

# Human Antibody against Shiga Toxin 2 Administered to Piglets after the Onset of Diarrhea Due to *Escherichia coli* O157:H7 Prevents Fatal Systemic Complications

Abhineet S. Sheoran,<sup>1</sup> Susan Chapman-Bonofiglio,<sup>1</sup> Barrett R. Harvey,<sup>2</sup> Jean Mukherjee,<sup>1</sup> George Georgiou,<sup>2</sup> Arthur Donohue-Rolfe,<sup>1</sup> and Saul Tzipori<sup>1\*</sup>

Division of Infectious Diseases, Tufts University School of Veterinary Medicine, 200 Westboro Rd., Building 20, North Grafton, Massachusetts 01536,<sup>1</sup> and Institute of Cellular and Molecular Biology, University of Texas, Austin, Texas 78712<sup>2</sup>

Received 9 December 2004/Returned for modification 17 January 2005/Accepted 22 March 2005

**Infection of children with Shiga toxin (Stx)-producing *Escherichia coli* (STEC) can lead to hemolytic-uremic syndrome (HUS) in 5 to 10% of patients. Stx2, one of two toxins liberated by the bacterium, is directly linked with HUS. We have previously shown that Stx-specific human monoclonal antibodies protect STEC-infected animals from fatal systemic complications. The present study defines the protective antibody dose in relation to the time of treatment after the onset of diarrhea in infected gnotobiotic piglets. Using the mouse toxicity model, we selected 5C12, an antibody specific for the A subunit, as the most effective Stx2 antibody for further characterization in the piglet model in which piglets developed diarrhea 16 to 40 h after bacterial challenge, followed by fatal neurological symptoms at 48 to 96 h. Seven groups of piglets received doses of 5C12 ranging from 6.0 mg/kg to 0.05 mg/kg of body weight, administered parenterally 48 h after bacterial challenge. The minimum fully protective antibody dose was 0.4 mg/kg, and the corresponding serum antibody concentration in these piglets was 0.7  $\mu\text{g}$  ( $\pm 0.5$ )/ml, measured 7 to 14 days after administration. Of 40 infected animals which received Stx2 antibody treatment of  $\geq 0.4$  mg/kg, 34 (85%) survived, while only 1 (2.5%) of 39 placebo-treated animals survived. We conclude that the administration of the Stx2-specific antibody was protective against fatal systemic complications even when it was administered well after the onset of diarrhea. These findings suggest that children treated with this antibody, even after the onset of bloody diarrhea, may be equally protected against the risk of developing HUS.**

Hemolytic-uremic syndrome (HUS), characterized by hemolytic anemia, thrombocytopenia, acute renal damage, and variable degrees of central nervous system (CNS) complications, can result in death or chronic, irreversible renal dysfunction (50). Infection with Shiga toxin (Stx)-producing *Escherichia coli* (STEC) is the most significant cause of HUS, the leading cause of renal failure in children (1, 9, 21, 26). There are two immunologically distinct Shiga toxins, of which Stx2 is directly linked with HUS. In contrast to Stx1, which is largely homogeneous, Stx2 is highly heterogeneous and is encoded by at least 10 Stx2 gene variants (8, 12, 20, 28, 29, 34, 40, 41, 54). The Stx2 genotype is the most prevalent genotype identified in STEC isolated from patients with HUS (7, 38). Stx2 is also about 400 times more lethal to mice than Stx1 when administered systemically (45). STEC strains producing Stx2 alone cause more severe neurologic symptoms in gnotobiotic piglets than strains producing both Stx1 and Stx2, whereas Stx1-producing strains induce only diarrhea and no systemic complications (4).

The Stx molecule consists of an A-subunit monomer and a B-subunit pentamer. The B subunit binds to its receptor globotriaosylceramide (Gb<sub>3</sub>) on the host's cell surface, and then Stx undergoes endocytosis (16, 39). The A subunit inactivates the 60S ribosomal subunit and thereby inhibits protein synthe-

sis, which leads to cell death (5, 25, 37). During infection, most STEC strains express intimin, a virulence factor responsible for the attaching and effacing lesions observed within the gastrointestinal (GI) tract (6, 33, 48), which is thought to facilitate Stx absorption from the gut (48). Though the mechanism by which Stx2, and possibly Stx1, mediates development of HUS in susceptible individuals is not understood, it is believed that endothelial cell injury within the kidney leads to HUS (51).

There is no effective treatment or prophylaxis for HUS available clinically. The systemic administration of Stx-specific neutralizing antibodies, we believe, is currently the most promising approach for the prevention or treatment of Stx-mediated systemic complications, including HUS (50) and edema disease in pigs (13). Murine Stx1- and Stx2-specific monoclonal antibodies (MAbs) have been shown to neutralize both toxins in vitro and in vivo (11, 27, 43). However, a murine MAb is not considered appropriate for human use. The reshaping of a murine antibody against Stx2 into a humanized form has recently been shown to completely protect mice against a lethal challenge with STEC when administered within 24 h after infection (55). The disadvantage of a humanized antibody is that it still has mouse components and reduced affinity (10).

We have previously reported the production, characterization, and evaluation of human monoclonal antibodies (HuMAbs) against Stx1 and Stx2 in transgenic mice (22, 23). Five highly effective Stx2-specific antibodies were selected for further characterization, which also included their relative neutralizing efficacies against Stx2 variants (42) using the mouse toxicity model (11,

\* Corresponding author. Mailing address: Tufts University School of Veterinary Medicine, Division of Infectious Diseases, 200 Westboro Rd., Building 20, North Grafton, MA 01536. Phone: (508) 839-7955. Fax: (508) 839-7911. E-mail: saul.tzipori@tufts.edu.

22, 23, 27, 43) and the streptomycin-treated mouse model of oral STEC infection (19, 52, 53).

This communication describes the selection and preclinical evaluation of one antibody (5C12) against the A subunit of Stx2 in the piglet model of infection in which, as in children, diarrheal symptoms precede systemic complications associated with Stx2 uptake from the gut. We have defined the minimum protective antibody dose, the optimal time it can be administered after bacterial challenge and onset of diarrhea, and the corresponding serum antibody level in the protected piglets.

## MATERIALS AND METHODS

**Bacteria and toxin.** Enterohemorrhagic *E. coli* O157:H7 strain 86-24, which produces Stx2, was isolated in 1986 from a patient in Seattle, Washington (44). Purified Stx2 was obtained as described previously (2).

