JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 9281–9286 9281
DOI:10.1021/jf101744z

Ingested Shiga Toxin 2 (Stx2) Causes Histopathological Changes in Kidney, Spleen, and Thymus Tissues and Mortality in Mice

Reuven Rasooly,*,† Paula M. Do,† Stephen M. Griffey,‡ Jose G. Vilches-Moure,‡ and Mendel Friedman†

†Western Regional Research Center, Agricultural Research Service, United States Department of Agriculture, Albany, California 94710, and ‡Comparative Pathology Laboratory, School of Veterinary Medicine, University of California, Davis, California 95616

The Shiga toxin (Stx)-producing bacterial strain, *Escherichia coli* O157:H7, colonizes the distal small intestine and the colon, initiating serious illness, including hemolytic-uremic syndrome (HUS), characterized by microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure. Although intravenous administration of purified Stx to primates has been able to reproduce the features of HUS, it has not been conclusively established as to whether ingestion of Stx alone without the bacterium poses a potential health risk. To help answer this question, in this study, we fed Shiga toxin 2 (Stx2) directly into the stomachs of mice via gavage. Our data show that ingestion of Stx2 at a concentration of 50 μg/mouse induces weight loss and kills the mice at 3−5 days post-gavage. Additional studies revealed that the toxin retains activity at low pH, that its activity is neutralized by treatment with toxin-specific antibody, and that about 1% of the fed toxin is absorbed into the blood circulation. Lethality by intraperitoneal (IP) injection of Stx2 occurred at much lower doses than by ingestion. Detailed histopathological evaluation of stained tissues by light microscopy revealed severe histopathological changes in kidneys, spleen, and thymus but not in the pancreas, lymph nodes, heart, lungs, trachea, esophagus, stomach, duodenum, jejunum, ileum, cecum, and colon. The pathological changes in the kidney appeared similar to those seen in humans with HUS. The cited data suggest that (a) most but not all of the toxin is inactivated in the digestive tract, (b) part of the oral-ingested toxin is absorbed from the digestive tract into the circulation, (c) enough active toxin reaches susceptible organs to induce damage, and (d) Stx2 in the absence of toxin-producing bacteria can be harmful to mice. The results are clinically relevant for food safety because we also found that heat treatments (pasteurization) that destroy bacteria did not inactivate the heat-resistant toxin produced and secreted by the bacteria.

KEYWORDS: Shiga toxin 2; oral-ingested; gavage; *Escherichia coli* O157:H7; histopathological; neutralizing antibody

INTRODUCTION

Shiga toxin (Stx)-producing *Escherichia coli* serotype O157:H7 colonizes the distal small intestine and colon (*1*, *2*). The combined actions of Stx and bacterial lipopolysaccharide on the renal glomeruli and tubules cause renal disease (*3*−*5*), characterized by fibrin deposition within small vessels, swelling of glomerular endothelial cells, and thrombotic occlusion of capillaries (*6*). Severe cases of hemolytic-uremic syndrome (HUS) exhibit renal cortical necrosis, pervasive inflammatory cell infiltrates in the kidney, and apoptosis of renal and cortical glomerular and tubular cells.

E. coli serotype O157:H7 produces a family of two major related toxins: Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) (*7*, *8*). The B subunits of Stx molecules attach to glycolipid globotriosylceramide Gb_3 receptors in the gut and kidney cell membranes (*9*). The toxin is internalized via retrograde transport through the Golgi complex. Then, the A and B subunits dissociate, and the A subunit is translocated to the cytosol (*4*, *9*), where it cleaves the N-glycosidic bond of adenine at nucleotide position 4324 in the 28s rRNA of the 60s ribosomal subunit (*10*). Elimination of this one adenine residue blocks peptide chain elongation and inhibits protein synthesis. These events can lead to cell death.

Human exposure to these toxins is generally through the consumption of bacterially contaminated red meats, milk, and their products (*11*). It has been shown that inoculating *E. coli* will produce 306 ng/mL of Stx in milk and 452 ng/g of Stx in meat (*12*). It was also shown that Stx2 is relatively heat-stable, that the pasteurization process that destroyed the bacteria did not reduce Stx2 biological activity, and that apple compounds inhibited the biological activity of the toxin in cell assays (*7*, *8*).

