MINI-REVIEW

Extracellular Vesicles and Renal Endothelial Cells

A Fatal Attraction in Hemolytic Uremic Syndrome

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This review focuses on typical hemolytic uremic syndrome (HUS), a life-threatening sequela of human infections caused, particularly in children, by Shiga toxin—producing Escherichia coli strains. Thrombotic microangiopathy of the brain and the kidney is the end point of toxin action, resulting in the hallmarks of HUS (ie, thrombocytopenia, anemia, and acute renal failure). A growing body of evidence points to the role of extracellular vesicles released in the blood of patients by toxin-challenged circulating cells (monocytes, neutrophils, and erythrocytes) and platelets, as a key factor in the pathogenesis of HUS. This review provides i) an updated description of the pathogenesis of Shiga toxin—producing E. coli infections; ii) an analysis of blood cell—derived extracellular vesicles, and of their parent cells, as triggering factors in HUS; and iii) a model explaining why Shiga toxin—containing vesicles dock preferentially to the endothelia of target organs. (Am J Pathol 2021, 191: 795–804; https://doi.org/10.1016/j.ajpath.2021.02.011)

The Basics of the Pathogenesis of STEC Infections

HUS is a severe sequela of STEC infections occurring after a prodromal intestinal phase, characterized by watery and bloody diarrhea. STEC are complex pathogens capable of tight and intimate interactions with the gut mucosa, culminating in the derangement of the absorption properties of the enterocytes (watery diarrhea). These Stx-independent injuries, known as attaching and effacing lesions, are critical for the subsequent pathogenetic steps, such as the synthesis and release of Stx, which cross the intestinal mucosa to reach the lamina propria. Bloody diarrhea is the consequence of toxin actions on the endothelial lining of the intestine, culminating in typical histopathologic changes, such as mucosal and submucosal edema, hemorrhage, focal necrosis, and thrombotic microangiopathy. Lesions to the

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microvasculature of the gut trigger bloody diarrhea during precocious toxemia, whereas HUS develops 5 to 7 days later when the endothelial cells of the kidney and brain are targeted by the toxin.² Endothelial damage/dysfunction related to Stx action causing thrombotic microangiopathic lesions in target organs is considered the major pathogenetic event in HUS and occurs when Stx cross the epithelial lining of the large bowel and are transported in circulation. These injuries narrow renal glomerular capillaries, thereby damaging passing erythrocytes, and consume platelets; therefore, acute renal failure, thrombocytopenia, and anemia ensue.¹,² The latter condition is exacerbated by the specific targeting of red cell precursor by Stx¹ and by complement-mediated Stx-triggered hemolysis of mature erythrocytes.⁵

Stx are a family of AB5 bacterial toxins consisting of two main types (Stx1 and Stx2) and many different subtypes (4 for Stx1 and 12 for Stx2).¹,⁷,⁸ Only a few of these have been found in STEC isolates associated with severe human illness [eg, Stx1a (bloody diarrhea and rarely HUS) or Stx2a, mucus-activatable Stx2d, and the controversial Stx2c (bloody diarrhea and HUS)].⁹ Stx2a is mainly involved in HUS because the STEC strains that produce and release this subtype are epidemiologically associated with HUS.¹,⁹

Stx are rarely found free in the bloodstream of patients during HUS¹⁰–¹⁴; rather, they are found free in blood during late toxemia and before the onset of HUS, as well as associated with circulating cells (neutrophils, monocytes, and erythrocytes) and platelets.¹⁰,¹¹,¹⁵–²² The release of extracellular vesicles containing Stx (and other virulence factors) by these stimulated cells is considered the trigger producing the transition from bloody diarrhea to life-threatening HUS.²³–²⁵

Stx activate the innate immune responses, resulting in immunopathologic changes related to inflammation and complement activation.²⁶ The multiple interactions of Stx with circulating cells, and with target epithelial and endothelial cells, induce the release of proinflammatory cytokines and chemokines capable of up-regulating the expression of specific toxin receptors (see the next paragraph), of exacerbating tissue damage in targeted organs, and of recruiting inflammatory cells in injured tissues.²⁶ Moreover, the activation of the alternative pathway of the complement system by Stx, as part of the innate immune response, is considered a crucial point in the development of renal injuries.²⁶,²⁷

