EspP, a Serine Protease of Enterohemorrhagic Escherichia coli, Impairs Complement Activation by Cleaving Complement Factors C3/C3b and C5

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Hemolytic-uremic syndrome (HUS) is a life-threatening disorder characterized by hemolytic anemia, thrombocytopenia, and renal insufficiency. It is caused mainly by infections with enterohemorrhagic Escherichia coli (EHEC). Recently, Shiga toxin 2, the best-studied virulence factor of EHEC, was reported to interact with complement, implying that complement may be involved in the pathogenesis of EHEC-induced HUS. The aim of the present study was to investigate whether or not the serine protease EspP, an important virulence factor of EHEC, interacts with complement proteins. EspP did not have any effect on the integrity of factor H or factor I. However, EspP was shown to cleave purified C3/C3b and C5. Cleavage of the respective complement proteins also occurred in normal human serum (NHS) as a source of C3/C3b or C5 or when purified complement proteins were added to the supernatant of an EspP-producing wild-type strain. Edman degradation allowed unequivocal mapping of all three main C3b fragments but not of the three main C5 fragments. Complement activation was significantly downregulated in all three pathways for C5-depleted serum to which C5, preincubated with EspP, was added (whereas C5 preincubated with an EspP mutant was able to fully reconstitute complement activation). This indicates that EspP markedly destroyed the functional activity, as measured by a commercial total complement enzyme-linked immunosorbent assay (Wieslab). Downregulation of complement by EspP in vivo may influence the colonization of EHEC bacteria in the gut or the disease severity of HUS.

EspP is further cleaved to iC3b by factor I (FI) in the presence of factor H (FH), CR1, or membrane cofactor protein. FI cleavage of C3b to iC3b inactivates and prevents C3b from functioning in the C3 or C5 convertase enzymes. Further cleavage of iC3b by FI in the presence of CR1 results in the formation of C3c. When C3b is not inactivated through cleavage by FI, the C3 convertase develops into a C5 convertase which cleaves C5 into C5a and C5b. Both C3a and C5a have anaphylatoxic and chemotactic activities, recruiting inflammatory cells and triggering degranulation of mast cells. C5b initiates the membrane attack pathway, which results in the terminal complement complex, termed the membrane attack complex when formed on a membrane, consisting of C5b, C6, C7, C8, and polymeric C9.

Implication of complement in diarrhea-negative, atypical HUS has been reported, and mutations in different complement regulator genes, such as those encoding FH, FI, and membrane cofactor protein, as well as gain-of-function mutations in C3 and factor B genes, have been described for affected patients (5, 10, 18). We recently showed that purified Stx2 activates the complement cascade (20) and demonstrated that Stx2 binds FH and that cofactor activity of surface-attached FH is delayed in the presence of Stx2. Thus, our observations indicate that complement may also be involved in the pathogenesis of EHEC-induced HUS (20).

Several reports suggest that in addition to Stxs, the serine protease EspP may be an important virulence factor of EHEC.

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bacteria (1, 14). We have shown that EspP is widely distributed among Shiga toxin-producing E. coli strains of human and animal origins and that EspP is associated with EHEC serogroups which cause HUS. This suggests that EspP contributes to the pathogenesis of this disorder (1, 14). However, the exact role of EspP as a virulence factor in the pathogenesis of EHEC-induced HUS is unknown.

The aim of this study was to investigate whether or not EspP interacts with complement. In particular, it was envisaged to extend our knowledge on the pathogenesis of EHEC-induced HUS by determining whether or not the serine protease EspP cleaves complement components and thereby modulates complement activity.

**MATERIALS AND METHODS**

**Proteins and antibodies.** EspP was purified from clone DH5α(pBB-5), which contains espP from E. coli O157:H7 EDL933, as described previously (1). An inactive EspP protein which served as a negative control was purified from the site-directed EspP S263A mutant in the same way (2). The concentration of both proteins was determined using the Bradford assay (3 mg/ml).

FH, FI, C3, C3b, C5, C5-depleted serum (CDS), and goat anti-human FH antibody were all purchased from Calbiochem, goat anti-human FI was from Qiudel, rabbit anti-human C3 was from Santa Cruz Biotechnology, rabbit anti-human C5 was from Dako-Cytomation, and alkaline phosphatase-conjugated anti-goat and anti-rabbit antibodies, as well as 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (NBT) substrate, were from Sigma-Aldrich. A rabbit anti-EspP antibody (Davids Biotechnology) directed against a peptide of the C-terminal part of the EspP passenger domain was employed for the detection of EspP and the S263A mutant.

