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(54) Title: METHODS, COMPOSITIONS, AND KITS FOR TREATING SHIGA TOXIN ASSOCIATED CONDITIONS

(57) Abstract: The invention features methods, compositions, and kits for treating a subject having a Shiga toxin associated disease with chimeric anti-Shiga Toxin 1 (cαStx1) and anti-Shiga Toxin 2 (cαStx2) antibodies.

## METHODS, COMPOSITIONS, AND KITS FOR TREATING SHIGA TOXIN ASSOCIATED CONDITIONS

### BACKGROUND OF THE INVENTION

In general, this invention relates to the field of treating and preventing Shiga toxin associated diseases.

In the United States, Shiga toxin (Stx)-producing *Escherichia coli* (STEC) account for about 110,000 infections per year. Enterohemorrhagic *E. coli* (EHEC), 10 most notably the serotype O157:H7, is a subset of STEC that is noted for producing Stx mediated disease. A possible complication from an infection with a Stx-producing organism is the hemolytic uremic syndrome (HUS), which is characterized by hemolytic anemia, thrombic thrombocytopenia, and renal failure. There is approximately a 5-10% fatality rate for those with HUS and survivors may have 15 lasting kidney damage. Currently there are no FDA approved therapies or vaccines to combat or prevent illness from a Stx-mediated disease, but several promising options for the future include: a humanized monoclonal antibody that binds to and neutralizes Stx2 and a chimeric StxA2/StxB1 toxoid that elicits a neutralizing response and provides protection against a lethal challenge of Stx1 or Stx2 or Stx1 and Stx2.

20 There are essentially two main types of Stxs: Stx/Stx1 and Stx2. Stx is produced from *Shigella dysenteriae* type 1, while Stx1 and Stx2 are produced from *Escherichia coli*. Stx and Stx1 are virtually identical, with only one amino acid difference in the A subunit. The mature A and B subunits of Stx1 and Stx2 have 68 and 73% similarity, respectively. Despite the amino acid sequence differences, the 25 crystal structures of Stx and Stx2 are remarkably similar (Figure 1). These toxins can be differentiated by polyclonal antisera: polyclonal antisera raised against Stx1 does not neutralize Stx2 and vice-versa. Variants of Stx1 and Stx2 exist and include Stx1c, Stx1d, Stx2c, Stx2d, Stx2d-activatable (Stx2-act.), Stx2e, and Stx2f.

30 Shiga toxins are complex holotoxins with an AB<sub>5</sub> structure. The active domain (A), contains an N-glycosidase that depurinates the 28S rRNA of the 60S ribosomal subunit, which stops proteins synthesis and eventually leads to cell death. The A subunit is ~ 32 kDa and is proteolytically cleaved by trypsin or furin into a ~

28 kDa A<sub>1</sub> subunit and a ~ 5 kDa A<sub>2</sub> peptide which are connected through a single disulphide bond. The A<sub>1</sub> subunit contains the active domain, and the A<sub>2</sub> peptide non-covalently tethers the active domain to the binding domain. The binding domain (B) consists of five identical ~ 7.7 kDa monomers that form a pentamer through which the 5 C-terminus of the A<sub>2</sub> peptide traverses. Each of the B subunit monomers has two cysteine residues that form a disulphide bond within each monomer (Figure 2). The B pentamer binds the eukaryotic receptor globotriaosyl ceramide (Gb<sub>3</sub>) (or Gb<sub>4</sub> as is the case for Stx2e).

Despite this knowledge about the results of exposure to these toxins, currently 10 there is no known cure or vaccine for HUS. The use of antibiotics may exacerbate the situation by increasing toxin release from bacteria. Thus, there is a need for a compound to prevent or to treat the complications of EHEC produced by Shiga toxin. Such a compound could be used to treat infected subjects and decrease the systemic effects of toxin on the CNS, blood, and kidneys. In addition, if the toxin could be 15 neutralized, antibiotics could be safely given to kill the bacteria in the GI tract. Such a compound could also be used to prevent infectious complications, by treating exposed or high risk individuals before they acquire EHEC infection. Such individuals would include children in day care or the elderly in nursing homes, where a case of EHEC diarrhea has been identified. These individuals are at increased risk 20 to develop EHEC, often with severe complications, and spread of EHEC in these environments is not unusual.

## SUMMARY OF THE INVENTION

We have discovered that chimeric anti-Stx1 and chimeric anti-Stx2, when 25 administered at 1 mg/kg or 3 mg/kg, are effective in treating Shiga toxin-associated diseases.

In one aspect, the invention features a method for the treatment of a Shiga toxin associated condition in a subject (e.g., a human) by administering an effective amount of chimeric anti-Stx1 and chimeric anti-Stx2 antibodies to the subject. Each 30 of the chimeric antibodies is administered at 1 mg/kg or 3 mg/kg (for example by intravenous infusion over a period of 15, 30, 45, 60, 90, 120 minutes, or more. In a

preferred embodiment, the antibodies are administered by intravenous infusion for between 30 minutes and 1 hour). In this aspect, the chimeric anti-Stx1 antibody includes a human IgG1-kappa immunoglobulin constant region, an immunoglobulin heavy chain variable region including the amino acid sequence set forth in SEQ ID NO:1, and an immunoglobulin light chain variable region including the amino acid sequence set forth in SEQ ID NO:2. The chimeric anti-Stx2 antibody includes a human IgG1-kappa immunoglobulin constant region, an immunoglobulin heavy chain variable region including the amino acid sequence set forth in SEQ ID NO: 3, and an immunoglobulin light chain variable region including the amino acid sequence set forth in SEQ ID NO: 4.

In another aspect, the invention features an article of manufacture. This article of manufacture includes chimeric anti-Stx1 and chimeric anti-Stx2 antibodies and a label. The label indicates that the chimeric anti-Stx1 and chimeric anti-Stx2 antibodies are for treating a Shiga-toxin associated disease (e.g., in a human less than 18 years of age, less than 6 months old, and between 6 months and 3 years old) and are to be administered at a dosage of 1 mg/kg or 3 mg/kg each. In this aspect, the chimeric anti-Stx1 antibody includes a human immunoglobulin constant region IgG1-kappa, an immunoglobulin heavy chain variable region including the amino acid sequence set forth in SEQ ID NO:1, and an immunoglobulin light chain variable region including the amino acid sequence set forth in SEQ ID NO:2. The chimeric anti-Stx2 antibody includes a human immunoglobulin constant region IgG1-kappa, an immunoglobulin heavy chain variable region including the amino acid sequence set forth in SEQ ID NO: 3, and an immunoglobulin light chain variable region including the amino acid sequence set forth in SEQ ID NO: 4.

In yet another aspect, the invention features a kit. This kit includes chimeric anti-Stx1 and chimeric anti-Stx2 antibodies, instructions, and a label. The instructions are for administering the chimeric anti-Stx1 and chimeric anti-Stx2 antibodies at a dosage of 1 mg/kg or 3 mg/kg. In this aspect the label indicates that the chimeric anti-Stx1 and chimeric anti-Stx2 antibodies are for treating a Shiga-toxin associated disease (e.g., in a human less than 18 years of age, less than 6 months old, and between 6 months and 3 years old). Further, the chimeric anti-Stx1 antibody includes

a human immunoglobulin constant region IgG1-kappa, an immunoglobulin heavy chain variable region including the amino acid sequence set forth in SEQ ID NO:1, and an immunoglobulin light chain variable region including the amino acid sequence set forth in SEQ ID NO:2. The chimeric anti-Stx2 antibody includes a human 5 immunoglobulin constant region IgG1-kappa, an immunoglobulin heavy chain variable region including the amino acid sequence set forth in SEQ ID NO: 3, and an immunoglobulin light chain variable region including the amino acid sequence set forth in SEQ ID NO: 4.

In any of the foregoing aspects, the chimeric anti-Stx1 and chimeric anti-Stx2 10 antibodies can be co-administered. Further, the subject being administered chimeric anti-Stx1 and chimeric anti-Stx2 antibodies can be less than 18 years old (e.g., less than 6 months old or between 6 months and 3 year old).

By "chimeric anti-Stx1" or "caStx1" is meant a humanized antibody that 15 specifically binds to Stx1 and includes an IgG1 kappa human immunoglobulin constant region and the murine 13C4 (ATCC Accession No. CRL 1794) variable region. This antibody is described in U.S. Patent Application Publication No. 20030170248, which is hereby incorporated by reference in its entirety.

By "chimeric anti-Stx2" or "caStx2" is meant a humanized antibody that 20 specifically binds to Stx2 and includes an IgG1 kappa human immunoglobulin constant region and the murine 11E10 (ATCC Accession No. CRL 1987) variable region. This antibody is described in U.S. Patent Application Publication No. 20030170248, which is hereby incorporated by reference in its entirety.

By the terms "specifically binds to" is meant an antibody that binds to a 25 protein (e.g., Stx1 or Stx2) with a  $K_d$  value of between 100 nM-1 pM. Antibody affinities may be determined using any of the assays known in the art including, but not limited to, surface plasmon resonance based assay, enzyme-linked immunoabsorbent assay (ELISA), and competition assays (e.g. RIA's).

By "co-administered" is meant introduction of the two antibodies 30 simultaneously. In one embodiment, each antibody is introduced at the appropriate dosage into single container (e.g., a container containing a buffer or solution (e.g., a

saline solution)). This mixture is then administered by intravenous infusion to the subject.

By "Shiga toxin associated disease" is meant any disease resulting from a pathogen expressing a Shiga toxin. The term "Shiga toxin associated disease" is 5 meant to include hemolytic uremia syndrome, shigellosis, and diseases resulting from Shiga toxin-producing *Escherichia coli* and *S. dysenteriae* infection.

The term "treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the condition as well as those in which the disorder is to be prevented.

10 The term "intravenous infusion"" refers to introduction of a drug into the vein of a subject over a period of time of 15, 30, 45, 60, 90, 120 minutes, or more. In a preferred embodiment, the antibodies are administered by intravenous infusion for between 30 minutes and 1 hour.

15

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is a graph showing the concentration of  $\alpha$ Stx1 and  $\alpha$ Stx2 as a function of time after intravenous administration to healthy adult volunteers.

20 Figure 2 is a graph showing the serum concentration of  $\alpha$ Stx1 with and without co-administration of  $\alpha$ Stx2 as a function of time.

Figure 3 is a graph showing the serum concentration of  $\alpha$ Stx2 with and without co-administration of  $\alpha$ Stx1 as a function of time.

Figure 4 is a diagram showing the study population and database.

25 Figure 5 is a series of graphs showing the number of days of hospitalization as a function of score on the STEC Disease Severity Scale for the indicated patients.

The STEC "bloody diarrhea" was correlated with the outcome "days of hospitalization" (error bars are standard errors of the means).

30 Figure 6 is a disease expression array showing four children with evolving STEC O157:H7 infection, two with incipient HUS (HUS-1, -2) and two with bloody diarrhea only (Colitis-1, -2), separated by bold lines. Each row represents a distinct clinical or laboratory item and its evolution over time, in the same order for each of

the four patients. Columns indicate consecutive days, starting with Day 1 (=disease onset) for each patient. Severity of symptoms or laboratory abnormalities are visualized by different intensities of shading such that the degree of darkening corresponds to the score (0-4) in the proposed STEC Disease Severity and

5 Progression scale (see Table 17). Empty fields represent days with missing information

### **DETAILED DESCRIPTION OF THE INVENTION**

10 In general the invention features methods, compositions, and kits for treating Shiga toxin associated diseases in a subject with chimeric anti-Shiga Toxin 1 ( $\alpha$ Stx1) and chimeric anti-Shiga Toxin 2 ( $\alpha$ Stx2) antibodies as defined herein.

