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Shiga Toxin–Induced Complement-Mediated Hemolysis and Release of Complement-Coated Red Blood Cell–Derived Microvesicles in Hemolytic Uremic Syndrome

Ida Arvidsson,* Anne-lie Ståhl,* Minola Manea Hedström,* Ann-Charlotte Kristoffersson,* Christian Rylander,† Julia S. Westman,‡ Jill R. Storry,‡ Martin L. Olsson,‡ and Diana Karpman*

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) cause hemolytic uremic syndrome (HUS). This study investigated whether Stx2 induces hemolysis and whether complement is involved in the hemolytic process. RBCs and/or RBC-derived microvesicles from patients with STEC-HUS ($n = 25$) were investigated for the presence of C3 and C9 by flow cytometry. Patients exhibited increased C3 deposition on RBCs compared with controls ($p < 0.001$), as well as high levels of C3- and C9-bearing RBC-derived microvesicles during the acute phase, which decreased after recovery. Stx2 bound to P_1^k and P_2^k phenotype RBCs, expressing high levels of the P^k Ag (globotriaosylceramide), the known Stx receptor. Stx2 induced the release of hemoglobin and lactate dehydrogenase in whole blood, indicating hemolysis. Stx2-induced hemolysis was not demonstrated in the absence of plasma and was inhibited by heat inactivation, as well as by the terminal complement pathway Ab eculizumab, the purinergic P2 receptor antagonist suramin, and EDTA. In the presence of whole blood or plasma/serum, Stx2 induced the release of RBC-derived microvesicles coated with C5b-9, a process that was inhibited by EDTA, in the absence of factor B, and by purinergic P2 receptor antagonists. Thus, complement-coated RBC-derived microvesicles are elevated in HUS patients and induced in vitro by incubation of RBCs with Stx2, which also induced hemolysis. The role of complement in Stx2-mediated hemolysis was demonstrated by its occurrence only in the presence of plasma and its abrogation by heat inactivation, EDTA, and eculizumab. Complement activation on RBCs could play a role in the hemolytic process occurring during STEC-HUS. ***The Journal of Immunology*****, 2015, 194: 2309–2318.**

Hemolytic uremic syndrome (HUS) is characterized by hemolytic anemia, thrombocytopenia, and acute renal failure. HUS is classified into two major subtypes based on etiology: one is associated with gastrointestinal infection usually caused by Shiga toxin (Stx)-producing *Escherichia coli* (STEC) strains, and the other is associated with complement disorders (atypical HUS) (1). The most common clinical isolate of STEC is *E. coli* O157:H7 (2). The strain isolated during the large German outbreak in 2011 was *E. coli* O104:H4 (3).

The presence of fragmented RBCs is a feature of the hemolysis occurring during HUS. It has been assumed that mechanical damage to RBCs occurs as a result of the formation of microthrombi, followed by narrowing of the vascular lumen in glomerular capillaries, or by oxidative damage (4, 5). Stx binds to a glycosphingolipid receptor known as globotriaosylceramide (Gb3) (6) or the P^k Ag (CD77) on RBCs (7). The P1 and P^k Ags of the P1PK blood group system combined with the P Ag from the GLOB blood group system give rise to five RBC phenotypes: P_1, P_2, P_1^k, P_2^k, and p (Supplemental Table I). All but the p phenotype express the P^k Ag, to a greater or lesser extent and, thus, can potentially bind Stx (8).

There is evidence for complement activation during STEC-HUS, even if the condition is not primarily a complement-mediated disorder. Patient samples may exhibit low serum levels of C3 (9, 10) and subsequently elevated complement-degradation products C3a, factor Bb, and soluble C5b-9 (11, 12). Furthermore, C3 and C9 were demonstrated on circulating platelet–leukocyte aggregates and on microvesicles derived from these blood cells (11). There have been no reports indicating that complement activation occurs on RBCs during STEC-HUS. This is of interest because complement activation on RBCs may induce hemolysis in vitro and in various clinical hemolytic conditions (13–15).

