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Shiga Toxin Activates Complement and Binds Factor H: Evidence for an Active Role of Complement in Hemolytic Uremic Syndrome[1]

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Infections with enterohemorrhagic *Escherichia coli* (EHEC) are a major cause of hemolytic uremic syndrome (HUS). Shiga toxins (Stxs), especially Stx2, are believed to represent major virulence factors of EHEC, contributing to HUS pathogenesis. Beside EHEC-associated HUS, there are hereditary atypical forms of HUS, which are mostly caused by mutations of complement regulators. The aim of the present study was to investigate whether or not complement is also involved in the pathogenesis of EHEC-induced typical HUS, by being activated either directly or indirectly by involvement of its inhibitors. Purified Stx2 markedly activated complement via the alternative pathway and was found to bind to factor H (FH), however, only when it was active. No apparent cleavage or destruction of FH was visible, and cofactor activity in fluid phase was unaffected, but clearly delayed for surface-attached FH, where it is essential for host cell protection. Binding studies using FH constructs revealed that Stx2 binds to short consensus repeats (SCRs) 6–8 and SCRs18–20, but not to SCRs16–17, i.e., to regions involved in the surface recognition function of FH. In conclusion, complement, and in particular FH, not only plays an important role in atypical HUS, but most probably also in EHEC-induced HUS. ***The Journal of Immunology,*** **2009, 182: 6394–6400.**

Hemolytic uremic syndrome (HUS)[4] is the most common cause of acute renal failure in childhood. It is defined by the triad of hemolytic anemia, thrombocytopenia, and acute renal failure, and results in a high rate of renal and non-renal sequelae in survivors (1).

HUS may be caused by numerous different underlying diseases, including infection with enterohemorrhagic *Escherichia coli* (EHEC). This pathogen typically produces Shiga toxins (Stxs) 1 and/or 2, which are believed to represent the most important virulence factors. In addition, the majority of EHEC possess the adhesin intimin, which is involved in adhesion to the intestinal epithelium (2). After intestinal colonization, Stxs are translocated into the circulation, allowing the target organs to be reached, the most vulnerable of which are the kidneys and brain (1). Although neutrophilic leukocytes have been proposed to act as Stx carriers (3), the major transport vehicle for Stxs has not yet been elucidated.

Epidemiological and experimental studies have shown that the *stx* genotype is associated with the severity of disease and, in particular, with the development of HUS. Patients infected with EHEC strains that harbor stx_2 as the sole *stx* gene have been found to develop HUS significantly more frequently than those infected with strains harboring stx_1 only or stx_1 in combination with stx_2 (4).

Stx is an AB5 holotoxin consisting of one enzymatically active A subunit, which is noncovalently associated with a pentameric B subunit. The pentameric B subunit is responsible for binding to the globotriaosylceramide receptors (Gb3, Gb4) present on different cells. The enzymatically active A subunit has been shown to cleave an adenine residue from the 28S rRNA of the 60S ribosome using its *N*-glycosidase, and thus to inhibit protein synthesis, eventually leading to cell death (5). However, whether this accounts for the majority of clinical symptoms found in EHEC-associated HUS or not is still unknown, with the consequence that an effective therapy is as yet unavailable (5). Armstrong et al. (6) have found that human serum amyloid P is binding to Stx2 and protecting mice against the lethal challenge by i.v. administered Stx2. Other human proteins circulating in the blood, such as complement proteins, have not yet been investigated for binding to Stx2.

Beside EHEC-associated, diarrhea-positive HUS, hereditary forms of HUS, called atypical HUS (aHUS), have been reported (7). aHUS is a rare disease; it is usually recurrent and is characterized by a more severe course of disease with a poorer outcome than in the case of EHEC-induced HUS. It has been shown that

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[4] Abbreviations used in this paper: HUS, hemolytic uremic syndrome; aHUS, atypical HUS; AP, alkaline-phosphatase; CHO, Chinese hamster ovary; EHEC, enterohemorrhagic *Escherichia coli*; FH, factor H; FI, factor I; hi-Stx, heat-inactivated Shiga toxin; iC3b, inactivated C3b; NHS, normal human serum; PMN, polymorphonuclear cell; SCR, short consensus repeat; Stx, Shiga toxin; TCC, terminal complement complex; VB, veronal buffer.

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aHUS is strongly associated with mutations of complement regulator genes, such as those for factor H (FH), factor I (FI), and membrane cofactor protein (CD46), as well as those in the factor B gene. However, incomplete penetrance of mutations in each of these genes has been described, suggesting that an additional trigger is required to unmask the complement regulatory deficiency (8).