Although several published studies demonstrated that IP-injected Stx's cause tissue damage and death of animals (*13*−*17*), to our knowledge, there are no studies that demonstrate that ingestion of Stx without bacteria is a health risk. The main objective of this study was to find out whether orally ingested Stx2 can survive the gastrointestinal tract environments of mice and whether it can be absorbed into the circulation, reach and damage the

*To whom correspondence should be addressed. Telephone: 510-559-6478. Fax: 510-559-6429. E-mail: reuven.rasooly@ars.usda.gov.

Published on Web 07/27/2010

kidney and other tissues, and cause lethality. The data suggest that this may be the case.

MATERIALS AND METHODS

Materials. Stx2 was obtained from Toxin Technology (Sarasota, FL), and the antibody against Stx2 subunit B was obtained from SIFIN (Berlin, Germany). Vero cells and African Green Monkey adult kidney cells (ATCC CCL-81) were obtained from American Type Culture Collection (Manassas, VA). Rodent Laboratory chow 5001 diet was obtained from Purina (Richmond, IN).

Cell Culture. The procedure was adapted from a previous study (*7*). Briefly, Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM) from Gibco (Carlsbad, CA) containing 10% fetal bovine serum (Hyclone, Waltham, MA) and 100 units/mL of both penicillin and streptomycin (Gibco). Cells were trypsinized (Gibco) when ready to harvest.

Effect of Low pH on Stx2 Activity. Phosphate-buffered saline (PBS) solutions of different pH values were made by adding HCl and different concentrations of Stx2 to PBS. The toxin in PBS solution was then incubated for 1 h at room temperature. An acidic PBS was neutralized to a pH of 7.0 with PBS of pH 8.3.

Methylthiazolyldiphenyl-tetrazolium Bromide (MTT) Assay. Vero cells were plated on black 96-well plates (Greiner 655090) at 1×10^4 cells in 100 μL of medium per well. The cells were incubated overnight to allow time for the cells to attach to the plate. Samples (5 μL) were added to individual wells and incubated for 48 h at 37 °C in a 5% CO_2 incubator. MTT was diluted in PBS to 2 mg/mL, and 25 μL was added to each well. Plates were incubated at 37 °C for 4 h, and the medium was removed. A total of 100 μL of dimethyl sulfoxide (DMSO) was added to each well, and plates were read at 450 nm. The MTT assay is a common assay for cell viability. Viable cells can reduce the yellow, water-soluble MTT reagent to a purple formazan salt, which is water-soluble and can be colorimetrically detected.

Inactivation of Stx2 Activity by Toxin-Specific Antibodies. The following experiment was undertaken to demonstrate that Stx2-specific antibodies can neutralize the biological effect of the toxin used in the present study. The anti-Stx2 subunit B antibody solution containing IgG_1 at a concentration of 4.3 mg/mL was used to prepare dilutions for the neutralization assay. A total of 1 μL of the dilutions shown in **Figure 1** was incubated for 1 h with 9 μL of the medium containing 1 ng of Stx2 in all cases to a final volume of 10 μL. The treated toxin was then added to Vero cells that had been seeded previously into 96-well plates. The accumulation of formazan formed after reduction of MTT was then monitored spectrophotometrically at 540 nm after 48 h.

Mice Feeding Study. Female Swiss Webster (CFW) mice, 6 weeks of age and weighing 22 ± 2 g each, were purchased from Charles River (Wilmington, MA). They were maintained under standard conditions of temperature of 25 ± 2 °C, relative humidity of 55 ± 5%, and 12 h of a light−dark cycle. Groups of five mice were used at each dosage level, and three dose levels (0.5, 1, and 50 μg) were tested by gavage with 100 μL of toxin in PBS. The mice had access to standard laboratory feed and tap water. Mice were observed (twice a day) for symptoms and were sacrificed when moribund. The study protocol was approved by our Animal Care and Use Committee, which follows guidelines for the humane use of laboratory animals.

Mice IP Study. Mice were IP-injected with 5 ng of Stx2 in a volume of 500 μL of PBS.

Histopathology. At 3−5 days after oral administration of Stx2, moribund mice were sacrificed for gross and histologic examination. Tissues were collected into 10% buffered formalin and processed routinely to paraffin blocks. Hematoxylin and eosin-stained sections (5 μM) were examined for pathologic changes. Tissues examined histologically included liver, spleen, kidneys, pancreas, lymph nodes, heart, lungs, trachea, thymus, esophagus, stomach, duodenum, jejunum, ileum, cecum, and colon. Two age-matched nontreated control mice were similarly examined. Injuries to tissues were evaluated by light microscopy to observe changes relative to control tissues.