**Neutral Glycolipids and Proteins as Stx Receptors**

Stx are bipartite exotoxins composed of a single A chain, which is endowed with the enzymatic activity, non-covalently bound to five B chains forming a pentameric ring that surrounds the COOH-terminus of the A subunit.¹,⁷ The latter is a proenzyme that is enzymatically cleaved into two fragments (A1 and A2) linked by a disulfide bond that is reduced within cells, permitting the A1 fragment to express its deadenylating activity on 28S RNA in ribosomes and on DNA in chromatin, leading to irreversible arrest of translation and to formation of nuclear apurinic sites, respectively.¹,⁷ Cleavage can occur during retrograde internalization in target cells by the protease furin or can be induced by extracellular or bacterial proteases.¹,⁷ It has been recently shown that cleaved unreduced Stx have changed their blood binding properties with respect to whole toxins.²⁸ Stx-intoxicated endothelial cells not only have impaired translation, but also activate a wide array of responses to injury, which have a great impact in the pathogenesis of HUS (eg, release of proinflammatory cytokines,²⁹ up-regulation of adhesion molecules,³⁰ and activation of the apoptotic program).³¹

Receptor-dependent toxicity is a key concept explaining why these powerful toxins cause damages to selected tissues in animals and humans. The B pentamers of the human subtypes Stx1a and Stx2a show a specific and preferential binding to globotriaosylceramide (Gb3Cer), a member of globoseries glycosphingolipids.¹,³² The same subtypes also interact, although weakly, with globotetraosylceramide.¹,³² On the other hand globotriaosylceramide is preferred by the Stx2e subtype associated with edema disease in pigs, that is capable of binding to Gb3Cer, globopentaosylceramide, and Forssman antigen.¹ Therefore, the tissue expression of these glycolipids and their relative amounts might affect the sensitivity of cells to specific Stx subtypes.

In humans, during HUS, several organs can be involved, with kidney and brain being predominantly targeted.¹,²,⁴ The focused expression of Gb3Cer by intestinal, renal, and cerebral endothelial cells and by other cells in the kidney (mesangial cells and tubular and glomerular epithelial cells) allows the toxin to target these organs preferentially by means of multivalent interactions of the glycolipid receptors with B subunits.¹,³² This is a crucial point to be considered because any hypothesis on the pathogenesis of HUS must explain the focused action of Stx on target cells expressing Gb3Cer.¹

Besides Gb3Cer-expressing target cells, circulating erythrocytes, monocytes, and platelets also express this receptor with similar lipoforms.¹,³³ Human neutrophils are peculiar in this respect because they specifically bind Stx even though they lack the set of enzymes necessary for the synthesis of these neutral glycolipids.¹,³⁴ In fact, they are not targeted by Stx; rather, they recognize these bacterial toxins through toll-like receptor 4 (TLR4).³⁵ Interestingly, TLR4 interacts with Stx A chain rather than with the B chains.³⁶ Moreover, in humans, the receptor pattern of circulating cells is complicated by the simultaneous expression of Gb3Cer and TLR4 by monocytes and platelets.³³

**Soluble Factors Present in Blood Affect Stx Activity**

A negative modulator of Stx2, known as human serum amyloid P (HuSAP) component, present in humans,
binds tightly to this toxin type\textsuperscript{38,39} and impairs the Gh3Cer-related cytotoxic activity.\textsuperscript{30} On the other hand, HuSAP fails to inhibit and rather stimulates Stx2 binding to TLR4-expressing human polymorphonuclear leukocytes.\textsuperscript{40} Soluble decoy forms of TLR4 have been described,\textsuperscript{41} deriving from alternative mRNA splicing or conversion of the internalized membrane receptor by proteolysis and subsequent release of the truncated form.\textsuperscript{41,42} This is useful in preventing excessive stimulation of innate immunity cells by lipopolysaccharide via TLR4.\textsuperscript{43} Stx2a interacts with soluble TLR4 \textit{in vitro}, and the formation of the complex Stx2a/soluble TLR4 allows the toxin to escape capture by HuSAP and to damage Gh3Cer-expressing human cells.\textsuperscript{43} Therefore, a similar protecting interaction of Stx2 with the extracellular domain of membrane-anchored TLR4 can be expected. The implications of these Stx2-modulating activities on the pathogenesis of HUS are discussed in the following paragraphs.