**Impact of EspP on FH, FI, C3/C3b, and C5 integrity, determined by Western blotting.** EspP (1 μg) and FH, FI, C3, C3b, or C5 (2 μg) were incubated in 10 mM HEPES buffer containing 150 mM NaCl and 20 mM CaCl₂ (pH 7.4) at 37°C for 24 h. For C3b and C5, a time course consisting of 5-, 12-, 24-, and 48-h incubations was performed, and different amounts of EspP (1, 0.5, 0.25, and 0.125 μg) were tested within a 5-h incubation. In addition, all complement components were incubated with the site-directed EspP S263A mutant (1 μg) or with HEPES buffer only, both of which served as negative controls. The reaction mixtures were separated by SDS-PAGE on 6 to 12% (wt/vol) gels (Bio-Rad), depending on the molecular weight of the investigated protein, under reducing and nonreducing conditions, and were transferred to a nitrocellulose membrane. Goat anti-human FH (1:1,000), goat anti-human FI (1:1,000), and rabbit anti-human C3 (1:5, both 1:1,000) or anti-human C5 (both 1:1,000) were used as primary antibodies, with alkaline phosphatase-conjugated anti-goat or anti-rabbit IgG (1:2,000) serving as the secondary antibody and with 5-bromo-4-chloro-3-indolyl phosphate/NBT as the substrate.

**Impact of EspP on integrity of C3/C3b and C5 in NHS.** In order to analyze whether or not EspP is able to cleave C3/C3b and C5 in NHS, EspP (1 μg) as well as EspP S263A (1 μg) and HEPES buffer, with the latter as negative controls, was incubated with 0.2 μl NHS obtained from healthy blood donors as a source of C3/C3b or C5 at 37°C for 5 and 24 h. In the case of C3, NHS had to be spiked with 0.5 μg C5 due to its low concentration in NHS. The reaction mixtures were analyzed by Western blotting with a rabbit anti-human C3 or anti-human C5 primary antibody and an alkaline phosphatase-conjugated anti-rabbit IgG secondary antibody, as described above.

**Impact of EspP-containing supernatant of a wild-type strain on integrity of purified C3b and C5.** One EspP-positive strain and one EspP-negative strain, both displaying comparable virulence profiles with identical types of stx₂, cnf₁, and hlyA genes and both obtained from patient samples, were grown overnight in 250 ml of Luria-Bertani broth (Sifin) at 37°C with vigorous shaking. The cultures were centrifuged (6,000 × g for 30 min at 4°C), and supernatants were passed through a 0.20-μm-pore-size filter (Corning). The filtrates were then added to an Amicon stirred-cell system (series 8000; Millipore) with a YM30 membrane (30-kDa molecular mass cutoff) and concentrated to 50 ml under nitrogen pressure (0.5 × 10⁻⁵ to 1 × 10⁻³ Pa) with stirring at 4°C. Finally, proteins were precipitated (1 h at 4°C) by adding ammonium sulfate (Merek) to 55% saturation. The precipitate was collected by centrifugation (6,000 × g for 30 min at 4°C), and the pellet was dissolved in 500 μl of 10 mM HEPES buffer containing 150 mM NaCl and 20 mM CaCl₂ (pH 7.4). Further buffer changes and concentration of the sample were achieved with a 10-kDa Vivaspin spin-down filter (Vivaspin-Sartorius).

The presence or absence of EspP in the resuspended pellet was evaluated by Western blotting with a rabbit anti-EspP (1:1,000) primary antibody and an alkaline phosphatase-conjugated anti-rabbit IgG secondary antibody (1:2,000) as described above.

In order to analyze whether or not the supernatant of an EspP-positive strain was capable of cleaving C3b and C5, 10 μl of the resuspended pellet of an EspP-positive or EspP-negative strain was incubated with 2 μl purified C3b or C5 (both 1 μg/μl) at 37°C for 24 h. The reaction mixtures were analyzed by Western blotting, employing a rabbit anti-human C3 or rabbit anti-human C5 primary antibody and an alkaline phosphatase-conjugated anti-rabbit IgG secondary antibody as described above.