Enzyme-Linked Immunosorbent Assay (ELISA) for Stx2 Levels in Blood. Blood (1 mL) was withdrawn from mice before morbidity for the ELISA. The blood was then diluted l:5 with PBS at pH 7.4 buffer. An aliquot (200 μL) of this dilution was then added to a 96-well plate, and Stx2 was determined by an ELISA as described in our previous study (*18*).

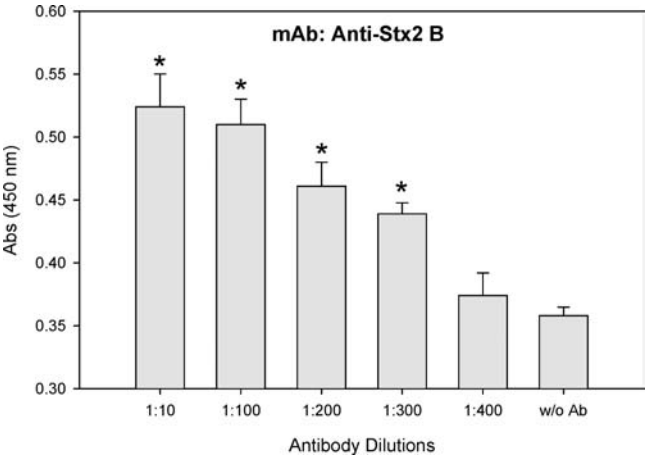

Figure 1. Neutralization of Stx2 by Stx2 B-specific antibodies. Stx2 was mixed with a dilution series of anti-Stx2 B antibodies. The cytotoxicity of Stx2 in Vero cells was then determined in the MTT assay. The accumulation of MTT−formazan produced after reduction of MTT was monitored at 450 nm. Error bars represent standard errors (n = 3), and an asterisk indicates significant differences.

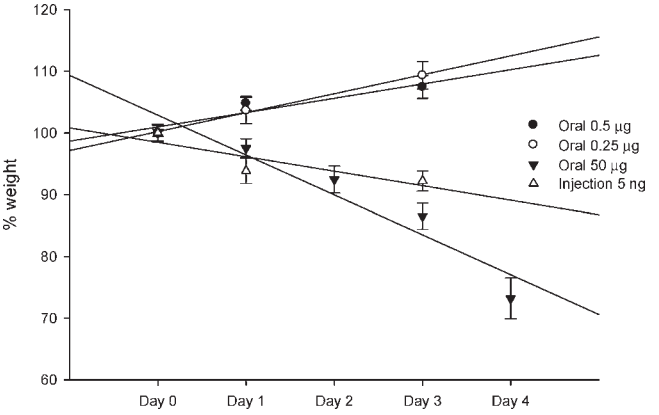

Figure 2. Effect of oral and IP Stx2 on body weights of mice [± standard error (SE)]. Oral administration of 0.25 and 0.5 μg of toxin resulted in weight gain, whereas oral administration of 50 μg and IP injection of 5 ng caused a continuous decrease in weight. Error bars (n = 5) represent standard errors. Generally, the data show significant differences after day 3.

Statistical Analysis. Statistical analyses of differences were determined by analysis of variance (ANOVA) using SigmaStat 3.5 for Windows (Systat Software, San Jose, CA). One-way ANOVA was used to compare the effects of different concentrations of antibody, Stx2, or pH on Vero cell cytotoxicity, percent weight, or inactivation of Stx2, respectively, illustrated in **Figures 1−3**. Results with (∗) $p < 0.05$ are considered statistically significant.

RESULTS

Anti-Stx2 B Antibodies Protected Vero Cells against Cytotoxicity of Stx2. Before proceeding with the *in vivo* studies, it was of interest to verify that adding Stx2 subunit B antibodies to the toxin will increase cell viability as measured by the MTT assay. We found that a dilution series ranging from 1:10 to 1:400 of antibodies neutralized the biological activity of the toxin in a concentration-dependent manner (**Figure 1**).

