GMO) has been a barrier to its use in humans, even though no other effective treatments for STEC infection are available. The present study seeks to overcome this roadblock by using bacterial ghost (BG) technology (12). BGs are empty, nonliving bacterial envelopes of Gram-negative bacteria produced by controlled expression of the cloned bacteriophage PhiX174 gene E. This forms a lysis tunnel structure within the envelope of the bacteria, expelling cytoplasmic contents but leaving the cell envelope, including the inner and outer membrane structure, intact. In the present study, we have constructed BG derivatives of *E. coli* CWG308 expressing the globotriose epitope and examined their capacity to neutralize Stx and protect mice from otherwise fatal challenge with virulent STEC. We also examined the Stx neutralization capacity of subbacterial particles (minicells and outer membrane blebs), as these might provide an alternative non-GMO therapeutic delivery platform.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** Strains and plasmids used in this study are listed in Table 1. All *E. coli* strains were routinely grown in Luria-Bertani (LB) medium with or without 1.5% Bacto agar.

**Preparation of Gb3-mimic minicells and blebs.** Minicell- and bleb-shedding host strains were constructed in the *E. coli* C600Δ*waaOB* strain, which, like CWG308, has a truncated LPS core terminating in Glc (13). For the generation of minicells (14), the *minB* operon (consisting of the *minC*, *minD*, and *minE* genes) and the *msbB* gene were deleted using the method described by Datsenko and Wanner (15); PCR primers used for mutagenesis are listed in Table 2. The mutations were verified by PCR and DNA sequencing, and the confirmed minicell-producing strain was designated the C600Δ*waaOB*Δ*minB*Δ*msbB* strain.

A conditional blebbing strain was constructed by first deleting *lpp* and *msbB* in the *E. coli* C600Δ*waaOB* strain (15). The *lpp* gene then was integrated into the chromosome of the C600Δ*waaOB*Δ*lpp*Δ*msbB* strain under the control of the arabinose-inducible promoter $P_{araB}$ using the CRIM plasmid method described by Haldimann and Wanner (16). The genes encoding the enzymes responsible for the utilization of arabinose (*araBAD*) and *ompA* subsequently were deleted in this strain. The genotype of the final strain C600Δ*waaOB*Δ*lpp*Δ*msbB att*λ::$P_{araB}$-*lpp* Δ*araBAD*Δ*ompA* was verified by PCR and DNA sequencing. The minicell and conditional blebbing host strains then were transformed with pGb3.

Minicells for use in toxin neutralization assays were isolated by diluting an overnight culture of the C600Δ*waaOB*Δ*minB*Δ*msbB*:pGb3 strain 1:50 in LB broth supplemented with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 200 μg/ml kanamycin and incubating at 37°C with shaking for 5 h. Whole cells were removed by sequential centrifugation at 3,900 × *g* for 15 min at 4°C. Minicells were harvested by centrifugation at 15,000 × *g* for 1 h at 4°C and purified by sequential sucrose density gradient centrifugation. The minicell phenotype was confirmed by scanning electron microscopy, and the purity of the isolated minicells was determined by fluorescence microscopy.

Blebs were isolated from the C600Δ*waaOB*Δ*lpp*Δ*msbB att*λ::$P_{araB}$-*lpp*Δ*ompA*:pGb3 strain for use in toxin neutralization assays after growth in LB broth supplemented with 0.2% L-arabinose, 0.1 mM IPTG, and 200 μg/ml kanamycin, where appropriate, at 37°C with shaking until an optical density at 600 nm ($OD_{600}$) of ~0.4 was reached. Cells were harvested by centrifugation at 3,900 × *g* for 15 min and were resuspended in the original volume of prewarmed LB broth supplemented with 0.2% glucose, 0.1 mM IPTG, and 200 μg/ml kanamycin where appropriate, and it was incubated for an additional 6 h at 37°C with shaking. Whole cells were removed by two rounds of centrifugation at 3,900 × *g* for 15 min. Blebs were isolated from the supernatant by centrifugation at 150,000 × *g* for 3 h at 4°C. The supernatant was carefully removed, and the pellet containing blebs was washed with phosphate-buffered saline (PBS) before a second centrifugation at 120,000 × *g* for 30 min at 4°C.