#### **I. INDICATIONS**

15 The compounds and methods of the invention are useful for treating subjects having, or at risk of developing a Shiga toxin associated disease. Such subject include subjects infected with *S. dysenteriae* or EHEC (Enterohemorrhagic *E. coli*). Such subjects would also include children in day care or the elderly in nursing homes. In one example, the subject is in a day care or in a nursing home where a case of EHEC  
20 diarrhea has been detected. In this example, the subject may or may not have developed the disease. Shiga toxin associated diseases include those resulting from infection with Shiga toxin producing *S. dysenteriae* or EHEC, most notably the serotype O157:H7. These infections often result in hemolytic uremic syndrome (HUS), which is characterized by hemolytic anemia, thrombotic thrombocytopenia,  
25 and renal failure.

#### **II. CHIMERIC $\alpha$ STX1 AND $\alpha$ STX2 ANTIBODIES**

The invention features methods and compositions that include chimeric  $\alpha$ Stx1 and chimeric  $\alpha$ Stx2 antibodies for the treatment of Shiga toxin associated diseases.

30 These antibodies are set forth in U.S. Patent Application Serial Nos. 09/215,163 and 11/471,420, each of which is incorporated by reference in its entirety.

caStx1 and caStx2 are chimeric monoclonal IgG1 antibodies that bind to Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) respectively. caStx1 recognizes the B subunit of Stx1 and caStx2 recognizes the A subunit of Stx2.

The chimeric anti-Stx1 antibody is a humanized antibody that specifically binds to Stx1 and includes an IgG1 kappa human immunoglobulin constant region and the murine 13C4 (ATCC Accession No. CRL 1794) variable region. In one embodiment, the murine heavy chain variable region includes the amino acid sequence set forth in SEQ ID NO:1 and the murine light chain variable region includes the amino acid sequence set forth in SEQ ID NO:2. This antibody is described in U.S. Patent Application Publication No. 20030170248, which is hereby incorporated by reference in its entirety.

The chimeric anti-Stx2 antibody is a humanized antibody that specifically binds to Stx2 and includes an IgG1 kappa human immunoglobulin constant region and the murine 11E10 (ATCC Accession No. CRL 1987) variable region. In one embodiment, the murine heavy chain variable region includes the amino acid sequence set forth in SEQ ID NO:3 and the murine light chain variable region includes the amino acid sequence set forth in SEQ ID NO:4. This antibody is described in U.S. Patent Application Publication No. 20030170248, which is hereby incorporated by reference in its entirety.

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### III. PHARMACEUTICAL FORMULATIONS

Therapeutic formulations of the antibodies used in accordance with the present invention can include an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (Remington: The Science and Practice of Pharmacy 21st edition, University of the Sciences in Philadelphia Ed. 2005), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers excipients, or stabilizers for intravenous administration are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium

chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone;

5 amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN<sup>TM</sup>,

10 PLURONICS<sup>TM</sup>, or polyethylene glycol (PEG).

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies that binds Stx1 or Stx2 (e.g. an antibody

15 which binds a different epitope on Stx1 or Stx2) in the one formulation.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example,

20 liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington: The Science and Practice of Pharmacy 21st edition, University of the Sciences in Philadelphia Ed. 2005.

The formulations to be used for in vivo administration must be sterile. This is

25 readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include

30 polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic

acid and  $\gamma$  ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT<sup>TM</sup> (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for 5 stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

10 Formulations for oral use include tablets containing the active ingredient(s) in a mixture with non-toxic pharmaceutically acceptable excipients. These excipients may be, for example, inert diluents or fillers (e.g., sucrose, sorbitol, sugar, mannitol, microcrystalline cellulose, starches including potato starch, calcium carbonate, sodium chloride, lactose, calcium phosphate, calcium sulfate, or sodium phosphate);

15 granulating and disintegrating agents (e.g., cellulose derivatives including microcrystalline cellulose, starches including potato starch, croscarmellose sodium, alginates, or alginic acid); binding agents (e.g., sucrose, glucose, sorbitol, acacia, alginic acid, sodium alginate, gelatin, starch, pregelatinized starch, microcrystalline cellulose, magnesium aluminum silicate, carboxymethylcellulose sodium, 20 methylcellulose, hydroxypropyl methylcellulose, ethylcellulose, polyvinylpyrrolidone, or polyethylene glycol); and lubricating agents, glidants, and antiadhesives (e.g., magnesium stearate, zinc stearate, stearic acid, silicas, hydrogenated vegetable oils, or talc). Other pharmaceutically acceptable excipients 25 can be colorants, flavoring agents, plasticizers, humectants, buffering agents, and the like.

The tablets may be uncoated or they may be coated by known techniques, optionally to delay disintegration and absorption in the gastrointestinal tract and thereby providing a sustained action over a longer period. The coating may be adapted to release the active drug substance in a predetermined pattern (e.g., in order 5 to achieve a controlled release formulation) or it may be adapted not to release the active drug substance until after passage of the stomach (enteric coating). The coating may be a sugar coating, a film coating (e.g., based on hydroxypropyl methylcellulose, methylcellulose, methyl hydroxyethylcellulose, hydroxypropylcellulose, carboxymethylcellulose, acrylate copolymers, polyethylene glycols and/or 10 polyvinylpyrrolidone), or an enteric coating (e.g., based on methacrylic acid copolymer, cellulose acetate phthalate, hydroxypropyl methylcellulose phthalate, hydroxypropyl methylcellulose acetate succinate, polyvinyl acetate phthalate, shellac, and/or ethylcellulose). Furthermore, a time delay material such as, e.g., glyceryl monostearate or glyceryl distearate may be employed.

15 The solid tablet compositions may include a coating adapted to protect the composition from unwanted chemical changes, (e.g., chemical degradation prior to the release of the active drug substance).

#### IV. TREATMENT WITH $\alpha$ STX1 AND $\alpha$ STX2 ANTIBODIES

20 It is contemplated that, according to the present invention,  $\alpha$ Stx1 and  $\alpha$ Stx2 antibodies may be used to treat various Shiga toxin associated diseases. The antibodies of the invention are administered to a subject, (e.g., a human patient), in accord with known methods, such as intravenous administration as a bolus or by 25 continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, rectal, topical, or inhalation routes. Intravenous administration of the antibody is preferred. In addition, the antibody or antagonist may suitably be administered by pulse infusion, e.g., with declining doses of the antibody.

30 For the prevention or treatment of disease, the appropriate dosage of  $\alpha$ Stx1 and  $\alpha$ Stx2 antibodies will depend on the type of disease to be treated, as defined above, the severity and course of the Shiga toxin associated disease, whether the

antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments.

5 According to the invention, dosage regimens may include a dose of 1 mg/kg, or more preferably 3 mg/kg delivered by intravenous or subcutaneous infusion for each of the  $\alpha$ Stx1 and  $\alpha$ Stx2 antibodies. These two antibodies may administered as a single formulation or separate formulations. Where the antibody is well-tolerated by the patient, the time of infusion may be reduced.

10 This initial dose may be followed by subsequent follow-up doses by intravenous infusion, intravenous bolus injection, subcutaneous infusion, solid oral doses, or subcutaneous bolus injection.

## V. ARTICLES OF MANUFACTURE

15 In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container, a label and a package insert. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a  
20 composition which is effective for treating the condition and may have a sterile access port (for example, the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is either the  $\alpha$ Stx1 or  $\alpha$ Stx2 antibody or both. The label on, or associated with, the container indicates that the composition is used for treating the  
25 condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes. In addition, the article of manufacture may  
30 comprise a package inserts with instructions for use.

Furthermore, kits of the invention may include one, or both  $\alpha$ Stx1 and  $\alpha$ Stx2, preferably formulated for intravenous administration. Such kits may further include instructions for administering the antibodies at either 1 mg/kg or 3 mg/kg to a patient having or at risk of developing a Shiga toxin associated disease.

5

## VI. EXAMPLES

### Example 1

#### Safety and Tolerability of Chimeric Anti-Shiga toxin 1 ( $\alpha$ Stx1) and Shiga toxin 2 ( $\alpha$ Stx2) in Healthy Adults

10

##### Introduction

Currently there is no causal treatment for Shiga toxin producing bacterial (STPB) infection and its complications. Chimeric monoclonal antibodies against Shiga toxin 1 and Shiga toxin 2 (designated  $\alpha$ Stx1 and  $\alpha$ Stx2) have been developed to address this unmet medical need. Since human STPB can produce either or both Shiga toxins, antibodies against both toxins are needed to maximize chances for clinical success. In preclinical studies, neither of the two monoclonal antibodies was found to be associated with any systemic toxicity in two species.

The safety, tolerability and pharmacokinetics of the anti-toxins  $\alpha$ Stx1 and  $\alpha$ Stx2 in healthy adults from two Phase I studies are presented below.

##### Objective

The primary objective of two clinical Phase I studies, conducted in healthy male and female adults, was to evaluate the safety and tolerability of chimeric monoclonal antibodies  $\alpha$ Stx1 and  $\alpha$ Stx2 following a single intravenous dose of the antibodies administered individually or concomitantly.

The secondary objectives of the studies were to:

- i) evaluate the pharmacokinetics of  $\alpha$ Stx1 and  $\alpha$ Stx2 and
- ii) assess the development of human anti-chimeric antibodies (HACA).

30

## Methods

Each study was a Phase I, single site, open label, non-randomized study. The primary study eligibility criteria are listed in Table 1. All study participants were healthy male and female volunteers.

5

Table 1.  
Primary Study Eligibility Criteria

**Inclusion criteria:**

- Male or female age 18 to 55 years inclusive
- BMI  $\geq 19.0$  and  $< 24.9 \text{ kg/m}^2$

**Exclusion criteria:**

- Chronic condition requiring daily prescription medication
- History or presence of diabetes, cancer, heart disease, autoimmune disease, mental illness, CNS disorder, seizure, respiratory disorder or renal disorder
- Abnormal laboratory tests judged clinically significant
- Positive screening tests for hepatitis B, C or HIV
- ECG abnormalities (clinically significant) or vital sign abnormalities (systolic blood pressure lower than 90 or over 140 mmHg, diastolic blood pressure lower than 50 or over 90 mmHg, or heart rate less than 50 or over 100 bpm) at screening

The chimeric antibodies were administered as a single dose at the concentrations listed in Table 2. The study treatment was diluted in 100 mL of saline and infused at 100 mL/hr for 1 hour through a dedicated intravenous line.

25

Table 2.  
Study Treatment

Number of Participants	Dose $\alpha$ Stx1 (mg/kg)	Dose $\alpha$ Stx2 (mg/kg)
4	1	-
4	3	-
4	-	1
4	-	3
5	1	1
5	3	3

30

The main study visit assessments are listed in Table 3.

Table 3.  
Study Visit Assessments

Assessment	Screening Day -30 to Day -1	Day -1	Day 1	Day 2	Day 4	Day 8	Day 15	Day 29	Day 43	Day 57 or ET
Physical Exam	X	X		X	X	X	X	X	X	X
ECG	X		X*	X		X	X			X
Safety Laboratory	X	X		X	X	X	X	X		X
Study Medication Administration			X							
PK			X*	X	X	X	X	X	X	X
HACA			X					X	X	X

ET = Early Termination \* = multiple assessments HACA = Human Anti-Chimeric Antibody

5

PK serum samples were collected at the following intervals: predose, 0.25 h, 0.5 h, 1 hour (end of study medication infusion), 1.25 h, 1.5 h, 2 h, 3 h, 4 h, 5 h, 7 h, 9 h, 12 h, 24 h (Day 2), 72 h (Day 4), 168 h (Day 8), 336 h (Day 15), 672 h (Day 29), 1032 h (Day 43), and 1344 h (Day 57) after start of study medication infusion. PK serum samples were analyzed to determine the  $\alpha$ Stx1 and  $\alpha$ Stx2 concentrations using validated ELISA methods. PK parameters (Cmax, Tmax, AUC (0 – t), AUC(0-inf),  $\lambda_z$ ,  $t_{1/2}$ , CL and Vz) were calculated by standard non-compartmental methods for each antibody.