Oral Ingestion of Stx2 Induces Weight Loss and Death of Mice. To study the effect of oral ingestion of Stx2 on mice, various concentrations of Stx2 were administered orally to three groups of five mice directly into the stomach via gavage. Our data demonstrated that a single oral dose of 50 μg/mouse of Stx2

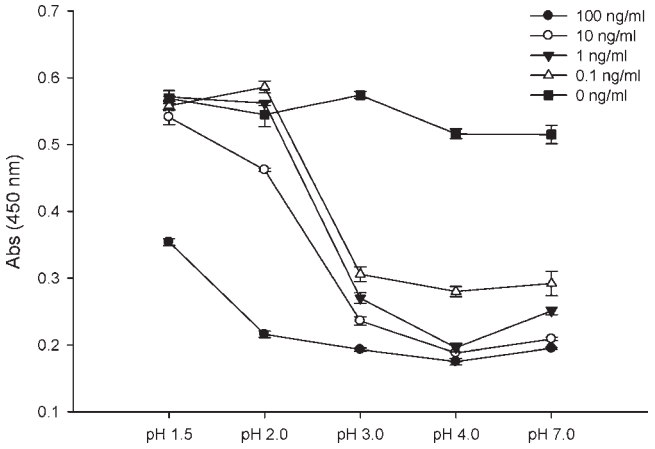

Figure 3. Inactivation of Stx2 at low pH values within the normal pH range of the stomach. Various concentrations of Stx2 were incubated for 1 h at various pH values. An aliquot of the neutralized sample was then added to Vero cells. Cell viability was quantified by measuring the activity of the dehydrogenase enzyme. Error bars show standard errors ($n = 3$). Generally, above pH 2, the data show significant differences among all dosage points.

resulted in significant ($p < 0.05$) weight loss and death of all mice. As seen in **Figure 2**, the weight of mice following IP injection with 5 ng of Stx2 and oral ingestion of 50 μg of Stx2 decreased over the course of the study. At day 3, the weight loss of 50 μg of orally dosed mice was statistically higher than 5 ng of IP-dosed mice. The IP-dosed mice became moribund 3 days after injection, and the oral-dosed mice became moribund 3−5 days post-feeding. No weight loss was observed at lower oral concentrations of 0.25 or 0.5 μg/mouse.

For comparison to other foodborne toxins, the oral toxicity of botulinum toxin A in the same strain of mice was 20 μg and the toxicity via IP injection was about 20 pg (*19*−*21*). These results suggest that some but not all of Stx and botulinum toxin withstand the acidic conditions in the stomach and retain *in vivo* activity.

Inactivation of Stx2 after Treatment at Low pH for 1 h. To study toxin stability under acidic conditions, we measured Stx2 inactivation *in vitro* in solutions of different pH values within the normal pH range of the mouse stomach. The activity of the toxin was measured by the MTT assay. As shown in **Figure 3**, at low pH, cytotoxic activity of Stx2 was reduced in a pH- and dose-dependent manner. Plotting dehydrogenase activity over the range of pH values from 1.5 to 7.0 gave reverse sigmoid-shaped curves (**Figure 3**). The data show that treatment of the toxin in the pH range of 3.0−7.0 did not change Stx2 cytotoxic activity. In this range, the toxin could be assayed in a dose-dependent response with a 0.1 ng/mL limit of detection. In contrast, at pH 1.5−3.0, the Vero cells were less sensitive to the toxin, increasing the limit of detection by 1000-fold. At pH 1.5, the detection limit increased to 100 ng/mL.

These results show that the cytotoxic activity significantly decreased at low pH values at all Stx2 concentrations, as indicated by the increase in absorbance of MTT−formazan. However, strong acid did not entirely eliminate toxin activity.

Detection of Stx2 in Blood after a Single Administration by Gavage. To trace the toxin beyond the digestive system, we assayed by sandwich ELISA for the presence of the toxin in the blood. Our data demonstrated that, when Stx2 (50 μg) was delivered directly into the stomachs of mice via gavage, the toxin concentration in the blood of moribund mice was 8 ng/mL on day 3 and 0.025 ng/mL on day 5. These results suggest that the toxin was not completely degraded after exposure to digestive enzymes, hydrochloric acid, and the low pH of the stomach.

Histopathology of Mice Administered 50 μg of Stx2 by Oral Gavage. To study the effect of the toxin on the animal organs (liver, spleen, kidneys, pancreas, lymph nodes, heart, lungs, trachea, thymus, esophagus, stomach, duodenum, jejunum, ileum, cecum, and colon), tissues were collected in 10% buffered formalin and processed routinely to paraffin blocks. Hematoxylin and eosin-stained sections (5 μM) were examined for pathologic changes. No overt pathology was noted on gross examination from Stx2-treated or control mice. Major histologic and pathologic changes were present in kidneys, thymus, and spleen of Stx2-treated mice, while no changes were noted in the control mice. The kidneys of treated mice had multifocal, cortical tubular dilation with epithelial vacuolation and necrosis (**Figure 4**). In affected tubules, the epithelial lining cells were sometimes absent, attenuated, or characterized by cytoplasmic pallor with clear vacuolation. Occasional cells had nuclei with pyknotic or fragmented nuclei (**Figure 5**). Lumina of affected tubules contained scattered sloughed epithelial cells. In treated mice, the thymus had moderate, diffuse cortical lymphoid necrosis characterized by cellular debris with pyknotic or fragmented nuclei (**Figure 6**). The spleens from treated mice had prominent germinal lymphoid centers with aggregates of pyknotic and fragmented nuclei partially phagocytized by foamy histiocytes (tingible body macrophages) (**Figure 7**).