**Sequence analyses of cleavage sites via Edman degradation.** A similar procedure for SDS-PAGE and Western blotting to that described above was followed for sequence analyses, with slight modifications. Briefly, 2 μg of C3b was incubated with 5 μg of purified EspP for 24 h at 37°C. Proteins were separated in a 12% SDS-PAGE gel under reducing conditions and blotted onto a polyvinylidene difluoride (PVDF) membrane (activated with methanol for 30 s prior to use) in the presence of borate buffer. The PVDF membrane was stained with Coomassie blue. Spots of interest were cut out of the membrane, and the N-terminal amino acids were sequenced via Edman degradation. Peptide sequencing was performed on an Applied Biosystems Inc. (ABI) model 492 Procise protein sequencer.

**Assessment of in vitro complement activity.** Subsequently, we analyzed whether cleavage of complement proteins resulted in reduced or enhanced complement activation by using a Wieslab complement system screening kit. This enzyme immunoassay was developed for the determination of a functionally active CP, MBL pathway, and AP in human serum, using deposition of the terminal C5-9 complex in microtiter wells as the readout. The wells of the microtiter plate strips are coated with specific activators of the three pathways. Patient serum is diluted in diluent containing specific blockers to ensure that only the respective pathway is activated.

For the purpose of our study, samples instead of sera were applied and were prepared in the following way. C5 (11.5 μg in 11.5 μl; Calbiochem) was incubated with EspP (28.75 μg) or the S263A mutant (28.75 μg), both in 11.5 μl HEPES, or with 11.5 μl of 10 mM HEPES buffer only (containing 150 mM NaCl and 20 mM CaCl₂), with the last two serving as negative controls, at 37°C for 24 h. CDS (15 μl) was added to each sample, thus restoring the former to a 10-fold excess of C5 (770 μg/ml). These samples were treated with a Wieslab complement system screening kit as described for serum for the CP, the MBL pathway, or the AP. The amount of complement activation correlates with the color intensity and was measured as absorbance (optical density) at 410 nm. The value for the positive control provided in the test kit is defined as 100% complement activation. All measured values are expressed as % complement activity, determined as follows: (sample value − negative-control value)/(positive-control value − negative-control value) × 100.

**Statistical analyses.** Student’s t-test was used for comparison of paired means of two groups of measurements. One-way analysis of variance (Graphpad Software Inc.) was applied. P values of <0.05 were considered significant.

**RESULTS**

EspP cleaves C3/C3b and C5 but not FH or FI. The complement factors C3, C3b, C5, FH, and FI were incubated with EspP for 5 to 48 h. SDS-PAGE and Western blotting were performed in order to assess the presence of any cleavage. In the case of C3, C3b, or C5, a clear degradation and the occurrence of additional bands were observed. For C3b, three major bands, with molecular masses of ~42 kDa, ~38 kDa, and ~37 kDa, were found in a reducing gel (Fig. 1). When the incubation mixture with EspP and C3b was run in a nonreducing gel, one major band, at ~140 kDa, and another strong band, at ~35 kDa, were observed (data not shown). In the case of incubation of EspP with C3, similar cleavage products to those with C3b were observed. However, in the controls, where C3 was incubated with the inactive EspP S263A mutant or with HEPES buffer, additional cleavage products were observed at other positions, implying that C3 is less stable than C3b in the
fluid phase (data not shown). Thus, for all further experiments, C3b was used instead of C3.

The time course experiment revealed that C3b was already cleaved after 5 h, but the intensity of cleavage increased with longer incubations, and the 42-kDa fragment appeared to be digested further (Fig. 1A to D). The intensity of the band representing the α-chain of C3b was visibly reduced after 12 h, and the band was almost absent after 48 h (Fig. 1A to D), whereas the β-chain appeared to be resistant to cleavage.

For C5, three fragments were gained, with molecular masses of 39 kDa, 37 kDa, and 33 kDa, and similar to the case with C3b, the α- but not the β-chain was cleaved (Fig. 2). Compared to that of C3b, cleavage of C5 appeared to be less pronounced. Minor additional bands were also detected, but they were the same as those after incubation with the inactive EspP S263A mutant or with HEPES buffer for the same period.

In order to exclude the possibility of C3b changing its configuration at 37°C, thus rendering it inaccessible for cleavage by EspP, C3b was also preincubated at 37°C for 24 h in buffer alone, followed by a further 24 h of incubation in the presence of EspP. C3b was still cleaved to a degree comparable to that with direct incubation of C3b and EspP (data not shown).