10 15 Serum samples were analyzed for anti- $\alpha$ Stx1 and anti- $\alpha$ Stx2 antibodies using validated ELISA methods.

All analyses were based on the Safety Population, which was comprised of any participant who enrolled and who received any amount of study medication.

## Results

20 Twenty-five (25) of the twenty-six (26) participants completed the study to Day 57. One participant in the combined 1 mg/kg  $\alpha$ Stx1 and 1 mg/kg  $\alpha$ Stx2 group was lost to follow-up after the Day 2 visit.

25 No serious adverse events were observed and 4 of the 26 (15%) participants did not experience any adverse event. The most frequent adverse events reported are presented in Table 4 and were mild to moderate in severity.

**Table 4.**  
**Most Frequent Adverse Events**

AE	No. Participants with AE / No. Participants per Dose Group						Total
	caStx1 1 mg/kg	caStx1 3 mg/kg	caStx2 1 mg/kg	caStx2 3 mg/kg	caStx1/caStx2 1/1 mg/kg	caStx1/caStx2 3/3 mg/kg	
Abd Cramps	0/4	0/4	0/4	0/4	1/5	1/5	2/26
Back Pain	1/4	1/4	1/4	0/4	0/5	0/5	3/26
Bloating	0/4	0/4	0/4	0/4	0/5	3/5	3/26
Cold Symptoms	0/4	1/4	0/4	0/4	1/5	0/5	2/26
Cough	2/4	0/4	0/4	1/4	0/5	0/5	3/26
Diarrhea	0/4	1/4	0/4	0/4	0/5	2/5	3/26
Fever	1/4	1/4	0/4	0/4	0/5	0/5	2/26
Headache	2/4	0/4	0/4	0/4	2/5	1/5	5/26
Nausea	0/4	1/4	0/4	1/4	0/5	1/5	3/26
Leucocyturia	0/4	0/4	0/4	1/4	0/5	2/5	3/26
Erythema at Blood Draw Site	0/4	0/4	0/4	0/4	2/5	0/5	2/26
Erythema at Infusion Site	0/4	0/4	0/4	0/4	1/5	1/5	2/26
Sleepiness	0/4	0/4	0/4	0/4	4/5	1/5	5/26
Sore Throat	1/4	0/4	1/4	1/4	0/4	0/4	3/26

5 There was no apparent relationship between the dose of antibodies and the number of participants who experienced an adverse event and, in general, abnormal laboratory parameters were not clinical significant. There was no trend between the dose of caStx1 or caStx2 and the occurrence of abnormal laboratory values and except the fever reported in Table 4, there were no clinically significant changes in  
10 body weight, vital signs or electrocardiogram measurements (heart rate, PR, QRS, and QT/QTc).

Mean serum concentrations of caStx1 and caStx2 in study participants administered the antibodies individually are shown in Figure 1.

15 The pharmacokinetic results for each study participant in the individual antibody administration study are shown in Table 5. The determination of the pharmacokinetic results for the combined antibody administration study is in progress.

Table 5.  
Pharmacokinetic Parameters for  $\alpha$ Stx1 and  $\alpha$ Stx2 Administered Individually

Participant No.	Dose Group	C <sub>max</sub> (ng/mL)	AUC <sub>inf</sub>	t <sub>1/2</sub> (hours)
001	1 mg/kg $\alpha$ Stx1	23155.29	1976510.68	97.701
002	1 mg/kg $\alpha$ Stx1	28636.93	N/A	N/A
003	1 mg/kg $\alpha$ Stx1	25562.79	2156130.90	85.588
004	1 mg/kg $\alpha$ Stx1	21644.56	2293940.55	112.164
005	3 mg/kg $\alpha$ Stx1	86707.01	10651193.50	155.691
006	3 mg/kg $\alpha$ Stx1	85035.24	18089272.33	184.153
007	3 mg/kg $\alpha$ Stx1	112543.33	N/A	N/A
008	3 mg/kg $\alpha$ Stx1	86116.34	8114842.87	282.396
009	1 mg/kg $\alpha$ Stx2	25086.41	7244993.38	314.921
010	1 mg/kg $\alpha$ Stx2	29603.41	7214392.22	430.969
011	1 mg/kg $\alpha$ Stx2	28666.08	2477386.00	60.832
012	1 mg/kg $\alpha$ Stx2	33610.09	6131600.07	418.828
013	3 mg/kg $\alpha$ Stx2	77715.58	18405696.29	251.760
014	3 mg/kg $\alpha$ Stx2	104325.34	13362783.90	188.628
015	3 mg/kg $\alpha$ Stx2	104839.75	23071589.07	219.575
016	3 mg/kg $\alpha$ Stx2	115883.56	17131825.36	211.623

5

Three (3) out of 26 participants exhibited an induction of human anti-chimeric antibody (HACA) at concentrations close to the detection limit as per Table 6.

Table 6.  
HACA Response

Participant No.	Dose Group	Sampling Day	Antibody Concentration (ng/mL)
016	3 mg/kg $\alpha$ Stx2	Day 57	56.94
007	3 mg/kg $\alpha$ Stx1 and 3 mg/kg $\alpha$ Stx2	Day 29	8.40
		Day 57	18.60
008	3 mg/kg $\alpha$ Stx1 and 3 mg/kg $\alpha$ Stx2	Day 29	8.19

The standard curve range was 7.81-750 ng/mL. None of the samples tested in either study yielded results above the lower limit of quantitation for binding against  $\alpha$ Stx1.

10

15

### **Conclusions**

No study participants had their study medication infusion interrupted or discontinued due to an adverse event (AE) or abnormal finding. No participants dropped out of the studies due to an AE and none experienced a SAE and there were 5 no consistent changes in body weight, vital signs or electrocardiogram measurements between the individual or combined antibody dose groups of  $\alpha$ Stx1 and  $\alpha$ Stx2.

There is an expected proportionality of an approximately 3-fold increase in the Cmax and AUC between the 2 doses for each antibody. At 3 mg/kg the half-life of  $\alpha$ Stx1 and  $\alpha$ Stx2 is approximately 9 days. Human anti-chimeric antibody (HACA) 10 response was minimal.

At the termination of each study a safety committee, which consisted of an independent medical monitor and the Principal Investigator, reviewed the laboratory results, ECG tracings and AEs of all study participants and concluded that  $\alpha$ Stx1 and  $\alpha$ Stx2 administered individually or concomitantly up to 3 mg/kg per antibody was 15 safe and well tolerated. Overall, anti-toxins  $\alpha$ Stx1 and  $\alpha$ Stx2 administered intravenously individually or concomitantly at a dose up to 3 mg/kg per antibody were determined to be safe and well tolerated in healthy adult males and females.

### **Example 2**

20 Toxicology and Immunogenicity of  $\alpha$ Stx1 and  $\alpha$ Stx2 in Mice and Marmosets

#### **Introduction**

Shiga toxin producing Escherichia coli (STEC) are zoonotic pathogens that cause potentially fatal and often epidemic and food or waterborne illness with a 25 clinical spectrum that includes diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (HUS)(Karmali MA, et al. J Infect Dis, 2003: 188 (1 Dec) 1724-1729). STEC produces two distinct Shiga toxin types, Shiga toxin 1 and Shiga toxin 2 (Karmali MA, et al. J Infect Dis, 2004: 189 (1 Feb) 355-359). Currently there is no specific treatment against STEC infection but two specific anti-toxins are in 30 development to treat infections due to Shiga toxin producing bacteria (STPB).

The toxicology and immunogenicity of the anti-toxins tested in two animal species are presented in this example. The mouse was tested because it is a

universally used model for evaluating the toxicity of various classes of chemicals and for which there is a large historical database. Additional toxicology was conducted in the non-rodent marmoset primate model.

## 5    **Objective**

The objective of two non-clinical studies conducted in mice and marmosets was to evaluate the potential acute toxicity and immunogenicity of each chimeric monoclonal antibody,  $\alpha$ Stx1 and/or  $\alpha$ Stx2, following a single or repeat dose of the antibodies administered individually or concomitantly to healthy animals.

10

## **Methods**

In the mouse study, one hundred sixty-seven male and female (167) CD1 mice experimentally naïve and approximately 7 weeks in age were included. All mice had body weights that fell within  $\pm$  20% of the mean body weight for each sex. Animals 15 were randomized by sex into treatment groups. Each animal was implanted with a microchip bearing a unique identification number. Mice were housed individually in suspended, stainless steel, wire-mesh type cages. Temperature was maintained at 18 to 26°C and humidity at 30 to 70%. A 12 hour dark cycle was controlled automatically to provide a cycle of 12 hours of light and 12 hours of dark, unless 20 other light/dark schedules were required by the experimental design. Mice were fed meal Lab Diet® Certified Rodent Diet #5002, PMI Nutrition International, Inc. and water ad libitum.

Dosing solutions were prepared on the day of dose administration. Mice were given a single bolus injection via the tail vein of  $\alpha$ Stx1,  $\alpha$ Stx2 or both  $\alpha$ Stx1 and 25  $\alpha$ Stx2 on Day 1 or Day 1 and Day 8 of the study per Table 7. Dosing solution samples were collected and analyzed for protein concentration by bicinchoninic acid (BCA). Mice serum samples collected on Day 29 were analyzed for anti- $\alpha$ Stx1 and anti- $\alpha$ Stx2 antibodies and isotyping using validated ELISA methods.

Cageside observation and a detailed clinical examination of the skin, fur, eyes, 30 ears, nose, oral cavity, thorax, abdomen, external genitalia, limbs, feet and respiratory, circulatory and nervous systems was performed daily.

Body weights for all mice were measured and recorded the day after arrival, just prior to randomization (Day -1) and weekly during the study.

Blood and urine samples were taken for clinical pathology and serum antibody evaluation as defined in Table 7.

5            Macroscopic, microscopic and organ weight evaluations were conducted post-sacrifice according to the times in Table 7. The mice were examined macroscopically for external abnormalities including masses. The abdominal, thoracic and cranial cavities were examined for abnormalities and the organs removed, examined and placed in fixative. Organ weights for the adrenal gland, brain, heart, kidney, liver, 10 lung, ovary, pituitary, testis, thymus, thyroid (with parathyroid) and uterus with cervix were recorded for all animals at the scheduled necropsies and appropriate organ weight ratios were calculated (relative to body and brain weights). Microscopic examinations were performed on the fixed sections of brain, heart, injection site, kidney, large intestine (cecum, colon, rectum), liver, lung, lymph node (inguinal), 15 small intestine (duodenum, ileum, jejunum), spleen, thymus and gross lesions per Table 7.