DISCUSSION

The results presented in this study have shown that oral ingestion of Stx by mice caused weight loss and damage to vital organs and death. Previous research has shown that IP injection of Stx's in the absence of bacteria is harmful, causing HUS and lethality, and that anti-Stx's neutralizing antibodies protected animals from lethality (*15*, *22*). In this study, we showed that the antibodies neutralized the biological activity of the toxin in a cell assay. These results indicate that the toxin was biologically active for use in the *in vivo* mice studies. Surprisingly, although Stx is listed by the National Institute of Health/Center of Disease Control (NIH/CDC) as a select biothreat agent (*23*), none of the published studies determined whether these effects can also result from orally ingested toxin without the presence of bacteria.

Our *in vitro* results demonstrated that the toxin retains some activity at low pH and that toxin-specific antibodies neutralized its biological activity in a cell assay. Our *in vivo* results showed that oral ingestion of 50 μg caused apoptosis of epithelial cells, resulting in major histologic/pathologic changes in the kidney, thymus, and spleen tissues, which may be responsible for the observed mortality of the mice.

Our data also show that, to achieve the same toxicity, an oral dose of Stx2 must be about 10 000-fold greater than the corresponding amount administered by IP injection. These results are in agreement with results from oral ingestion of botulinum neurotoxin (BoNT). As mentioned earlier, to achieve the same result, oral administration of BoNT needed to be 10^6 times higher than IP injection (*19*−*21*). These observations suggest that the method of toxin delivery significantly affects toxicity and that the two toxins vary widely in their ability to induce lethality in mice following oral consumption.

After ingestion, Stx will be exposed to digestive enzymes in the small intestine and to the protective mechanisms of the intestine, such as high pH, protein defenses, mucins, trefoil peptides, filamentous brush border glycocalyx, and secretory IgA. This enzyme as well as the low physiological pH of the stomach leads to a large reduction in the dehydrogenase enzymatic activity. We

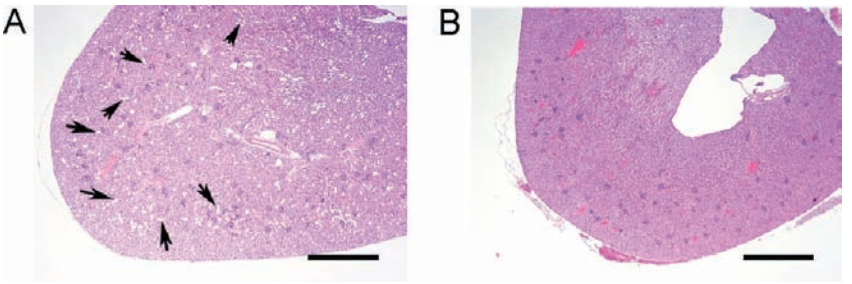

Figure 4. (A) Kidney of a Stx2-treated mouse has multifocal, cortical tubular dilation with epithelial vacuolation and necrosis (arrows). (B) Kidney from a nontreated control shows no tubular changes. Hematoxylin and eosin-stained sections; bar = 500 μM.