**TABLE 2** PCR primers used in construction of blebbing and minicell-producing strains

| Primer name | Sequence[a] (5′–3′) |
|---|---|
| lpp-FRTKm-F | ACTTGTAACGCTACATGGAGATTAACTCAATCTAGAGGGTATTAATAATGTTAACCCTCACTAAAGGGCG |
| lpp-FRTKm-R | GTGCGCCATTTTTCACTTCACAGGTACTATTACTTGCGGTATTTAGTAGCAATACGACTCACTATAGGGC |
| msbB-FRTKm-F | CGCTACACTATCACCAGATTGATTTTTGCCTTATCCGAAACTGGAAAAGCATGTTAACCCTCACTAAAGGGCG |
| msbB-FRTKm-R | GCGAAGGCCTCTCCTCGCGAGAGGCTTTTTTATTTGATGGGATAAAGATCAATACGACTCACTATAGGGC |
| lppNdeI-F | CATATGAAAGCTACTAAACTGGTA |
| lppXmaI-R | CCCGGGTTACTTGCGGTATTTAGT |
| pLAKm-FRTCm-F | CCGCAAAAATTAAAAATGAAGTTTTGACGGTATCGAACCCCAGAGTCCCGGTGTAGGCTGGAGCTGCTTC |
| pLAKm-FRTCm-R | ATGCTTCAATAATCTAGTGGATCAAGAGACAGGATGAGGATCGTTTCGCACATATGAATATCCTCCTTAG |
| araBAD-FRTCm-F | GCAACTCTCTACTGTTTCTCCATACCCGTTTTTTTGGATGGAGTGAAACGGTGTAGGCTGGAGCTGCTTC |
| araBAD-FRTCm-R | TTCGTTTGATTGGCTGTGGTTTTATACAGTCATTACTGCCCGTAATATGCCATATGAATATCCTCCTTAG |
| ompA-FRTCm-F | CTCGTTGGAGATATTCATGGCGTATTTTGGATGATAACGAGGCGCAAAAAGTGTAGGCTGGAGCTGCTTC |
| ompA-FRTCm-R | GCAGCGGGGTTTTTCTACCAGACGAGAACTTAAGCCTGCGGCTGAGTTACCATATGAATATCCTCCTTAG |
| minC-FRTKm-F | ATCATCGCGCGCTGGCGATGATTAATAGCTAATTGAGTAAGGCCAGGATGTTAACCCTCACTAAAGGGCG |
| minE-FRTKm-R | TTGAAGATAAATGCGCTTTTACAGCGGGCTTATTTCAGCTCTTCTGCTTCAATACGACTCACTATAGGGC |

[a] Restriction sites are underlined.

**Construction of BGs.** Production of BGs was carried out according to Langemann et al. (12). BGs were prepared from *E. coli* CWG308:pK184 (for control BG) or CWG308:pGb3 (for Gb3 BG); pGb3 is a pK184 derivative with cloned *Neisseria lgtC* and *lgtE* genes, directing expression of the Gb3-mimic LPS on the cell surface (9). Both *E. coli* strains were transformed with lysis plasmid pAWJ (17) carrying the lysis gene E from bacteriophage PhiX174 under a temperature-inducible promoter/repressor system and a tetracycline resistance cassette. Bacteria were grown in animal-free LB medium supplemented with tetracycline (10 μg/ml) and kanamycin (50 μg/ml) until mid-logarithmic phase, followed by E-lysis induction via temperature upshift from 35°C to 42°C. After inactivation with β-propiolactone (BPL) and washing with deionized water, the BGs were lyophilized, weighed, and stored at room temperature. Prior to use, BGs were resuspended in PBS (for *in vitro* toxin neutralization) or 10% sucrose, 10% $NaHCO_3$ (for *in vivo* studies) at the indicated density, using three 30-s pulses at 6,000 rpm in a Precellys 24 homogenizer (Bertin Technologies).