The complement system is part of the innate immunity and consists of a set of plasma and membrane-bound proteins that, among other functions (9), protect the body against invading organisms. Complement is activated by three different pathways, as follows: the classical pathway, the lectin pathway, and the alternative pathway, finally resulting in the formation of the terminal complement complex (TCC) (9).

Several regulatory proteins control this complex cascade system. FI plays a major role by inactivating C3b, a central protein of the cascade. FI-mediated processing of C3b into inactivated C3b (iC3b) requires the cofactor activity of one of several molecules, including FH, complement receptor 1, and membrane cofactor protein (CD46) (9).

FH, composed of 20 short consensus repeats (SCRs) that are also termed complement control protein repeats, is a serum glycoprotein predominantly synthesized by the liver. FH is the most important fluid-phase regulator of the alternative pathway. Apart from being an essential cofactor in the FI-mediated proteolytic conversion of plasma C3b to iC3b (iC3b may then be cleaved into further fragments (C3dg and C3c)), FH is able to block the formation of the alternative pathway C3 convertases by binding to C3b, thereby inhibiting the interaction between C3b and factor B. In addition, FH possesses decay acceleration activity, i.e., it promotes the dissociation of these C3 convertases once they have been formed (10).

Whereas excessive complement activation and deposition of complement fragments on target membranes in aHUS are due to mutations leading to dysfunctional complement regulator proteins, the mechanisms of the development of EHEC-associated HUS have been attributed to a direct effect of Stx, mainly on renal cells. The aim of our study was to investigate whether complement is also involved in the development of the EHEC-associated HUS or not. In particular, the aim was to verify or refute our hypothesis that Stx, rather than having a sole direct effect on kidney cells, also exerts an effect via complement activation that subsequently destroys the kidney or, alternatively, interferes with complement inhibitors leading to complement activation due to lack of appropriate control (Fig. 1).

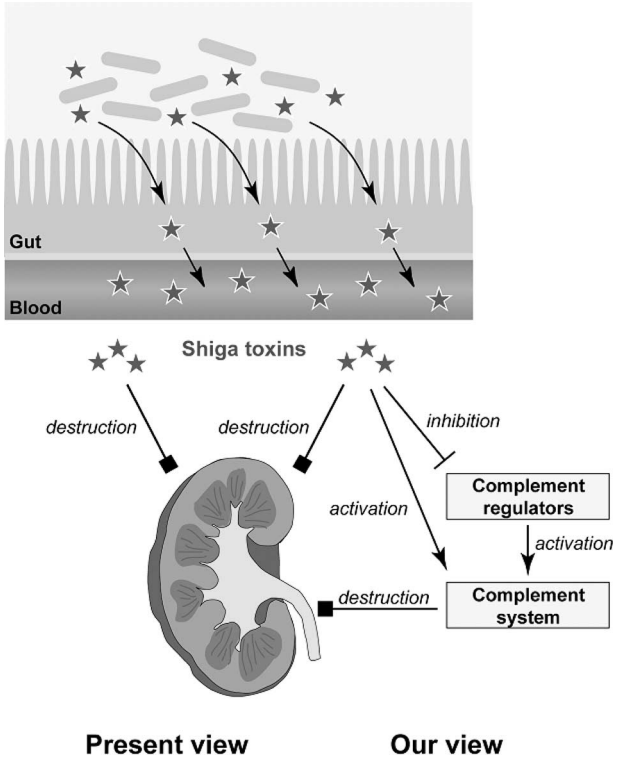


FIGURE 1. Effect of Stxs on renal cells. We hypothesized that Stx, rather than having a sole direct effect on kidney cells, also mediates complement activation, which subsequently destroys the kidney or, alternatively, interferes with complement regulators leading to complement activation due to lack of appropriate control. The *left side* shows the present view, and the *right side* our hypothesis.

Materials and Methods

Purification of Stx2

The toxin was purified, as described previously (11). Purity of Stx2 was evaluated by SDS-PAGE (data not shown).

Cytotoxic activity on Vero cells was found to be 100% for purified Stx2 and <11% for heat-inactivated (hi; 15 min at 80°C)-Stx2 used as negative control (4) (data not shown).

Proteins and Abs

FH, FI, C3b, and goat anti-human FH Ab were all purchased from Calbiochem; sheep anti-human FH from The Binding Site; mouse anti-human FI from Cedarlane Laboratories; rabbit anti-human C3c from Dako-Cytomation; anti-Stx2 from Hycult Biotechnology; and alkaline phosphatase (AP)-conjugated anti-goat, anti-mouse, and anti-sheep Abs, as well as 5-bromo-4-chloro-3-indolyl phosphate/NBT and *p*-nitrophenyl phosphate substrates, all from Sigma-Aldrich.

