Fabry Disease in Mice Protects against Lethal Disease Caused by Shiga Toxin–Expressing Enterohemorrhagic *Escherichia coli*

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Fabry disease is an X-linked recessive disorder in which affected persons lack α-galactosidase A (α-GalA), which leads to excess glycosphingolipids in tissues, mainly globotriaosylceramide (Gb3). Gb3 is the cellular receptor for Shiga toxin (Stx), the primary virulence factor of enterohemorrhagic *Escherichia coli. α-GalA*–knockout mice were significantly protected against lethal intraperitoneal doses of Stx2 or oral doses of Stx2-expressing bacteria, compared with wild-type *(wt)* control mice. Kidneys of moribund *wt* mice revealed tubular necrosis, but no histopathologic changes were observed in Gb3-overexpressing mice. Reducing Gb3 levels in *α-GalA*–knockout mice by the intravenous injection of recombinant human α-GalA restored the susceptibility of knockout mice to lethal doses of Stx2. These results suggest that excess amounts of Gb3 in *α-GalA*–deficient mice may impair toxin delivery to susceptible tissues.

Enterohemorrhagic *Escherichia coli* (EHEC) strains are a significant cause of intestinal foodborne illness worldwide. An estimated 20,000 cases of EHEC infection occur in the United States alone each year [1]. Infection may be complicated by hemorrhagic colitis and hemolytic-uremic syndrome (HUS), which is characterized by hemolytic anemia, thrombocytopenia, and renal failure. HUS is the most common cause of acute renal failure in children in the United States [2]. At present, there is no effective therapy for EHEC disease; treatment consists of supportive care and the management of disease complications.

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EHEC strains differ from other pathogenic *E. coli* strains in that they produce toxins related to the Shiga toxin (Stx) of *Shigella dysenteriae* type 1 [2]. EHEC strains implicated in human disease may produce the immunologically distinct Stx1 and/or Stx2, which are heterodimeric proteins consisting of 5 B receptor–binding subunits and 1 A catalytic subunit [3]. After release from the bacterium, the B subunits of Stx bind to their eukaryotic cell-surface receptor, the glycolipid globotriaosylceramide (Gb3). After the toxin-receptor complex is internalized by susceptible cells, vesicles with toxin undergo retrograde transport via the Golgi apparatus to the endoplasmic reticulum, where the A subunit cleaves a specific N-glycosidic bond in the 28S rRNA, thus blocking protein synthesis [3].

Recently developed therapeutic approaches to EHEC disease have concentrated on inhibiting the binding of toxin to susceptible target cells. Stx-specific human monoclonal antibodies have been demonstrated to protect animals from systemic complications of EHEC infection [4]. In addition, carbosilane dendrimers with high densities of trisaccharides that mimic Gb3 have been tested successfully in mice as intravenous agents [5]. With this latter therapy in mind, we noted that a mouse model in which Gb3 is overexpressed in all tissues had been developed for the study of Fabry disease, an X-linked recessive disorder in humans in which affected males are deficient in the lysosomal enzyme α-galactosidase A (α-GalA) [6, 7]. This deficiency leads to the accumulation of neutral glycosphingolipids, especially Gb3, in multiple tissues. These mice appear to be clinically normal, but lipid analysis demonstrates excess ceramidetrihexosides in numerous tissues [6]. Quantitation of Gb3 in tissues of these mice using an StxB binding assay revealed ~200-, 100-, 100-, 40-, and 20-fold higher concentrations of Gb3 in spleen, kidneys, plasma, liver, and heart, respectively, compared with concentrations in wild-type *(wt)* mice [8]. We speculated that the increased expression of Gb3 in these mice would affect their susceptibility to Stx and might provide insights both into the role that endogenous Gb3 plays and into the use of Gb3 analogues in human disease. Therefore, we challenged male *α-GalA*–knockout mice and their *wt* littermates with intraperitoneal (ip) injections of Stx and oral doses of an Stx2d-expressing EHEC strain.