**Extracellular Vesicles Bearing Stx and Their Parent Cells**

In STEC-infected patients, Stx come in contact with and bind to platelets\textsuperscript{21} and leukocytes, and erythrocytes during early toxemia,\textsuperscript{10,11,16–18,22} thereby leading to their activation.\textsuperscript{20} This impacts the formation of circulating platelet-monocyte and platelet-neutrophil aggregates containing Stx, as observed in children with HUS during the acute phase of disease, but not after recovery.\textsuperscript{16} Experimental models with normal whole blood treated with Stx2 and/or STEC-lipopolysaccharide confirm these findings.\textsuperscript{16} Under normal conditions, platelets and leukocytes do not interact in the circulation. Hence, the formation of aggregates between these blood components in STEC-infected patients could play an important role in thrombogenesis and inflammation.

The second more important consequence is the generation of blood cell–derived extracellular vesicles bearing Stx detected in plasma of patients during overt HUS by flow cytometry and visualized within renal endothelial cells by electron microscopy.\textsuperscript{24} The toxins within or on the surface of host blood cell–derived extracellular vesicles can be transferred to target endothelial cells, as shown in \textit{in vitro} studies (ie, extracellular vesicles containing Stx and derived from human platelets and leukocytes undergo endocytosis in human glomerular endothelial cells and release the toxin, leading to inhibition of protein synthesis and cell death).\textsuperscript{24}

The extracellular vesicles involved in the pathogenesis of HUS, analyzed and characterized by transmission electron microscopy, exhibit a rounded shape and 1 μm diameter.\textsuperscript{24} This is an important point because this technique allows direct visual inspection of the vesicles, giving a more accurate measure of the size compared with other methods, such as flow cytometry or nanoparticle tracking.\textsuperscript{45,46} A weak point of transmission electron microscopy is that it is difficult to ascertain whether the detected vesicles are typical or rare structures. Therefore, it is recommended to supplement transmission electron microscopy with an additional method, allowing detection and analysis of the whole vesicle population.

Besides apoptotic bodies, the other known extracellular vesicles may play a pivotal role in intercellular communication, providing receptors and soluble molecules (mRNA, lipids, and proteins) to recipient cells.\textsuperscript{45,46} The classification of extracellular vesicles is based on their size and structure and on the mechanism of generation.\textsuperscript{46} Microvesicles are shedding vesicles released directly from the plasma membrane by budding, and their diameter (100 to 1000 nm) is generally greater than that of exosomes.\textsuperscript{23,46} The latter are small vesicles (30 to 100 nm) formed by budding into early endosomes and multivesicular bodies.\textsuperscript{23,46} Exosomes are released by fusion of the external membrane of multivesicular bodies with the plasma membrane.\textsuperscript{23,46} According to the above-described findings, the extracellular vesicles involved in the pathogenesis of HUS more closely resemble microvesicles.

An analysis of the features of the extracellular vesicles found in HUS patients and of the circulating parent cells that release them is crucial to understand their contribution in the pathogenesis of HUS. In a 2-year–old child, platelets (7.9 fL),\textsuperscript{47} erythrocytes (78 fL),\textsuperscript{48,49} neutrophils (299 fL),\textsuperscript{50,51} and monocytes (355 fL)\textsuperscript{52} greatly differ in size. Considering the size of HUS-related rounded 1-μm–diameter microvesicles (0.52 fL), the volumetric ratio of microvesicle/parent cell is roughly in the range 1:150 to 1:700 for all of the cells while platelets show a 1:15 ratio. Therefore, the number of extracellular vesicles potentially released by a single thrombocyte appears to be limited by the small size of these cell fragments deriving from megakaryocytes. Moreover, the different blood half-lives of erythrocytes (months), platelets (few days), classic monocytes (1 day), and neutrophils (few hours) also affect the daily availability of new vesicle-generating cells entering in circulation from bone marrow.