FH constructs SCRs6–8, with a histidine variant at aa 402 in SCR7 (402H; corresponding to reside 384 in the mature form of the protein), or with a tyrosine variant at the same position (402Y), were expressed and purified, as described previously (12). The 402H allele has been reported to be associated with a higher probability of acquiring age-related macular degeneration (13) and has different ligand-binding properties when compared with the nondisease-associated 402Y variant (12, 14). FH constructs SCRs16–17 and SCRs18–20 were generated according to the method described by Giannakis et al. (10). SCRs6–8 and SCRs18–20 are involved in the surface recognition function of FH (10).

Assessment of complement activation by TCC-ELISA

A sandwich ELISA, based on a neoepitope-specific mAb (15) directed against C9, was used to measure the concentration of TCC generated in normal human serum (NHS) preincubated with purified Stx2 (10–0.5 μg) for 24 h. Biotinylated polyclonal anti-C7 IgG served as detecting reagent (16). Measurements were compared with a standard of zymosan-activated human serum with a TCC concentration of 500 μg/ml. Hi-Stx2 and veronal buffer (VB; Virion/Serion) were used as negative controls.

Determination of the complement pathway involved

NHS preincubated with Stx2 and either EDTA (25 mM) for blocking both the classical and the alternative pathway, or EGTA (15 mM) in the presence of Mg^{2+} (7.5 mM) for blocking the classical pathway only, served as Ag in the TCC-ELISA described above. NHS preincubated with only Stx2 was used as positive control; NHS with only VB served as negative control.

Impact of Stx2 on FH and FI integrity determined by Western blot

Stx2 (2.5 or 1.25 μg) and 2.5 μg of either FH or FI were incubated at 37°C for 24 h; PBS alone was used as a negative control, whereas FH or FI alone served as positive controls. The samples were analyzed by SDS-PAGE using a 6% (w/v) gel (Bio-Rad) under reducing conditions and transferred to a nitrocellulose membrane. Goat anti-human FH or mouse anti-human FI (both 1:2000) were used as the primary Abs, with AP-conjugated anti-goat or anti-mouse IgG as secondary Abs, respectively, and 5-bromo-4-chloro-3-indolyl phosphate/NBT as the substrate.

Analysis of binding of Stx2 to FH and FI by ELISA

Stx2 (2.5, 1.25, or 0.625 μg), hi-Stx2 (2.5 μg), or C3b (0.5, 0.25, or 0.125 μg), serving as positive control, was immobilized in 100 μl of coating buffer (15) onto the wells of a microtiter plate, with VB as the negative control. Following blocking with 1% (w/v) gelatin at room temperature for 30 min, 2 μg of either FH or FI was added for 24 h at 37°C. After washing, bound FH was detected with a primary sheep anti-human FH Ab (1:2000) and a secondary AP-conjugated anti-sheep IgG. Bound FI was detected, as described above, for the Western blot. The plates were developed with *p*-nitrophenyl phosphate as substrate.

Analysis of binding of Stx2 to FH by coimmunoprecipitation

A total of 100 μl of anti-Stx2 was coupled to AminoLink Plus coupling gel (Pierce), as described in the manufacturer's protocol. Stx2 (20 μl; 0.5 μg/μl), preincubated with 20 μl of NHS at 37°C for 24 h, was applied to the anti-Stx2 Ab-coupled gel in a Handee spin cup column (Pierce) and incubated with end-over-end mixing at room temperature for 2 h. An inactive form of the gel was prepared and incubated with Stx2-NHS complex and served as a negative control. After washing, bound proteins were eluted, as described in the manufacturer's protocol. The eluted protein complexes were resolved by SDS-PAGE, followed by Western blotting with an anti-FH Ab, as described above.

Analysis of binding of Stx2 to FH constructs SCRs6–8 (402H, 402Y), SCRs16–17, and SCRs18–20 by ELISA

For the ELISA-assay, 2.5 μg of Stx2 or VB (serving as negative control) was coated onto the wells of a microtiter plate (100 μl) and blocked, as described above. SCRs6–8 (402H and 402Y), SCRs16–17, and SCRs18–20 were added at a range of concentrations (2, 1, 0.5, or 0.25 μg; in 100 μl) and incubated at 37°C for 24 h. Detection was performed in the same way as for the FH-binding ELISA, because sheep anti-human FH Ab detects SCRs6–8 (402H and 402Y), SCRs16–17, and SCRs18–20 to a comparable extent (data not shown).