Materials and methods. The streptomycin-resistant *E. coli* O91:H21 strain B2F1 [9, 10] was a gift from Alison O'Brien (Uniformed Services, University of the Health Sciences, Be-

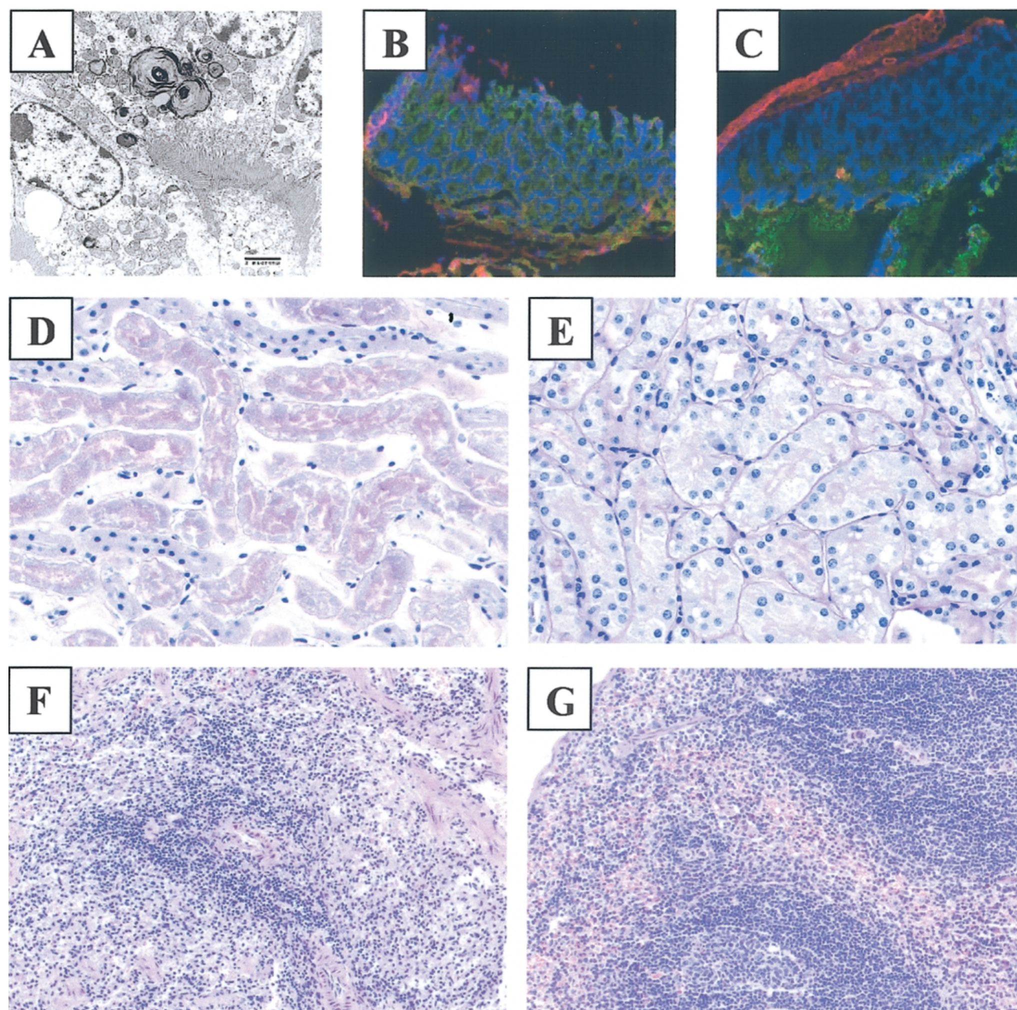

Figure 1. *A,* Electron micrograph of a renal cell from an α-*GalA*–knockout mouse. Magnification, ×5700. Bar, 2 μm. *B* and *C,* Immunofluorescent staining of frozen sections of colon from α-*GalA*–knockout *(B)* and wild-type *(wt; C)* mice. Staining is for actin *(red),* nuclei *(blue),* and Gb3 *(green).* Magnification, ×100. *D–G,* Histopathologic results of a periodic acid–Schiff–stained kidney (*D* and *E*) and a hematoxylin-eosin–stained spleen (*F* and *G*) from moribund *wt* (*D* and *F*) and α-*GalA*–deficient mice (*E* and *G*) given 1×10^5 cfu of the enterohemorrhagic *Escherichia coli* strain B2F1. Magnification, ×200.

thesda, MA). Stx2 holotoxin was a gift from Anne Kane (Tufts University School of Medicine, Boston, MA). In vitro cytotoxicity assays were performed as described elsewhere [11].