Table 7.  
Mouse Study Design

Grp No.	Number of mice		Dose mg/Kg of each antibody (IV bolus)	Time of Dose	Clinical Pathology collection times (Hematology, serum biochemistry, urinalysis and serum antibody evaluation)				Necropsy/ Organ collection times				Microscopic Pathology			
	Projected				Actual		Actual		Actual		Actual		Actual			
	M	F			M	F	M	F	M	F	M	F	M	F		
1a	10	10	0 (Fb)	Day 1	Day 8*		10	10	Day 8		10	10	AR	1	2	
1b	10	10	0 (Fb)	Day 1, 8	Day 15*		10	10	Day 15		10	10	Day 15	10	10	
1c	10	10	0 (Fb)	Day 1, 8	Day 29**		10	10	Day 29		10	10	Day 29	10	10	
2a	10	10	30	Day 1	Day 8*		10	10	Day 8		10	10	AR	-	2	
2b	10	10	30	Day 1	Day 29**		10	10	Day 29		10	10	AR	1	-	
3a	10	10	30 x 2	Day 1, 8	Day 15*		10	3*	Day 15		10	3	Day 15	10	3	
3b	10	10	30 x 2	Day 1, 8	Day 29**		10	4*	Day 29		10	4	Day 29	10	10	
4	10	10	caStx1@30	Day 1	Day 29**		10	10	Day 29		10	10	AR	-	-	
5	10	10	caStx2@ 30	Day 1	Day 29**		10	10	Day 29		10	10	AR	-	-	

20 Fb = formulation buffer, Group 1 dosed 2 x 6 mL/Kg of vehicle except for animals sacrificed on Day 8 (1 dose).

\* : 5/sex/group used for hematology analysis and 5/sex/group used for clinical chemistry.

\*\* : 5/sex/group used for hematology & serum antibody analysis and 5/sex/group used for clinical chemistry.

AR: Microscopic evaluation was performed as required based on microscopic pathology evaluations of Group 3.

**a, b:** Due to lack of product only 7 of the 20 females dosed on Day 1 received the second dose on Day 8 (all animals survived).

**a:** 2 of the 3 females dosed for Group 3a were used for hematology analysis and 1 was used for clinical chemistry.

**5 b:** 2 of the 4 females dosed for Group 3b were used for hematology & serum antibody anal & 2 were used for clinical chem.

Statistical comparisons were conducted between the mouse controls in group 1 and the treated mice in groups 2, 3, 4 and 5.

**10** In the marmoset study twenty-two male and female marmosets (44 in total) experimentally naïve and approximately 1 to 5 years old (*Callithrix jacchus*) were used in the study after 9 weeks acclimation. Males placed on the study had body weights from 299 to 372 g and females 313 to 435 g. Animals were randomly allocated to dosing groups based on stratified body weight. Each animal was  
**15** implanted with a microchip bearing a unique identification number. Marmosets were housed individually by sex in a climate controlled room. Temperature was maintained at 24 to 28°C and relative humidity at 30 to 70%. A 12 hour light/dark cycle was maintained. Each animal was offered a variety of food which was prepared fresh each day and given twice a day according to a meal plan. Animals were also  
**20** given food rewards immediately after each handling/manipulation. Tap water was provided ad libitum via an automatic water system or bottles except during urine collection.

Dosing solutions were prepared on the day of dose administration. Male and female marmosets were administered  $\alpha$ Stx1,  $\alpha$ Stx2 or both  $\alpha$ Stx1 and  $\alpha$ Stx2 on  
**25** Day 1 or Day 1 and Day 8 of the study per Table 8. The test item was administered by an ambulatory infusion pump over 30 minutes through an indwelling catheter in the leg or arm vein. Dosing solution samples were collected and analyzed for protein concentration by bicinchoninic acid (BCA).

All marmosets were observed cageside twice daily throughout the duration of  
**30** the study for morbidity, mortality, injury, behavior, appearance and feces. Body weights for all marmosets were measured and recorded at least once predose, just prior to randomization (Day -1) and weekly during the study.

Indirect ophthalmoscopy was performed on all animals once during the predose phase and before necropsy.

5 Heart rate, RR, PR, QRS, QT and QTc intervals were measured by electrocardiography once during the predose phase and directly after the end of the 30 minute infusion on Day 1 or Day 8.

10 Blood samples for clinical pathology (hematology and chemistry) and serum antibody evaluation were taken from the femoral vein/artery for designated marmosets on Days 8, 15 or 29 according to the sampling schedule in Table 8. Samples for urinalysis over a 16 hour period were also collected as per Table 8. Marmoset serum samples were analyzed for anti- $\alpha$ Stx1 and anti- $\alpha$ Stx2 antibodies and isotyping using validated ELISA methods.

15 Macroscopic, microscopic and organ weight evaluations were conducted postmortem according to the times in Table 8. A full macroscopic examination was performed. Organ weights for all the same organs as listed in the mice study along with eyes and optic nerve, epididymides, prostate, seminal vesicles and spleen were recorded for all surviving animals at the scheduled necropsies and appropriate organ weight ratios were calculated (relative to body and brain weights). Microscopic examinations were performed on the same fixed sections as listed in the mice study along with that of the eyes and optic nerve, skin and stomach per Table 8.

20 Table 8.  
Marmoset Study Design

Grp No.	Number of Marmosets		Antibody (30 minutes IV infusion)	Dose mg/kg, each Mab	Time of Dose	Antibody & Clinical Pathology Blood & Urine collection times		Necropsy/Organ collection times		Microscopic Pathology		
	Projected					Actual		Actual		Actual		
	M	F	$\alpha$ Stx1	$\alpha$ Stx2		M	F	M	F	M	F	
1a	2	2	-	-	0 (Fb)	Day 1	Day 8	2	2	Day 8	2	2
1b	2	2	-	-	2 x 0 (Fb)	Day 1, 8	Day 15	2	2	Day 15	2	2
1c	2	2	-	-	2 x 0 (Fb)	Day 1, 8	Day 29	2	2	Day 29	2	2
2a	2	2	Yes	Yes	30	Day 1	Day 8	2	2	Day 8	2	2
2b	2	2	Yes	Yes	30	Day 1	Day 29	2	2	Day 29	2	2
3a	2	2	Yes	Yes	2 x 30	Day 1, 8	Day 15	2	2	Day 15	2	2
3b	2	2	Yes	Yes	2 x 30	Day 1, 8	Day 29	2	2	Day 29	2	2
4a	2	2	Yes	-	30	Day 1	Day 8	2	2	Day 8	2	2
4b	2	2	Yes	-	30	Day 1	Day 29	2	2	Day 29	2	2
5a	2	2	-	Yes	30	Day 1	Day 8	2	2	Day 8	2	2
5b	2	2	-	Yes	30	Day 1	Day 29	2	2	Day 29	2	2

Fb = formulation buffer, Mab = Monoclonal antibody

AR: Microscopic evaluation was performed As Required based on microscopic pathology evaluations of Group 3.

YES: performed after end of in-life – All animals survived.

\*: Microscopic pathology was not required based on pathology evaluations of Group 3.

Data from marmosets that were administered the monoclonal antibodies were compared with the control data. No statistical analyses were performed.

## Results

5 All dosing solution concentrations were within acceptance values for slope, intercept and R2 and reproducibility of sample values were within 6%. All animals from both studies survived the in-life phase of the study until their scheduled termination. Test article related changes for the endpoints observed are presented in Table 9 for the mice and marmosets respectively.

10

Table 9.  
Mice and Marmosets Test Article Related Effects

Endpoints observed	Test article-related changes	
	Mice	Marmosets
<b>Cage side observations:</b>		
Body weights:	None	None
Food consumption:	None	None
Feces:	N/A	None
Indirect ophthalmoscopy:	N/A	None
Electrocardiography:	N/A	None
Detailed clinical examinations:	None	None
<b>Clinical pathology:</b>		
Hematology:	None	None
Clinical chemistry:	None	None
Urine analysis:	None	None
<b>Necropsy examinations:</b>		
Macroscopic observations:	None	None
Organ weights:	None	None
Microscopic examinations:	None	None
<b>Comparisons:</b>		
Controls versus treatment groups:	No test article-related changes	No test article-related changes
Gender difference:	No gender difference	No gender difference

15 In the mouse study 2 animals exhibited a low mouse anti-human antibody (MAHA) response to  $\alpha$ Stx2 on Day 29. None of the mice that received  $\alpha$ Stx1 yielded results above the lower limit of quantitation (LLOQ) for binding against  $\alpha$ Stx1. The concentrations and isotyping of the positive responses are presented in Table 10. Of the 44 marmoset samples tested, none were found to contain anti-therapeutic antibodies above the LLOQ.

20

**Table 10.**  
**Mouse Serum Antibody Concentration Analysis for Binding Against anti- $\alpha$ Stx2**

Sample ID	Gender	Dose	Antibody Conc.	Isotype
6067	Male	60/60 mg/kg ( $\alpha$ Stx1/ $\alpha$ Stx2)	159.25ng/mL	IgG1, IgG2b
6180	Female	30mg/kg ( $\alpha$ Stx2)	373.80ng/mL	IgG1, IgG2b

## 5 Conclusions

$\alpha$ Stx1 and  $\alpha$ Stx2, administered intravenously individually or concomitantly, at a single or repeat dose of 30 mg/kg or 60 mg/kg per antibody respectively, were well tolerated and did not show any consistent adverse treatment-related effects in male and female mice or marmosets. The anti-therapeutic response was minimal and 10 only limited to mice.

In conclusion, the monoclonal antibodies against Shiga toxins 1 and 2 were tested in two species at doses up to 20 times above the intended clinical dose and were not associated with any systemic toxicity.

15

## Example 3

### Pharmacokinetics of $\alpha$ Stx1 and $\alpha$ Stx2 in Mice

#### Introduction

Shiga toxins 1 and 2 are the virulence factors that are responsible for the 20 complications that come from infection by Shiga toxin producing bacteria (STPB) (Gavin PJ, et al. J Clin Microbiology, Apr 2004, p.1652-1656). Shiga toxin producing Escherichia coli (STEC) strains represent the most important recently 25 emerged group of food-borne pathogens (Blanco JE, et al. J Clin Microbiology, Jan 2004, p. 311-319). STEC causes a potentially fatal foodborne illness whose clinical spectrum includes asymptomatic carriage, nonspecific diarrhea, bloody diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (HUS) (Karmali MA, Molecular Biotechnology, Vol. 26, 2004, p. 117-122; Klein EJ, et al. J Pediatr, Aug 2002, Vol 141, No. 2, p. 172-177). Two specific monoclonal antibodies against Shiga toxin 1 and 2 have been developed to treat STPB infection.

$\text{caStx1}$  and  $\text{caStx2}$  are chimeric monoclonal IgG1 antibodies that bind with Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) respectively.  $\text{caStx1}$  recognizes the B subunit of Stx1 and  $\text{caStx2}$  recognizes the A subunit of Stx2.

5 The pharmacokinetics of these specific antitoxins in mice are presented in this example.

### Objective

10 The objective of this study was to evaluate the pharmacokinetic profile of each monoclonal antibody following a single dose of antibodies  $\text{caStx1}$  and/or  $\text{caStx2}$  administered individually or concomitantly to healthy CD-1 mice.

### Methods

One hundred thirty male and female CD1 mice were used in the study after 1 week of acclimation. All mice placed on the study had body weights that fell within  $\pm$  15 20% of the mean body weight for each sex. Animals were randomized by sex into treatment groups using a standard block randomization procedure. Each animal was implanted with a microchip bearing a unique identification number. Mice were housed in suspended, stainless steel, wire-mesh type cages. Temperature was maintained at 64 to 79°F and humidity at 30 to 70%. A 12 hour dark cycle was 20 provided via an automatic timer for approximately 12 hours per day. Mice were fed meal Lab Diet® Certified Rodent Diet #5002, PMI Nutrition International, Inc. and water ad libitum.

Dosing solutions were prepared on the day of dose administration. Male and female mice were given a single bolus injection via the tail vein of  $\text{caStx1}$ ,  $\text{caStx2}$  or 25 both  $\text{caStx1}$  and  $\text{caStx2}$  on Day 1 of the study per Table 11. Blood samples were taken via the maxillary vein for the first designated time points and via cardiac puncture under anesthesia for the last or only designated time point according to the sampling schedule in Table 11.