**Stx2-specific HuMAbs.** We have previously described the production of 37 hybridomas secreting Stx2-specific HuMAbs (22), from which we selected five HuMAbs for further evaluation. Three HuMAbs against the A subunit (3E9, 2F10, and 5C12) and two against both the A and the B subunits (5H8 and 6G3) were selected (22, 42). All five HuMAbs were of the human immunoglobulin G1( $\kappa$ ) [IgG( $\kappa$ )] isotype. HuMAbs were used either in the form of mouse ascites (22) or as concentrated protein-free cell culture media (HyQ ADCF-MAB; HyClone, Logan, UT) in CELLLine 1000 flasks (BD Biosciences, Bedford, MA).

**Quantitation of Stx2-specific HuMAbs by enzyme-linked immunosorbent assay.** The IgG1( $\kappa$ ) concentration of each HuMAb in mouse ascites fluid and from CELLLine 1000 flasks was measured by enzyme-linked immunosorbent assay. Briefly, 96-well plates were coated overnight at 4°C with 100  $\mu$ l of the mouse anti-human  $\kappa$  light-chain MAB at 2.5  $\mu$ g/ml. Plates were washed with phosphate-buffered saline–Tween 20 (PBS-T; PBS, 0.05% Tween 20) and blocked with 100  $\mu$ l/well of 2% nonfat dried milk powder in PBS-T at 37°C. Following washing, samples (ascites fluid or cell culture supernatants or pig serum) diluted 1:10 or 1:100 in PBS-T were diluted serially twofold in duplicate rows of the plate (100  $\mu$ l/well). The human IgG1( $\kappa$ ) (Sigma, St. Louis, MO) standard was similarly titrated on each plate from a starting concentration of 1  $\mu$ g/ml. The plates were incubated at 37°C for 1 h and washed again. Horseradish peroxidase-conjugated goat anti-human IgG (Southern Biotech, Birmingham, AL), which is affinity purified and cross-adsorbed with human IgA, IgM, and IgD, was added at 100  $\mu$ l/well at a dilution of 1/1,000. Following incubation at 37°C for 1 h and washing, the plates were developed with substrate solution (0.2% *o*-phenylenediamine, 0.05% hydrogen peroxide in citric acid-phosphate buffer, pH 5.0). The chromogenic reaction was stopped using 50  $\mu$ l of 2 M sulfuric acid, and the absorbance was read at 490 nm. Using the linear portion of the IgG1( $\kappa$ ) standard curve, the total IgG1( $\kappa$ ) content of each HuMAb was determined and expressed as mg or  $\mu$ g of IgG1( $\kappa$ )/ml.

**HeLa cell cytotoxicity neutralization assay.** The in vitro HeLa cell cytotoxicity assay was used to evaluate the efficacy of each HuMAb to neutralize the toxic effects of Stx2 exerted against HeLa cells. Briefly, HeLa cells were plated at  $1.4 \times 10^4$ /well on 96-well plates in McCoy's 5A medium (Mediatech, Inc., Herndon, Va.) containing 10% fetal bovine serum (Harlan Bioproducts for Science, Inc., Madison, Wis.) and incubated overnight at 37°C in 5% CO<sub>2</sub>. Stx2 was titrated on HeLa cells to determine the concentration that killed ~70% of HeLa cells. Dead cells were removed by washing with PBS, and crystal violet was used to stain viable cells (14). Stx2 at a dilution that killed ~70% of HeLa cells was preincubated with the HuMAbs or Fab fragments at various concentrations for 1 h at 37°C in 5% CO<sub>2</sub> and then added to the cells and incubated overnight at 37°C in 5% CO<sub>2</sub>. Plates were developed by crystal violet staining, and the absorbance (optical density [OD]) was read at 690 nm. The percent neutralization of Stx2-mediated HeLa cell cytotoxicity by the HuMAbs was calculated by the following formula:  $[(OD_{\text{toxin} + \text{HuMAb}} - OD_{\text{toxin only}})/(OD_{\text{no toxin}} - OD_{\text{toxin only}})] \times 100$ , where the ODs were determined for plates containing toxin and HuMAb, toxin only, and no toxin.

**Mouse toxicity model.** The mouse toxicity model (11, 22, 23, 27, 43) was used to select the most protective among the five HuMAbs against a lethal Stx2 dose in mice. Dose-response studies were performed with groups of five 3- to 4-week-old, female Swiss Webster mice (Taconic) to determine the amount of Stx2 required to induce 100% mortality in untreated animals. A concentration of 25 ng of Stx2 was sufficient (data not shown). The efficacies of the HuMAbs were evaluated by administering every Stx2-specific HuMAb intraperitoneally (i.p.) to each of the five 3- to 4-week-old Swiss Webster mice at a dose of 5, 10, 20, 35, or 50  $\mu$ g/mouse in 200  $\mu$ l of PBS followed 18 h later by i.p. administration of 62.5

ng (250% lethal dose) of Stx2. A control group of five mice received human myeloma IgG1( $\kappa$ ) (20  $\mu$ g/mouse; Sigma, St. Louis, MO), and another control group received 200  $\mu$ l of PBS alone. Both control groups were also challenged with 62.5 ng of Stx2. The mice were observed twice daily for survival.

**Gnotobiotic piglet model of *E. coli* STEC infection.** The selected antibody, 5C12, was then further characterized in the gnotobiotic piglet model of STEC infection, previously described, using *E. coli* strain 86-24 (22). A total of 117 piglets were used to determine the minimum protective dose of the HuMAb 5C12, given i.p., either 24 h (17 treated, 17 placebo) (Table 2) or 48 h (39 treated, 44 placebo) (Table 3) after bacterial challenge with  $\sim 10^{10}$  CFU of strain 86-24. This large inoculum usually induces neurological signs and lesions associated with Stx2 activity in approximately 90% of control piglets within 48 to 96 h postinfection (4). The placebo group received either human myeloma IgG1( $\kappa$ ) (Sigma, St. Louis, MO) or PBS. Piglets were monitored several times daily for symptoms of diarrhea, dehydration, and CNS complications, which included ataxia, paresis, headpressing, paddling, convulsions, and opisthotonos. Piglets were rehydrated i.p. twice daily (20 to 30 ml/injection) with Aminosyn II 3.5% M plus 5% Dex Inj NTRMX (Hospira, IL), an amino acid injection with maintenance electrolytes in a dextrose injection, immediately after the appearance of diarrhea. Surviving animals with severe CNS symptoms, or animals that survived 7 to 14 days after the bacterial challenge, were humanely euthanized. Animals that succumbed before administration of the antibody were excluded from analysis. Those animals were not part of the 117 piglets included in this study. Brain tissue (cerebral cortex and cerebellum) and gut sections were fixed in formalin and processed for histology, and blood was collected for measuring serum antibody concentration.