**Toxin neutralization assay.** The capacity to neutralize Stx was determined by incubating BG, minicell, bleb, or live bacterial suspensions with various concentrations of purified Stx2 or a crude lysate of the Stx1-producing STEC strain CB168 (18) in a final volume of 700 μl of PBS for 1 h at 37°C with gentle agitation. The mixtures then were centrifuged and the supernatants were filter sterilized. Cytotoxicity of the supernatant fraction then was assayed using Vero (African green monkey kidney) cells, which are highly susceptible to all Stx-related toxins (4). Serial 2-fold dilutions were prepared in tissue culture medium (Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin). Fifty microliters of each dilution was transferred onto washed Vero cell monolayers in 96-well tissue culture trays, and after 30 min of incubation at 37°C, a further 150 μl of culture medium was added to each well. Cells were examined microscopically after 72 h of incubation at 37°C and scored for cytotoxicity. The endpoint Stx titer (cytotoxic doses [CD] per milliliter) was defined as the reciprocal of the highest dilution resulting in cytotoxicity in at least 10% of the cells in a given monolayer. The percentage of Stx adsorbed/neutralized was calculated using the formula $100 - (100 \times CD_{BG}/CD_{PBS})$, where $CD_{BG}$ is the Stx titer in the extracts incubated with the BGs (or other subbacterial particles) and $CD_{PBS}$ is the Stx titer in the respective Stx extract treated only with PBS.

***In vivo* studies.** Animal experimentation was approved by the University of Adelaide Animal Ethics Committee. The streptomycin-treated mouse model of STEC-induced renal injury has been described previously (19, 20). Two groups of 10 BALB/c female mice were given 5 mg/ml streptomycin in drinking water for 24 h before oral challenge with $10^8$ CFU of streptomycin-resistant STEC 97MW1 (9) suspended in 50 μl of 10% sucrose, 10% $NaHCO_3$. Mice then were given oral doses of 10 mg control BG or Gb3 BG suspended in 75 μl of 10% sucrose and 10% $NaHCO_3$, twice daily for up to 12 days, commencing approximately 30 min after STEC challenge. All STEC and BG doses were administered by micropipette into the mouth and swallowed voluntarily. Oral streptomycin (5 mg/ml) in drinking water was continued throughout the experiment. The numbers of 97MW1 were monitored in fecal samples from two representative mice from each group 2 days and 10 days after challenge. These fecal samples were homogenized and serially diluted in PBS and then plated onto LB agar supplemented with 50 μg/ml streptomycin. Mice were weighed daily, and the survival times of mice in each group also were recorded. Animals were euthanized if they became moribund, were exhibiting signs of distress, or had greater than 15% weight loss. The difference in survival rate between groups was analyzed using the Fisher exact test. Kidneys also were removed from mice that succumbed to challenge for histological examination, as well as from surviving mice at the end of the experiment (day 12). Blood samples were collected on day 12 from surviving mice for hematological and biochemical analyses.

## RESULTS

***In vitro* Stx neutralization by BGs.** The capacity of BGs derived from CWG308:pK184 (control BG) and CWG308:pGb3 (Gb3 BG) to neutralize crude Stx1 extracts or purified Stx2 was compared with that of the respective live cell suspensions. In a preliminary experiment, 12 mg BG or live cell suspension was mixed with 100 μl of CB168 lysate in a final volume of 700 μl PBS, and the cytotoxicity of filtered supernatants was assayed as described in Materials and Methods. The undiluted CB168 lysate had an Stx1 cytotoxicity titer of approximately $3.3 \times 10^6$ CD/ml. Gb3 BG neutralized 99.6% of this cytotoxicity, compared with 99.9% neutralization for live CWG308:pGb3 cells. No neutralization of Stx1 whatsoever was detected using control BG or CWG308:pK184 cells.

The neutralization capacities of BGs and whole cells then were compared using purified Stx2. Serial dilutions of control BG and Gb3 BG suspensions, as well as the respective live cell suspensions, were incubated with 50 μg purified Stx2 (specific cytotoxicity of 2,300 CD/μg) in a final volume of 700 μl PBS, and the cytotoxicity of filtered supernatants was determined after incubation as before. No neutralization of Stx2 was detected using either control BG or CWG308:pK184 cell suspensions. However, for Gb3 BG, neutralization ranged from 99.6% at 4 μg Stx2 per mg BG to 87.5% at 32 μg Stx2 per mg BG. For live CWG308:pGb3 cells, neutralization ranged from 99.8% at 4 μg Stx2 per mg cells to 98.4% at 32 μg Stx2 per mg cells (Table 3).

**TABLE 3** *In vitro* Stx2 neutralization by Gb3 BGs

| Toxin dose (μg Stx2 per mg BG/cells) | Stx2 neutralization (%) for: | |
|---|---|---|
| | Gb3 BG | CWG308:pGb3 live cells |
| 4 | 99.6 | 99.8 |
| 8 | 99.2 | 99.8 |
| 16 | 96.8 | 99.6 |
| 32 | 87.5 | 98.4 |

***In vitro* Stx neutralization by minicells and blebs.** The capacity of minicells and outer membrane blebs prepared from Gb3 mimic-expressing *E. coli* strains to neutralize purified Stx2 also was examined (Table 4). Although significant neutralization of Stx2 was observed for the whole-cell suspensions from the respective $Gb_3$-expressing minicell- and bleb-shedding strains, no significant toxin neutralization was observed for the isolated Gb3-minicell or Gb3-bleb preparations.