Cofactor activity of FH when bound to Stx2 by assessment of cleavage of C3b

Stx2 (2.5 μg), FH (4 μg; serving as the positive control), or PBS (serving as the negative control) was immobilized onto the wells of a microtiter plate. FH (4 μg) or PBS in the case of the positive control was added at 37°C for 24 h, followed by 40 μl of PBS containing 1.2 μg of C3b and 2 μg of FI for 90 min at 37°C. The contents of the wells were collected, resolved on an 8% SDS-PAGE, and transferred to a nitrocellulose membrane by electroblotting. Rabbit anti-human C3c and AP-conjugated anti-rabbit IgG were used for the detection of C3b degradation products.

Cofactor activity of FH treated with Stx2 on Chinese hamster ovary (CHO) cells

CHO cells were seeded in a 96-well plate at 2×10^6 cells/ml and cultured at 37°C till a confluent (>90%) cell layer was formed. Cells were washed and fixed with ice-cold methanol for 10 min at −20°C. FH (1 μg) was incubated with 2 μg of Stx2 or hi-Stx2, respectively, for 24 h at 37°C, and added to the CHO-coated wells for 1 h at 37°C. For the negative control, cells were incubated with Stx2 only. Cells were washed with PBS, and subsequently, 2 μg of FI and 1.5 μg of C3b were added and incubated at 37°C. Supernatants were collected at different time points (5, 15, and 45 min), and C3b degradation products were detected by SDS-PAGE, as described above.

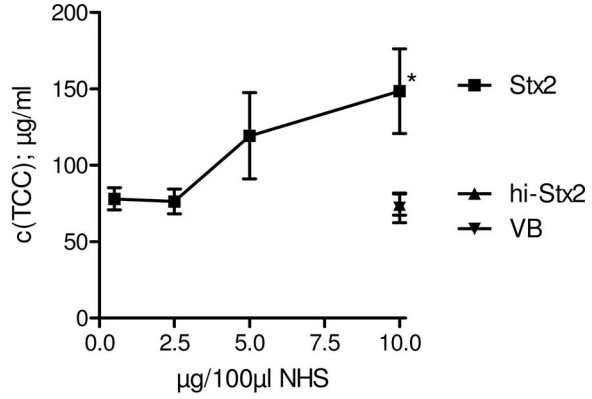
Statistical analyses

Student's *t* test was used for comparison of paired means of two groups of measurements. One-way ANOVA (GraphPad) was applied. Values of $p < 0.05$ were considered significant.

Results

Stx2 activates complement

Purified Stx2 activated complement in the fluid phase in vitro, as evidenced by formation of TCC when coincubating NHS with Stx2 (Fig. 2). Complement activation by Stx2 was clearly concentration dependent. Interestingly, hi-Stx2 only activated complement to background levels.



* $P < 0.05$

FIGURE 2. Complement activation by Stx2. TCC concentrations, (c(TCC)), induced by purified Stx2 or hi-Stx2 at different concentrations, after coincubation in 100 μl of NHS for 24 h at 37°C. The TCC concentration was significantly higher for Stx2 at the highest concentration (10 μg) when compared with hi-Stx2 ($p < 0.05$). The results are the means ± SEM of four separate experiments using duplicates ($n = 8$).

Complement activation proceeds predominantly via the alternative pathway

High concentrations of TCC were detected when NHS was incubated with Stx2 in the presence of Mg^{2+}-EGTA, with comparable levels being observed in the positive control (NHS and Stx2). In contrast, coincubation with EDTA (which blocks complement activation completely) showed only very low concentrations of TCC, even lower than those observed in the negative control (NHS and VB). The fact that the blocking of the classical pathway (by adding EGTA) resulted in nearly as much TCC formation as observed in