**Affinity determination of HuMAbs.** The binding kinetics of the HuMAbs with Stx2 were determined by surface plasmon resonance (BIAcore 3000; Biacore Inc., Piscataway, NJ) using the procedure of indirect capture of MAbs (24, 35). Rabbit anti-human IgG Fc (Jackson Immuno Laboratories, West Grove, PA) was amine coupled to a CM5 (carboxymethylated dextran matrix) chip as the capture antibody, and then the HuMAb to be tested was run over it. The capture antibody and the HuMAb complex remained stable over the duration of each run. Kinetics analysis was performed at a flow rate of 100  $\mu$ l/min to reduce mass transport limitations. Injections were performed with twofold dilutions in duplicate, with Stx2 concentrations ranging from 100 nM to 6.25 nM in HEPES-buffered saline with 3 mM EDTA and 0.005% Tween 20. The surface was regenerated with 4 M MgCl<sub>2</sub> to disrupt the interaction between the capture antibody and the HuMAb before the HuMAb was reapplied for the next run.

## RESULTS

**Concentration of HuMAbs in ascites fluid and in concentrated cell culture supernatant.** Since the five HuMAbs were produced at only ng/ml quantities by conventional tissue culture propagation of hybridomas in 75-cm<sup>2</sup> flasks, they were produced as ascites fluid for mouse toxicity and in vitro neutralization assays. The mean concentrations of 5H8, 6G3, 3E9, 2F10, and 5C12 in ascites fluid were 1.8 mg/ml, 308  $\mu$ g/ml, 532  $\mu$ g/ml, 226  $\mu$ g/ml, and 459  $\mu$ g/ml, respectively. For the experiments with piglets, 5C12 was produced as concentrated cell culture supernatant in CELLLine 1000 flasks (BD Biosciences, Bedford, MA). The mean concentration of 5C12 in cell culture supernatant was 250  $\mu$ g/ml.

**Antibody neutralization of Stx2 in HeLa cells.** All HuMAbs completely neutralized cytotoxicity at the highest concentration of 5  $\mu$ g/ml, except HuMAbs 3E9 and 6G3, which manifested partial neutralization (Fig. 1). Differences in neutralizing efficiency were apparent in all HuMAbs at lower doses. As Fig. 1 indicates, at 8 ng/ml, 5C12, reactive with the A subunit, was superior among the five HuMAbs tested, with 40% neutralization of Stx2 cytotoxicity for HeLa cells.

**Antibody neutralization of Stx2 in the mouse assay.** Although all five HuMAbs were effective at protecting mice against a lethal dose of Stx2, differences in relative potency were apparent at the lowest doses of 10 and 5  $\mu$ g/mouse (5C12 > 5H8 > 6G3 = 2F10 = 3E9) (Fig. 2). 5C12, directed against

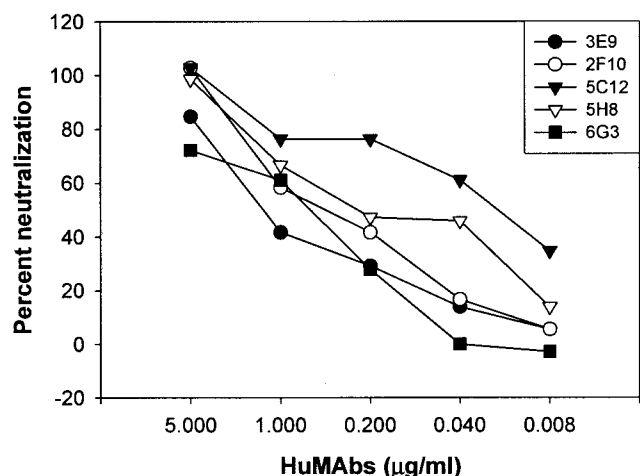


FIG. 1. Neutralization of Stx2-mediated HeLa cell cytotoxicity by Stx2-specific HuMAbs. All Stx2-specific HuMAbs neutralized Stx2 at the highest concentration of 5 µg/ml and showed dose dependency. The HuMAb 5C12 was the best neutralizing HuMAb as it neutralized cytotoxicity by about 40% at the lowest tested concentration (8 ng/ml).

the A subunit of the toxin, was the most effective, protecting all five mice at the lowest dose of 5 µg/mouse. Although less effective at 5 µg/mouse, 5H8, which is directed against the B subunit, showed results indicating that antibodies against both toxin subunits are capable of effective neutralization in vivo.

**Affinity of the HuMAbs.** Affinities of the Stx2 A subunit-specific HuMAbs were in the order of 2F10 > 3E9 > 5C12 (Table 1). The HuMAb 2F10 had Stx2-binding kinetics of  $k_{on}$  (association rate) equal to  $2.3 \times 10^5 M^{-1} s^{-1}$  and  $k_{off}$  (dissociation rate) equal to  $8.8 \times 10^{-5} s^{-1}$ , giving a  $K_d$  of 0.39 nM. The HuMAbs 3E9 and 5C12 had 1.7- and 2.17-fold higher  $K_d$ s, respectively.

**Experiments with 5C12 in gnotobiotic piglets.** Animals that either died or became severely ill and were euthanized before the onset of treatment at 48 h were excluded from this investigation. Piglets received antibody treatment either 24 h (Table 2) or 48 h (Table 3) after bacterial challenge. This was well

TABLE 1. Relative affinities of three HuMAbs directed against the A subunit of Stx2

HuMAb	$k_{off}$	$k_{on}$	$K_d$ (nM)
2F10	$8.8 \times 10^{-5}$	$2.3 \times 10^5$	0.39
3E9	$4.3 \times 10^{-5}$	$6.2 \times 10^4$	0.69
5C12	$1.1 \times 10^{-4}$	$1.3 \times 10^5$	0.85

after the onset of diarrhea in all animals but before or at onset of CNS symptoms. Table 2 shows that treatment with 5C12 at either 1.5 or 3 mg/kg of body weight administered 24 h after challenge was protective (78% and 75%, respectively), compared with treatment with the placebo. The placebo for the 3-mg/kg group included an equal dose (3 mg/kg) of an irrelevant human IgG1( $\kappa$ ) given i.p. to eight animals, which showed that a specific antibody against Stx2 was needed for protection against challenge with STEC. Because of the expense involved, all subsequent experiments were performed using PBS as the placebo.

