SHIGA TOXIN MODE OF ACTION IN E. *COLI* O157:H7 DISEASE.

Tom G. Obrig

Department of Microbiology and Immunology, University of Rochester, Box 672, 601 Elmwood Avenue, Rochester, NY 14642

TABLE OF CONTENTS:

1. ABSTRACT

Shiga toxins (Stx) are virulence factors produced by selected bacteria pathogenic for humans. These multicomponent protein complexes are among the more potent toxins known. As inhibitors of eukaryotic protein synthesis, these toxins selectively inactivate ribosomes in an enzymatic manner. Specificity of cell targeting is determined by the high-affinity binding of Stx to its receptor, a glycosphingolipid (Gb3) located in the plasma membrane or some eukaryotic cells. Elaborated by food-borne *E. coli* O157:H7 bacteria, isotypes of Stx (Stx1 & Stx2) are required for the ensuing vascular changes in humans, including hemorrhagic colitis and renal hemolytic uremic syndrome. Experimental therapeutic intervention of Stx-associated disease includes the Stx receptor immobilized on biologically inert particles designed for oral presentation.

2. THE SHIGA TOXINS (Stx)-OVERVIEW

2.1 Production by gram-negative pathogenic bacteria

Discovery of the Shiga toxins was a result of a need to know more about how gram negative bacterial pathogens cause disease (1,2). This class of toxins has been proven to be fundamental to the pathogenicity of both *Shigella dysenteriae* type 1 and to the more well-known *E. coli* O157:H7. Because the *E. coli* -derived toxins were cytotoxic to Vero cells, the term "verotoxin" was suggested. Most recently, there has been an attempt to unify the terminology of the Shiga toxins such that: Stx is from *Shigella dysenteriae* type 1, and Stx1, Stx2, etc. from *E. coli*. The *E. coli* toxins were previously called Shiga-like toxins, type 1 and 2. As discussed below, the Shiga toxins are responsible for the systemic complications of infections and are not required for the initial colonization of the gut by these bacteria. *Shigella dysenteriae* type 1 is a tropical pathogen endemic in some areas of Indo-Asia, such as Bangladesh. In contrast, the enterohemorrhagic *E. coli* (EHEC), of which *E. coli* O157:H7 is the prototype, are common to more temperate areas of the world. Recent outbreaks of EHEC have occurred, for example, in the U.S., Canada, Germany, England, Switzerland, France,

and Japan. Although *Shigella* and *Escherichia* represent different genera of bacteria, they are considered to be very close, both genetically and biochemically. The isoforms of Shiga toxins produced by these different pathogens are also very closely related in structure and function. However, some of the differences among members of the Shiga toxin group may be related to specific clinical developments in EHEC-related disease. Overall, the emphasis here is that these unique toxins are required for the vascular complications which occur following infection of humans with these bacterial pathogens.

2.2 Evolution of Stxs and the "emerging pathogen", *E. coli* O157:H7

Although the exact origin of O157:H7 is not known, a thorough comparison of the enzyme-encoding genes common to virtually all *E. coli* revealed the closest relative to be another pathogen, *E. coli* O55:H7, that is capable of colonizing the gut, but which causes only a mild diarrhea in infants (3). It is important to note that in addition to a difference in serotypes (a property of surface lipopolysaccharides), the O157:H7 bacteria now harbor the genes for Shiga toxins. The Shiga toxin genes are encoded by bacteriophage in EHEC, but reside on the chromosome in *Shigella dysenteriae* type 1. The evolutionary origin of Shiga toxin genes remains somewhat of a mystery. However, as mentioned below a portion of Shiga toxin protein structure is closely related to other proteins found in higher plants, all of which inactivate eukaryotic ribosomes enzymatically (4). It appears that the fate of these protein toxins in animals and humans is determined by the additional associated protein subunits which have an affinity for specific carbohydrate molecules expressed on the surface of various eukaryotic cells.

2.3 Stx is a vascular acting toxin

A comprehensive review of both clinical and basic science aspects of this topic has appeared recently (5). Although the virulence of *E. coli* O157:H7 without its toxin genes, i.e. an isogenic tox-minus mutant, has yet to be characterized in animals, it is likely that this pathogen would more resemble the virulence ability of *E. coli* O55:H7. A study of this type was conducted with *Shigella dysenteriae* 1, the results clearly showing that an isogenic tox-minus mutant could colonize lower primates, but had lost the ability to cause systemic vascular damage (6). Direct evidence of Shiga toxin action at the endothelial level was provided by a study in which purified Stx was administered to rabbits (i.v.) with ensuing vascular damage similar to that observed in humans, although this was restricted to the colon and brain in rabbits (7).