***In vivo* protection from lethal STEC challenge.** In view of the inability of Gb3-minicells and Gb3-blebs to neutralize sufficient Stx2 *in vitro*, animal studies were conducted only with BGs. The streptomycin-treated mouse model was used to assess protective efficacy against lethal challenge with the highly virulent O113:H21 STEC strain 97MW1, which carries three $stx_2$ genes. Groups of 10 mice were challenged with 97MW1 and then treated orally with 10 mg Gb3 BG or control BG twice daily for 12 days (see Materials and Methods). Examination of fecal pellets from two mice from each group on days 3 and 11 confirmed stable gut colonization by 97MW1 at approximately $4 \times 10^8$ CFU per g feces for both groups. All 10 mice in the Gb3 BG group were alive and well at the end of the experiment (day 12). However, 6 of the 10 mice treated with control BG succumbed to the STEC challenge (Fig. 1). The difference in overall survival rate between groups is highly significant ($P = 0.0059$). Moreover, unlike the Gb3 BG group, the four surviving mice in the control BG group were clearly sick, with dull, ruffled fur, hunched appearance, and reluctance to move freely.

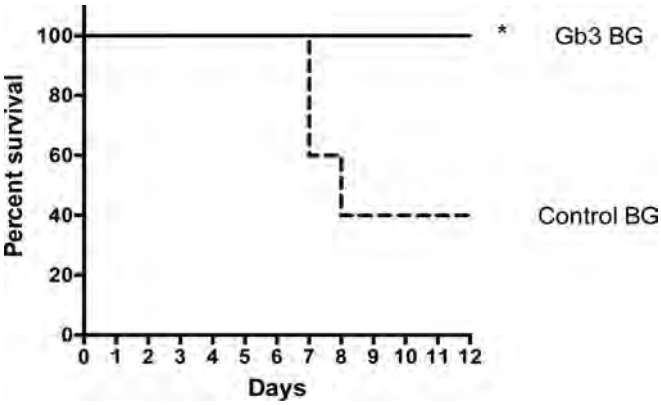


**FIG 1** Survival curve showing *in vivo* protective efficacy of Gb3 BG. Streptomycin-treated mice were challenged with STEC 97MW1 and then treated twice daily with 10 mg control BG or Gb3 BG, as described in Materials and Methods. Mice were weighed daily, and the survival times of mice in each group ($n = 10$ per group) also were recorded. An asterisk indicates significant difference in survival rates between groups ($P = 0.0059$) by Fisher exact test.

There also was a significant difference in body weight between the two groups by the end of the experiment (Fig. 2). The mean body weight of the Gb3 BG group increased steadily during the experiment from 15.5 g on day 1 to approximately 17.3 g on day 12. In contrast, the control BG group maintained or slightly increased body weight until day 5 and then steadily lost weight, and by day 12, the mean weight of the four surviving mice had dropped to below 14 g, approaching the threshold for euthanasia. The difference in body weight between the two groups reached statistical significance on days 6, 7, and 8 ($P < 0.05$), as well as on days 11 and 12 ($P < 0.02$).

Blood films collected on day 12 from all of the surviving mice showed evidence of leukocytosis in three of the four surviving control BG mice, unlike the case for the Gb3 BG group (Fig. 3). Differential cell counts showed that these three control BG mice