Table 3 summarizes the dose response of piglets given doses of 5C12 ranging from 6 mg/kg down to 0.05 mg/kg and challenged 48 h later. When administered 48 h after infection, 6 or 3 mg/kg of 5C12 protected all piglets against fatal systemic complications. The corresponding mean serum concentrations of antibody were 16.9 and 11.4 µg/ml, respectively. The lowest protective dose was 0.4 mg/kg, with a corresponding mean serum concentration of 0.7 µg/ml. The doses below 0.4 mg did not provide reliable protection. The mean survival time of piglets in the PBS control groups ranged from 3.4 to 4.6 days. Two of 23 piglets in groups which received  $\geq 0.4$  mg/kg of 5C12 succumbed (91% survival rate), while 10 of 16 piglets in the groups which received  $\leq 0.2$  mg/kg 5C12 succumbed (37% survival rate). The mean survival time of piglets that received  $\leq 0.2$  mg/kg 5C12 ranged from 4.0 to 7.0 days (Table 3). When the results of Tables 2 and 3 are combined, 34 of the 40 animals (85%) that received  $\geq 0.4$  mg/kg of 5C12 survived STEC challenge, while only 1 (2.5%) of 39 piglets which the received placebo of either irrelevant human antibody (8 animals) or PBS (31 animals) survived.

**Clinical observations.** Piglets challenged orally with STEC typically develop profound GI tract symptoms within 2 days, including diarrhea, anorexia, depression, dehydration, and moderate but rapid loss of body weight. To avoid mortalities

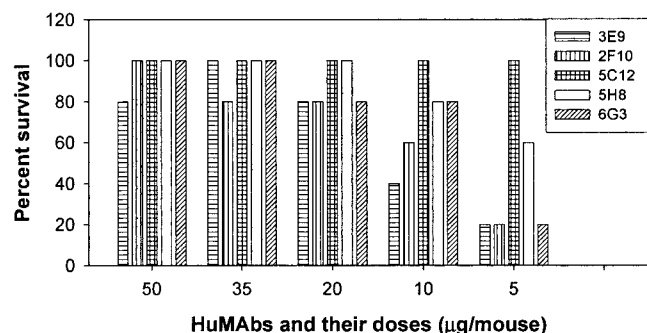


FIG. 2. Percent survival of mice given i.p. 50, 35, 20, 10, or 5 µg of HuMAbs 3E9, 2F10, 5C12, 5H8, or 6G3 followed 18 h later with i.p. administration of a 250% lethal dose of Stx2. Mice in PBS and IgG1( $\kappa$ ) control groups died within 3 days of Stx2 injection. All HuMAbs protected mice but showed dose dependency except 5C12, which protected 100% of the mice even at the lowest dose administered (5 µg/mouse).

TABLE 2. Survival of piglets treated i.p. with 5C12 antibody or control after oral challenge with STEC 86-24, an Stx2 producer<sup>a</sup>

Treatment type	Antibody concn (mg/kg)	Total no. of piglets	No. (%) of surviving piglets <sup>b</sup>	Serum IgG1( $\kappa$ ) concn (µg/ml) <sup>c</sup>
5C12	3	9	7 (78)	$4.4 \pm 1.0$
IgG1( $\kappa$ )	3	8	0 (0)	$9.2 \pm 2.5$
PBS	0	2	1 (50)	0
5C12	1.5	8	6 (75)	$3.1 \pm 1.8$
PBS	0	7	0 (0)	0

<sup>a</sup> Irrelevant human IgG( $\kappa$ ) or PBS was used as the control. Challenge was with  $10^{10}$  CFU of STEC 86-24.

<sup>b</sup> Piglets were either euthanized due to severe CNS-related illness or found dead.

<sup>c</sup> Results reflect the concentration in serum of the human IgG1( $\kappa$ ) isotype, which is the isotype of 5C12. Values are means  $\pm$  standard deviations.

TABLE 3. Survival of piglets orally challenged with  $10^{10}$  CFU of STEC 86-24 followed by i.p. administration of PBS (placebo) or 5C12 antibody at 48 h after bacterial challenge

Treatment	Dose (mg/kg)	Total no. of piglets	No. (%) of surviving piglets <sup>a</sup>	Days of survival after infection (mean $\pm$ SD) <sup>b</sup>	Serum IgG1( $\kappa$ ) concn ( $\mu$ g/ml) <sup>c</sup>
5C12	6	8	8 (100)		$16.9 \pm 7.4$
Placebo	0	7	0 (0)	$4.6 (\pm 1.9)$	0
5C12	3	4	4 (100)		$11.4 \pm 1.2$
Placebo	0	3	0 (0)	$4.6 (\pm 0.6)$	0
5C12	0.75	6	5 (83)	3.0	$2.0 \pm 1.2$
Placebo	0	5	0	$3.4 (\pm 0.5)$	0
5C12	0.4	5	4 (80)	4.0	$0.7 \pm 0.5$
Placebo	0	7	0	$3.4 (\pm 0.5)$	
5C12	0.2	5	1 (20)	$4.0 (\pm 1.4)$	$0.6 \pm 0.1$
Placebo	0	6	0	$3.5 (\pm 0.8)$	
5C12	0.1	7	4 (57)	$7.0 (\pm 3.4)$	$0.18 \pm 0.04$
Placebo	0	8	0	$4.2 (\pm 1.7)$	
5C12	0.05	4	1 (25)	$7.0 (\pm 3.4)$	$0.07 \pm 0.03$
Placebo	0	8	0	$4.2 (\pm 1.7)$	

<sup>a</sup> Piglets that did not survive either died overnight or were euthanized due to severe illness.

<sup>b</sup> Data for surviving piglets in the 5C12 group who were euthanized at the end of the experimental period (7 to 14 days) are included. Data for the piglets who were euthanized or died in the 5C12 group at another time are not included.

<sup>c</sup> Results reflect the concentration in serum of the human IgG1( $\kappa$ ) isotype, which is the isotype of 5C12. Values are means  $\pm$  standard deviations.

due to dehydration, all piglets received supplemental fluid therapy at and subsequent to the onset of diarrhea. Of the 83 piglets (Table 3) which had received the antibody or placebo at 48 h after bacterial challenge, 38 developed GI tract symptoms within 24 to 26 h, and all 83 developed them within 40 h of infection. This shows that all animals had serious GI tract symptoms that required rehydration well before the onset of antibody therapy at 48 h. As expected, while parenteral administration of Stx2 antibody had a major impact on animal survival, it had no effect on the severity of the GI tract symptoms (Table 4).