3. BIOCHEMICAL FEATURES OF THE SHIGA TOXINS

3.1 Subunit protein structure

Structurally, toxins in the Stx family each consist of one A-subunit responsible for the inhibitory activity and five identical B-subunits that determine binding specificity to eukaryotic cells (2,8). These toxins have been the subject of comprehensive review articles (2,8,9). Stx has been purified from *Shigella dysenteriae* 1 and shown to be a 68,000 kDa multi-subunit complex Shiga toxin comprised of a single 32kDa "A-subunit" and 5 x 7.7kDa "B-subunits" (10,11). Shiga-like toxins (Stx1, Stx2, and Stx2c) have also been purified from *E. coli* and appear similar to Shiga toxin (2). Despite minor differences between the A-subunit amino acid sequences of the Stx subspecies (Stx1, Stx2, Stx2c, etc.), all appear to exhibit identical enzymatic activity. However, differences in the B-subunit amino acid sequences are important in determining the receptor to which the Stx will bind, and thus the target cell type. Stx, Stx1, Stx2 and Stx2c all bind to the same receptor, the glycosphingolipid, Gb3 (9). Antigenically, Stx1 from *E. coli* is considered to be almost identical to Shiga toxin (Stx) from *S. dysenteriae* 1, but is distinct from *E. coli* Stx2, and Stx2c (2). Another member of the Stx family , Stx2e, is produced by unique isolates of *E. coli* that infect pigs, and thus is also called pig edema factor. Stx2e is not only antigenically distant from the other toxins, but prefers to bind to a different glycolipid, i.e. Gb4 (12,13).

3.2 Stx removal of a single base from 28S rRNA

The A-subunit of the Stx inhibits protein synthesis by inactivating ribsomes. The A subunit enzymatically removes a purine base from the 28S rRNA within the 60S ribosomal subunit (14). This modification of the ribosome prevents interaction of peptide elongation factors (EF-1 and EF-2) with the altered ribosome and stops protein synthesis at the level of peptide elongation (15,16).

3.3 Stx is a member of the ribosome inactivating protein family

Stx A-subunits work biochemically in a manner identical to that of a series of other ribosome inactivating proteins (RIPs) derived from higher plants. It has been 25 years since the first reports appeared defining the RIPs as ribosome-inactivating proteins (17,18). Primary amino acid structure is highly conserved within the enzymatic site of the RIPs (19). In contrast to the Stxs, most of the plant-derived RIPs exist as a single peptide without associated B-subunit proteins. An exception to this is the toxin, ricin. It should be emphasized that Stx and ricin are much more potent than the single peptide RIPs because their B-subunits direct the toxins to specific high affinity receptors on eukaryotic cells, whereas the single peptide RIPs interact weakly and less discriminately to certain sugar residues on cells. The natural importance of RIPs to bacteria is questionable with the exception that they may help in spread of the pathogen by increasing bacterial shedding during the disease process. Person-to-person spread of EHEC is a well-documented problem within a household and in day care centers (5). Plant-derived RIPs exhibit natural antiviral activity against plant RNA viruses, and likely serve a natural role in protecting plants from their viral pathogens. To date, attempts to harness

the power of of RIPs by biotechnology for use as immunotoxins for antitumor purposes has been a general disappointment. Although there have been some partial successes, the failures far outnumber the successes.

4. STX ISOTYPES PRODUCED BY *E. COLI* O157:H7

E. coli strains isolated from HUS patients produce moderate to high levels of Stxs (20). Such *E. coli* O157:H7 isolates normally produce either Stx2 alone or the combination of Stx1 and Stx2. Rarely do these strains produce Stx1 alone. Stx-producing *E. coli* serotypes other than O157:H7 have been associated with human disease, particularly outside the US, but toxin production by these isolates does not fit the pattern for O157:H7. A significant number of these non-O157:H7 isolates produce Stx1 alone, thus resembling *Shigella dysenteriae* 1 that elaborates Shiga toxin only (21). Whether either one of these toxin isotypes is more efficient in causing vascular damage in the clinical setting not known. However, Stx2 was shown to be approximately 1,000-times more potent than Stx1 in mice (22). Stx2 was also more potent, by three orders of magnitude, as a cytotoxic agent than Stx1 when incubated with human renal microvascular endothelial cells (23,24). These cells are the putative target of Stx in the hemolytic uremic syndrome caused by Stx-producing *E. coli* (25).