**TABLE 4** Neutralization of Stx2 by whole cells, minicells, and blebs[a]

| Strain–preparation | Stx2 titer | % Neutralization |
|---|---|---|
| C600Δ*waaOB*–whole cells | 16,384 | |
| C600Δ*waaOB*/pGb3–whole cells | 512 | 96.9 |
| C600Δ*waaOB*Δ*minB*Δ*msbB*–whole cells | 16,384 | |
| C600Δ*waaOB*Δ*minB*Δ*msbB*/pGb3–whole cells | 1,024 | 93.75 |
| C600Δ*waaOB*Δ*minB*Δ*msbB*–minicells | 16,384 | |
| C600Δ*waaO*Δ*minB*Δ*msbB*/pGb3–minicells | 16,384 | 0 |
| C600Δ*waaOB*Δ*lpp*Δ*ompA*Δ*msbB*Δ*araBAD attλ*::$P_{ara}$*lpp*–whole cells | 16,384 | |
| C600Δ*waaOB*Δ*lpp*Δ*ompA*Δ*msbB*Δ*araBAD attλ*::$P_{ara}$*lpp*/pGb3–whole cells | 4,096 | 75 |
| C600Δ*waaOB*Δ*lpp*Δ*ompA*Δ*msbB*Δ*araBAD attλ*::$P_{ara}$*lpp*–blebs | 16,384 | |
| C600Δ*waaOB*Δ*lpp*Δ*ompA*Δ*msbB*Δ*araBAD attλ*::$P_{ara}$*lpp*/pGb3–blebs | 16,384 | 0 |
| C600Δ*waaOB*Δ*lpp*Δ*ompA*Δ*msbB*Δ*araBAD attλ*::$P_{ara}$*ompA*–whole cells | 16,384 | |
| C600Δ*waaOB*Δ*lpp*Δ*ompA*Δ*msbB*Δ*araBAD attλ*::$P_{ara}$*ompA*/pGb3–whole cells | 1,024 | 93.75 |
| C600Δ*waaOB*Δ*lpp*Δ*ompA*Δ*msbB*Δ*araBAD attλ*::$P_{ara}$*ompA*–blebs | 16,384 | |
| C600Δ*waaOB*Δ*lpp*Δ*ompA*Δ*msbB*Δ*araBAD attλ*::$P_{ara}$*ompA*/pGb3–blebs | 8,192 | 50 |
| CWG308–fresh whole cells | 16,384 | |
| CWG308:pGb3–fresh whole cells | 64 | 99.6 |

[a] Aliquots of whole cell/minicell/bleb suspensions (0.5 mg dry weight equivalent) were incubated with 10 μg purified Stx2 in a final volume of 0.7 ml PBS for 60 min with gentle rocking at room temperature. Suspensions then were centrifuged (at high speed), and filter-sterilized supernatant was serially diluted and assayed for Stx2 activity on Vero cell monolayers. Endpoint Stx2 titers were determined microscopically at 72 h. Freshly prepared CWG308 and CWG308:pGb3 cells also were tested as additional positive and negative controls. Percent Stx2 neutralization was determined by comparing Stx2 titers for a given Gb3-expressing preparation (whole cells, minicells, or blebs) and the respective preparation from control cells.

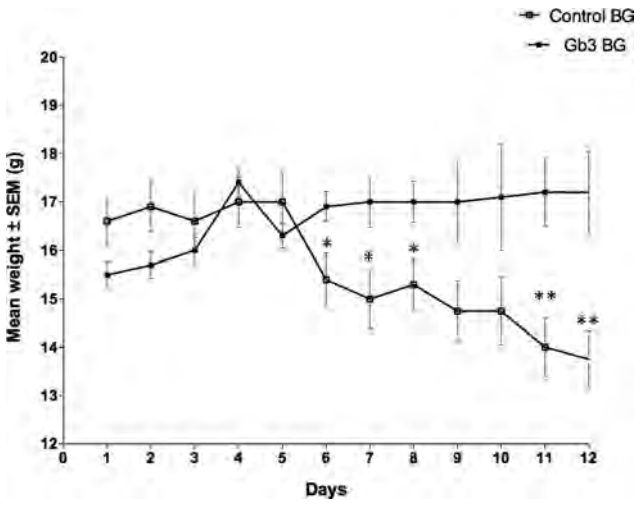


**FIG 2** Treatment with Gb3 BG prevents STEC-induced weight loss. Data are weights of STEC-challenged mice (means ± standard errors of the means) for the groups of mice from Fig. 1 treated with control BG or Gb3 BG. Significant differences between control BG and Gb3 BG groups are indicated: *, $P < 0.05$; **, $P < 0.02$ (unpaired 2-tailed *t* test).

had marked neutrophilia (Fig. 4), a typical feature of Stx2 intoxication in murine models (21) and human cases of HUS (22). Blood samples also were tested for plasma urea and creatinine as a measure of renal function. Unfortunately, insufficient specimen remained for the three control BG mice that had neutrophilia, but the remaining control BG mouse had higher urea and creatinine levels than any of the 10 Gb3 BG mice (Fig. 4).