Typically, all piglets challenged with STEC develop diarrhea and other GI tract symptoms due to an intimate attachment of

TABLE 4. Dose response in piglets orally challenged with  $10^{10}$  CFU of STEC strain 86-24 and treated 48 h later with 5C12 antibody or placebo<sup>a</sup>

Treatment	Total no. of piglets (no. of piglets euthanized <sup>b</sup> or found dead) with diarrhea onset at:			
	16–28 h		29–40 h	
	0.4–6.0 <sup>c</sup>	0.05–0.2	0.4–6.0	0.05–0.2
5C12	9 (0)	6 (4)	14 (2)	10 (6)
PBS	12 (12)	11 (11)	10 (10)	11 (11)

<sup>a</sup> All 83 animals developed diarrhea 8 to 32 h before treatment. The time of onset of diarrhea had no impact on the outcome of treatment, nor did antibody therapy improve the severity of the GI tract symptoms (see text).

<sup>b</sup> Severely ill animals were euthanized.

<sup>c</sup> Dose of 5C12 antibody in milligrams/kilogram.

bacteria to the mucosal surfaces of the terminal ileum and the entire large bowel, which include the colon and cecum. The nature, distribution, and extent of the mucosal lesions in gnotobiotic piglets induced by STEC strain 86-24 were consistent with those amply described in the past by our group (4, 47, 49) and by others (7).

Of the 83 animals whose results are given in Table 3, 16 were found dead in the morning; of these, none were among the piglets which received  $\geq 0.4$  mg/kg of antibody, and only 3 were among those which received a lower dose of 5C12. Among the 23 which received  $\geq 0.4$  mg/kg of antibody, only 2 were euthanized due to systemic complications (one had neurological symptoms, and another was comatose). In contrast, 18 of the 30 euthanized animals which received the placebo developed typical fatal neurological symptoms of ataxia, paddling, head-pressing, and recumbency. The remaining 12 placebo-treated animals were comatose when they were first observed. These neurological symptoms are accompanied histologically by cerebellar vascular lesions of petechial hemorrhages in the molecular and cortex layers, with evidence of infarction and extensive shrinkage of the neuronal nuclei, as previously described in some detail (4, 47, 49).

Eleven of the surviving antibody-treated animals, kept alive for 14 days after bacterial challenge, continued to have diarrhea but at a reduced intensity after 6 days. They became more alert and very active and gained weight but continued to excrete strain *E. coli* 86-24 in their feces until euthanasia.

## DISCUSSION

The HuMAb 5C12 was the most effective among the five HuMAbs selected for initial characterization in cell culture and in the mouse toxicity assay, as shown in the present study. The lowest protective dose when administered 48 h after bacterial challenge or 8 to 32 h after the onset of diarrhea was 0.4 mg/kg of body weight. Doses below 0.4 mg/kg protected some of the animals, indicating that many factors impact the degree of protection observed, including the rate of bacterial colonization and toxin uptake from the gut and the individual genetic variation among outbred animals, among others. Antibody 5C12 was also shown previously to have the widest spectrum of neutralizing activity against Stx2 variants. In these studies, 5C12 protected mice for up to 48 h after oral challenge with STEC strains producing Stx2c, Stx2vha, and Stx2vhb (42).

HUS occurs in 5 to 10% of children with STEC infections, resulting in 5 to 10% of deaths due to renal failure and other complications, including neurological ones (50). In earlier studies using the piglet model of STEC, we showed that fatal systemic complications occurred in 90% of animals challenged with the Stx2 producer strain 86-24 (4). In the present study, the mortality rate among the control piglets was close to 100% (1 of 61 survived). Diarrhea in these piglets occurred between 16 and 40 h after bacterial challenge, and fatal systemic complications occurred within 48 to 96 h after challenge. Both results are consistent with earlier observations (4). In those earlier reports, we described protection of piglets either with five Stx2-specific HuMAbs given i.p. at 12 h (22) or with swine polyclonal antibodies given at 24 h (3) after STEC challenge. The current study demonstrated that piglets are still fully protected when the interval between bacterial challenge and an-

tibody administration is extended from 12 to 48 h. This is significant since it shows that the administration of 5C12 antibody occurred well after the onset of diarrhea and close to the onset of CNS symptoms. CNS symptoms in piglets are manifestations of the systemic impact of Stx2 uptake from the gut and correspond to the onset of HUS in children. While piglets do not develop the acute kidney lesions attributed to Stx2, which is the hallmark of HUS, they do develop characteristic, vascular system-mediated brain lesions which result in profound and fatal CNS symptoms. Despite this difference, we believe that the piglet model offers several advantages over the infected mouse model. The pathogenic pathways as they relate to Stx2 are similar in humans and pigs, as outlined previously in some detail (50). This is particularly relevant to systemic therapy, since the mechanisms and dynamics of toxin uptake from a severely injured colonic mucosa by attaching-effacing bacteria are the same for both species (50). Consequently, a circulating human antibody that is effective in neutralizing Stx2 in piglets is likely to be even more effective in the homologous human species. The relevance of this model is substantiated further because (i) piglets are the only species in addition to humans that are naturally susceptible to the systemic effects of Stx produced by *E. coli* organisms that proliferate in the gastrointestinal tract (17, 18); (ii) piglets exhibit upon infection with STEC characteristic attaching-effacing lesions in the colonic mucosa (33, 48), which are absent in mice (15); (iii) piglets and humans develop symptoms of diarrhea following infection with STEC, symptoms that the mouse model lacks; (iv) piglets are anatomically and physiologically similar to infants and children; and (v) after the onset of diarrhea, there is a prodromal period in children of 5 to 7 days before the onset of HUS and of about 2 to 3 days in piglets before the onset of CNS symptoms (3, 22, 50). We have utilized this window between the onset of diarrhea and the onset of systemic complications in our study because this is the most likely time in which children with bloody diarrhea will be seeking medical intervention. This study showed that piglets were protected when the antibody was administered well after the onset of diarrhea, close to the onset of systemic complications.

Not surprisingly, the parenteral administration of Stx2 antibody had no effect on the severity or duration of the GI tract symptoms. The 11 antibody-treated animals, while fully protected against systemic complications, continued to have diarrhea and excrete strain 86-24 for at least 14 days. They did not require rehydration therapy 8 to 10 days after bacterial challenge.

The results of the *in vivo* efficacy studies, however, did not correlate with the results of the affinity measurement studies in which the rank order was 2F10 > 3E9 > 5C12. This suggests that 5C12 is either directed against an epitope which is more important for functional activity of the toxin or directed more specifically against the "functionally active site" of Stx2 than the epitopes recognized by the other HuMabs. Since 5C12 is directed against the A subunit, it may either inhibit binding of B subunits with Gb<sub>3</sub> by steric hindrance or neutralize its protein synthesis-blocking effect following internalization of the 5C12/Stx2 complex. A clear understanding of the mechanism by which A subunit-specific antibodies neutralize Stx2 *in vivo* awaits studies on the mechanism of Stx2 neutralization by these HuMabs and epitope mapping studies. The likely neu-

tralizing mechanism of HuMabs directed against the B subunit, on the other hand, is most likely blocking of the binding of Stx2 with Gb<sub>3</sub>.