5. STX RECEPTORS EXPRESSED ON EUKARYOTIC CELLS

5.1 The Stx receptor is a glycosphingolipid, Gb3

The receptor for Stx, Stx1 and Stx2 on the cell surface of eukaryotic cells is the neutral glycolipid, globotriaosylceramide, Gb3 (26). A comprehensive review of this topic exits (27). Gb3 consists of a ceramide long chain fatty acid embedded in the plasma membrane, and a short extracellular trisaccharide chain terminated by a digalactose residue. The B-subunits of Shiga toxins facilitate high-affinity (Kd= 0.1 nM) binding of holotoxin to the terminal digalactose residue of Gb3. This terminal disaccharide contains the unusual carbohydrate linkage, galactose(alpha 1,4)-galactose. Gb3 is also known as cell differentiation marker, CD77 and is a tumor marker for a B-cell lymphoma. Gb3 trisaccharide also occurs on red blood cells of the Pk blood group.

5.2 Specificity of Stx-receptor interaction

Recently, the interaction between Stx molecules and Gb3 has been elucidated. Combined information from the crystal structure of Stx B-subunit and site-directed mutations in the subunit has led to the concept of two sites within the B-subunit that interact with the digalactose of Gb3 (27,28). Another important finding is that the chemical structure of the ceramide portion of Gb3 strongly influences which Stx isotypes bind to Gb3 (29). More exactly, the length of

the ceramide fatty acids and their chemical substitutions were shown to dictate binding of Stx isotypes to Gb3. This phenomenon appears to be due to conformational changes in the extracellular carbohydrate portion of Gb3 which are dictated, in part, by how the ceramide is arranged in the plasma membrane of the target cell.

5.3 Potential receptor-based therapeutics for EHEC disease

Therapeutic intervention for EHEC-associated disease does not exist at this time. Traditional antibiotics are largely ineffective against EHEC, and as the disease is a toxemia rather than a bacteremia, a different type of intervention is required. Current treatment which has been effective in sharply reducing the death rate due to EHEC infections has been limited to renal-based supportive care aimed at the symptoms of HUS, i.e. hemolytic uremic syndrome (30). However, there exists a real need for a method of treatment early in the EHEC infection process. Indeed, a novel treatment currently in phase III trials in the US and Canada is a toxin receptor-based agent consisting of the carbohydrate moiety of Gb3 attached to the surface of silica particles administered orally to potential victims of EHEC on admission. This approach is aimed at binding of the free Stx when released by the pathogenic bacteria in the distal small intestine and the large intestine. The results of this "SYNSORB" clinical study should be available in late 1998.

Stx receptor does exist on cells within the human kidney and human endothelial cells are capable of producing large quantities of cell surface Gb3 (24,31,32). Whether receptor-based intervention would be feasible once Stx enters the bloodstream is highly questionable, but systemic receptor-based treatments for other diseases do exist, and more are being developed for future use.

6. INTERNALIZATION AND PROCESSING OF STX BY EUKARYOTIC CELLS

6.1 Stx enters by receptor-mediated endocytosis

Toxin internalization and processing is fundamental in dictating the effect of Stxs' on individual cell types. Stx enters and is processed in eukaryotic cells by a series of steps which are collectively referred to as receptor mediated endocytosis (33). This process involves trafficking of Stx between intracellular vesicles, endosomes and lysosomes to the golgi and finally to the endoplasmic reticulum where it is released into the cytoplasm. It is in the cytoplasm where Stx comes into contact with and enzymatically inactivates the ribosomes. The Gb3 receptor is required, but not sufficient for Stx action. Examples have been described of cells being resistant to Stx action because Stx could bind to, but not be internalized by the cells (34). In contrast, part of the reason that human renal microvascular endothelial cells are 1,000-times more sensitive to Stx2 vs. Stx1 is likely due to the efficiency of toxin internalization and processing by this cell type (23). Routing of Stx within

cells is also dictated by the chemical structure of the Gb3 receptor itself. For example, it has recently been demonstrated that shorter fatty acids (i.e. C16) in the ceramide portion of Gb3 direct the internalized Stx-Gb3 complexes directly to the nucleus in certain cell types (29). This is important because in the latter case, the cells succumb to apoptotic cell death rather than to necrosis through inhibition of cytoplasmic protein synthesis.

6.2 Processing and activation of Stx in target cells

Processing of Stx within the target cell by trypsin-like action releases the Stx-A1 peptide fragment (the enzymatic portion) from a shorter C-terminal Stx-A2 fragment. It is thought that this action, along with reduction of a disulfide bond within the same region of the Stx-A subunit is required for "activation" of Stx biological activity. However, changes in the trypsin cleavage site or in the cysteines of the disulfide bond yield Stx that is toxic to cells because the cells remain capable of cleaving and releasing the Stx-A1 subunit (35,36).