Table 11.  
Study Design

Table 1. Study Design						
Group	Test Article	# Male	# Female	Dose Level (mg/kg)	Dose Volume (mL/kg)	Sample Time Post Dose
i	FB	5	5	0	1.0	1 hr
ii	caStx1	5	5	3	1.0	10 min
iii	caStx1	5	5	3	1.0	1 hr, 72 hrs, Day 7
iv	caStx1	5	5	3	1.0	24 hrs, Day 14, Day 28 <sup>a</sup>
v	caStx1	5	5	3	1.0	48 hrs, Day 21 <sup>b</sup>
vi	caStx2	5	5	3	1.0	10 min
vii	caStx2	5	5	3	1.0	1 hr, 72 hrs, Day 7
viii	caStx2	5	5	3	1.0	24 hrs, Day 14, Day 28 <sup>a</sup>
ix	caStx2	5	5	3	1.0	48 hrs, Day 21 <sup>b</sup>
x	caStx1 / caStx2	5	5	3/3	1.0	10 min
xi	caStx1 / caStx2	5	5	3/3	1.0	1 hr, 72 hrs, Day 7
xii	caStx1 / caStx2	5	5	3/3	1.0	24 hrs, Day 14, Day 28 <sup>a</sup>
xiii	caStx1 / caStx2	5	5	3/3	1.0	48 hrs, Day 21 <sup>b</sup>

FB = formulation buffer

<sup>a</sup> Female animals (3/5 in Group iv, 3/5 in Group viii and 1/5 in Group xii had samples drawn on Day 22).

<sup>b</sup> Female animals (4/5 in Group v, 3/5 in Group ix and 1/5 in Group xiii had samples drawn on Day 16).

Samples of dosing formulations at each concentration were collected pre and 5 post dose administration on Day 1 and analyzed for protein concentration by bicinchoninic acid (BCA). Serum samples were analyzed to determine the caStx1 and caStx2 concentrations using validated ELISA methods. Nominal sample collection times were used in the pharmacokinetic data analysis. Serum concentrations of caStx1 and caStx2 were used to construct semi-logarithmic serum concentration 10 versus time curves. Pharmacokinetic parameters were determined by non-compartmental methods. Values below the limit of quantitation were treated as 0.

Observations for mortality, morbidity, injury and availability of food and 15 water were conducted twice daily for all animals. Mice body weights were measured on Day -1, Day 1, and weekly thereafter. Necropsy examinations were performed on animals that died on study.

## Results

No basal diet or water contaminants likely to interfere with the results of this 20 study were reported. There was no test article related mortality or adverse clinical observations. All dosing solution concentrations were within acceptance values for slope, intercept and R2 and reproducibility of sample values were within 6%.

The minimum concentration of  $\alpha$ Stx1 and  $\alpha$ Stx2 in the serum samples that could be accurately determined by ELISA was 130 ng/mL and 150 ng/mL respectively. Serum concentrations of  $\alpha$ Stx1 and  $\alpha$ Stx2 in male (22.5 – 37.0 g) and female (21.6 – 29.9 g) mice administered alone or in combination are shown in

5 Figures 2 and 3.

The pharmacokinetic parameters of area under the serum concentration – time curve from time 0 to infinity (AUC<sub>0-∞</sub>), the terminal half-life (t<sub>1/2</sub>), serum clearance (CL) and volume of distribution at steady state (V<sub>ss</sub>) are presented in Table 12.

10

Table 12.  
Pharmacokinetic Parameters for  $\alpha$ Stx1 and  $\alpha$ Stx2 Administered Alone or in Combination

	AUC <sub>0-∞</sub> (hr·ng/mL)		CL (mL/kg/min)		t <sub>1/2</sub> (hr)		V <sub>ss</sub> (mL/kg)	
	M	F	M	F	M	F	M	F
$\alpha$ Stx1 alone	1,760,000	2,310,000	0.0284	0.0216	89.2	159	156	109
$\alpha$ Stx2 alone	2,570,000	1,880,000	0.0195	0.0266	99.0	113	159	240
$\alpha$ Stx1 (co-administered with $\alpha$ Stx2)	2,230,000	2,260,000	0.0225	0.0222	90.6	98.1	103	129
$\alpha$ Stx2 (co-administered with $\alpha$ Stx1)	2,640,000	2,460,000	0.0189	0.0203	117	116	193	211

15

Conclusions

Serum concentrations of  $\alpha$ Stx1 and  $\alpha$ Stx2 were similar in male and female mice whether administered alone or in combination. There were no consistent differences between males and females in the other pharmacokinetic parameters calculated. CL was low for both  $\alpha$ Stx1 and  $\alpha$ Stx2 which is consistent with the long

20

half-lives and expected for monoclonal antibodies. V<sub>ss</sub> was also low for both  $\alpha$ Stx1 and  $\alpha$ Stx2 which is indicative that the antibodies are retained within the blood volume. Combined administration of  $\alpha$ Stx1 and  $\alpha$ Stx2 did not alter AUC<sub>0-∞</sub>, t<sub>1/2</sub>, CL or V<sub>ss</sub>.

25

PK results are consistent with the structure of the anti-Shiga toxin monoclonal antibodies and with the known absence of cross-reactivity with human or animal tissues.

**Example 4**  
**Renal Involvement in Children with STEC Colitis**

**Introduction**

5 The definition of hemolytic uremic syndrome (HUS) includes hemolytic anemia, thrombocytopenia and acute nephropathy/renal failure. Up to 25% of children who survive STEC associated (enteropathic) hemolytic uremic syndrome (eHUS) develop long-term renal abnormalities. As Garg et al pointed out, the prognosis of acute, self-limited Escherichia coli O157:H7 gastroenteritis has never been previously 10 studied (Garg et al., Kidney Int. 2006; 70: 807-812). A recent cohort-control study, initiated after the Walkerton outbreak due to contaminated drinking water, which affected about 2000 people, demonstrated slightly increased rates of hypertension and reduced kidney function in affected adults four years after outbreak-associated STEC (plus *C. jejuni*) diarrhea (Garg et al., CMAJ. 2005;173: 261-268; Garg et al., Kidney 15 Int. 2005; 67: 1476-1482). These studies do not include laboratory analyses at the time of the STEC infection. The current analysis was undertaken to address the question whether children with STEC diarrhea without HUS demonstrate disease-associated renal abnormalities.

**20 Objectives**

To determine whether children with acute STEC colitis demonstrate urinary/renal abnormalities.

**Design**

25 Retrospective chart review and data analysis. Data are from a single, tertiary care university-affiliated pediatric hospital with an active emergency medicine department. The hospital serves >1 million total population.

**Methods**

30 Patients with STEC isolates between 1992 and 2006 were identified from the hospital microbiology laboratory records. Available clinical and demographic information was extracted from medical charts (paper and electronic, if available)

using a standardised questionnaire and entered into the Montreal Children's STEC Disease database, which now comprises 186 children with culture-proven STEC O157 infection.

5 Data were analysed with respect to final diagnosis (STEC diarrhea, HUS), demographic information

For the purposes of this study, the diagnostic criteria for indicated diseases are as follows: haematuria, urine RBC (red blood cells) > 5 per HPF (high power field) or dipstick analysis greater than "trace"; proteinuria, urine protein  $\geq 0.3$  g/l (30 mg/dl) or urine protein:creatinine ratio (U p/c) > 0.2 g/g; pyuria, urine WBC (white blood cells) >5 per HPF or positive dipstick analysis; and azotaemia, serum creatinine level exceeding 1.5 x reference limit for age and gender. Day of first STEC infection-related symptoms (generally diarrhea or abdominal cramps), referred to as DDO (Day of disease onset)

15 **Results**

Matching urinalyses, plasma creatinine and hematological profiles were available from 103/186 patients with *E. coli* O157 isolates (mostly O157:H7). Twenty-two patients developed HUS. Hematuria, defined as positive dipstick analysis or >5 RBC per high power field (HPF), was noted in 14 patients with non-20 HUS colitis (16.4%) on day 6 of onset (median; range 1-10 days); nine patients (10.6%) had proteinuria  $\geq 0.3$  g/l on day 6 (4-15). Conversely, 3/6 patients with matching laboratory studies prior to apparent HUS presented with hematuria on day 5 (3-6), and 2/6 with proteinuria on days 3, corresponding to two days before HUS. Age-adjusted plasma creatinine levels were normal in all patients except those with 25 manifest HUS. These data are set forth in Tables 13-16.

Table 13.

Diagnosis	N	Urinalysis <sup>1</sup>	Results	
			Haematuria	Proteinuria
STEC colitis/diarrhea only	163	85	14 (16.4%)	9 (10.6%)
HUS	22	18	18 (100%)	17 (94.4%)
Total	186	103	32 (31.1%)	25 (24.3%)

Table 14

Diagnosis	N (positive test result)	Urinalysis <sup>1</sup>	First Positive Test Result (Day of Disease Onset) <sup>1</sup>	
			Haematuria	Proteinuria
STEC colitis/diarrhea only	14	Mean (SD)	6.2 ± 2.6	7.1 ± 3.9
		Median (range)	6 (1-10)	5.5 (4-15)
HUS	18	Mean (SD)	7.1 ± 3.4 (n = 18)	7.3 ± 3.0 (n = 17)
		Median (range)	8 (2-14)	8 (2-14)

<sup>1</sup> Within 15 days of disease onset

5

Table 15  
Scoring of urinalysis results in patient with STEC infection<sup>1</sup>

		Most Abnormal Test Result	
		Haematuria	Proteinuria
Non-HUS	Mean (SD)	2.1 ± 0.9 (n = 14)	1.8 ± 1.0 (n = 9)
	Median (range)	2 (0-3)	1 (1-3)
HUS	Mean (SD)	2.9 ± 0.5 (n = 18)	2.9 ± 0.25 (n = 16)
	Median (range)	3 (1-3)	3 (2-3)

<sup>1</sup> Scores as defined in Table 16

10

**Table 16**  
**STEC Disease Severity and Progression (SDSP) Scale**

<b>Nephropathy</b>						
<b>Category</b>	<b>Test item</b>	<b>0</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
<b>Renal function</b>	Serum creatinine <sup>1</sup>	< 1.5 x upper normal	1.5 to 2x upper normal	>2 to 4x upper normal	>4x upper normal	Dialysis
<b>Haematuria</b>	RBC [per HPF]	? 5	>5-10	>10-30	>30	Gross haematuria
	Dipstick analysis	Negative or trace	Small	Moderate	Large	
<b>Proteinuria</b>	Dipstick [g/l] (mg/dl)	<0.3 (30)	0.3 - <1 (30-100)	1 - <3 (100-<300)	?3.0 (?300)	-
	U prot/creat [g/g]	<0.2	0.2 - <1	1 - <3	?3.0	-
<b>Pyuria</b>	WBC [per HPF]	?6	6-10	>10-50	>50	-
	Leukocyte esterase (dipstick)	Negative	Positive 1+	Positive 2+	Positive 3+	-

<sup>1</sup> relative to age and gender

## 5 Conclusion and Discussion

16% of children with non-HUS STEC colitis were found to have proteinuria and/or haematuria, compared to 50% of pre-HUS patients  $\geq 2$  days before microangiopathic hemolytic anemia became evident (falling hemoglobin and/or platelets or increasing serum creatinine). These results indicate that STEC may induce HUS-independent subtle renal injury mirroring findings in follow-up studies of children with bona fide HUS. The clinical significance and pathomechanism of STEC-colitis associated urinary abnormalities, if any, is unclear. Similar arguments apply to patients with “incomplete” HUS lacking evidence of renal failure.