For affinity measurements of HuMabs, we preferred capturing the antibody onto the amine-coupled rabbit anti-human IgG Fc over coupling a HuMAb directly onto the chip because the former gives a more uniform and natural orientation since the two Fab arms of every HuMAb are free to interact with the Stx2. Also, since the antibody was reapplied after each run, it was not subjected to regeneration conditions, which are detrimental over time. Lastly, the capture antibody/HuMAb complex remained stable over the duration of each run and thus did not affect evaluation. Though this method was well suited for affinity measurements of anti-Stx2 A subunit antibodies (2F10, 3E9, and 5C12), it could not measure affinities of the Stx2 B subunit-specific antibodies accurately, because the B subunit is pentameric and therefore pentavalent and can potentially bind with more than one antibody molecule on the chip, producing an avidity effect. For this reason, we have only reported affinity data for the A-subunit binders. It will not be easy to perform affinity measurement studies on B-subunit binders because the recombinant monomeric subunit polymerizes even in the absence of the A subunit.

The observation that a dose of 0.4 mg/kg administered 48 h after bacterial challenge protected piglets suggests that the 5C12-based therapy is more than likely to protect children not only when they present with bloody diarrhea but possibly also when the antibody is given at the onset of HUS. Therefore, the time window of 48 h for immunotherapeutic intervention has direct implications for children at risk of developing HUS, e.g., those presenting with bloody diarrhea or excreting STEC or for contact individuals. The development of rapid and sensitive methods has made it possible to detect STEC infection almost a week before symptoms of HUS become apparent (31, 32). Adoption of these methods by clinics for diagnosis would greatly benefit immunotherapy. Clinical diagnosis is currently limited to the detection of *E. coli* O157. Since the serum level of 0.7 µg/ml of 5C12 was measured at the time of euthanasia, some 7 to 14 days after antibody therapy, it is well below the expected protective serum level. While not determined, the protective serum level can be extrapolated from half-life studies. An accurate serum protective level should be measured 4 to 6 h after antibody administration.

Stx2 is the most prevalent genotype identified in STEC organisms isolated from patients with HUS (7, 38, 50). Stx2c and Stx1, in contrast, are infrequently linked with HUS (7), and Stx2 variants other than Stx2c are rarely linked with HUS (12, 30, 36, 46). Given that STEC can produce any combination of Stx1, Stx2, and/or Stx2c (7), an ideal therapeutic formulation should, in our view, include antibodies that can neutralize all three. Since 5C12 neutralizes Stx2 and Stx2c, the addition of an antibody against Stx1 to 5C12 will provide the widest spectrum of protection against HUS. We have previously reported the production of several neutralizing Stx1-specific HuMabs evaluated in the mouse toxicity model (23). Such an antibody could be included in future formulations.

In this model, it was possible to reverse the impact of the GI tract symptoms in most piglets by assisted oral feeding and parenteral rehydration. In the current investigation, we made no attempt to reverse the development of systemic complica-

tions. All treated animals were given 5C12 antibody before the development of such symptoms. Our approach was intended to mimic the protection of children who may benefit from treatment when they present between the onset of bloody diarrhea and just before hospitalization due to HUS. We do not believe that an antibody is capable of reversing the process of HUS. It may, however, modify the outcome if given early enough. Studies to address this aspect will be conducted with piglets in the future.

In conclusion, our study on the Stx2-specific HuMAb 5C12, which also effectively neutralizes Stx2 variants (42), protected piglets against fatal CNS symptoms, even when the antibody was administered 48 h after bacterial challenge. The minimum protective dose was established at 0.4 mg/kg body weight. We are confident that this antibody, administered at an optimal dose (to be determined in human volunteers) and given immediately after the onset of bloody diarrhea, to patients with confirmed cases of STEC infection, or after exposure to sources contaminated with STEC, will be equally effective in protecting children at risk for developing HUS.

#### ACKNOWLEDGMENTS

This study was supported by Public Health Service NIH grants RO1-AI41326 and R01-DK58993.

We thank Donald Girouard, Rachel Sora, Rui Wu, and Catherine McCann for technical assistance.