7. A ROLE FOR SHIGA TOXINS IN VASCULAR DISEASE

7.1 Evidence for Stxs' role in EHEC-associated vascular disease

Some evidence indicating a role for the Shiga toxins at the vascular level are: 1) the production of active Shiga toxin is required for *S. dysenteriae* 1 to cause bloody dysentery, but not for watery diarrhea, in monkeys (6). 2) Stx has been detected in serum of HUS patients (3,37). 3) When purified Stx is presented to animals *i.v.*, it targets to the endothelium, producing hemorrhagic lesions (7,38). 4) an *E. coli* 0157:H7 isolate was obtained from a patient with hemorrhagic colitis (HC) and when presented to rabbits intragastrically caused symptoms characteristic of HC in humans (39). In this study, gastrointestinal bleeding abnormalities appeared only if the bacteria synthesized Stx. 5) the overwhelming evidence, first presented by Karmali and others, of the incidence of Stx-production by E. coli isolates and the occurrence of HC and HUS in humans (20). 6) the association of HUS with a Stx-producing *Citrobacter freundii* isolate (41). 7) many patients exposed to Stx-producing EHEC produce circulating anti-Stx antibodies, indicating the toxin entered the blood. 8) the correlation that individuals belonging to the P1 blood group are less likely to get EHEC-associated HC and HUS because Stx binds to these erythrocytes (42,43). 9) isolated glomerular endothelial cells of humans are capable of expressing Gb3 and are exquisitely sensitive to the Stxs (24,44). 10) Gb3 is expressed on glomerular endothelial cells of young children (45). 11) purified Stx induces cytokine production in isolated monocyte/macrophage (46). 12) Stx1 presented to mice causes a renal-specific induction of a TNF promoter-encoded gene (47). 13) Stx1 induces expression of endothelial adhesion molecules for neutrophil adherence in static endothelial cell cultures and under laminar flow conditions (48).

7.2 Additional bacterial factors in EHEC-associated vascular disease

It is unlikely that Shiga toxins are the only factors produced by EHEC that lead to vascular damage. For example, *E. coli* O157:H7 elaborates an hemolysin, the gene for which is encoded on the large (60 mDa) plasmid (49). This toxin is capable of lysing eukaryotic cells (50). However, it is not known to what extent this toxin contributes to the virulence of EHEC. Other factors may be encoded by EHEC, but these will soon be identified as part of the *E. coli* genome sequencing project which has been extended to include EHEC bacteria (51).

8. SENSITIVITY OF HUMAN ENDOTHELIAL CELLS TO Stxs

8.1 Endothelial cells are the putative target of Stxs

It has been 10 years since the first report indicating that Stx was cytotoxic to endothelial cells (52). Those studies were prompted by previous reports that endothelial cells were the site of initial damage in HUS (25,53,54). Histopathologic examination of kidney samples from HUS patients revealed glomerular vascular capillaries with swollen endothelial cells (53,55). In this study, microvascular angiopathy was present in all patients diagnosed with HUS and having an infection with Stx-producing *E. coli*. The authors concluded that endothelial cells are the putative primary target cells of Stx. Others have demonstrated that human kidney contains the Gb_3 receptor for Shiga toxin, particularly in young children (31). Endothelial cells isolated from human kidney also express Gb3 and are very sensitive to Shiga toxin (24).

Although glomerular endothelial cells appears to represent the primary target of the Stxs, more evidence is accumulating, both direct and indirect, that endothelial cells from other vascular beds are also subject to Stx damage. It is now certain that microvascular (vs. large vessel) endothelial cells from a number of locations are very sensitive to the Stxs. This would explain the nature of hemorrhagic colitis, and changes in some other organs (e.g. pancreas, lung), and why neurological complications occur in over 30 to 50% of HUS patients. These may all be due to the preference of Stxs to recognize microvascular endothelial cells.

New evidence shows that Stxs can directly interact with vascular cell types other than endothelial cells in a way that may be important to HUS (9,56,57). In an earlier report, it was suggested that Stx interacts directly with macrophage to elicit cytokines (56). Indeed, this has now been demonstrated directly by murine macrophage responding to Stx1 with increased IL-1 and IL-6 production (46). Although macrophage are considerably less sensitive to Stx, these data are relevant and point out that release of cytokines from macrophage would add to the validity of the hypothesis described below that Stx action on the endothelium is enhanced by cytokines in developing HUS (58,59,60,61).