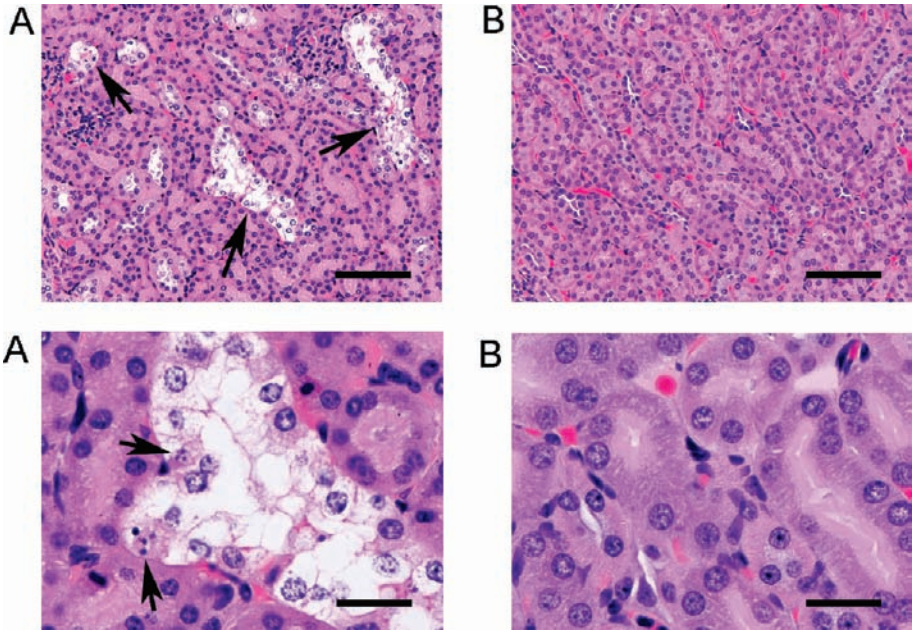

Figure 5. (A) Higher magnification of an affected cortical tubule in a Stx2-treated mouse showing marked diffuse tubular cell vacuolation and individual necrotic epithelial cells (arrows). (B) Nontreated control shows no tubular changes. Hematoxylin and eosin-stained sections; bar = 25 μM.

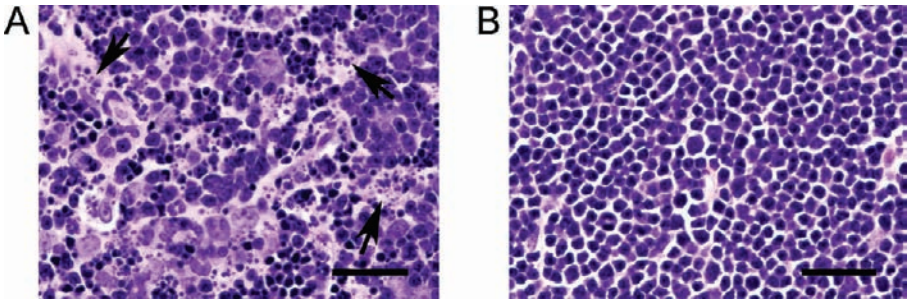

Figure 6. (A) Thymus of a Stx2-treated mouse has moderate cortical lymphoid necrosis with scattered fragmented (apoptotic) nuclei (arrows). (B) Thymic cortex from a nontreated control shows normal viable lymphocytes. Hematoxylin and eosin-stained sections; bar = 25 μM.

also measured the activity of the toxin in the blood by the MTT assay.

Analysis by ELISA of blood samples withdrawn on days 3 and 5 of the feeding study showed the presence of 16 and 0.5 μg/mL, respectively, of the toxin in the blood samples. These results suggest that the toxin is absorbed in the circulation and is depleted from the blood over time.

The concentration of Stx needed to cause illness and death is quite probably much lower in humans than in mice. This is because the distribution of Gb_3 toxin receptor has a major impact on the delivery of the toxin and the pathogenesis of the disease (*9*) and because higher levels of Gb_3 receptors are found in human kidneys than in mouse kidneys (*24*). Because a much higher concentration of Stx2 is needed to compensate for the low abundance of Gb_3 receptors in mice, humans are likely to be more susceptible to the toxin than mice and may require less Stx2 to cause illness. Our results may explain an outbreak in which HUS was linked to drinking pasteurized milk after the bacteria that secreted the toxin were killed and no live bacteria were found (*25*). However, this suggestion is inconclusive because the authors did not determine the Stx content in the pasteurized milk.

The results of the present oral and IP injection studies as well as related published studies on IP injection mentioned in the Introduction show that injection of Stx alone can cause death.

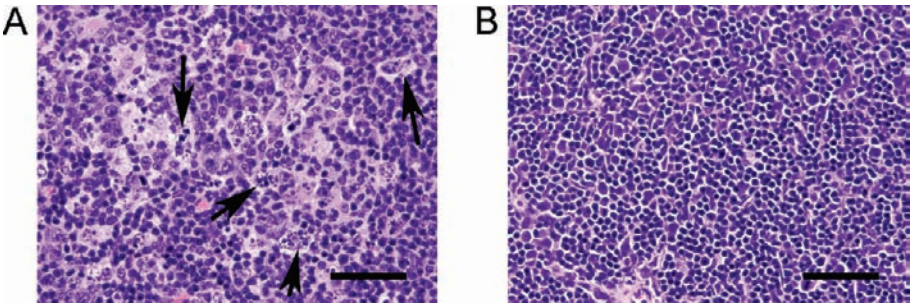

Figure 7. (A) Area of the germinal lymphoid center containing aggregates of nuclear debris and histiocytes undergoing phagocytosis (arrows) in the spleen from a Stx2-treated mouse. (B) No nuclear debris or reactive histiocytes are present in the germinal lymphoid center of a nontreated control. Hematoxylin and eosin-stained sections; bar = 50 μM.