Taking into account the pediatric blood count ranges of circulating cells (1 to 4 years; Mayo Clinic reference value, http://a1.mayomedicallaboratories.com/webjc/attachments/110/30a2131-complete-blood-count-normal-pediatric-values.pdf, last accessed February 19, 2021),\textsuperscript{47,49,53–56} the ratio between the number of vesicles found in the blood of 13 HUS patients (median age, 3 years)\textsuperscript{24} to the number of vesicle-generating cells is maximal for monocytes (0.4 to 1.3 vesicles per cell), intermediate for neutrophils (4 to 22 × 10\textsuperscript{12} vesicles per cell), low for platelets (2.7 to 3.1 × 10\textsuperscript{12} vesicles per cell), and extremely low for erythrocytes (7 to 9 × 10\textsuperscript{12} vesicles per cell). Only a small percentage of microvesicles are Stx2-positive (ie, approximately 25% to 30%, except erythrocytes (9%)).\textsuperscript{44} The calculated number for neutrophils is probably overestimated because of the known neutrophilia found in HUS patients.\textsuperscript{2} The steady-state blood count of extracellular vesicles is
determined by a balance between their biogenesis and their delivery to target cells. Nevertheless, when whole blood samples from healthy donors (normal adult blood count ranges; Royal Wolverhampton Trust Pathology Services, https://www.royalwolverhampton.nhs.uk/services/service-directory-a-z/pathology-services/departments/haematology/ haematology-normal-adult-reference-ranges, last accessed February19, 2021) or isolated red cells were treated in vitro with Stx2 in the absence of recipient target cells, similar values were obtained (monocytes, 0.3 to 1.1 vesicles per cell; neutrophils, 2 to 9 × 10⁻² vesicles per cell; platelets, 1.8 to 5.5 × 10⁻³ vesicles per cell; erythrocytes, 1 × 10⁻⁴ vesicles per cell). Therefore, although a number of platelet-derived microvesicles prevail in patients’ blood during overt HUS, the efficiency of vesicular shedding shown by these cell fragments is lower than that of monocytes and neutrophils. The same ranking in vesicle generation efficiency among human circulating cells was found when unstimulated human blood was analyzed by imaging flow cytometry (ImageStream, Amnis Corporation, Seattle, WA) after simultaneous staining of circulating cells and cell-derived vesicles,25 excluding any interference caused by microvesicle isolation procedures in the data obtained with patients’ blood. In conclusion, Stx2 enhances the basal level of microvesicle release by circulating cells but does not selectively change the vesicle-generating ability of a specific circulating cell. Besides Gb3Cer, TLR4 appears to contribute to vesicle generation by interacting with Stx, as demonstrated in Gb3Cer-lacking human neutrophils.24

Extracellular Vesicles Bearing Stx Contain Further Virulence Factors Involved in the Pathogenesis of HUS

Leukocyte-platelet aggregates and microvesicles involved in HUS contain additional pathogenic factors apart from Stx. The deposition of C3 and C9 on the surface of circulating blood cell—derived microvesicles, originated from platelets, monocytes, and neutrophils, was observed in the plasma of 12 HUS patients in the acute phase.25 Accordingly, C3 bound to platelet-leukocyte complexes (on 30% of platelet-monocyte aggregates and on 15% of platelet-neutrophil aggregates) was demonstrated in a 3-year STEC-infected patient with HUS.25 These findings have been confirmed in experimental models with whole blood stimulated with Stx and/or STEC-lipopolysaccharide, showing that these virulence factors induced the deposition of surface-bound C3 and C9 on platelet-leukocyte aggregates and derived microvesicles.25