8.2 Host cytokine regulation of Stx-sensitivity in endothelial cells

To date, all evidence suggests that Stxs combine with other bacterial and host factors to cause HUS. Workers in this field tend to agree that induction of cytokines by LPS and Stx in macrophage does take place and this may be more localized to the kidney (46,62,63). As stated above, Stx interacts directly with other cell types such as macrophage to elicit cytokines and adherence molecules. Cytokines may act alone or in combination to directly alter the physiological state of endothelial cells. Firstly, these factors tilt the endothelial hemostatic balance towards a more procoagulant/antifibrinolytic state (62,64,65,66,67,68) which is a hallmark of HUS. Secondly, LPS and some cytokines induce Gb3 on endothelial cells (24,59,69), through a series of signal-transduction steps involving protein kinase C (70,71). The author views EHEC-associated HUS as a gram negative bacterial response which has been diverted by Stx. Future research should reveal the signal transduction pathways activated during induction of Gb3 by LPS, cytokines, and other agents. The more difficult task will be to determine the individual events that take place in humans during development of Stx-associated HUS. This is particularly true in the absence of a reproducible animal model for this disease.

9. ACKNOWLEDGMENTS

10. REFERENCES

1. Kononwalchuk, J., J.I. Speirs. & S. Stavric: Vero response to a cytotoxin of *Escherichia coli*. *Infect. Immun* 18,775-779 (1977).

2. O'Brien, A.D., V.L. Tesh, A. Donohue-Rolfe, M.P. Jackson, S. Olsnes, K. Sandvig, A.A. Lindberg & G.T. Keusch: Shiga toxin: Biochemistry, genetics, mode of action, and role in pathogenesis. *Curr Topics Microbio. Immunol* 180,65-94 (1992)

3. Whittam, T.S., M.L. Wolfe, I.K. Wachsmuth, F. Orskow, I. Orskov & R.A. Wilson: Clonal relationships among *Escherichia coli* strains that cause hemorrhagic colitis and infantile diarrhea. *Infect Immun* 61,1619-1629 (1993)

4. Girbes, T., J.M. Ferreras, R. Iglesias, L. Citores, C. De Torre, M.L. Carbajales, P. Jiminez, F.M. Benito & R. Munoz: Recent advances in the uses and applications of ribosome-inactivating proteins from plants. *Cellular Molec Biol* 42,461-471 (1996)

5. Pickering, L.K, T.G. Obrig & F.B. Stapleton: . Hemolytic uremic syndrome and enterohemorrhagic *Escherichia coli* (CME review article). *Ped Infect Dis J* 13,459-476 (1994)

6. Fontaine, A., J. Arondel, & P.J. Sansonetti: Role of Shiga toxin in the pathogenesis of bacillary dysentery, studied by using a tox⁻ mutant of *Shigella dysenteriae* 1. *Infect Immun* 56,3099-3109 (1988)

7. Richardson, S.E., T.A. Rotman, V. Jay, C.R. Smith, L.E. Becker, M. Petric, N.F. Olivieri & M.A. Karmali: Experimental verocytotoxemia in rabbits. *Infect Immun* 60,4154-4167 (1992)

8. Jackson, M. P.: Structure-function of Shiga toxin and the Shiga-like toxins. *Microbiol Path* 8,235-242 (1990)

9. Lingwood, C. A.: Verotoxins and their glycolipid receptors. *Adv Lipid Res* 25:189-211 (1993)

10. O'Brien, A.D., G.D. LaVeck, D.E. Griffin & M.R. Thompson: Characterization of *Shigella dysenteriae* 1 (Shiga) toxin purified by anti-Shiga toxin affinity chromatography. *Infect Immun* 30,170-179 (1980)

11. Olsnes, S., R. Reisbig & K. Eiklid: Subunit structure of *Shigella* cytotoxin. *J Biol Chem*, 256, 8732-8738 (1981)

12. Keusch, G.T., M. Jacewicz, D.W. Acheson, A. Donohue-Rolfe, A. V. Kane & G. McCluer: Globotriaosylceramide, Gb3, is an alternative functional receptor for Shiga-like toxin 2e. *Infect Immun* 63,1138-1141 (1995).

13. Weinstein, D.L., M.P. Jackson, J.E. Samuel, R.K. Holmes & A.D. O'Brien: Cloning and sequencing of a Shiga-like toxin type II variant from an *Escherichia coli* strain responsible for edema disease of swine. *J Bact* 170,4223-4230 (1988)

14. Endo, Y., K. Tsurugi, T. Yutsudo, Y. Takeda, T. Ogasawara & K. Igarashi: Site of action of Vero toxin (VT2) from *Escherichia coli* O157:H7 and of Shiga toxin on eukaryotic ribosomes. *Eur J Biochem* 171,45-50 (1988).