#### REFERENCES

- Boerlin, P., S. A. McEwen, F. Boerlin-Petzold, J. B. Wilson, R. P. Johnson, and C. L. Gyles. 1999. Associations between virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans. *J. Clin. Microbiol.* **37**:497–503.
- Donohue-Rolfe, A., D. W. Acheson, A. V. Kane, and G. T. Keusch. 1989. Purification of Shiga toxin and Shiga-like toxins I and II by receptor analog affinity chromatography with immobilized P1 glycoprotein and production of cross-reactive monoclonal antibodies. *Infect. Immun.* **57**:3888–3893.
- Donohue-Rolfe, A., I. Kondova, J. Mukherjee, K. Chios, D. Hutto, and S. Tzipori. 1999. Antibody-based protection of gnotobiotic piglets infected with *Escherichia coli* O157:H7 against systemic complications associated with Shiga toxin 2. *Infect. Immun.* **67**:3645–3648.
- Donohue-Rolfe, A., I. Kondova, S. Oswald, D. Hutto, and S. Tzipori. 2000. *Escherichia coli* O157:H7 strains that express Shiga toxin (Stx) 2 alone are more neurotropic for gnotobiotic piglets than are isotypes producing only Stx1 or both Stx1 and Stx2. *J. Infect. Dis.* **181**:1825–1829.
- Endo, Y., K. Tsurugi, T. Yutsudo, Y. Takeda, T. Ogasawara, and K. Igarashi. 1988. 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.* **171**:45–50.
- Francis, D. H., J. E. Collins, and J. R. Duimstra. 1986. Infection of gnotobiotic pigs with an *Escherichia coli* O157:H7 strain associated with an outbreak of hemorrhagic colitis. *Infect. Immun.* **51**:953–956.
- Friedrich, A. W., M. Bielaszewska, W. L. Zhang, M. Pulz, T. Kuczus, A. Ammon, and H. Karch. 2002. *Escherichia coli* harboring Shiga toxin 2 gene variants: frequency and association with clinical symptoms. *J. Infect. Dis.* **185**:74–84.
- Gannon, V. P., C. Teerling, S. A. Masri, and C. L. Gyles. 1990. Molecular cloning and nucleotide sequence of another variant of the *Escherichia coli* Shiga-like toxin II family. *J. Gen. Microbiol.* **136**:1125–1135.
- Griffin, P. M., and R. V. Tauxe. 1991. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. *Epidemiol. Rev.* **13**:60–98.
- Halloran, P. F., and S. Prommool. 1998. Humanized monoclonals and other biological initiatives. *Clin. Biochem.* **31**:353–357.
- Islam, M. S., and W. H. Stimson. 1990. Production and characterization of monoclonal antibodies with therapeutic potential against Shiga toxin. *J. Clin. Lab. Immunol.* **33**:11–16.
- Ito, H., A. Terai, H. Kurazono, Y. Takeda, and M. Nishibuchi. 1990. Cloning and nucleotide sequencing of Vero toxin 2 variant genes from *Escherichia coli* O91:H21 isolated from a patient with the hemolytic uremic syndrome. *Microb. Pathog.* **8**:47–60.
- Johansen, M., L. O. Andresen, L. K. Thomsen, M. E. Busch, H. Wachmann, S. E. Jorsal, and C. L. Gyles. 2000. Prevention of edema disease in pigs by passive immunization. *Can. J. Vet. Res.* **64**:9–14.
- Keusch, G. T., A. Donohue-Rolfe, M. Jacewicz, and A. V. Kane. 1988. Shiga toxin: production and purification. *Methods Enzymol.* **165**:152–162.
- Lindgren, S. W., A. R. Melton, and A. D. O'Brien. 1993. Virulence of enterohemorrhagic *Escherichia coli* O91:H21 clinical isolates in an orally infected mouse model. *Infect. Immun.* **61**:3832–3842.
- Lingwood, C. A. 1996. Role of verotoxin receptors in pathogenesis. *Trends Microbiol.* **4**:147–153.
- MacLeod, D. L., C. L. Gyles, and B. P. Wilcock. 1991. Reproduction of edema disease of swine with purified Shiga-like toxin-II variant. *Vet. Pathol.* **28**:66–73.
- Marques, L. R. M., J. S. M. Peiris, S. J. Cryz, and A. D. O'Brien. 1987. *Escherichia coli* strains isolated from pigs with edema disease produce a variant of Shiga-like toxin II. *FEMS Microbiol. Lett.* **44**:281–283.
- Melton-Celsa, A. R., J. E. Rogers, C. K. Schmitt, S. C. Darnell, and A. D. O'Brien. 1998. Virulence of Shiga toxin-producing *Escherichia coli* (STEC) in orally-infected mice correlates with the type of toxin produced by the infecting strain. *Jpn. J. Med. Sci. Biol.* **51**:S108–S114.
- Meyer, T., H. Karch, J. Hacker, H. Bocklage, and J. Heesemann. 1992. Cloning and sequencing of a Shiga-like toxin II-related gene from *Escherichia coli* O157:H7 strain 7279. *Zentbl. Bakteriol.* **276**:176–188.
- Milford, D. V., C. M. Taylor, B. Guttridge, S. M. Hall, B. Rowe, and H. Kleanthous. 1990. Haemolytic uraemic syndromes in the British Isles 1985–8: association with verocytotoxin producing *Escherichia coli*. Part 1. Clinical and epidemiological aspects. *Arch. Dis. Child.* **65**:716–721.
- Mukherjee, J., K. Chios, D. Fishwild, D. Hudson, S. O'Donnell, S. M. Rich, A. Donohue-Rolfe, and S. Tzipori. 2002. Human Stx2-specific monoclonal antibodies prevent systemic complications of *Escherichia coli* O157:H7 infection. *Infect. Immun.* **70**:612–619.
- Mukherjee, J., K. Chios, D. Fishwild, D. Hudson, S. O'Donnell, S. M. Rich, A. Donohue-Rolfe, and S. Tzipori. 2002. Production and characterization of protective human antibodies against Shiga toxin 1. *Infect. Immun.* **70**:5896–5899.
- Myszka, D. G. 1999. Improving biosensor analysis. *J. Mol. Recognit.* **12**:279–284.
- Ogasawara, T., K. Ito, K. Igarashi, T. Yutsudo, N. Nakabayashi, and Y. Takeda. 1988. Inhibition of protein synthesis by a Vero toxin (VT2 or Shiga-like toxin II) produced by *Escherichia coli* O157:H7 at the level of elongation factor 1-dependent aminoacyl-tRNA binding to ribosomes. *Microb. Pathog.* **4**:127–135.
- Ostroff, S. M., P. I. Tarr, M. A. Neill, J. H. Lewis, N. Hargrett-Bean, and J. M. Kobayashi. 1989. Toxin genotypes and plasmid profiles as determinants of systemic sequelae in *Escherichia coli* O157:H7 infections. *J. Infect. Dis.* **160**:994–998.
- Padhye, V. V., T. Zhao, and M. P. Doyle. 1989. Production and characterization of monoclonal antibodies to Verotoxins 1 and 2 from *Escherichia coli* of serotype O 157:H7. *J. Med. Microbiol.* **30**:219–226.
- Paton, A. W., J. C. Paton, P. N. Goldwater, M. W. Heuzenroeder, and P. A. Manning. 1993. Sequence of a variant Shiga-like toxin type-I operon of *Escherichia coli* O111:H. *Gene* **129**:87–92.
- Paton, A. W., J. C. Paton, M. W. Heuzenroeder, P. N. Goldwater, and P. A. Manning. 1992. Cloning and nucleotide sequence of a variant Shiga-like toxin II gene from *Escherichia coli* OX3:H21 isolated from a case of sudden infant death syndrome. *Microb. Pathog.* **13**:225–236.
- Paton, A. W., J. C. Paton, and P. A. Manning. 1993. Polymerase chain reaction amplification, cloning and sequencing of variant *Escherichia coli* Shiga-like toxin type II operons. *Microb. Pathog.* **15**:77–82.
- Paton, A. W., R. M. Ratcliff, R. M. Doyle, J. Seymour-Murray, D. Davos, J. A. Lanser, and J. C. Paton. 1996. Molecular microbiological investigation of an outbreak of hemolytic-uremic syndrome caused by dry fermented sausage contaminated with Shiga-like toxin-producing *Escherichia coli*. *J. Clin. Microbiol.* **34**:1622–1627.
- Paton, A. W., M. C. Woodrow, R. M. Doyle, J. A. Lanser, and J. C. Paton. 1999. Molecular characterization of a Shiga toxinigenic *Escherichia coli* O113:H21 strain lacking *eae* responsible for a cluster of cases of hemolytic-uremic syndrome. *J. Clin. Microbiol.* **37**:3357–3361.
- Phillips, A. D., S. Navabpour, S. Hicks, G. Dougan, T. Wallis, and G. Frankel. 2000. Enterohaemorrhagic *Escherichia coli* O157:H7 target Peyer's patches in humans and cause attaching/effacing lesions in both human and bovine intestine. *Gut* **47**:377–381.
- Piérard, D., G. Muylderma, L. Moriau, D. Stevens, and S. Lauwers. 1998. Identification of new verocytotoxin type 2 variant B-subunit genes in human and animal *Escherichia coli* isolates. *J. Clin. Microbiol.* **36**:3317–3322.
- Pless, D. D., E. R. Torres, E. K. Reinke, and S. Bavari. 2001. High-affinity, protective antibodies to the binding domain of botulinum neurotoxin type A. *Infect. Immun.* **69**:570–574.
- Ramachandran, V., M. A. Hornitzky, K. A. Bettelheim, M. J. Walker, and S. P. Djordjevic. 2001. The common ovine Shiga toxin 2-containing *Escherichia coli* serotypes and human isolates of the same serotypes possess a Stx2d toxin type. *J. Clin. Microbiol.* **39**:1932–1937.
- Reisbig, R., S. Olsnes, and K. Eiklid. 1981. The cytotoxic activity of Shigella toxin. Evidence for catalytic inactivation of the 60 S ribosomal subunit. *J. Biol. Chem.* **256**:8739–8744.