In whole blood from four children with HUS, circulating platelet-monocyte and platelet-neutrophil aggregates expressing surface-bound tissue factor (TF) were observed during the acute phase of disease but not after recovery.16 In plasma from the same HUS patients, levels of functional TF and TF-expressing microparticles were also considerably higher than those in pediatric controls.16 In in vitro experiments, Stx2 and/or STEC-lipopolysaccharide induced TF expression on platelet-monocyte aggregates compared with phosphate-buffered saline—treated samples.16 In addition, an increased number of TF-positive microparticles derived from platelets or monocytes was observed.16

The contribution of complement factors and TF to the thrombotic microangiopathy of HUS is clear-cut. Activated complement factors fixed on the surface of extracellular vesicles can be transferred to endothelial target cells, causing injuries. Stx2 activates complement alternative pathway in the fluid phase and binds to factor H, thereby reducing its protective effect on cellular membranes.27 In this respect, it is important to stress that a minority of HUS cases (atypical HUS) are related to genetic defects in regulatory molecules of the complement pathway.17 TF carried by extracellular vesicles and delivered to target endothelial cells can bind and activate coagulation factor VII, leading to a cascade of reactions culminating in thrombin generation and thus moving the scale toward a prothrombotic state.

Pathotype of HUS-Triggering Extracellular Vesicles

An emerging concept in microbiology of STEC infections is that of pathotype. To describe strains commonly associated to HUS, it is recommended to refer to the cocktail of virulence factors involved in pathogenesis rather than to the E. coli serogroup, such as O157 or O26. It is becoming clear that the specific host clinical symptoms depend on the peculiar combinations of virulence factors (pathotype) produced by a specific STEC strain as well as on their allelic types.57 Likewise, extracellular vesicles involved in the pathogenesis of HUS should not be considered, based on the type of generating cells, as neutrophil-, monocyte-, or platelet-derived microvesicles nor based on the absolute blood count. Paradoxically, a specific cell-derived microvesicle can have the highest blood count in HUS patients because of delivery systems for target cells. On the other hand, a sparsely represented circulating microvesicle can be promptly cleared from the blood by interactions with target cells. Although the parent cell may confer some of its features to the microvesicles, the specific combination of virulence factors (pathotype) present in the microvesicle/s mainly responsible for the transition from bloody diarrhea to HUS in STEC-infected patients should be investigated. HUS-triggering microvesicles are expected to bear TF, complement factors, and Stx2 on their surface and within the vesicle as toxic cargo. It is conceivable that the most poisoning HUS-triggering extracellular vesicle/s would have a golden ratio among the different necessary and interactive virulence factors, allowing targeting and full expression of the pathologic effects.
Is Cerebral Endothelium Differently Targeted in HUS?

Central nervous system involvement in STEC-infected patients and the role of Stx in this process are intriguing. Neurologic symptoms are more closely related to the direct action of Stx in the brain rather than to the consequences of renal failure (uremic encephalopathy), as demonstrated by their appearance before overt HUS in 15% of patients. The underlying mechanisms could be thrombotic microangiopathy in the brain (weak evidence), direct toxic action of Stx2 on neural cells after blood-brain barrier disruption, or indirect inflammatory-mediated damage to the brain. Patients with an elevated toxic cargo on neutrophils during the acute phase of HUS are more prone to develop neurologic complications, whereas HUS ensues in STEC-infected patients during the toxin-decreasing phase on circulating cells, as demonstrated by the reduced toxic cargo on neutrophils in patients with the most severe forms of HUS. It can be hypothesized that higher amounts of blood Stx are necessary to induce sudden brain endothelial injuries before the toxin-decreasing phase on circulating cells or that the mechanisms of endothelial damage by Stx are not related to microvesicle release by circulating cells. This interesting topic needs to be further investigated, keeping in mind that fenestrated endothelial cells (renal glomeruli) and those composing the robust blood-brain barrier (brain) differ considerably.