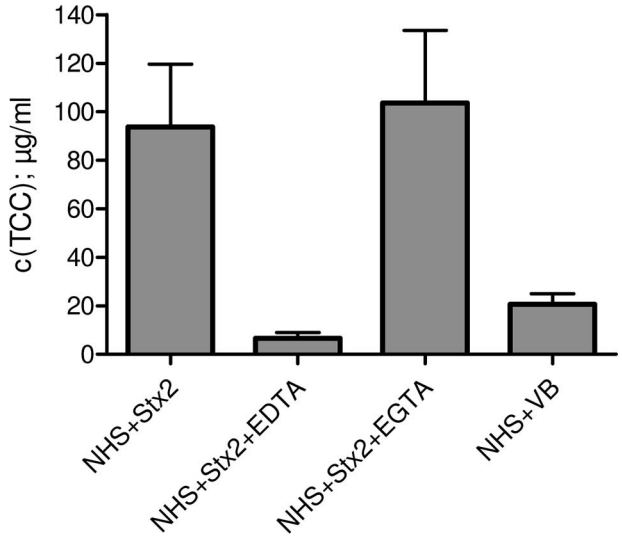


FIGURE 3. Stx2 activates complement via the alternative pathway. TCC concentrations (c(TCC)), induced by purified Stx2 in the presence of different complement inhibitors: NHS + Stx2, complement activation by Stx2; NHS + Stx2 + EDTA, EDTA blocks complement activation by Stx2; NHS + Stx2 + EGTA, EGTA blocks the classical pathway, allowing Stx2 to activate complement via the alternative pathway only; NHS + VB, complement activation by buffer. The results are the means ± SDs of three separate experiments.

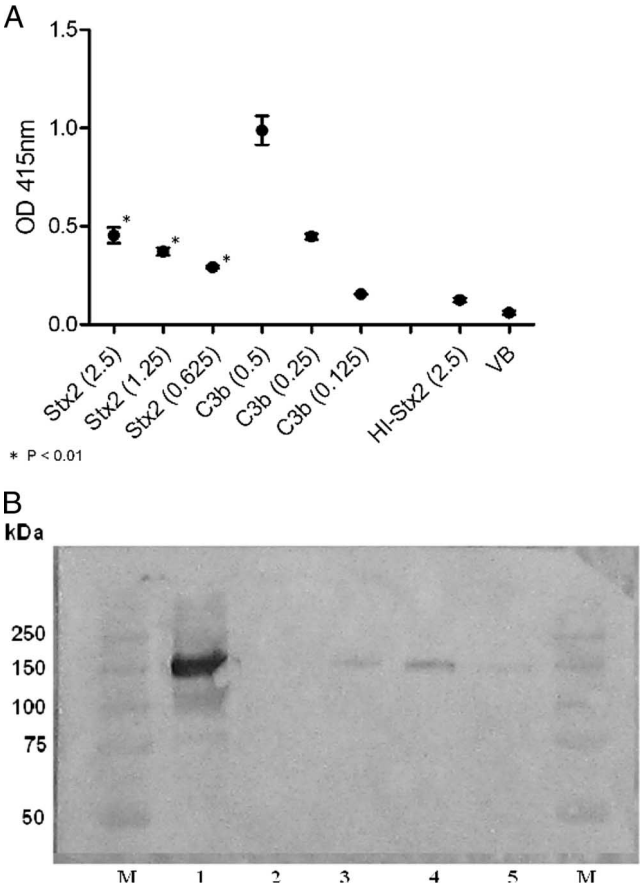


FIGURE 4. *A*, Binding of Stx2, C3b, and hi-Stx2 to FH. The binding capacity of Stx2 was significantly higher at all concentrations when compared with hi-Stx2 ($p < 0.01$ for all). The binding capacity of 2.5 μg of Stx2 was comparable to that of 0.25 μg of C3b. VB served as negative control. The results are the means ± SDs of four separate experiments using duplicates ($n = 8$). *B*, Binding of FH to Stx2 by coimmunoprecipitation. The binding capacity of FH to Stx2 was confirmed by coimmunoprecipitation. M, precision plus protein standard marker; *lane 1*, flow through (Stx2 + NHS); *lane 2*, control gel (inactive form) elution with Stx2 + NHS; *lane 3*, coupled gel elution 1 with Stx2 + NHS; *lane 4*, coupled gel elution 2 with Stx2 + NHS; *lane 5*, coupled gel elution 3 with Stx2 + NHS.

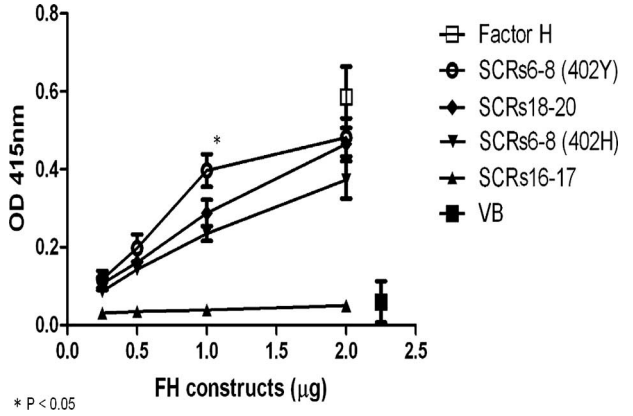


FIGURE 5. Binding of Stx2 to FH constructs. Binding capacity of Stx2 to FH construct 402Y was significantly ($p < 0.05$) higher than to FH construct 402H. Stx2 also bound to SCRs18–20, but not SCRs16–17. VB served as negative control. The results are the means ± SDs of three separate experiments using duplicates ($n = 6$).

the case without the blocking of any complement pathway indicates that Stx2 activates complement mainly via the alternative pathway (Fig. 3).