All mouse experiments were done in accordance with the institutional protocols of Massachusetts General Hospital. Breeding pairs of B6;129-Gla^{tm1Kul}/J heterozygous (+/−) female and *wt* (+/0) male mice (Jackson Laboratory) were mated, and their offspring were genotyped by polymerase chain reaction with the primer pair IMR0506 (5′-CGGCGAGGATCTCGTCG-TGACCCA-3′) and IMR0507 (5′-GCGATACCGTAAAGCAC-GAGGAAG-3′), amplifying a 200-bp DNA fragment from the knockout allele, and the primer pair IMR1338 (5′-TCCACAGC-AAAGGATTGAAGC-3′) and IMR1339 (5′-TTTTAGCAGATC-TACGCCCCA-3′), amplifying a 150-bp DNA fragment from the *wt* allele.

Stx2 holotoxin was prepared in doses of 200, 20, 10, and 2 ng/mouse in PBS. Then, 28–36-week-old α-*GalA*–knockout mice and their *wt* littermates were injected ip with Stx2 on day 0 and observed 3 times a day for evidence of illness. Moribund mice were euthanized. All surviving mice were killed on day 13. The heart, lung, spleen, kidney, liver, small and large intestine, and brain from each mouse were collected and fixed with 2.5% formalin in PBS in preparation for analysis by light microscopy or with Karnovsky's electron microscopy buffer (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 mol/L sodium cacodylate buffer [pH 7.4], and 0.025% $CaCl_2$) for analysis by electron microscopy. Formalin-fixed and paraffin-processed tissues were prepared on glass slides and stained with hematoxylin-eosin, Giemsa, or periodic acid–Schiff.