15

## Example 5

### A Quantitative Disease Scale for Shiga Toxin Producing Escherichia coli Infections

#### Introduction

Infections by Shiga toxin (Stx) producing *E. coli* (STEC) can lead to severe complications and result in significant human morbidity and economic losses. Hemorrhagic colitis (HC), haemolysis, nephropathy and central nervous system involvement are thought to result from the absorption of *in vivo* produced Stx into the regional (colon) or systemic circulation. Evidence suggests that Stx-mediated injury

to the endothelium and other tissue causes a disordered microvascular and inflammatory response.

A limited set of signs and symptoms has been linked to Stx-inflicted tissue injury based on clinical observation, animal experimentation and cell biology 5 experiments. Some of these presumed Stx-mediated events (STMEs) precede the appearance of the enteropathic haemolytic uremic syndrome (eHUS): cramping abdominal pain, bloody diarrhoea or hemorrhagic colitis. Others are part of HUS, such as thrombocytopenia, hemolytic anemia and various degrees of renal injury, but have also been noted in patients not fulfilling the diagnostic criteria of HUS 10 ("incomplete" HUS). Rare extraintestinal and extrarenal manifestations (cerebral ischemia/stroke, seizures, cardiomyopathy, diabetes mellitus) are also thought to be caused by Stx or Stx-induced effects. Stx (or STEC infection) imparts a distinct inflammatory response associated with the induction of IL-8, TNF $\alpha$ , acute phase reactants and vasoactive mediators (Tarr PI et al. 2005; Bitzan M et al. 1998), which 15 may explain why elevated peripheral neutrophil counts and C-reactive protein correlate positively with increased HUS risk.

While the clinical diagnosis of haemolytic uraemic syndrome (HUS) and HC is straightforward, criteria to define the severity or progression of STMEs and other clinical signs, or to evaluate the effect of therapeutic or preventive measures are 20 lacking.

### Objectives

To generate and test a quantitative disease severity scale reflecting STEC and Stx-induced microvascular and tissue injury and to apply the resultant disease scale in 25 a retrospective analysis using a well-described cohort of children with culture-proven STEC infection.

## Methods

- Literature search for descriptions of STEC infections and markers/predictors of (complicated) HUS
- Selection and adaptation of relevant items of the Common Terminology Criteria for Adverse Events (CTCAE V.3.0), based on known manifestations of STEC infections including HUS and expert opinion.
- Identification of all patients with available STEC isolates from medical microbiology laboratory records.
- Review of patients' medical files (paper charts and electronic records) and extraction of pertinent demographic, laboratory and clinical information using a standardized questionnaire.
- Construction of a dedicated STEC disease database.

## Results

### 15 Patient population and Database

The presence and evolution of relevant clinical signs and symptoms, commonly available biological markers, and outcomes, if documented, of 186 patients with a wide spectrum of STEC disease manifestations, diagnosed between 1992 and 2006, were captured to create the comprehensive Montreal Children's STEC Disease 20 (MCSD) database. Patient population and generation of the database are depicted in Figure 4.

### Creation of a Disease Severity Scale

A set of disease-related clinical and biological parameters was selected and 25 assigned linear numerical values, in analogy to the Common Terminology Criteria for Adverse Events. Items were grouped into four pathological categories: enteropathy, inflammation and vasculopathy, microangiopathic hemolytic anemia and nephropathy (Table 17), plus extraintestinal and extrarenal organ involvement (not shown). The instrument is called "Shiga toxin/STEC Disease Severity and Progression" (SDSP) 30 scale.

Table 17.  
STEC/Shiga toxin Severity and Progression Scale (abbreviated)

Grade	0	1	2	3	4
<b>A Enteropathy</b>					
<b>Diarrhea (stool frequency)</b>	No diarrhea (baseline)	<5	5 - <10	10 - <15	? 15
<b>Abdominal pain or cramps Age-adjusted pain scale</b>	No pain (0)	Mild pain (1-3)	Moderate pain (4-6)	Severe pain (7-9)	Unbearable pain (10)
<b>Bloody diarrhea (hemorrhage)</b>	No visible blood	Occasional streaks of blood	Blood mixed with stool	Frank blood (colorectal hemorrhage)	Hemorrhage requiring intervention
Grade	0	1	2	3	4
<b>B Inflammation &amp; Vasculopathy</b>					
<b>WBC [N/nl] &lt; 2 yrs</b>	<15	-	15 - <18	18 - <22	? 22
2-5.9 yrs	<12	12 - <14	14 - <18	18 - <22	? 22
? 6 yrs	< 10.0	10 - <14	14 - <18	18 - <22	? 22
<b>Cerebrovascular ischemia</b>	None	-	Migraine-type headache	Transient ischemic attacks (TIA)	Stroke, neurological deficit or blindness
Grade	0	1	2	3	4
<b>B Inflammation &amp; Vasculopathy</b>					
<b>WBC [N/nl] &lt; 2 yrs</b>	<15	-	15 - <18	18 - <22	? 22
2-5.9 yrs	<12	12 - <14	14 - <18	18 - <22	? 22
? 6 yrs	< 10.0	10 - <14	14 - <18	18 - <22	? 22
<b>Cerebrovascular ischemia</b>	None	-	Migraine-type headache	Transient ischemic attacks (TIA)	Stroke, neurological deficit or blindness
Grade	0	1	2	3	4
<b>D Nephropathy</b>					
<b>Hematuria (RBC per HPF or dipstick)</b>	None (<6)	Small (6-10)	Moderate (>10-30)	Large (>30)	-
<b>Proteinuria ([g/l] or pro/creat)</b>	<0.3 (<0.2 g/g)	0.3 - <1 (0.2 - <1 g/g)	1 - <3 (1 - <3 g/g)	? 3 (>3 g/g)	-
<b>Pyuria (WBC/HPF)</b>	? 3	3-10	>10-50	>50	-
<b>Serum creatinine (relative for age, gender)</b>	Normal	? 1.5 -2 x upper normal	>2.4 x upper normal	>4 x upper normal	Dialysis
<b>Hyponatremia (μM)</b>	? 135	<134-131	<131-127	<127	Hyponatremic seizure

#### Validation of the STEC Disease Severity Scale

To validate the "Scale", scores for presumed STMEs and other clinical markers were compared with relevant outcome measures. An example is shown in Figure 5. The STME "bloody diarrhea" was correlated with the outcome "days of hospitalization". We demonstrate that a high score for bloody diarrhea translates into increased hospitalization. Not unexpectedly, however, this correlation is lost in patients with HUS due to secondary events dictating their hospital stay (compare the two lower panels in Figure 5).

### Utility of the STEC Disease Severity Scale

The “Scale” was conceived to “measure”, in numeric, unified terms, the severity and evolution of STEC infections, e.g. in intervention studies. An additional, novel application is demonstrated in Figure 6. We used the scale to visually display 5 the dynamics of the disease. We tentatively labeled this method “Disease Expression Array.”

### Summary and Conclusions

We developed a new instrument, the “Shiga toxin/STEC Disease Severity and 10 Progression” (SDSP) scale, as a tool to quantify STMEs and other clinical and laboratory abnormalities in patients with a wide spectrum of STEC infections.

### Example 6

#### 15 Epidemiological Evaluation of Shiga Toxin Producing Escherichia coli (STEC) Infections in the European Community

### Background

The majority of published reports on the epidemiology of STEC infections focus on outbreaks and the haemolytic uraemic syndrome (HUS). Publications 20 providing global epidemiological figures, particularly of incident STEC infection rates in Europe, are scarce. Diagnostic challenges and reporting bias resulted in variable and distorted estimates of STEC infection and of extraintestinal complications hampering risk/benefit analyses for future preventive or therapeutic strategies.

25

### Objective

To establish the incidence of STEC infections in the European Community utilizing a variety of publicly available data sources and modelling strategies.

### 30 Methods

We have estimated the prevalence of STEC infections in Europe based on the literature using three different approaches:

- Extrapolation of STEC infection incidence from STEC registries and other estimates reported as such in the literature.
- Extrapolation of the number of STEC infections based on literature assessment of HUS incidence, assuming that HUS represents 15% of the total STEC infections; a sensitivity analysis was also made using a 5% rate of HUS in STEC infections.
- Extrapolation of the number of STEC infections based on the literature reported incidence of childhood gastroenteritis assuming that a certain proportion of them should be STEC as reported by a few prospective papers.

10

## Results

The three different approaches lead to different estimates – not a surprising situation given the clinical context. No evaluation of the prevalence of the condition in the adult and elderly has been made given the absence of information in the literature

15 literature

### *Extrapolation of STEC infection incidence from STEC registries STEC in Finland*

Study reported by Keskimaki in 1998 on the 1990–1997 period; in the first 20 part of that period the prevalence of STEC was evaluated to be 0.013 cases per 100 000 per year; it was later realized that the majority of STEC infections are due to non-O157:H7 strains; the incidence was evaluated to 0.73 cases per 100 000 (Keskimäki et al., J Clin Microbiol 36, 3641-3646 (1998)).

25 STEC infections in Spain

Blanco et al. published in 2004 a study on stool specimens of patients from the Lugo Hospital in Spain. STEC strains were detected in 2.5% of the 5,054 cases; O157:H7 represented 0.5% of cases and non-O157:H7 represented the balance (Blanco et al., J Clin Microbiol 42, 311-319 (2004)).

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### STEC infections in Denmark

Ethelberg reported 425 patients with STEC infections in Denmark from January 1997 to May 2003 (Ethelberg et al., Emer Infect Dis 10, 842-847 (2004)). Most of the cases were sporadic.

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### STEC infections in Netherlands

In 2004, Havelaar evaluated the median number of incident cases of STEC to be 1250 cases per year for the Netherlands (Havelaar et al., Epidemiol Infect 132, 467-484 (2004)). The estimate was derived from a case-control study which found 4 out of 798 faecal samples tested to be positive for STEC – a rate close to that reported in Spain.

### STEC infections in Ireland

Garvey and McKeown published in 2003 data derived from the National Disease Surveillance Centre (NDSC) for the 1999-2003 period (Garvey and McKeown, Epidemiology of Verotoxigenic E. coli O157 in Ireland, 2003. NDSC (2003)). The surveillance was limited to O157:H7 (Table 18).

20

**Table 18.**  
**Crude Incidence Rate of O157:H7 infections in Ireland**

Year	Numbers of cases (incl. non-residents)	Crude incidence rate (95% CI) per 100,000 population
1999	51	1.4 (1.0-1.8)
2000	37(42)	0.9 (0.6-1.3)
2001	50 (52)	1.3 (0.9-1.6)
2002	68 (70)	1.7 (1.3-2.2)
2003	82 (86)	2.1 (1.6-2.6)

### *Extrapolation of the number of STEC infections based on HUS incidence*

25 HUS incidence in France

Incidence of HUS in 1998 was 0.7 cases per 100 000 children aged under 15 years (Haeghebaert et al., Eurosurv 5, 68-73 (2000)). Population under 15 years of age represents 11 121 100 persons over 59 630 100 or 18.65% of the French

population (Eurostat 2004). Incidence of HUS would therefore be estimated to be 0.131/100 000 in the overall population.

#### HUS incidence in Germany and Austria

5 A four-year prospective multicentre study across Germany and Austria was conducted between 1997 and 2000 (Gerber et al., *J Infect Dis* 186, 493-500 (2002)). 394 paediatric HUS reported translate into an annual incidence in Germany of 0.71 cases per 100 000 children <15 years (range 0.69-0.75); in Austria the number were respectively 0.51 cases for 100 000 children.