In order to determine the amount of EspP necessary for cleavage of C3b or C5, different concentrations of EspP were incubated with 2 μg of C3b or C5. Cleavage of C3b or C5 was visible in the Western blot at all concentrations of EspP investigated, with 0.125 μg being the smallest amount of EspP necessary for cleavage to still be observed (data not shown).

When FH or FI was incubated with EspP, only the expected and predicted bands were detected, with no visible cleavage products (data not shown).

EspP cleaves C3/C3b and C5 in NHS. NHS containing approximately 1,200 μg/ml C3 and 80 μg/ml C5 was incubated with purified EspP (1 μg). In the case of C5, NHS had to be spiked with 0.5 μg C5 due to its low concentration in NHS. Subsequent Western blot analysis with an anti-C3 or anti-C5 antibody was performed in order to determine whether or not EspP was able to cleave C3/C3b or C5, not only in purified form but also in NHS. When NHS was incubated with EspP, similar C3/C3b and C5 cleavage products to those obtained with the purified protein were detected, but not when NHS was incubated with the inactive EspP S263A mutant (Fig. 3 and 4).

C3b and C5 are cleaved by supernatant of an EspP-producing wild-type strain. In order to analyze whether or not cleavage of C3b and C5 also occurred in the supernatant of an EspP-producing wild-type strain, i.e., whether the cleaving activity was still active in a more physiological setup, one EspP-positive strain and one EspP-negative strain were cultured, and the supernatants were crudely purified and concentrated. Finally, the processed supernatants were incubated with both complement proteins. Supernatant of the EspP-positive strain showed cleavage of C3b and C5, whereas supernatant of the EspP-negative strain did not show any cleavage (Fig. 5 and 6).
Determination of C3b cleavage sites via Edman degradation. The N-terminal sequences of all three fragments of C3b were determined via Edman degradation, and a map of the fragments and cleavage sites was generated (Fig. 7). One fragment resembled the first part of C3c and the very N-terminal portion of C3dg. A second fragment resembled the second part of C3c, starting close to the N-terminal end, and the third fragment resembled C3dg, also starting close to the N-terminal end, C3f, and the very N-terminal portion of the second part of C3c.

Determination of C5 cleavage sites by Edman degradation was not possible due to weaker cleavage than in the case of

FIG. 2. Cleavage of C5 by EspP. C5 (2 µg) incubated with EspP (1 µg) for 5 h (A), 12 h (B), 24 h (C), and 48 h (D) is cleaved into three fragments (indicated by arrows), with molecular masses of ~39 kDa, ~37 kDa, and ~33 kDa. Lanes M, protein marker; lanes 1, C5 + EspP; lanes 2, C5 + S263A mutant; lanes 3, C5 + buffer.

FIG. 3. Cleavage of C3 in NHS. C3b is cleaved not only in the purified form but also when NHS, as a source of C3/C3b, is incubated with EspP for 24 h (cleaved fragments are indicated by arrows). Lane M, protein marker; lane 1, NHS + EspP; lane 2, NHS + S263A mutant.

FIG. 4. Cleavage of C5 in NHS. NHS, used as a source of C5, was spiked with C5 (0.5 µg) and then incubated with EspP for 24 h. Similar cleavage products (indicated by arrows) to those observed with the purified form can be seen. Lane M, protein marker; lane 1, NHS + EspP; lane 2, NHS + S263A mutant.
C3b, with diffuse bands of low intensity representing the cleavage fragments.

**Cleavage of C5 by EspP results in reduced complement activation.** Since cleavage of C5 by EspP was weaker than that of C3/C3b, it was unclear whether or not the cleavage of C5 was sufficient to result in impaired complement activation. Hence, the effect of cleavage of C5 on complement activation, but not that of C3/C3b, was investigated using a Wieslab complement system screening kit. In order to determine whether cleavage of C5 by EspP resulted in reduced complement activation, purified C5 was preincubated with EspP and subsequently added to C5DS. Since the optimal temperature for EspP activity is 37°C, the same temperature at which complement activation proceeds, we used this experimental setup instead of direct incubation of EspP and NHS with a following determination of complement activation in the Wieslab complement system screening kit. With the latter experimental setup, we would have seen complement activation during incubation of the controls in NHS.