Oral inoculation of mice with EHEC strain B2F1 was per-

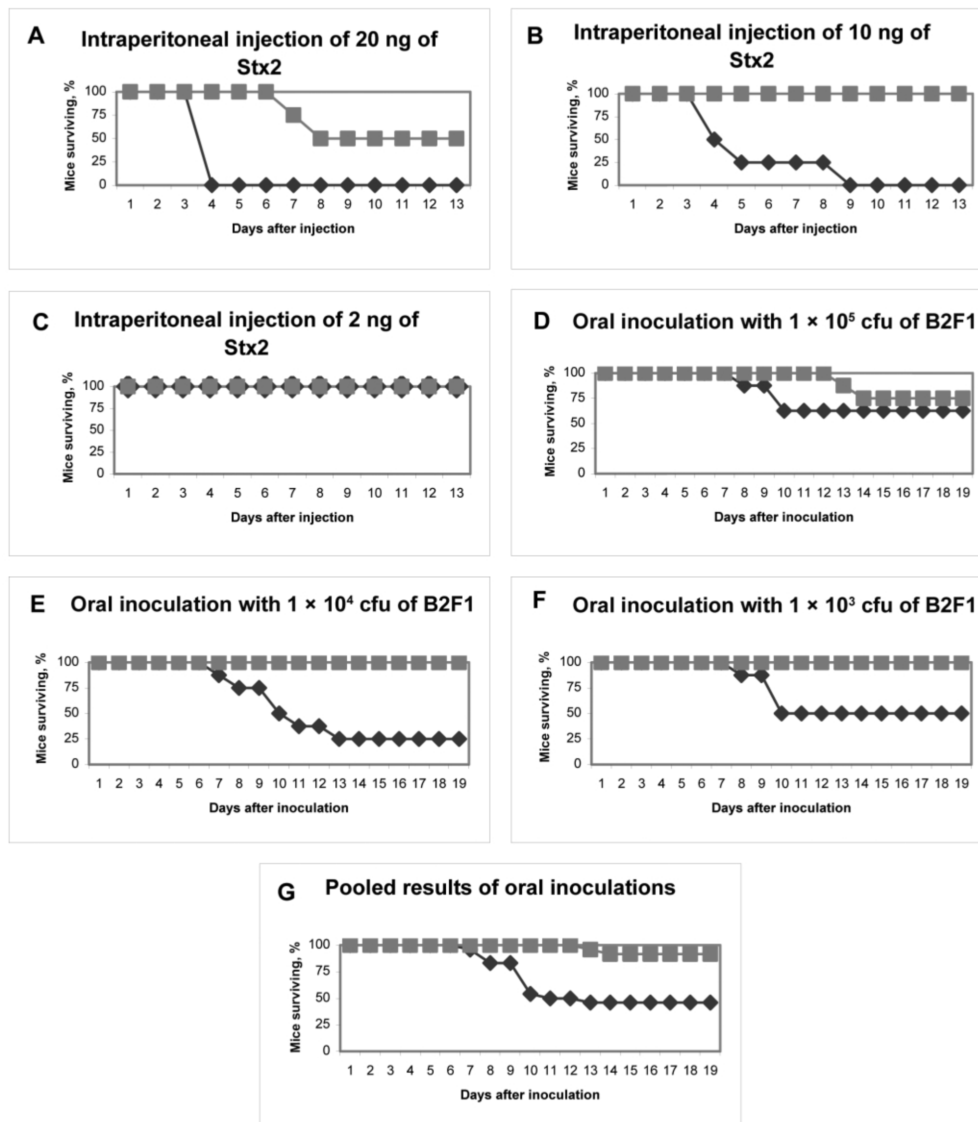

Figure 2. Survival curves of wild-type *(diamonds)* and α-*GalA*–knockout mice *(squares)* after the intraperitoneal injection of Shiga toxin 2 (Stx2) *(A–C)* and oral challenge with the enterohemorrhagic *Escherichia coli* strain B2F1 *(D–G).* No. of mice/group: 4 in *A–C* and 8 in *E–G.*

formed using the streptomycin-treated mouse model [9, 10]. Mice were provided with water that contained 5 g/L streptomycin for 72 h before oral inoculation, to deplete their normal intestinal flora and allow the overgrowth and colonization of streptomycin-resistant EHEC. On the day of infection, bacteria were diluted in 1 mol/L $NaHCO_3$ that contained 20% sucrose to doses of 1×10^5, 1×10^4, and 1×10^3 cfu/mouse in 100-μL samples. Mice were fed orally through micropipette tips and were then observed for illness; they were removed from the study when they became ill, as described above. Mice were bled on day 3 of infection to obtain serum for the analysis of concentrations of blood urea nitrogen and creatinine. All mice that survived to day 19 were euthanized. Tissues were recovered and fixed as described above.

wt and α-*GalA*–knockout mice were injected intravenously (iv) via the tail vein with either PBS or 3 mg/kg of recombinant human α-GalA diluted in PBS (Fabrazyme; Genzyme). Seven days after the iv injection, all mice were injected ip with 10 ng of Stx2. Mice were observed for illness and were removed from the study when they became ill, as described above. All mice that survived to day 10 were euthanized. Tissues were fixed and examined as described above.

Colons from *wt* and α-*GalA*–knockout mice were prepared for analysis by immunofluorescence microscopy. Frozen sections were washed in PBS, then incubated with rat monoclonal antibody to Gb3 (Immunotech) at a 1:10 dilution in PBS. Slides were then washed with PBS and incubated with a mixture of Texas Red phallacidin (5 μL/sample) for actin (Molecular Probes;

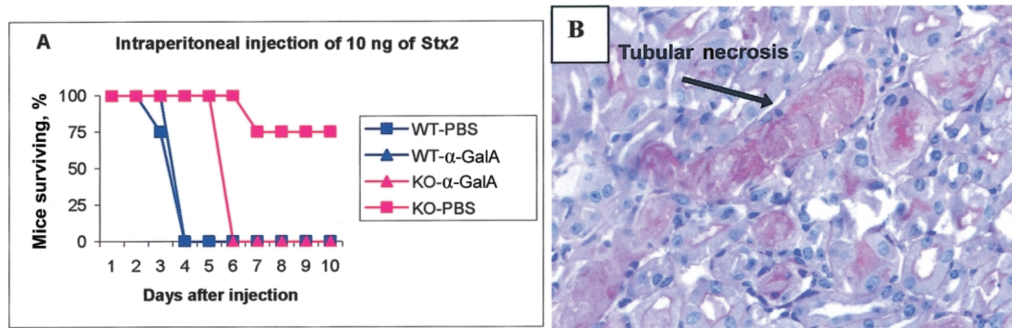

Figure 3. *A,* Survival curves of wild-type *(wt; blue)* and *α-GalA*–knockout (KO; *pink*) mice treated with PBS *(triangles)* or with recombinant human α-galactosidase A (α-GalA [Fabrazyme]; *squares*) after the intraperitoneal injection of 10 ng of Shiga toxin 2 (Stx2). *B,* Representative periodic acid–Schiff–stained kidney section from an *α-GalA*–knockout mouse treated with recombinant human α-GalA and then challenged with Stx2. Magnification, ×200.