Histological examination of kidneys collected at the time of euthanasia from four of the mice in the control BG group that succumbed to STEC challenge (euthanized on day 7 or 8) showed extensive evidence of necrosis in renal tubules and glomeruli. There also was glomerular capillary collapse and formation of microthrombi, as well as interstitial hemorrhage in the renal cortex. A representative example is shown in Fig. 5B. In the four control BG mice that were still alive on day 12, albeit with signs of morbidity, kidney tissue showed blood congestion in the glomeruli, swelling of tubular epithelial cells (some showing nuclear fading, suggesting cell death), and extensive interstitial hemorrhage (Fig. 5C). In contrast, kidney tissue appeared normal in all 10 mice in the Gb3 BG group, which were alive and well at the end of the experiment (Fig. 5A). Kidney slides also were scored for the above-described histopathological damage by a blinded observer using a 12-point scheme (Fig. 6). The 10 surviving Gb3 BG mice had a mean score of 0.25, compared with 6.25 for the 4 surviving control BG mice and 10.5 for the 4 euthanized control BG mice. Scores for both control BG groups were significantly higher than those for the Gb3 BG group ($P < 0.01$ in both cases).

## DISCUSSION

Large outbreaks of STEC disease, such as that which occurred in Europe in 2011, have the potential to overwhelm clinical acute

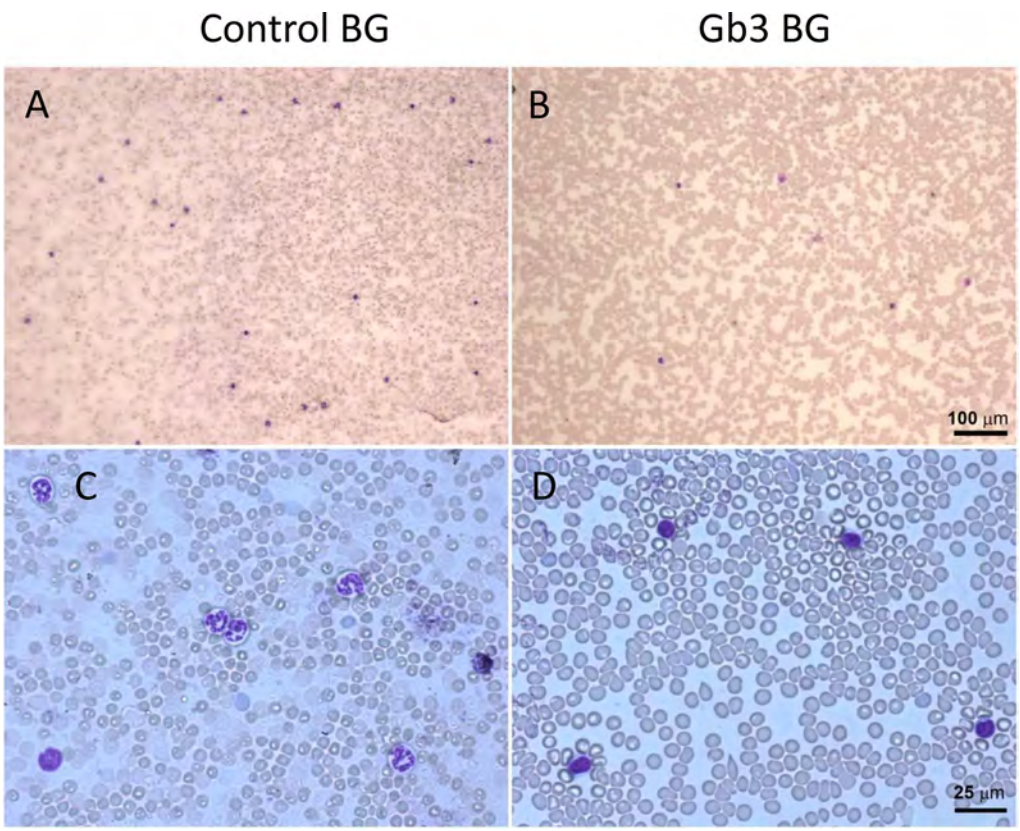


**FIG 3** Blood films from STEC-challenged mice treated with control BG or Gb3 BG. Blood was collected from surviving mice on day 12, and films were examined microscopically after Giemsa staining. (Upper) Low-power field showing leukocytosis in control BG-treated mice (A) and Gb3 BG-treated mice (B). (Lower) High-power field showing neutrophilia in control BG-treated mice (C) and Gb3 BG-treated mice (D).