15. Obrig, T.G., T.P. Moran & J.E. Brown: The mode of action of Shiga toxin on peptide elongation of eukaryotic protein synthesis. *Biochem J* 244:287-294 (1987)

16. Obrig, T.G.: Toxins that inhibit host protein synthesis. In: Bacterial Pathogenesis: Part A, Identification and Regulation of Virulence Factors. Eds: Clark V.L., Bavoil P.M., *Methods in Enzymol* 235,647-656 (1994)

17. Olsnes, S. and A. Phil: Different biological properties of the two constitutent peptide chains of ricin, a toxic protein inhibiting protein synthesis. *Biochemistry* 12,3121-3126 (1973)

18. Obrig, T.G., J.D. Irvin & B. Hardesty: The effect of an antiviral peptide on elongation enzymes EF-1 and EF-2. *Arch Biochem Biophys* 155,278-289 (1973)

19. Calderwood, S.B.J., F. Auclair, A. Donohue-Rolfe, G.T. Keusch & J.J. Mekalanos: Nucleotide sequence of the Shiga-like toxin genes of *Escherichia coli*. *Proc Natl Acad Sci USA* 84,4364-4368 (1987)

20. Karmali, M.A., M. Petric, C. Lim, P.C. Fleming, G.S. Arbus & H. Lior: The association between idiopathic hemolytic uremic syndrome and infection by Verotoxin-producing *Escherichia coli*. *J Infect Dis* 151,775-778 (1985).

21. Kleanthous, H., H.R. Smith & S.M. Scotland: Haemolytic uraemic syndromes in the British Isles, 1985-8: association with verocytotoxin producing *Escherichia coli* Part 2:microbiological aspects. *Arch Dis Child* 65,722-727 (1990)

22. Tesh, V.L., J.A. Burris, J.W. Owens, V.M. Gordon, E.A. Wadolkowski, A.D. O'Brien & J.E. Samuel: Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. *Infect Immun* 61,3392-402 (1993)

23. Louise, C.B. & T.G. Obrig: Specific interaction of *E. coli* O157:H7-derived Shiga-like toxin-2 with human renal endothelial cells. *J Infect Dis* 172:1397-1401 (1995)

24. Obrig, T.G., C.B. Louise,C.A. Lingwood, B. Boyd, L. Barley-Maloney & T.O. Daniel: Endothelial heterogeneity in Shiga toxin receptors and responses. J *Biol Chem* 268,15484-15488 (1993)

25. R. Habib: Pathology of the hemolytic uremic syndrome. In: Hemolytic-uremic syndrome and thrombotic thrombocytopenic purpura. Eds: Kaplan B.S., Trompeter R.S. & Moake, J.L. Marcel Dekker, New York, (1992)

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

27. Lingwood, C.A.: Verotoxins and their glycolipid receptors. *Adv Lipid Res* 25,189-211 (1993)

28. Stein, P.E., A. Boodhoo, G.J. Tyrell, J.L. Brunton & R.J. Read: Crystal structure of the cell-binding B oligomer of verotoxin-1 from *E. coli*. *Nature* 355,748-750 (1992)

29. Kiarash A, B. Boyd & C.A. Lingwood: Glycosphingolipid receptor function is modified by fatty acid content. Verotoxin 1 and verotoxin 2c preferentially recognize different globotriaosyl ceramide fatty acid homologues. J Biol Chem 269,11138-11146 (1994)

30. Kaplan, B.S., R.S. Trompeter & J.L. Moake: Hemolytic uremic syndrome and thrombotic thrombocytopenic purpura. Marcel Dekker, New York (1992)

31. Boyd, B. and C. Lingwood: Verotoxin receptor glycolipid in human renal tissue. *Nephron* 51,207-210 (1989)

32. Obrig, T.G., C.B. Louise, C.A. Lingwood, T.O. Daniel, S.A. Kaye, B. Boyd & M.P. Jackson: Shiga toxin-endothelial cell interactions. In: Recent Advances in Verocytotoxin-producing *E. coli* Infections. Eds: Karmali, M.A. & Goglio, A.G., Elsevier, Amsterdam (1994)

33. Sandvig, K, O. Garred, K Prydz, J.V. Kozlov, S.H. Hansen & B. van Deurs: Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. *Nature* 358,510-512 (1992)

34. Eiklid, K & S. Olsnes: Interaction of *Shigella shigae* cytotoxin with receptors on sensitive and insensitive cells. *J Receptor Res* 1, 199-213 (1980)

35. Garred, O, E. Dubinina, A. Polesslaya, S. Olsnes, J. Kozlov & K. Sandvig: Role of the disulfide bond in Shiga toxin-A chain for toxin entry into cells. *J Biol Chem* 272:11414-11419 (1997)

36. Garred, O, E. Dubinina, P.K. Holm, S. Olsnes, B. van Deurs, J. Kozlov & K. Sandvig: Role of processing and intracellular transport for optimal toxicity of Shiga and toxin mutants. *Exptl Cell Res* 218,39-49 (1995)