The Observation of STEC-Infected Patients before HUS Provides a Higher Vantage Point

The important observations described in the previous paragraphs on the role of extracellular vesicles in the pathogenesis of HUS have been obtained after the diagnosis of HUS. A recent study analyzed the journey of Stx in the blood of 20 STEC-infected patients after the onset of the prodromal intestinal symptoms (bloody diarrhea) and before the development of HUS. In this setting, the recognized virulence factors act during the evolution of the pathogenic process, before the end point (HUS). Contrary to studies on patients in the acute phase of HUS in whom free Stx are rarely found in serum, STEC-infected patients with bloody diarrhea show free Stx in blood detected by enzyme-linked immunosorbent assay (at ng/mL concentrations) and by functional assay on human cells (inhibition of protein synthesis). Few patients develop HUS (15%); therefore, the mere presence of free Stx in serum (100% of patients) is not a sufficient condition for the development of the syndrome. Consistently, Lopez et al, by analyzing about 100 patients with STEC-associated gastroenteritis, found Stx2 in blood by enzyme-linked immunosorbent assay in patients who developed HUS and in some who recovered. According to other studies on HUS patients, in most children, Stx were also found by flow cytometry on the surface of the analyzed circulating cells (neutrophils and monocytes). A quantitative investigation of neutrophil-associated Stx2 obtained by flow cytometry (0.2 to 1.7 × 10^15 mol/L, see equation below) or of cell-free circulating Stx2 detected by enzyme-linked immunosorbent assay (3.3 to 8.9 × 10^-11 mol/L) points to the presence of a huge reservoir of cell-associated toxic molecules ready for delivery.

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\frac{A \times B 	imes E}{D \times C} = \text{molarity of Stx2 bound to neutrophils}
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A = 2.1 × 10^5 number of Stx2 receptors per neutrophil.
B = 3.5 × 10^9 pediatric neutrophil count per liter, 2 years, mean.
C = 3.3 Stx2 bound to neutrophils at saturation, mean channel value of fluorescence.
D = 6.022 × 10^23 Avogadro’s number.
E = 0.6 to 4.5 Stx2 bound to neutrophils in STEC-infected patients, mean channel value of fluorescence range.

Kinetic analyses show that the toxin amounts in circulating leukocytes decrease over time with an increase in the concentration of functionally active Stx in patients’ sera, as well as that of the soluble decoy TLR4. Given the large excess of circulating Stx2-neutralizing HuSAP (10^-27 mol/L) over cell-free Stx2 (see above), the toxicity found in patients’ sera can be ascribed to Stx2/TLR4 complexes or to microvesicle-associated Stx2. Furthermore, detachment of Stx from leukocytes was also observed in patients who recovered (even showing proteinuria); thereby, this process, although representing the first step in the delivery to target organs, is not sufficient per se to trigger HUS.

The distinctive feature observed in patients who developed HUS was the sudden appearance of the vesicular form of Stx2 detected by enzyme-linked immunosorbent assay (capturing antibody against B-chains) in blood the day before the onset of HUS during the decreasing phase of toxin on leukocytes. As with antigens, vesicular toxin was obtained by centrifuging patients’ sera at the g-force required to isolate 1-µm microvesicles and was labeled particulate toxin in contrast to free soluble serum toxin. In these patients, the particulate toxin peak was accompanied by a corresponding peak of particulate TLR4 (microvesicle-associated TLR4), having a stoichiometry ratio with particulate Stx2 close to 1.

The amount of Stx associated with the circulating cells of the patients can decrease over time because of direct transfer of the toxic cargo to Gb3Cer-expressing recipient cells, as observed experimentally during neutrophil transmigration across endothelial monolayers or release of free Stx (cleared by HuSAP), Stx-TLR4 complex (escaping HuSAP), or microvesicle-associated Stx (within vesicles or Gb3Cer and TLR4 bound). Given the low vesicle generation efficiency of circulating cells (maximal for monocytes, approximately 1 vesicle per cell) and the small amount of...
microvesicles found in patients' blood,\textsuperscript{18,24} it is unlikely that a sudden decrease in the circulating toxic reservoir could be ascribed to vesicle generation alone. Despite this, there is a good correlation in patients between the toxic activity (translation inhibition in cells) of serum Stx2 and the amount of particulate toxin, but not of whole serum toxin (free toxin + particulate toxin).\textsuperscript{18} Therefore, microvesicle-associated Stx2 stands out as the most ominous toxin form\textsuperscript{16,24,25} found in patients' blood during the transition from bloody diarrhea to HUS.\textsuperscript{18}