38. **Russmann, H., H. Schmidt, J. Heesemann, A. Caprioli, and H. Karch.** 1994. Variants of Shiga-like toxin II constitute a major toxin component in *Escherichia coli* O157 strains from patients with haemolytic uraemic syndrome. *J. Med. Microbiol.* **40**:338–343.
39. **Sandvig, K., and B. van Deurs.** 1996. Endocytosis, intracellular transport, and cytotoxic action of Shiga toxin and ricin. *Physiol. Rev.* **76**:949–966.
40. **Schmidt, H., J. Scheef, S. Morabito, A. Caprioli, L. H. Wieler, and H. Karch.** 2000. A new Shiga toxin 2 variant (Stx2f) from *Escherichia coli* isolated from pigeons. *Appl. Environ. Microbiol.* **66**:1205–1208.
41. **Schmitt, C. K., M. L. McKee, and A. D. O'Brien.** 1991. Two copies of Shiga-like toxin II-related genes common in enterohemorrhagic *Escherichia coli* strains are responsible for the antigenic heterogeneity of the O157:H<sup>-</sup> strain E32511. *Infect. Immun.* **59**:1065–1073.
42. **Sheoran, A. S., S. Chapman, P. Singh, A. Donohue-Rolfe, and S. Tzipori.** 2003. Stx2-specific human monoclonal antibodies protect mice against lethal infection with *Escherichia coli* expressing Stx2 variants. *Infect. Immun.* **71**:3125–3130.
43. **Strockbine, N. A., L. R. Marques, R. K. Holmes, and A. D. O'Brien.** 1985. Characterization of monoclonal antibodies against Shiga-like toxin from *Escherichia coli*. *Infect. Immun.* **50**:695–700.
44. **Tarr, P. I., M. A. Neill, C. R. Clausen, J. W. Newland, R. J. Neill, and S. L. Moseley.** 1989. Genotypic variation in pathogenic *Escherichia coli* O157:H7 isolated from patients in Washington, 1984–1987. *J. Infect. Dis.* **159**:344–347.
45. **Tesh, V. L., J. A. Burris, J. W. Owens, V. M. Gordon, E. A. Wadolkowski, A. D. O'Brien, and J. E. Samuel.** 1993. Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. *Infect. Immun.* **61**:3392–3402.
46. **Thomas, A., T. Cheasty, H. Chart, and B. Rowe.** 1994. Isolation of Vero cytotoxin-producing *Escherichia coli* serotypes O9ab:H- and O101:H-carrying VT2 variant gene sequences from a patient with haemolytic uraemic syndrome. *Eur. J. Clin. Microbiol. Infect. Dis.* **13**:1074–1076.
47. **Tzipori, S., C. W. Chow, and H. R. Powell.** 1988. Cerebral infection with *Escherichia coli* O157:H7 in humans and gnotobiotic piglets. *J. Clin. Pathol.* **41**:1099–1103.
48. **Tzipori, S., R. Gibson, and J. Montanaro.** 1989. Nature and distribution of mucosal lesions associated with enteropathogenic and enterohemorrhagic *Escherichia coli* in piglets and the role of plasmid-mediated factors. *Infect. Immun.* **57**:1142–1150.
49. **Tzipori, S., F. Gunzer, M. S. Donnenberg, L. de Montigny, J. B. Kaper, and A. Donohue-Rolfe.** 1995. The role of the *eaeA* gene in diarrhea and neurological complications in a gnotobiotic piglet model of enterohemorrhagic *Escherichia coli* infection. *Infect. Immun.* **63**:3621–3627.
50. **Tzipori, S., A. Sheoran, D. Akiyoshi, A. Donohue-Rolfe, and H. Trachtman.** 2004. Antibody therapy in the management of Shiga toxin-induced hemolytic uremic syndrome. *Clin. Microbiol. Rev.* **17**:926–941.
51. **Waddell, T., A. Cohen, and C. A. Lingwood.** 1990. Induction of verotoxin sensitivity in receptor-deficient cell lines using the receptor glycolipid globotriosylceramide. *Proc. Natl. Acad. Sci. USA* **87**:7898–7901.
52. **Wadolkowski, E. A., J. A. Burris, and A. D. O'Brien.** 1990. Mouse model for colonization and disease caused by enterohemorrhagic *Escherichia coli* O157:H7. *Infect. Immun.* **58**:2438–2445.
53. **Wadolkowski, E. A., L. M. Sung, J. A. Burris, J. E. Samuel, and A. D. O'Brien.** 1990. Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shiga-like toxin type II. *Infect. Immun.* **58**:3959–3965.
54. **Weinstein, D. L., M. P. Jackson, J. E. Samuel, R. K. Holmes, and A. D. O'Brien.** 1988. Cloning and sequencing of a Shiga-like toxin type II variant from *Escherichia coli* strain responsible for edema disease of swine. *J. Bacteriol.* **170**:4223–4230.
55. **Yamagami, S., M. Motoki, T. Kimura, H. Izumi, T. Takeda, Y. Katsuura, and Y. Matsumoto.** 2001. 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.* **184**:738–742.

Editor: J. D. Clements