37. Stenger, K-O., F. Windler, H. Karch, H. von Wulffen & J. Heesemann: Hemolytic uremic syndrome associated with an infection by verotoxin producing *Escherichia coli* O111 in a woman on oral contraceptives. *Clin Nephrol* 29,153-158 (1988)

38. Bridgewater, F.A.J., R.S. Morgan, K.E.K. Rowson & G.P. Wright: The neurotoxin of *Shigella shigae*. Morphological and functional lesions produced in the central nervous system of rabbits. *Br J Exp Pathol* 36,447-453 (1955)

39. Pai, C.H., J.K. Kelly & G.L. Meyers: Experimental infection of infant rabbits with Vero toxin-producing *Escherichia coli*. *Infect Immun* 51,16-23 (1986)

40. Riley, L.W., R.S. Remis, S.D. Helgerson, H.B. McGee, J.G. Wells, B.R. Davis, R.J. Herbert, E.S. Olcott, L.M. Johnson, N.T. Hargrett, P.A. Blake, & M.C. Cohen: Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *N Engl J Med* 308,681-685 (1983)

41. Tschape, H., R. Prager, W. Streckel, A. Fruth, F. Tietze & G. Rohme: Verotoxinogenic *Citrobacter freundii* associated with severe gastroenteritis and cases of hemolytic uremic syndrome in a nursery school: green butter as the infection source. *Epidemiol and Infect* 114,441-450 (1995)

42. Taylor, C.M., D.V. Milford, P.E. Rose, T.C.F. Roy & B. Rowe: The expression of blood group P1 in post-enterpathic haemolytic uraemic syndrome. *Ped Nephrol* 4,59-61 (1990)

43. Newburg, D.S., P. Chaturvedi, E.L. Lopez, S. Devoto, A. Fayad & T.G. Cleary: Susceptibility to hemolytic-uremic syndrome relates to erythrocyte glycosphingolipid patterns. *J Infect Dis* 168,476-479 (1993)

44. Mahan, J.D. , P.A. van Setten, C. McAllister, L.P.W. van den Huvel & L.A.H. Monnens: Effect of verotoxin-1 on human glomerular capillary endothelial cells. In: Recent Advances in Verocytotoxin-producing *E. coli* Infections. Eds: Karmali M.A. & Goglio A.G., Elsevier, Amsterdam (1994)

45. Lingwood, C.A. Verotoxin binding in human renal sections. *Nephron* 66, 21-28 (1994)

46. van Setten, P.A., H.G.G. Verstraten, L.P.W.J van der Heuvel, M.A. Karmali & L.A.H. Monnens: The role of monocytes in the pathogenesis of the haemolytic uremic syndrome (HUS). In: Recent Advances in Verocytotoxin-producing *E. coli* Infections. Eds: Karmali, M.A. & Goglio A.G., Elsevier, Amsterdam (1994)

47. Harel, Y., M. Silva, B. Giroir, A. Weinberg, T.G. Cleary & B. Beutler: A reporter transgene indicates renal-specific induction of tumor necrosis factor (TNF) by Shiga-like toxin. *J Clin Invest* 92,2110-2116 (1993)

48. Morigi, M., G. Micheletti, M. Figliuzzi, B. Imberti, M.A.. Karmali, A. Remuzzi, G. Remuzzi & C. Zoja: Verotoxin-1 promotes leukocyte adhesion to cultured endothelial cells under physiological flow conditions. *Blood* 86,4553-4558 (1996)

49. Schmidt, H., E. Maier, H. Karch & R. Benz: Pore-forming properties of the plasmid-endoded hemolysin of enterohemorrhagic *Escherichia coli* O157:H7.. *Eur J Biochem* 241,594-601 (1996)

50. Bauer, M. & R.A. Welch: Characterization of an RTX toxin from enterohemorrhagic *Escherichia coli* O157:H7. *Infect Immun* 64,167-175 (1996)

51. Blattner, F.R., G. Plunkett III, C.A. Bloch, N.T. Perna, V. Burland, M. Rilewy, J. Collado-Vides, J.D. Glasner, C.K. Rode, G.F. Mayhew, J. Gregor, N.W. Davis, H.A. Kirkpatrick, M.A. Goeden, D.J. Rose, B. Mau & Y. Shao. The complete genome sequence of *Escherichia coli* K-12. *Science* 277,1453-1462 (1997)

52. Obrig, T.G., P.J. Del Vecchio, J.E. Brown, T.P Moran,., B.M. Rowland, T.K. Judge & S.W. Rothman: Direct cytotoxic action of Shiga toxin on human vascular endothelial cells. *Infect Immun* 56, 2373-2378 (1988)

53. Koster, F., V. Boonpucknavig, S. Sujaho., R. Gilman & M. Rahaman: Renal histopathology in the hemolytic-uremic syndrome following shigellosis. *Clin Nephrol* 21,126-133 (1984)