Stx2 does not destroy FH or FI

Because FH and FI are the most important regulators of the alternative pathway, we investigated, by Western blot analysis, whether or not Stx2 destroys FH or FI when incubated with different amounts of Stx2 for 24 h. In the samples (FH plus Stx2 and FI plus Stx2, respectively), as well as in the positive controls, we detected only a single band at 155 kDa for FH and at 88 kDa for FI, and no cleavage products. Thus, Stx2 did not cleave FH and FI (data not shown).

Stx2 binds to FH, but not to FI

Binding of purified Stx2 to FH or FI was analyzed by ELISA. Stx2 was coated onto the solid phase for this assay and, after addition of FH or FI, the binding of these proteins was assessed by an anti-FH or anti-FI Ab. We showed that purified Stx2 efficiently bound FH in a concentration-dependent manner. Even in the presence of only 0.625 μg of Stx2, a higher FH-binding activity was observed than for the negative control (with VB) or hi-Stx2. Comparison of the binding capacities of C3b, which is known to be a very well-characterized ligand for FH (17), and Stx2 to FH revealed that 2.5 μg of Stx2 showed a binding capacity comparable to that of 0.25 μg of C3b (Fig. 4*A*). Such comparisons are, however, rather difficult to make, given that one does not know how much active protein is immobilized in plate assays. Nevertheless, they confirm that Stx2 is clearly binding to FH.

In contrast, Stx2 did not bind to FI in any of the investigated concentrations (data not shown). Coimmunoprecipitation experiments were performed to confirm binding of Stx2 to FH. For this purpose, NHS was incubated with purified Stx2 and applied to an anti-Stx2 Ab-coupled gel. The three elution fractions were separated by SDS PAGE following Western blot with anti-FH Ab. All three elution fractions (fraction 3 to a lesser extent) showed a 155-kDa band as evidence for binding of FH to Stx2 (Fig. 4*B*).

Stx2 binds to FH SCRs6–8 and SCRs18–20

To determine the region of FH that binds to Stx2, two common variants of FH constructs SCRs6–8 (402Y and 402H) were used in addition to SCRs16–17 and SCRs18–20. Stx2 was found to bind both, SCRs6–8 and SCRs18–20, which are involved in the surface recognition function of FH. However, Stx2 bound significantly better to the 402Y variant than to 402H ($p < 0.05$). Stx2 did not bind to FH constructs SCRs16–17 (Fig. 5).

FH remains functionally active upon binding

We investigated whether or not FH retained its function, namely its ability to inhibit the alternative complement pathway, when bound to Stx2. FH was observed to retain functional activity when bound to Stx2 and still acts as a cofactor for the serum protease FI in the cleavage of C3b, as shown by the presence of the characteristic cleavage of C3b α-chain into α' 46-kDa and α' 43-kDa fragments (17) (data not shown).

Cofactor activity of FH on CHO cells is delayed upon binding with Stx2

In a cell assay, we determined whether or not FH also retains its cofactor activity on the cell surface when bound to Stx2. When compared with the experiments conducted with hi-Stx2, functionally active Stx2 delayed cofactor activity of FH. In the presence of