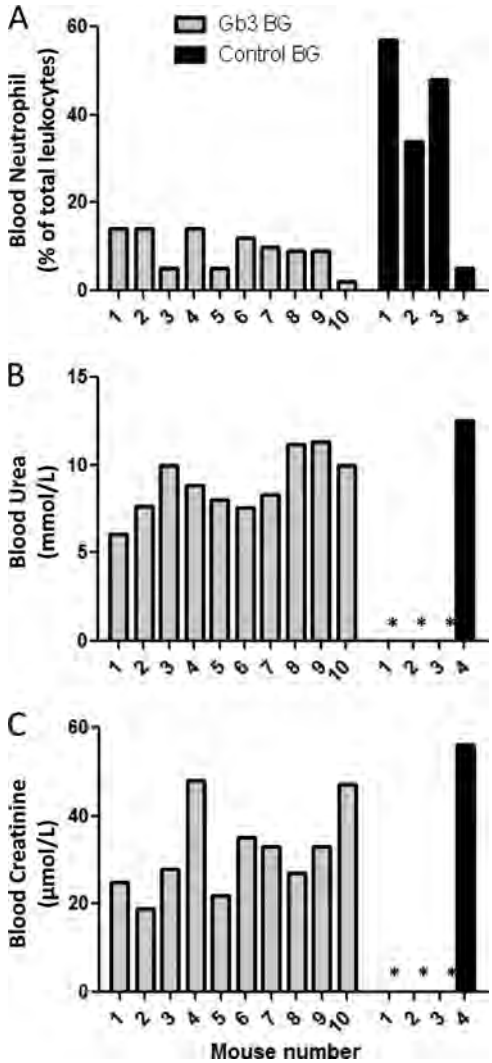


**FIG 4** Analysis of day 12 blood samples. Blood samples from all 10 Gb3 BG-treated mice and the four surviving control BG mice were examined by differential cell count (A); plasma urea (B) and creatinine (C) also were determined. Data for individual mice are shown; an asterisk denotes insufficient sample for analysis.

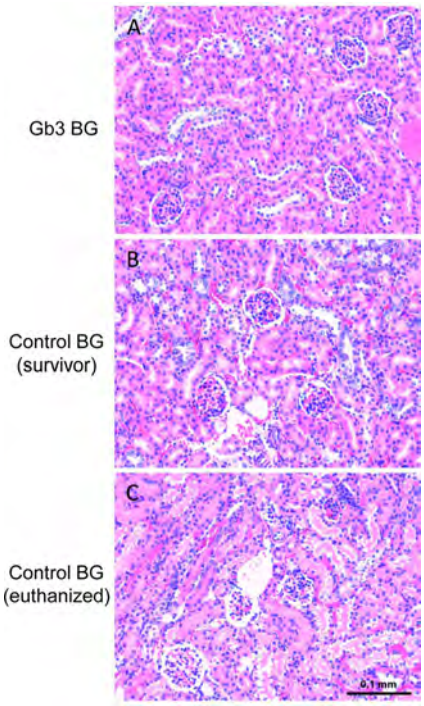


**FIG 5** Histopathological analysis of kidneys from STEC-challenged mice. Fixed kidney sections from all of the mice that had survived the experiment on day 12 (i.e., 10 Gb3 BG- and 4 control BG-treated mice), as well as from four of the six control BG-treated mice that were euthanized on day 7 or 8, were stained with hematoxylin and eosin and examined by light microscopy. Representative high-power fields from one mouse from the Gb3 BG group (A), a control BG survivor (B), and a euthanized control BG mouse (C) are shown. Histopathological features were similar within each group (not shown).

care facilities in even the most advanced economies. A feature of the 2011 outbreak was the high proportion of patients who progressed from gastrointestinal disease to systemic complications, such as HUS, and the higher than usual mortality (5, 23). While the etiological agent had an atypical genetic background (enteroaggregative *E. coli* O104:H4), it expressed the same phage-encoded toxin (Stx2) as classical O157:H7 enterohemorrhagic and other STEC strains associated with severe human disease. The early administration of agents capable of neutralizing Stx in the gut lumen would very likely have reduced the severity of gastrointestinal disease and prevented progression to HUS in many cases. However, no such agents were approved for human use at the time (23).