10 In Germany the population <16 years of age is 12 415 500, out of a total population of 82 536 700 or 15.04% (Eurostat 2004); the incidence of HUS in the general population would be 0.107/100 000.

In Austria the population <16 years of age is 1 335 200 out of a total population of 8 067 300 or 16.55%; the incidence of HUS would be 0.060/100 000.

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#### HUS incidence in the UK and Ireland

The average annual incidence (Lynn et al., *Emerg Infect Dis* 11, 590-596 (2005)) was 0.71/100 000 in children <16 years of age in the most recent cohort compared to 0.79 in a previous cohort. UK population <16 years of age is 11 126 200 out of a UK total population of 59 328 900 or 18.75%; the incidence of HUS being 0.71/100 000 in this population, the overall incidence rate would be 0.133/100 000.

#### HUS incidence in Italy

25 Tozzi published in 2003 an incidence of HUS in Italian children <15 years of 0.28 per 100 000 over a 13 year surveillance period (Tozzi et al., *Emerg Infect Dis* 9, 106-108 (2003)).

30

Data regarding HUS incidence in Europe are set forth in Table 19.

**Table 19:**  
HUS incidence per 100 000 in Europe

	Population < 15 years of age (000's)	Total population (000's)	% of population <15 years of age	Incidence in <16 years of age population per 100 000	Incidence in the entire population per 100 000
EU-25	75 415	453 685	16.62%	0.79	0.13132
EU-15	62 666	379 484	16.51%	0.79	0.130456
Eurozone	48 915	305 831	15.99%	0.79	0.126354

5 HUS is a complication of approximately 15% of STEC infections, however the rate of HUS following STEC infection varies greatly in the different literature reports. To take this variation into account we have used a 5% rate of HUS  
10 occurrence to serve as a sensitivity analysis as displayed in Table 20 below.

**Table 20**  
Incidence of STEC infections per 100 000 in Europe – derived from HUS rates

	Total population (000's)	STEC Incidence if 15% HUS rate (per 100,000)	STEC incidence if 5% HUS rate (per 100,000)	Total number of STEC cases if 15% HUS rate	Total number of STEC cases if 5% HUS rate
EU-25	453 685	0.875466	2.626397	3 972	11 916
EU-15	379 484	0.86971	2.609129	3 300	9 901
Eurozone	305 831	0.842357	2.527072	2 576	7 729

15 *Extrapolation of the number of STEC infections based on the literature reported  
incidence of childhood gastroenteritis*

The prospective assessment of community acquired gastroenteritis published by Frühwirth et al. in 2001 was our proxy reference for the incidence of  
20 gastroenteritis in Europe (Frühwirth et al., Arch Dis Child 84, 393-397 (2001).). The study population at risk comprised 6969 children up to the age of 4 years followed-up in prospective, population based, multicentre study. The incidence of acute  
gastroenteritis was 4.67 per 100 children per year. We extrapolated that incidence to  
the <15 years old age group of the three different European populations defined by  
25 Eurostat to evaluate the maximum number of gastroenteritis that could be observed in the age group. Rate of STEC infections in community-acquired gastroenteritis was based on the Blanco 2004 report where the rate of STEC infections was 2.5% of the

stools. These numbers are comparable to the 4 cases of STEC reported in a 798 stools series in Netherlands (Table 21).

5 **Table 21**  
Sensitivity analysis based on STEC rate in community acquired gastroenteritis

	Population <15 years of age (000's)	Total population (000's)	% of population <15 years of age	Gastroenteritis Incidence per 100 000	Extrapolated incidence in the entire population per 100 000	STEC incidence per 100 000 (If STEC rate is 2.5%)	Number of cases
EU-25	75415	453685	16.62%	4670	776.28	19.41	88,047
EU-15	62666	379484	16.51%	4670	771.18	19.28	73,163
Eurozone	48915	305831	15.99%	4670	746.93	18.67	57,108

#### Prevalence in Europe

10 STEC incidence range using the direct reports of STEC incidence in Europe or derived from the HUS incidence is 0.09 –0.26/10,000 cases. The sensitivity analysis gives an incidence of 1.94/10 000 cases. In conclusion a conservative estimate of Shiga toxin producing bacterial infection in the entire EU is 2 in 10 000.

15 **Prevalence in comparable jurisdictions**

Epidemiological data reported in the USA (Mead et al., Emer Infect Dis 5, 607-625 (1999)). 110-220 cases of STEC infections was the annual incidence estimated by the CDC. The Population Reference Bureau estimated the US population to be 300 million in 2006. The annual number of cases reported to this 20 population gives an incidence of 3.68/10 000 inhabitants.

In Australia, the incidence of HUS in children under 5 years (1.35 (95% CI 1.06 to 1.72) per 105) was lower than that reported in Canada (3.11 per 105 children <5 years) and the British Isles (3.3 per 105 children) (Elliott et al., Arch Dis Child 85, 125-131 (2001)).

25 In Canada, the annual rate of STEC infections was reported to be 1,21/10 000 cases (for the general population) in Alberta in the late 1980's (Waters et al., Clin Infect Dis. Nov;19(5):834-43 (1994)), which seems to have the highest rate of STEC infections in Canada. No other comprehensive epidemiological review was recently published.

All together the data from non-European developed countries is consistent with the European epidemiological data.

### **Summary**

The first approach, based on data for the years 1999-2003, yielded an 5 incidence of STEC infections of 0.73-2.1/100,000 population. Using HUS frequencies (second approach), the incidence of STEC infections was calculated as 0.88 (for a HUS rate of 15%) to 2.63/100,000 population (HUS rate 5%). Using the third approach, a STEC infection rate of 7.8 to 19.4 per 100,000 population was calculated assuming that 1 - 2.5% of all diarrhea cases are due to STEC.

10

### **Conclusion**

Based on literature analysis, registry data and extrapolation from incidence figures for HUS and childhood diarrhoea, we propose that the demonstrated discrepancies in STEC infection rates are due to underreporting and that the maximal, 15 true incidence in the EU-25 population is 19.4 per 100,000.

## **OTHER EMBODIMENTS**

Various modifications and variations of the described methods and compositions of the invention will be apparent to those skilled in the art without 20 departing from the scope and spirit of the invention. Although the invention has been described in connection with specific desired embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out 25 the invention that are obvious to those skilled in the fields of medicine, immunology, pharmacology, endocrinology, or related fields are intended to be within the scope of the invention.

All patents, patent applications, and publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication was specifically and individually incorporated by reference.

30

What is claimed is:

**Claims**

1. A method for the treatment of a Shiga toxin associated condition in a subject comprising administering an effective amount of a chimeric anti-Stx1 and a chimeric anti-Stx2 antibody to said subject, wherein each of the chimeric antibodies is administered at 1 mg/kg or 3 mg/kg, wherein said chimeric anti-Stx1 antibody comprises:

(a) a human IgG1-kappa immunoglobulin constant region;

(b) an immunoglobulin heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO:1; and

(c) an immunoglobulin light chain variable region comprising the amino acid sequence set forth in SEQ ID NO:2;

and wherein said chimeric anti-Stx2 antibody comprises:

(d) a human IgG1-kappa immunoglobulin constant region;

(e) an immunoglobulin heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 3; and

(f) an immunoglobulin light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 4.

2. The method of claim 1, wherein said chimeric anti-Stx1 and chimeric anti-Stx2 antibodies are co-administered.

3. The method of claim 1, wherein the chimeric antibodies are administered by intravenous infusion over a period of at least 30 minutes.

4. The method of claim 3, wherein the chimeric antibodies are administered by intravenous infusion over a period of between 30 minutes and 1 hour.

5. The method of claim 1, wherein said subject is a human.
6. The method of claim 5, wherein said human is less than 18 years old.
7. The method of claim 6, wherein said human is between 6 months and 3 years old.
8. The method of claim 6, wherein said human is less than 6 months old.
9. The method of claim 2, wherein the chimeric antibodies are each administered at a dosage of 1 mg/kg.
10. The method of claim 2, wherein the chimeric antibodies are each administered at a dosage of 3 mg/kg.
11. An article of manufacture comprising a chimeric anti-Stx1 and a chimeric anti-Stx2 antibody, and a label, wherein said label indicates that said chimeric anti-Stx1 and chimeric anti-Stx2 antibodies are for treating a Shiga-toxin associated disease and are to be administered at a dosage of 1 mg/kg or 3 mg/kg each, wherein said chimeric anti-Stx1 antibody comprises:
  - (a) a human IgG1-kappa immunoglobulin constant region;
  - (b) an immunoglobulin heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO:1; and
  - (c) an immunoglobulin light chain variable region comprising the amino acid sequence set forth in SEQ ID NO:2;and wherein said chimeric anti-Stx2 antibody comprises:
  - (d) a human IgG1-kappa immunoglobulin constant region;
  - (e) an immunoglobulin heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 3; and

(f) an immunoglobulin light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 4.

12. The article of manufacture of claim 11, wherein said label indicates that said chimeric anti-Stx1 and chimeric anti-Stx2 antibodies are to be administered at a dosage of 1 mg/kg each.

13. The article of manufacture of claim 11, wherein said label indicates that said chimeric anti-Stx1 and chimeric anti-Stx2 antibodies are to be administered at a dosage of 3 mg/kg each.

14. A kit comprising a chimeric anti-Stx1 and a chimeric anti-Stx2 antibody, instructions, and a label, wherein said instructions are for administering said chimeric anti-Stx1 and chimeric anti-Stx2 antibodies at a dosage of 1 mg/kg or 3 mg/kg, wherein said label indicates that said chimeric anti-Stx1 and chimeric anti-Stx2 antibodies are for treating a Shiga-toxin associated disease, wherein said chimeric anti-Stx1 antibody comprises:

(a) a human IgG1-kappa immunoglobulin constant region;

(b) an immunoglobulin heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO:1; and

(c) an immunoglobulin light chain variable region comprising the amino acid sequence set forth in SEQ ID NO:2;

and wherein said chimeric anti-Stx2 antibody comprises:

(d) a human IgG1-kappa immunoglobulin constant region;

(e) an immunoglobulin heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 3; and

(f) an immunoglobulin light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 4.

15. The kit of claim 14, wherein said instructions are for administering said chimeric anti-Stx1 and chimeric anti-Stx2 antibodies at a dosage of 1 mg/kg.

16. The kit of claim 14, wherein said instructions are for administering said chimeric anti-Stx1 and chimeric anti-Stx2 antibodies at a dosage of 3 mg/kg.

Figure 1.

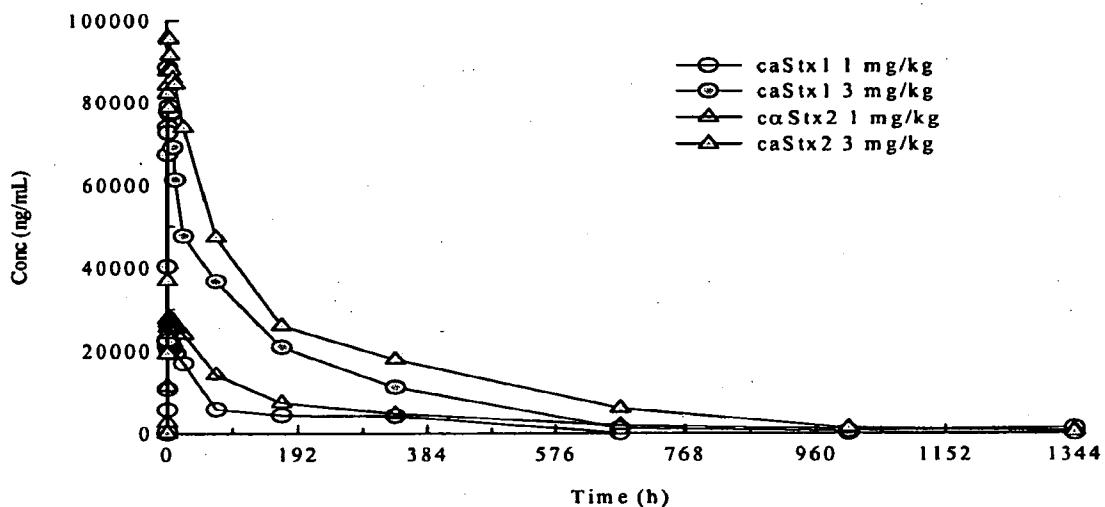


Figure 2.