Here, we demonstrate that the ingestion of Stx2 without bacteria can be harmful. This is clinically relevant because pasteurization destroys the bacteria but does not inactivate the heat-stable toxin produced and secreted by the bacteria (*7*, *8*). The results of the *in vitro* pH study suggest that some of the toxin might overcome the defense barrier of gastric acid, retaining its activity. The fact that Stx2 appears to be not completely inactivated under acid conditions may be of special concern for the elderly, because humans tend to produce less stomach acid as they age (*26*), possibly allowing even more of the toxin to survive this first line of defense. Such residual toxin could be absorbed by the intestine into the blood circulation and delivered to organs, leading to the observed histopathological changes and mortality of the mice.

These concerns demonstrate a need to prevent early contamination of food, because it may be impractical to eliminate the toxin from the food by thermal treatment once it has formed. These aspects merit further study.

ACKNOWLEDGMENT

We thank Dr. Louisa Cheng for her help with the experimental part of this study and Daphne Tamar and Sharon Abigail for helpful discussions.

Note Added after ASAP Publication

This article published ASAP July 27, 2010 without noting the keywords of the paper. The correct version published on August 3, 2010.

LITERATURE CITED

(1) Hicks, S.; Candy, D. C. A.; Phillips, A. D. Adhesion of enteroaggregative *Escherichia coli* to pediatric intestinal mucosa in vitro. *Infect. Immun.* **1996**, *64*, 4751–4760.

(2) Phillips, A. D.; Navabpour, S.; Hicks, S.; Dougan, G.; Wallis, T.; Frankel, G. Enterohaemorrhagic *Escherichia coli* O157:H7 target Peyer's patches in humans and cause attaching/effacing lesions in both human and bovine intestine. *Gut* **2000**, *47*, 377–381.

(3) Bitzan, M.; Moebius, E.; Ludwig, K.; Muller-Wiefel, D. E.; Heesemann, J.; Karch, H. High incidence of serum antibodies to *Escherichia coli* O157 lipopolysaccharide in children with hemolytic-uremic syndrome. *J. Pediatr.* **1991**, *119*, 380–385.

(4) Noris, M.; Remuzzi, G. Hemolytic uremic syndrome. *J. Am. Soc. Nephrol.* **2005**, *16*, 1035–1050.

(5) Tarr, P. I.; Gordon, C. A.; Chandler, W. L. Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. *Lancet* **2005**, *365*, 1073–1086.

(6) Habib, R. Pathology of the haemolytic uremic syndrome. In *Hemolytic Uremic Syndrome and Thrombotic Thrombocytopenic Purpura*; Kaplan, B., Trompeter, R., Moake, J., Eds.; Marcel Dekker, Inc.: New York, 1992; pp 315−353.

(7) Rasooly, R.; Do, P. M. Shiga toxin Stx2 is heat-stable and not inactivated by pasteurization. *Int. J. Food Microbiol.* **2010**, *136*, 290–294.

(8) Rasooly, R.; Do, P. M.; Levin, C. E.; Friedman, M. Inhibition of Shiga toxin 2 (Stx2) in apple juices and its resistance to pasteurization. *J. Food Sci.* **2010**, *75*, M296–M301.

(9) Lingwood, C. A.; Law, H.; Richardson, S.; Petric, M.; Brunton, J. L.; De Grandis, S.; Karmali, M. Glycolipid binding of purified and recombinant *Escherichia coli* produced verotoxin in vitro. *J. Biol. Chem.* **1987**, *262*, 8834–8839.

(10) Endo, Y.; Tsurugi, K.; Yutsudo, T.; Takeda, Y.; Ogasawara, T.; Igarashi, K. Site of action of a Vero toxin (VT2) from *Escherichia coli* O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins. *Eur. J. Biochem.* **1988**, *171*, 45–50.

(11) Rangel, J. M.; Sparling, P. H.; Crowe, C.; Griffin, P. M.; Swerdlow, D. L. Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982−2002. *Emerging Infect. Dis.* **2005**, *11*, 603–609.