**A Receptor-Based Model for the Specific Targeting of Gb3Cer-Expressing Endothelial Cells by Stx2-Containing Microvesicles**

The role of microvesicles in the pathogenesis of HUS was first explained by the specific binding of Stx2 to circulating monocytes, platelets, and red cells through Gb3Cer followed by a release phase and a targeting phase.\textsuperscript{23} Extracellular vesicles are capable of interacting with target cells in several ways (ie, binding and dissociation, stable binding, fusion, endocytosis, or receptor-mediated endocytosis).\textsuperscript{23,46} In this model, the toxic cargo, membrane-bound and internalized Stx2, and the associated pathogenic factors, TF and activated complement components, reach the target cells through non—receptor-mediated endocytosis or fusion. Both uptake mechanisms are largely unspecific; therefore, this model failed to explain why the histopathologic lesions observed in HUS patients are focused on few organs and tissues whose cells express Gb3Cer.\textsuperscript{1,2,4,5} HUS is not considered a multi-organ disease. On the other hand, non—receptor-mediated uptake mechanisms have been invoked to explain why other organs apart from intestine, kidney, and brain are affected, albeit rarely, by the thrombotic microangiopathy (eg, pancreas, musculoskeletal system, and heart).\textsuperscript{1,4}

Receptor-mediated uptake, favored by mutual molecular interactions between extracellular vesicles and recipient cells, is well-described.\textsuperscript{23,46} In this case, the specific targeting represents the main advantage. Stx associated with microvesicles are primarily localized within the microvesicles because permeabilization is required for maximal detection\textsuperscript{24}; nevertheless, the capture of Stx2-containing pathogenic microvesicles circulating in the blood of STEC-infected patients by means of monoclonal antibodies to Stx2 has been described.\textsuperscript{18} This means that at least part of the toxin is surface bound. It is worth noting that Stx interplay with circulating cells is mediated by two different receptors (Gb3Cer and TLR4), which specifically engage two different parts of the bipartite AB5 toxin (Figure 1).\textsuperscript{1,35}

If Stx are bound to microvesicle surface through the glycolipid receptor, their B chains are engaged and presumably the A chain is exposed. Conversely, when the toxins are bound through TLR4 via A chain, the B subunit pentamer is available for specific binding to target cells. Typically microvesicles captured by B-chain specific antibodies are the ones found in the blood of patients the day after infection.

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**Figure 1** Snapshot of the cells challenged by Shiga toxins (Stx) in the blood of Shiga toxin—producing *Escherichia coli* (STEC)—infected patients and of their toxin-specific receptors. Stx (left) consisting of a single A chain (yellow rectangle) and five B chains (blue circles) interact with (zigzag arrow) blood circulating cells and platelets through two different receptors: globotriaosylceramide (Gb3Cer; red rectangle), present on monocytes and platelets; and Toll-like receptor 4 (TLR4; green rectangle), present on monocytes, platelets, and neutrophils. The Gb3Cer/Stx B chain and TLR4/Stx A chain interactions on the surface of circulating cells and platelets are shown. A dangerous consequence of these multiple interactions is the budding of microvesicles from Stx-activated cells (right).
before the full fulfillment of the criteria for HUS diagnosis. These findings are sustained by results obtained in vitro, suggesting that uptake of Stx2-positive microvesicles is not sufficient to induce cell death and that the recipient cell needs to possess Gb3Cer for the toxin to exert its toxic effect. The binding of Stx2 to membrane-associated TLR4 on microvesicles would prevent the interaction of the toxin to HuSAP, a specific inhibitor of the Gb3Cer-dependent toxicity of Stx2, hence favoring the docking to Gb3Cer-expressing renal endothelial cells.

The features of the microvesicles derived from platelets or monocytes are similar because these cells are endowed with both Stx2-interacting receptors (Figure 2). This means that both TLR4-anchored B pentamer—exposing Stx2 and Gb3Cer-anchored A chain—exposing Stx2 are present on the vesicle surface. Renal endothelial cells also express both these receptors, and when they are targeted by soluble Stx in vitro, TLR4 acts as coreceptor, hence cooperating with Gb3Cer during binding and intoxication. In this light, the presence of surface-bound Stx2 exposing A or B chains alternatively would confer an advantage to the microvesicle in intoxicating the target cell. Finally, microvesicles derived from neutrophils expose on their surface only the TLR4-anchored B pentamer—exposing Stx2.