Invitrogen), Hoechst 33258 (20 ng/mL) for nuclei (Acros Organics), and goat anti-rat fluorescence-conjugated IgG for Gb3 (5 μL/sample; Sigma-Aldrich). Sections were viewed using a Nikon epifluorescence microscope.

Results. The phenotype of the male *α-GalA*–knockout mice was confirmed by electron and immunofluorescence microscopy. Electron-microscopic analysis of kidney sections from the *α-GalA*–knockout mice revealed concentric, lipid-rich, lamellar inclusions in lysosomes (figure 1*A*), which was consistent with previous reports and with the pathologic characteristics observed in human Fabry disease [6]. Similar structures were also observed in the spleens of *α-GalA*–knockout mice, but no inclusions were detected in kidneys or spleens of their male *wt* littermates (data not shown). Immunofluorescent staining of frozen sections of colon from *α-GalA*–knockout male mice showed an overabundance of Gb3 throughout the colon, compared with sections from male *wt* littermates (figure 1*B* and 1*C*). Both *wt* and knockout mice had shown no signs of clinical disease after 6 months of life, which is consistent with reports elsewhere [7].

To determine the susceptibility of *wt* and *α-Gal A*–knockout mice to Stx, we chose to use Stx2, which is both more toxic than Stx1 in animal models and clinically more significant in human disease [13]. Approximately 1, 5, and 10 times the LD_{50} of Stx2 (2, 10, and 20 ng) were injected into groups of 4 mice each. The ip injection on day 0 of 20 ng of Stx2 into *wt* mice resulted in 100% mortality by day 4 (figure 2*A*). The ip injection of 10 ng of Stx2 into *wt* mice resulted in the death of 2 mice on day 4, 1 mouse on day 5, and 1 mouse on day 9 (figure 2*B*). The ip injection of 2 ng of Stx2 into *wt* mice resulted in no deaths (figure 2*C*). By contrast, the ip injection of 20 ng of Stx2 into *α-GalA*–knockout mice resulted in both fewer and delayed deaths, with 1 death on day 7 and 1 death on day 8 (figure 2*A*). No *α-GalA*–knockout mice died from injections of 10 or 2 ng of Stx2 (figure 2*B* and 2*C*). When the results of all 3 groups were pooled, 8 of 12 *wt* mice died, whereas only 2 of 12 *α-GalA*–knockout mice died (figure 2*D*). Notably, when 2 *wt* and 2 *α-GalA*–knockout mice were injected ip with 200 ng of Stx2 (∼100 LD_{50}), no difference in survival was noted—all mice had died by day 4 (data not shown).

Given the relative resistance of *α-GalA*–knockout mice to ip injection of Stx2, we challenged these mice orally with B2F1, an Stx2d-producing EHEC strain that causes renal tubular necrosis in streptomycin-treated mice [10]. This particular EHEC strain was specifically chosen for its virulence properties in mice. Because the LD_{50} of B2F1 in CD-1 mice is as low as <10 organisms/mouse [14], challenge doses of 1×10^3–1×10^5 cfu/mouse were injected into groups of 8 mice each. At day 3 after infection, all mice had normal concentrations of blood urea nitrogen and serum creatinine (data not shown). In total, 12 of 24 *wt* mice died, with no detectable increase in mortality with increasing doses over the range examined. Of note, fewer *wt* mice died in the experiments with a dose of 1×10^5 cfu/mouse; however, in this model, the number of colonizing organisms was similar in all groups a few days after infection [9]. By contrast, only 2 of 24 *α-GalA*–knockout mice died: both in the group that received 1×10^5 cfu (figure 2*E*–2*G*). The combined survival curves for the oral inoculation experiments were similar to those for the ip injections, but deaths after oral infection occurred ∼5 days later (figure 2*D* vs. 2*H*).

Pathologic examination revealed renal tubular necrosis in all *wt* B6 mice that developed illness after oral infection with B2F1 and after ip injection with Stx2d. The most significant pathologic results were observed in *wt* mice that died from oral infection with 1×10^5 cfu of B2F1; these mice had severe tubular necrosis and tissue edema (figure 1*D*). Tubules lacked nucleated epithelia and contained necrotic cellular debris. These mice also showed lymphocyte depletion and a loss of architecture in the spleen (figure 1*F*). By contrast, the 2 *α-GalA*–knockout mice that died from oral infection with 1×10^5 cfu of B2F1 had normal kidneys and spleens (figure 1*E* and 1*G*). Similarly, the *α-GalA*–knockout mice that died after the ip

injection of Stx2 showed no renal or splenic changes. Examination of other organ systems identified no histopathologic changes in the heart, lungs, brain, liver, or small or large intestines of either *wt* or knockout mice.