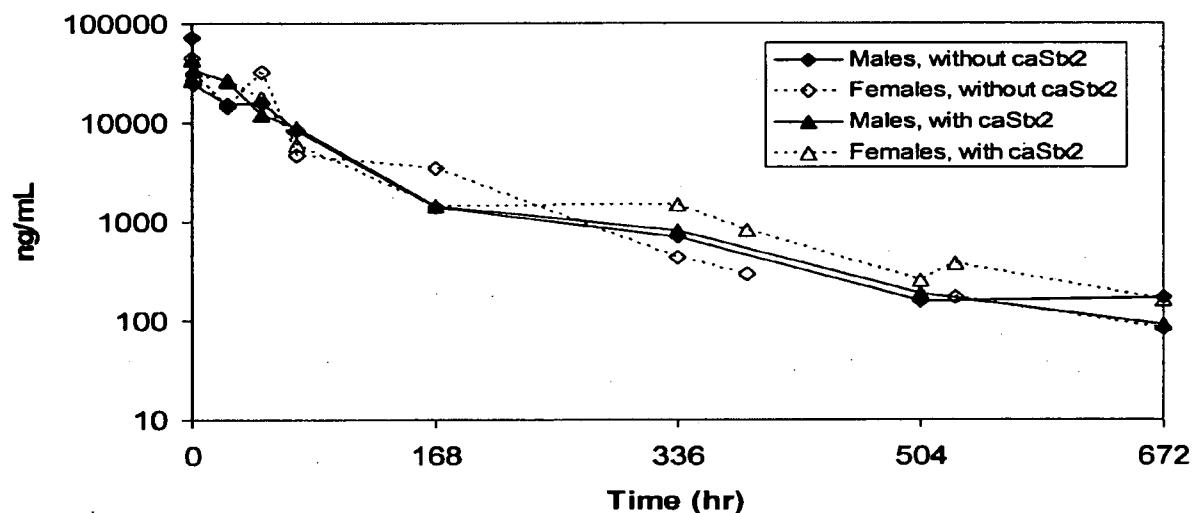


Figure 3.

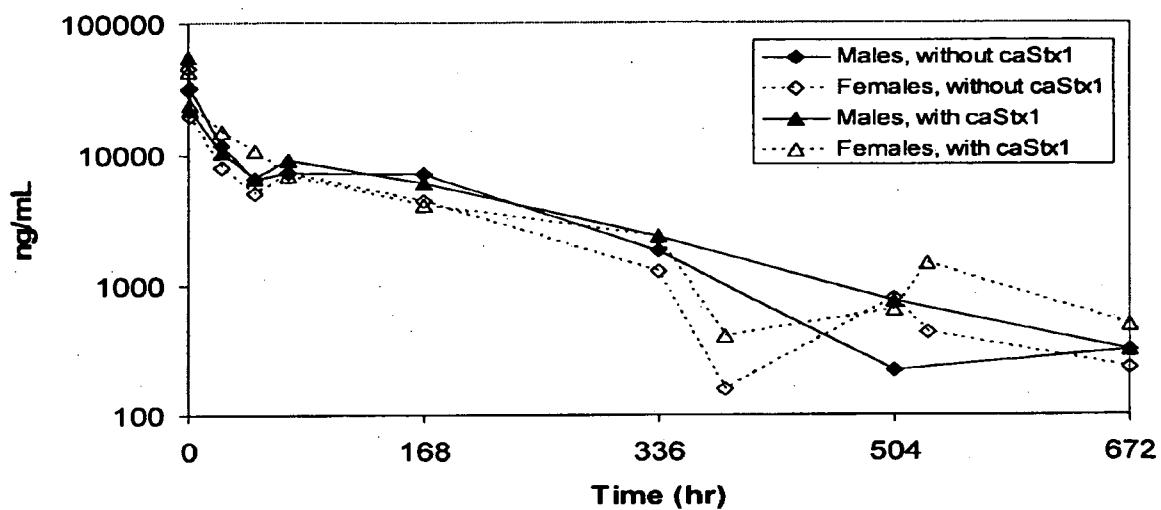
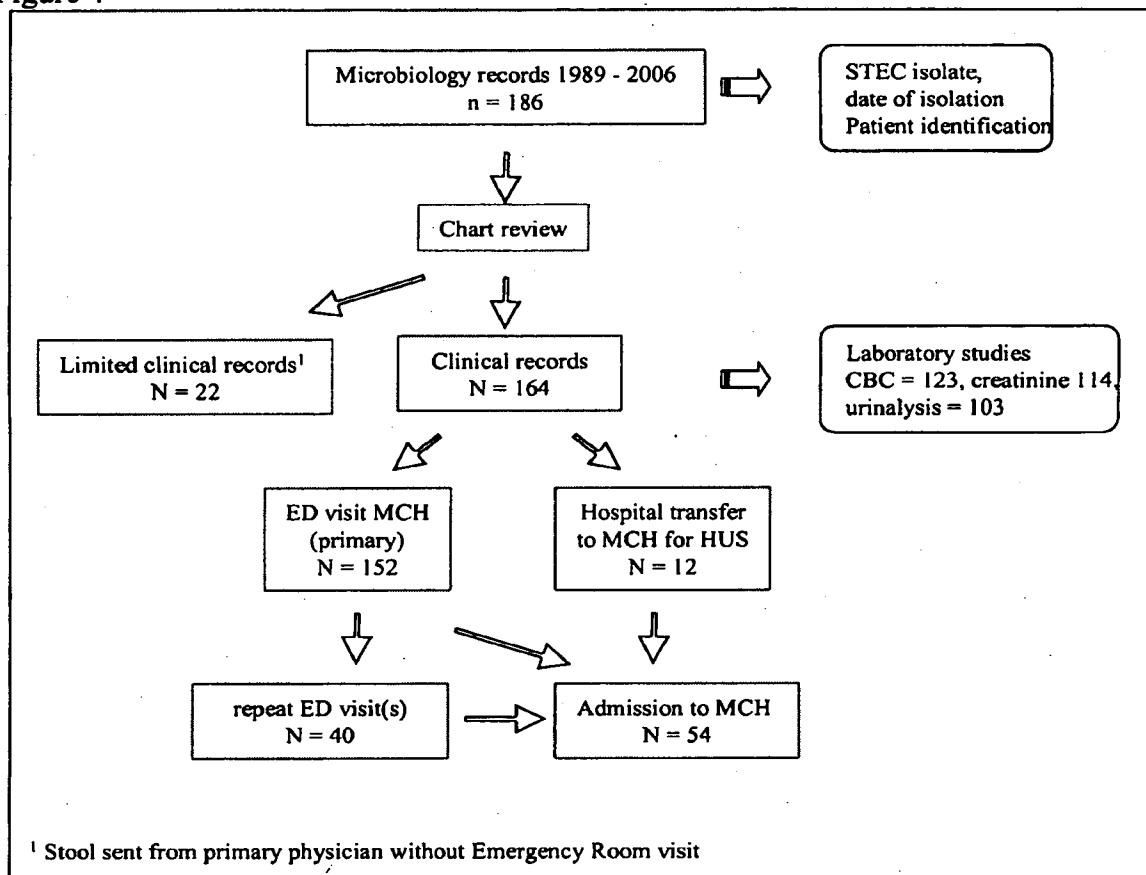


Figure 4



All patients had culture-proven STEC O157 infection (22 with *bona fide* HUS).

Figure 5

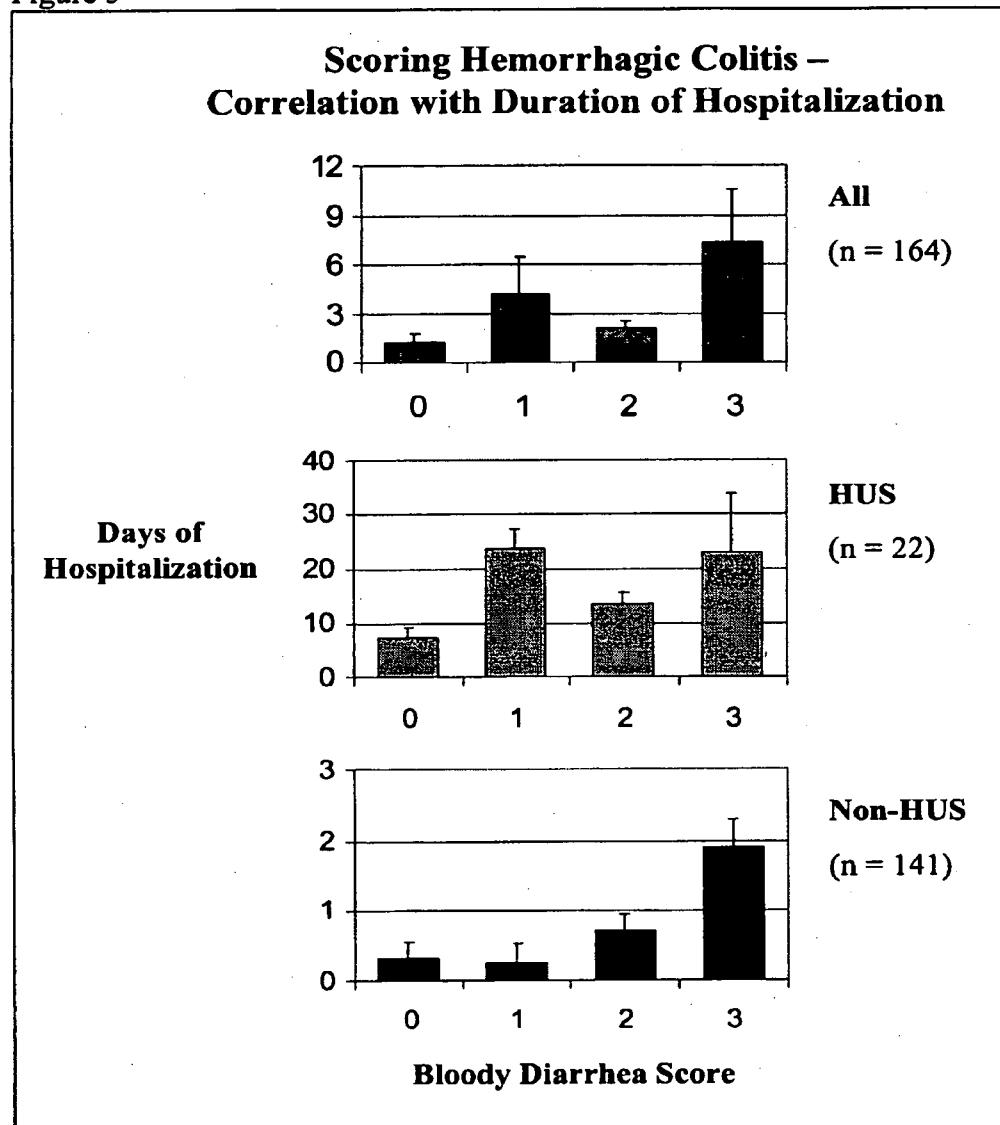


Figure 6