In conclusion, although the pathogenic cargo depends on the presence, amount, and specific proportion of the different virulence factors associated with the microvesicle, the delivering process is likely to be determined by the presence and spatial orientation of Stx2 exposed on the vesicle membrane. Similar to delivering a package, the delivery of Stx2 to target organs through microvesicles requires a stamp, a price (binding to blood cells); an address, (TLR4-anchored B pentamer—exposing Stx2); and a post box, that is Gb3Cer on target cells. Indeed, Stx are toxic components of the package as well as the leading factors necessary for the docking of the other virulence factors to renal endothelial cells.

This review focuses on the involvement of blood cell—or platelet-derived microvesicles in early pathogenesis of HUS (ie, the initial interactions of virulence factors, including Stx, with renal endothelial cells). In vitro experiments point to the involvement in HUS not only of microvesicle but also of Stx-containing exosomes released by target cells (Vero or HeLa cells) or innate immune cells (macrophage-like differentiated THP-1 cells) after toxin internalization and intracellular processing. Recipient cells bind and internalize Stx-containing exosomes through Gb3Cer receptors because of the presence of Stx on exosome surface. If confirmed in HUS patients, this might be an important mechanism for toxin spreading once the first cells have been targeted through the microvesicle-centered mechanisms reviewed herein. Moreover, Stx-containing exosomes might confer to adjacent nonintoxicated cells not only the toxic cargo but also ancillary factors that exacerbate tissue...

Figure 2  Snapshot of the microvesicles released by platelets, monocytes, and neutrophils after Shiga toxins (Stx) challenge and of their interactions with renal endothelial cell. Microvesicles are endowed with pathogenic factors, such as complement factors 3 (orange triangle) and 9 (orange circles) and tissue factor (blue cup), besides Stxs present within and on the surface of the vesicles. Stx A chain (yellow rectangle) binds to Toll-like receptor 4 (TLR4; green rectangle), whereas Stx B chains (blue circles) interact with globotriaosylceramide (Gb3Cer; red rectangle). These receptors are present on microvesicle surface and expressed by target endothelial cells. Targeting of Gb3Cer-expressing endothelial cells by Stx and/or Stx associated with microvesicles is considered the key event in the pathogenesis of hemolytic uremic syndrome. Docking of microvesicles to renal endothelial cells depends on the nature of microvesicle (neutrophil or monocyte/platelet derived) and on the Stx chain involved in the interaction with microvesicle receptors. Monocyte/platelet-derived microvesicles can engage endothelial Gb3Cer through the B chains of TLR4-anchored Stx and endothelial TLR4 through the A chain of Gb3Cer-anchored Stx. Only the former mechanism occurs in neutrophil-derived Gb3Cer-lacking microvesicles. The initial interaction of microvesicles with the endothelial surface is the basis for the successful release of the toxic cargo (Stx and other pathogenic factors) to target cells.

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damage, as mRNAs coding for proinflammatory cytokine and chemokines.65

Future research perspectives would include the definition of the pathotype of Stx2-bearing HUS-triggering extracellular vesicles (microvesicles and exosomes) and a direct demonstration of their involvement in the targeting of cerebral endothelia. These results will stimulate the development of new strategies for therapeutic interventions aimed at preventing the transition from bloody diarrhea to HUS in STEC-infected children. Blocking the interactions of Stx with circulating cells or the release of the toxic cargo from the circulating or target cells reservoirs by impairing the budding of pathogenic microvesicles and the release of Stx-containing exosomes would achieve this goal. Glycovesicles spiked with Gb3 neoglycolipids have been developed, which inhibit the binding of Stx to Gb3Cer.67 This novel approach would be useful to prevent the targeting of renal endothelium by microvesicle- or exosome-associated Stx in STEC-infected children.

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