We have previously reported that oral administration of recombinant *E. coli* expressing a molecular mimic of the oligosaccharide receptor for Stx is highly protective against otherwise fatal challenge with virulent STEC in a murine model (9). Subsequent studies demonstrated that protection also could be elicited using formalin-killed receptor mimic bacteria and that treatment was still efficacious when commencement was delayed for up to 48 h after STEC challenge (24). Nevertheless, these killed bacteria still

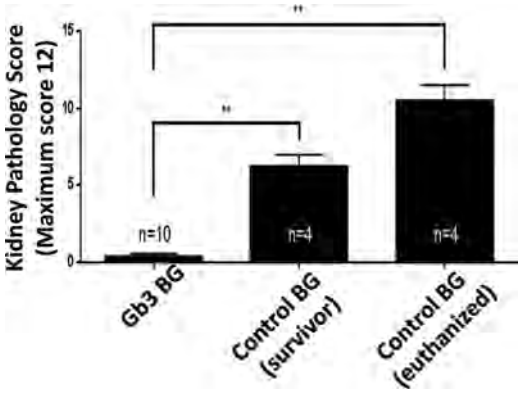


**FIG 6** Histopathological scores of kidney sections from STEC-challenged mice. Kidney section slides from the 10 surviving Gb3 BG mice, the 4 surviving control BG mice, and 4 of the control BG mice that were euthanized were scored (out of a possible total of 12) using the following scheme: blood congestion in renal glomeruli (score, 1), renal tubular epithelial swelling (score, 1), interstitial hemorrhage (focus-only score, 1; extensive score, 2), sign of cell death in tubular epithelium (focus-only score, 2; extensive score, 4), glomerular capillary collapse and microthrombus formation (focus-only score, 2; extensive score, 4). **, $P < 0.01$ by Student's unpaired two-tailed *t* test.

contain genomic DNA and do not satisfactorily quell regulatory concerns regarding exposure of humans to GMOs. The use of BG technology is a potential game changer, as BGs are empty shells devoid of cytoplasmic contents (12). Final treatment of BG preparations with BPL ensures sterility and alkylates any residual traces of DNA, leaving it nonfunctional and nonreplicable (25). Thus, unlike previous nonviable derivatives of the receptor mimic constructs, BGs can be classified as non-GMO. In the present study, we demonstrated that unlike Gb3-mimic minicells and blebs, Gb3 BGs bind significant amounts of Stx1 and Stx2 *in vitro* and are highly efficacious against STEC challenge. All treated mice survived challenge and steadily gained weight, even though the virulent O113:H21 challenge strain was maintained at high levels in the gut throughout the 12-day experiment. In the Gb3 BG-treated mice, pathological findings at the end of the experiment were unremarkable. In contrast, 6/10 control BG-treated mice succumbed to challenge, with the four survivors exhibiting significant weight loss, as well as pathological features consistent with Stx-induced renal damage.

Antibiotic therapy is contraindicated in cases of human STEC infection, owing to the risk of induction of phage *stx* gene expression by the bacterial SOS response and/or increased release of Stx into the gut lumen due to antibiotic-induced bacterial lysis (26). Management of patients is principally supportive, and early intravenous blood volume expansion is recommended, as it may provide protection against anuric HUS (26). During the 2011 European outbreak, the anti-C5 monoclonal antibody eculizumab, which has been used for treating atypical (non-Stx-related) HUS, was used empirically in some patients but without clear evidence of benefit (26). More encouragingly, intravenous administration of mouse-human chimeric monoclonal antibodies to Stx1 and Stx2 has been shown to protect mice against intraperitoneal injection of purified Stx or oral challenge with Stx2-producing STEC (27). Such antibodies previously have been shown to be safe in phase I clinical trials (28). Notwithstanding their likely efficacy in humans with STEC disease, monoclonal antibody-based therapeutics are likely to be expensive to manufacture on a large scale, and parenteral administration will mandate close clinical supervision. On the other hand, Gb3 BGs are likely to be extremely cheap to produce on a large scale and have a long shelf life, particularly in dried form. This will permit presumptive oral treatment of persons with suspected STEC disease, pending the results of laboratory analysis of stool samples or those deemed to be at high risk of infection, such as close contacts of confirmed cases. This is an important consideration, since early commencement of therapy will be more likely to prevent progression of STEC disease to life-threatening systemic complications.

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