Given the 100-fold increase in plasma concentrations of Gb3 in α-*GalA*–knockout mice, serum from *wt* and knockout mice was obtained to examine their ability to neutralize Stx2 in an in vitro cytotoxicity assay [11]. Plasma from neither *wt* nor knockout mice protected Vero cells from Stx-mediated killing (data not shown).

Treatment of α-*GalA*–knockout mice with recombinant human α-GalA (Fabrazyme) has been shown to reduce levels of Gb3 in multiple tissues [12]. One week after a single iv dose of 3 mg/kg, levels of Gb3 in the spleen, heart, and liver of α-*GalA*–knockout mice were dramatically reduced, with plasma levels of Gb3 becoming undetectable [12]. Groups of 4 *wt* and 4 knockout mice each were injected iv with either PBS or 3 mg/kg of recombinant human α-GalA 7 days before the ip injection of 10 ng of Stx2. All 8 of the *wt* mice injected either with PBS or with recombinant human α-GalA died by day 4, and all 4 of the knockout mice injected with recombinant human α-GalA died by day 6 (figure 3*A*). By contrast, 3 of 4 knockout mice injected with PBS remained alive at day 10. No pathologic findings were detected in the kidneys of knockout mice injected with PBS and then challenged with Stx2, whereas knockout mice treated with recombinant human α-GalA demonstrated increased sensitivity to subsequent Stx2 challenge, with acute tubular necrosis observed in the kidneys of these mice (figure 3*B*).

Discussion. In the present study, we used the α-*GalA*–knockout mouse, which was originally developed as a model of Fabry disease, to examine susceptibility to Stx-induced disease in the setting of excess Gb3, the cellular receptor for Stx, in tissues. We originally speculated that these mice might have an increased sensitivity to Stx, given the increased amount of Gb3 available for toxin binding and cellular translocation. However, our results have demonstrated that the Fabry phenotype decreases both pathologic changes and mortality after the ip injection of purified Stx2 or oral infection with an Stx2d-expressing strain of EHEC.

One possible mechanism for this protection is that the excess Gb3 in the Fabry mouse acts as a toxin sink, which allows increased binding of Stx to tissues that do not normally express high concentrations of receptor and thus compromises the ability of circulating Stx to reach susceptible organs. In this way, the Fabry mouse provides a natural counterpart of the Gb3 mimics that have been designed for parenteral therapy. In support of this conjecture, iv treatment of α-*GalA*–knockout mice with recombinant human α-GalA (Fabrazyme), which has been demonstrated to substantially reduce the amount of Gb3 in tissues of humans with Fabry disease, restored the susceptibility of knockout mice to lethal doses of Stx2. In addition, we observed that very high concentrations of toxin (100 LD_{50}) were lethal in Fabry mice, which suggests that receptors remain functional and can bind Stx but that their increased density can be saturated by excess amounts of toxin. This ability to overcome increased concentrations of receptor also argues against a potential alternative mechanism of protection from Stx-induced damage in Fabry tissues, in which the increased cell-surface density and intracellular inclusions of Gb3 act to interfere with the normal intracellular processing of Stx.

The tissues of the Stx-challenged α-*GalA*–deficient mice that were not treated with recombinant human α-GalA were surprisingly normal, even in mice that died after ip Stx2 and the 2 that died after oral infection with B2F1. No obvious pathologic changes were detected to explain the death of these mice, but they did not have the same organ dysfunction as their *wt* counterparts. No evidence was found for lethal sepsis or toxemia, but we cannot discount undetected microvascular events in the central nervous system, which have previously been described in the setting of Stx-induced disease, as the cause of death [15, 16].

The relative protection of Gb3-overexpressing mice from Stx-induced disease is an intriguing observation. Although the Fabry mouse will not provide a murine model for EHEC disease, this mouse strain may allow new insights into the complex roles of Stx and its eukaryotic cell-surface receptor in the pathogenesis of disease.

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