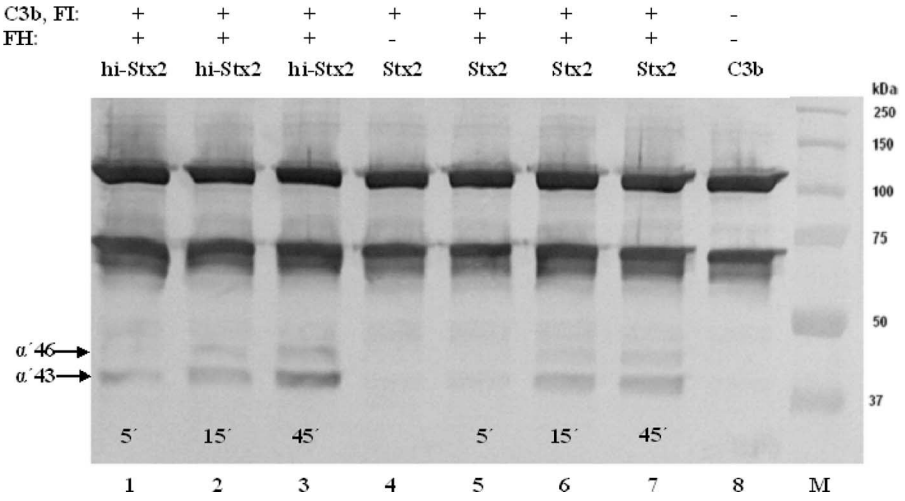


FIGURE 6. Cofactor activity of FH on coated CHO cells in the presence of Stx2. In the setup with hi-Stx2, cleavage products of C3b (α' 43 and α' 46) were already visible after an incubation time of 5 min, with active Stx2 only after 15 min. *Lanes 1–3*, Cells treated with preincubated FH and hi-Stx2 for 1 h and subsequent addition of C3b and FI for 5 min (*lane 1*), 15 min (*lane 2*), and 45 min (*lane 3*); negative control, cells treated with Stx2 and subsequent addition of C3b and FI for 45 min (*lane 4*). *Lanes 5–7*, Cells treated with preincubated FH and Stx2 and subsequent addition of C3b and FI for 5 min (*lane 5*), 15 min (*lane 6*), and 45 min (*lane 7*); cells treated with C3b only (*lane 8*); precision plus protein marker (lane M). The results show a representative experiment of four.

hi-Stx2, the characteristic cleavage products of C3b (43- and 46-kDa fragments) were already detected after 5-min incubation with C3b and FI, whereas cleavage products were only visible after 15 min when FH was incubated with functionally active Stx2 (Fig. 6).

Discussion

Pathogenesis of EHEC-induced HUS has not yet been fully elucidated; however, Stx has been found to damage endothelial and epithelial cells by inhibition of protein synthesis and apoptosis (18). Activation of the complement cascade as shown in this study also results in destruction of the kidney and other organs, not only by direct lysis of cells, but also via the release of chemotactic anaphylatoxins C3a and C5a (9). These chemotactic substances are engaged in the regulation of acute inflammatory reaction, including extravasation of leukocytes and chemotaxis. Anaphylatoxins C5a and to a lesser extent C3a interact with the receptors expressed on effector cells, such as monocytes and macrophages, thereby attracting and activating them. This activation leads to the release of various cytokines and chemokines, including TNF-α and different ILs (19). Histological and electron microscopic studies of HUS biopsies have shown the presence of mononuclear as well as polymorphonuclear cells (PMNs) in the glomeruli (20). Furthermore, the chemokines monocyte chemoattractant protein-1 and IL-8, which is a powerful activator and chemoattractant of PMNs, have been shown to be present at elevated levels in urine samples taken from HUS patients, whereas only very limited amounts were found in the urine samples obtained from 17 healthy control subjects. In addition, serum IL-8 levels were significantly increased and serum monocyte chemoattractant protein-1 levels were slightly increased in HUS patients (20) when compared with the levels observed in the control subjects. Renal epithelial cells can be stimulated to release IL-8 both into the urine and the interstitial space by several proinflammatory mediators (e.g., TNF-α) and activate PMN migration (21). These findings corroborate the hypothesis that chemokines, such as monocyte chemoattractant protein-1 and IL-8, may be implicated in the pathogenesis of HUS through the recruitment and activation of monocytes and PMNs, respectively.

On the one hand, inflammatory mediators cause exposure of the subendothelial extracellular matrix, which lacks endogenous complement receptors and thus allows the further deposition of C3b and the activation of the alternative pathway, resulting in further tissue damage.

In contrast, several studies have reported a significant influence of inflammatory mediators on local and peripheral homeostasis (19). It has been shown that IL-1 and TNF-α markedly increase tissue factor-like procoagulant activity in cultured human umbilical endothelial cells, which then acquire the capacity to bind factor VIIa and to initiate the extrinsic clotting pathway (22). Thus, IL-1 and TNF have profound effects on the endothelial coagulant-anticoagulant balance and enhance surface thrombogenicity and intravascular coagulation (22), which is a key component of HUS. Ikeda et al. (23) have demonstrated that human rC5a induces tissue factor activity in HUVECs.

These findings are significant in light of other studies showing that human endothelial cells become more sensitive to the cytotoxic action of Stx2 when coincubated with TNF-α or IL-1 (24). Ramegowda et al. (25) have reported that treatment of human glomerular microvascular endothelial cells and human brain microvascular endothelial cells with TNF-α resulted in increased expression of the toxin-binding Gb3 receptor, which might explain the increased Stx susceptibility.