When C5DS (alone) was investigated, complement activity was found to be <1% and thus was comparable to that of the negative control. In order to reach a complement activity of ~100%, C5DS had to be restored with a 10-fold excess of C5. When C5 was preincubated with HEPES buffer, complement activity was found to be >100% for both the CP and the AP and ~50% for the MBL pathway. When C5 was preincubated with the inactive EspP S263A mutant, complement activity was found to be ~100% for both the CP and the AP and ~60% for the MBL pathway. However, when C5 was preincubated with EspP, complement activity and formation of the terminal complement complex were found to be reduced significantly compared to the results in the setup with the mutant or HEPES buffer, with approximately 60% activity for both the CP and the AP and 5% activity for the MBL pathway (Fig. 8A to C).

**DISCUSSION**

Bacterial proteases are thought to act as virulence factors, enabling survival in the host (27). For example, a 56-kDa protease of *Serratia marcescens* was reported to inactivate the chemotactic activity of C5a (19). A zinc metalloprotease, StcE of *E. coli* O157:H7, was reported to cleave C1-esterase inhibitor and mucous-related glycoproteins (11, 16). Later, it was proposed that StcE mediates binding of C1-esterase inhibitor to bacterial and host cell surfaces, protecting them from complement-mediated lysis (11, 17). However, the interference of these and other virulence factors with the complement cascade is not completely understood.

EspP belongs to the serine protease autotransporters of Enterobacteriaceae (SPATE) family. Several other proteins of this family are present in pathogenic *E. coli* and other enteric pathogens, such as EspC in enteropathogenic *E. coli*, Tsh in avian-pathogenic *E. coli*, and SepA in *Shigella flexneri* (4). These serine protease autotransporters of *Enterobacteriaceae* proteins serve multiple functions for many pathogenic bacteria (8). EspP has been reported to cleave pepsin A and human coagulation factor V, and it was suggested that cleavage of factor V could result in decreased coagulation, leading to prolonged bleeding and increased hemorrhage in the gastrointestinal tract (3). A possible role of EspP in disease is also supported by the fact that patients suffering from EHEC infections show an antibody response to this protein (3).

In this study, we tested the proteolytic activity of purified EspP against several components and regulators of the complement system. It was observed that EspP was able to cleave C3/C3b and C5 in vitro, but not FH or FI. Mostly C3b rather than C5 was used in this study, as the former is more stable in the fluid phase (24) and represents the major cleavage product for opsonization and lysis. EspP cleaved both C3/C3b and C5 into several fragments. Several other bacterial proteases have been shown to cleave C3. Potempa and coworkers reported that interpain A, a cysteine protease of *Prevotella intermedia*, degrades the α-chain of C3 (22). Kuo and coworkers (15) have shown streptococcal pyrogenic exotoxin B to degrade α, α’ (a 41-kDa fragment of the α-chain), and the β-chain of C3 effectively. However, in their study, the exact cutting sites by streptococcal pyrogenic exotoxin B on the α- and β-chains of C3 were not presented (15). Similar protease cleavages of C3 were also demonstrated by a chymotrypsin-like surface protease.
(dentilisin) of *Treponema denticola* (28), a trypsine-like protease of *Porphyromonas gingivalis* (23), an elastase and alkaline protease of *Pseudomonas aeruginosa* (12), and a gelatinase of *Enterococcus faecalis* (21). However, looking at the information regarding cleavage sites in the aforementioned studies, we cannot assume that these are identical in all cases or comparable to our observations.

In the present study, C3b was shown to be cleaved by EspP, and subsequent Edman degradation allowed mapping of all fragments. Under reducing conditions, three additional bands were visible in the Western blot, resembling the first part of C3c, most of the second part of C3c, and most of C3dg. Under nonreducing conditions, only two fragments could be detected: one, with a molecular mass of ~140 kDa, comprised the C3b β-chain and most of both parts of C3c, all linked by disulfide bonds; and the other, with a molecular mass of ~35 kDa, resembled most of C3dg.

In contrast to the case for C3, where several bacterial proteases are known to cleave this complement protein, there are only a few reports on cleavage of C5 by proteases. Discipio and coworkers have shown that cysteine proteases (gingipain-R and gingipain-K) of *Porphyromonas gingivalis* cleave C5 (7). Cleary and coworkers have found a streptococcal peptidase which cleaves C5a but not native C5 (6). Furthermore, Oda and coworkers have shown that treatment of human recombinant C5a with a 56-kDa protease from *Serratia marcescens* results in a complete loss of chemotactic activity (19). In the present study, we have shown that another bacterial protease, EspP, cleaves human recombinant C5 into several fragments, presumably only affecting the α-chain, but an unequivocal assignment was not possible.