54. Cavanagh, J.B., J.G. Howard & J.L. Whitby: The neurotoxin of *Shigella shigae*. A comparative study of the effects produced in various laboratory animals. *Br J Exp Med* 37,272-278 (1956)

55. Richardson, S.E., M.A Karmali, L.E Becker & C.R. Smith: The histopathology of the hemolytic uremic syndrome associated with verotoxin-producing *Escherichia coli* infections. *J Human Pathol* 19,1102-1108 (1988)

56. Barrett, T.J., M.E. Potter & N.A. Strockbine: Evidence for participation of the macrophage in Shiga-like toxin-induced lethality in mice. *Microb Pathog* 9,95-103 (1990)

57. Eiklid, K. & S. Olsnes: Interaction of *Shigella shigae* cytotoxin with receptors on sensitive and insensitive cells. *J Receptor Res* 1,199-213 (1980)

58. Louise, C.B. & T.G. Obrig: Shiga toxin-associated hemolytic uremic syndrome: combined cytotoxic effects of Shiga toxin, interleukin-1 beta, and tumor necrosis factor alpha on human vascular endothelial cells *in vitro*. *Infect Immun* 59,4173-4179 (1991)

59. Louise,C.B. & T.G. Obrig: Shiga toxin-associated hemolytic uremic syndrome: combined cytotoxic effects of Shiga toxin and lipopolysaccharide (endotoxin) on human vascular endothelial cells *in vitro*. *Infect Immun* 60,1536-1543 (1992)

60. Tesh, V.L., J.E. Samuel, L.P. Perera, J.B. Sharefkin & A.D. O'Brien: Evaluation of the role of Shiga and Shiga-like toxins in mediating direct damage to human vascular endothelial cells. *J Infect Dis* 164,344-352 (1991)

61. van de Kar, N. C. A. J., L.A.H. Monnens, M.A. Karmali & V.W.M Van Hinsbergh: Tumor necrosis factor and interleukin-1 induce expression of the verocytotoxin receptor globotriaosylceramide on human endothelial cells: implications for the pathogenesis of the hemolytic uremic syndrome. *Blood* 80,2755-2764. (1992)

62. Cybulsky, M.I., M.K.W Chan & H.Z. Movat: Acute inflammation and microthrombosis induced by endotoxin, IL-1, and TNF and their implication in gram-negative infection. *J Lab Invest* 58,365-378 (1988)

63. Shands, J.W., Jr.: Macrophage procoagulants. *Hemostasis* 14,373-377 (1984)

64. Bevilacqua, M.P., R.R Schleef, M.A. Gimbrone & D.J. Loskutoff: Regulation of the fibrinolytic system of cultured human vascular endothelium by IL-1. *J Clin Invest* 78,587-591 (1986)

65. Bevilacqua, M.P., J.S. Pober, G.R Majeau, W. Friers, R.S. Cotran & M.A. Gimbrone Jr.: Recombinant TNF induces procoagulant activity in cultured human vascular endothelium: Characterization and comparison with the actions of IL-1. *Proc Natl Acad Sci USA* 83,4533-4537 (1986)

66. Nawroth, P.P. & D.M. Stern: Modulation of endothelial cell hemostatic properties by tumor necrosis factor. *J Exp Med* 163,740-745 (1986)

67. Pober, J.S. & R.S. Cotran: Cytokines and endothelial cell biology. *Physiol Rev* 70,427-451 (1990)

68. van de Kar, N.C.A.J., V.W.M. van Hinsbergh, F.T. Brommer & L.A.H. Monnens: The fibrinolytic system in the hemolytic uremic syndrome: *in vivo* and *in vitro* studies. *Ped Res* 36,257-264 (1994)

69. Kaye, S.A., C.B. Louise, B. Boyd, C.A Lingwood & T.G. Obrig: Shiga toxin-associated hemolytic uremic syndrome: Interleukin-1 beta enhancement of Shiga toxin cytotoxicity toward human vascular endothelial cells, *in vitro*. *Infect Immun* 61,3886-3891 (1993)

70. van de Kar, N.C.A.J., T. Kooistra, M. Vermeer, W. Lesslauer, L.A.H. Monnens & V.W.M. van Hinsbergh: Tumor necrosis factor induces endothelial galactosyl transferase activity and verocytotoxin receptors. Role of specific tumor necrosis factor receptors and protein kinase C. *Blood* 85,734-743 (1995)

71. Louise, C.B., M.C. Tran & T.G. Obrig: Sensitization of human umbilical vein endothelial cells to Shiga toxin: involvement of protein kinase C and NF-kappa B. *Infect Immun* 65,3337-3344 (1997)