It can be assumed that Stx2 activates complement, generates anaphylatoxins and TCC, and also triggers monocytes, which secrete factors such as IL-1 and TNF-α. Thus, TCC and secreted factors augment the destructive effect of Stx on target cells. However, there are no reports of the serum Stx level in humans with EHEC infection, and thus, it is difficult to estimate the appropriate amount of Stx required for in vitro studies.

To our knowledge, this is the first report of complement activation by Stx. Few other groups have studied the role of complement in EHEC-induced HUS, but could not find any association. Paixão-Cavalcante et al. (26) and Barrett et al. (27) did not find an increased host susceptibility to Stx-associated HUS in heterozygous FH- or homozygous C5-deficient mice, respectively, pointing against a role of complement in EHEC-associated HUS. Proulx et al. (28) reported that deficiency in mannan-binding lectin did not predispose to EHEC infection and associated HUS. This is not contradictory to our results showing that activation proceeds via the alternative pathway. Furthermore, increased breakdown and low levels of C3 have been demonstrated in HUS patients; however, no definite explanation for these complement abnormalities was given (29, 30). To further support our hypothesis of a role of complement in EHEC-induced HUS, it would be of great value to study renal biopsies of HUS patients for the presence of C3 deposits.

In our study, we have shown that the activation of complement by Stx2 proceeds predominantly via the alternative pathway. The absent requirement of the classical pathway excludes the possibility that the presence of Abs in the serum that served as a source of complement may account for this activation. This is further corroborated by the fact that hi-Stx2 activated complement in the very same serum to only background levels.

Because FH is one of the most important regulators of the alternative pathway, we have investigated the effect of Stx2 on FH. FH was not destroyed by Stx2, which cannot necessarily have been assessed by Western blot, but certainly by functional assays (see below). However, Stx2 has been shown to bind to FH by two independent methods, an ELISA and a coimmunoprecipitation assay.

In the case of aHUS, several studies have reported mutations in FH (8). These mutations tend to cluster in the C-terminal region of FH, predominantly within SCRs16–20 (8). Individuals with these mutations express FH molecules that show normal regulatory capacity in the fluid phase, but a limited capacity to bind to host cells and to protect them against complement lysis. In the case of complement activation induced by bacterial or viral infections or other triggers, the imbalance of an active complement system in plasma and a defective protection of cells results in an uncontrolled deposition of C3b on cell surfaces and finally in tissue damage (31).

In our study, we have demonstrated that Stx2 binds to FH at SCRs6–8. The binding capacity of Stx2 to FH constructs SCRs6–8 with the tyrosine variant at aa 402 in SCR7 (402Y) was significantly higher than to FH constructs with the histidine variant at the same position (402H), the latter being associated with age-related macular degeneration (13).

A number of other ligands, i.e., C-reactive protein, fibromodulin, and chondroadherin (32), were found to bind better to the 402Y variant than to the 402H construct; the latter, however, bound better to DNA and necrotic cells (32). Furthermore, the 402H and 402Y variants have greatly different specificities for sulfated glycosaminoglycans (12), the molecular basis for this having been determined by x-ray crystallography (14). These findings corroborate that the Y to H change has a major effect on the activity of FH.

Interestingly, the 402H allotype also leads to diminished binding of FH to group A streptococci, and thus, increased opsonophagocytosis of the bacteria in blood (33), implying that survival from streptococci in younger years is at the price of age-related macular degeneration when getting old.

Thus, we speculate that human beings with variants of FH, in particular in the surface recognition regions, are more vulnerable for EHEC-associated HUS.

We have, in addition, shown that Stx2 also binds to SCRs18–20, which are, similar to SCRs6–8, involved in the interaction of FH with cellular surfaces (10). Cofactor activity of FH preincubated with Stx2 was still present in the fluid phase; however, on the surface of CHO cells, selected because they lack endogenous complement regulators, its activity was clearly delayed.

This finding may be a consequence of an impaired binding of FH to the cell surface due to binding of Stx2 to FH at those regions that are involved in the interaction with cellular surfaces. A delayed cofactor activity may possibly lead to enhanced complement activation, contributing to an altered susceptibility of the target cell with subsequent host cell damage.

In conclusion, we have demonstrated that purified Stx2 has a dual influence on the complement cascade by a direct activation of complement in the fluid phase and a delayed cofactor activity of FH on the cell surface when bound to Stx2. These findings suggest that complement activation by Stx2 may represent an important trigger for cell damage, and thus for the pathogenesis of EHEC-associated HUS, similar to that of aHUS.

Disclosures

The authors have no financial conflict of interest.

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