(12) Weeratna, R. D.; Doyle, M. P. Detection and production of verotoxin 1 of *Escherichia coli* O157:H7 in food. *Appl. Environ. Microbiol.* **1991**, *57*, 2951–2955.

(13) Dran, G. I.; Fernández, G. C.; Rubel, C. J.; Bermejo, E.; Gomez, S.; Meiss, R.; Isturiz, M. A.; Palermo, M. S. Protective role of nitric oxide in mice with Shiga toxin-induced hemolytic uremic syndrome. *Kidney Int.* **2002**, *62*, 1338–1348.

(14) Paixão-Cavalcante, D.; Botto, M.; Cook, H. T.; Pickering, M. C. Shiga toxin-2 results in renal tubular injury but not thrombotic microangiopathy in heterozygous factor H-deficient mice. *Clin. Exp. Immunol.* **2009**, *155*, 339–347.

(15) Sauter, K. A. D.; Melton-Celsa, A. R.; Larkin, K.; Troxell, M. L.; O'Brien, A. D.; Magun, B. E. Mouse model of hemolytic-uremic syndrome caused by endotoxin-free Shiga toxin 2 (Stx2) and protection from lethal outcome by anti-Stx2 antibody. *Infect. Immun.* **2008**, *76*, 4469–4478.

(16) Siegler, R. L.; Pysher, T. J.; Tesh, V. L.; Taylor, F. B. J. Response to single and divided doses of Shiga toxin-1 in a primate model of hemolytic uremic syndrome. *J. Am. Soc. Nephrol.* **2001**, *12*, 1458–1467.

(17) Wang, H.; Paton, J. C.; Paton, A. W. Pathologic changes in mice induced by subtilase cytotoxin, a potent new *Escherichia coli* AB_5 toxin that targets the endoplasmic reticulum. *J. Infect. Dis.* **2007**, *196*, 1093–1101.

(18) Rasooly, R.; Raccah, B.; Klein, M. Laboratory transmission of the citrus stubborn disease agent by a leafhopper from the *Circulifer tenellus* complex from the Jordan valley. *Phytoparasitica* **1994**, *22*, 209–218.

(19) Cheng, L. W.; Onisko, B.; Johnson, E. A.; Reader, J. R.; Griffey, S. M.; Larson, A. E.; Tepp, W. H.; Stanker, L. H.; Brandon, D. L.; Carter, J. M. Effects of purification on the bioavailability of botulinum neurotoxin type A. *Toxicology* **2008**, *249*, 123–129.

(20) Ohishi, I.; Sugii, S.; Sakaguchi, G. Oral toxicities of *Clostridium botulinum* toxins in response to molecular size. *Infect. Immun.* **1977**, *16*, 107–109.

(21) Rasooly, R.; Stanker, L. H.; Carter, J. M.; Do, P. M.; Cheng, L. W.; He, X.; Brandon, D. L. Detection of botulinum neurotoxin-A activity in food by peptide cleavage assay. *Int. J. Food Microbiol.* **2008**, *126*, 135–139.

(22) Yamagami, S.; Motoki, M.; Kimura, T.; Izumi, H.; Takeda, T.; Katsuura, Y.; Matsumoto, Y.-I. Efficacy of postinfection treatment with anti-Shiga toxin (Stx) 2 humanized monoclonal antibody TMA-15 in

mice lethally challenged with Stx-producing *Escherichia coli*. *J. Infect. Dis*. **2001**, *184*, 738–742.
(23) Atlas, R. M. Biosecurity concerns: Changing the face of academic research. *Chem. Health Saf*. **2005**, *12*, 15–23.
(24) Boyd, B.; Lingwood, C. Verotoxin receptor glycolipid in human renal tissue. *Nephron* **1989**, *51*, 207–210.
(25) Goh, S.; Newman, C.; Knowles, M.; Bolton, F. J.; Hollyoak, V.; Richards, S.; Daley, P.; Counter, D.; Smith, H. R.; Keppie, N. *E. coli* O157 phage type 21/28 outbreak in North Cumbria associated with pasteurized milk. *Epidemiol. Infect*. **2002**, *129*, 451–457.
(26) Feldman, M.; Cryer, B. Effects of age on gastric alkaline and nonparietal fluid secretion in humans. *Gerontology* **1998**, *44*, 222–227.

Received for review May 6, 2010. Revised manuscript received July 14, 2010. Accepted July 19, 2010.