In order to further characterize the cleavage of C3b and C5 by EspP, we investigated whether EspP also degrades C3/C3b and C5 in NHS. NHS contains several protease inhibitors that could potentially inhibit the activity of EspP. However, we found that the proteolytic activity of EspP to cleave C3/C3b and C5 was still present in NHS, but apparently to a slightly lesser extent than in the purified form. Brunder and coworkers have previously shown that EspP is able to cleave factor V in human serum to the same extent as in the purified setup (3).

We could also confirm a possible role of these cleavages in vivo by demonstrating that both C3b and C5 are cleaved not only by purified recombinant EspP but also by the supernatant of an EspP-producing strain. The specificity of this cleavage could be corroborated, as an EspP-negative supernatant was not able to degrade C3b or C5, demonstrating that EspP appears to be the only major EHEC complement C3b- and C5-cleaving enzyme.

Since C5 cleavage was less pronounced than that of C3b, we assessed whether this cleavage influences complement activation. C5 plays an essential role in all three pathways of complement activation (25), and extended cleavage is likely to result in a loss of activation. Indeed, in the presence of EspP, the complement activities of all three pathways were significantly reduced compared to those in the presence of the inactive EspP S263A mutant, as measured by a functional enzyme-linked immunosorbent assay (ELISA) evaluating all three pathways. Interestingly, complement activation was generally weaker in the MBL pathway, for HEPES buffer, the S263A mutant, and EspP, than the activation observed for both the CP and the AP. This finding may be due to the fact that the low serum concentration in our setup (only ~40%) may primarily affect the MBL pathway. Since C3b is cleaved to a much greater extent than C5, it is likely that a similar setup with C3-deficient serum and addition of C3 after incubation with EspP would show an even more pronounced downregulation of complement activation. Our results also show that the Wieslab complement system screening kit, designed to detect deficiencies in human serum, can also be used to assess the functional activity of a complement protein.

Downregulation of complement activation may protect both EspP-producing EHEC bacteria and the host cells to which bacteria adhere in the intestinal lumen from opsonization and complement-mediated lysis, as well as potentially damaging inflammatory events. Thus, EspP may facilitate colonization of EHEC bacteria in the gut. Dziva and coworkers have shown that EspP influences the intestinal colonization of calves. They demonstrated that adherence of EHEC O157:H7 to a bovine primary rectal epithelial cell line was significantly impaired due to an espP mutation but was restored upon addition of highly purified exogenous EspP (9).

Brunder and coworkers have demonstrated an antibody response against EspP in sera of patients suffering from HUS (3). This could be due solely to contact with the gut immune system, but it possibly also indicates that besides Stx, EspP may also reach the blood circulation during HUS pathogenesis. However, there are no reports of the serum EspP levels in

**FIG. 7. Mapping of C3b cleavage sites.** Both cleavage sites of C3b by EspP (indicated by arrows) could be determined by Western blotting and Edman degradation, and a map showing the cleavage sites and all three fragments visible in a reducing gel was generated. The shaded areas indicate the composition of the ~140-kDa band in the nonreducing gel.
FIG. 8. Downregulation of complement activation due to cleavage of C5 by EspP. When C5 preincubated with EspP was added to C5DS, complement activity was significantly downregulated in the CP (A), the MBL pathway (B) and the AP (C) compared to the activities in a similar setup with the inactive EspP S263A mutant or HEPES buffer instead of EspP (P < 0.05), as measured by a commercial ELISA (Wieslab). C5DS, C5-depleted serum only was applied in the test; HEPES, C5 preincubated with HEPES buffer was added to C5DS and applied to the test; S263A, C5 preincubated with the S263A mutant was added to C5DS and applied to the test; EspP, C5 preincubated with EspP was added to C5DS and applied to the test. The results are the means ± standard deviations for three separate experiments. * P < 0.05; ** P < 0.01.

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humans with EHEC infection, and thus it is difficult to estimate the appropriate amount of EspP required for in vitro studies.

In a recent study, we showed that Sxt activates complement and binds FH, resulting in a delayed cofactor activity on cells (20). Thus, we assume that complement is involved in the pathogenesis of EHEC-associated HUS in more than one way. However, the net effect of complement activation by Stx2 and downregulation of complement by EspP is not clear at present. A series of complement-mediated effects caused by different virulence factors may contribute to the severity of the disease, and EspP appears to play a major detrimental role.