Purinergic signaling was shown to be involved in complement-mediated hemolysis (16). The purinergic P2 receptors are divided into P2X and P2Y. $P2X_1$, $P2X_7$, and P2Y13 receptors are expressed by human RBCs (17, 18). Complement-induced hemolysis was amplified by P2 receptor activation and inhibited by the P2 antagonists suramin and PPADS (16).

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This study was presented at the 8th International Symposium on Shiga toxin (Verocytotoxin) Producing *Escherichia coli* infections "VTEC," May 6–9, 2012, Amsterdam, the Netherlands; the 4th International Conference "HUS-MPGN-TTP and Related Disorders," June 9–11, 2013, Innsbruck, Austria; and the 14th European Meeting on "Complement in Human Disease," August 17–21, 2013, Jena, Germany.

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The online version of this article contains supplemental material.

Abbreviations used in this article: Gb3, globotriaosylceramide; HUS, hemolytic uremic syndrome; LDH, lactate dehydrogenase; RT, room temperature; STEC, Stx-producing *Escherichia coli*; Stx, Shiga toxin.

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Because complement activation on RBCs may induce hemolysis, this study focused on complement deposition on RBCs and RBC-derived microvesicles from patients with STEC-HUS. In vitro studies were performed using Stx2-stimulated RBCs to determine whether Stx2 induced hemolysis and complement activation on RBCs and whether this activation could be inhibited by blocking complement and purinergic signaling.

Materials and Methods

Subjects

Blood samples were available from pediatric (n = 16, median age 2.5 y) and adult (n = 9, median age 62 y) patients with STEC infection and HUS (Table I). Patients 3–14 and 17–25 were reported previously (11, 19). STEC infection was detected as previously described (20). HUS was defined as hemolytic anemia (hemoglobin levels $<$ 100 g/l), thrombocytopenia (platelet counts $< 140 \times 10^9$/l), and acute renal failure. Blood samples also were obtained from 12 of the pediatric patients 2–9 mo after recovery (patients 3–14).

As controls and for in vitro experiments, blood samples were obtained from 20 healthy adult volunteers (12 females and eight males) and four pediatric controls (two boys and two girls; median age 5 y) who were treated at the outpatient clinic of the Department of Pediatrics, Skåne University Hospital for unrelated conditions. Samples from the patients, pediatric controls, and healthy adult volunteers were taken with the informed written consent of the subjects or their parents. The study was performed with the approval of the Ethics Committee of the Medical Faculty, Lund University.

RBCs from anonymous donors (n = 16) were obtained from waste buffy coat in citrated tubes from the Department of Clinical Immunology and Transfusion Medicine, Division of Laboratory Medicine, Region Skåne Office of Medical Service in Lund. This provision complies with current national regulations regarding the use of superfluous material from blood donations for which the donor origin cannot be traced, and written consent was obtained at the time of donation. Anonymized waste buffy coat material also was provided from other centers in accordance with national regulations and the Declaration of Helsinki and with informed written consent.

Blood collection, isolation of RBCs, and RBC-derived microvesicles

Whole blood and three sources of RBCs were used in this study (Table II). Whole blood from healthy volunteers was collected in citrate-containing tubes. RBCs were isolated from patients (RBC-pat), from healthy volunteers (RBC-control), and from donor waste buffy coats (RBC-BC).

Blood samples from patients and controls were drawn by venipuncture into tubes containing EDTA (1.8 mg/ml; Becton Dickinson, Plymouth, U.K.) for RBC and DNA analysis or into tubes containing sodium citrate (0.129 M; Becton Dickinson) for microvesicle analysis. Blood samples from healthy volunteers used for in vitro stimulation were drawn by venipuncture via a butterfly needle (Terumo Medical Products, Hangzhou, China) into plastic tubes containing sodium citrate (0.129 M) or into glass tubes (Becton Dickinson) with added lepirudin (5 mg/ml; Refludan; Celgene, Windsor, U.K.). Anticoagulation with citrate gave the lowest background and was used in all in vitro experiments.

For isolation of RBCs, samples were centrifuged, washed three times at $830 \times g$ for 5 min in PBS (pH 7.4; Medicago, Uppsala, Sweden), and used for in vitro stimulation or stored in Alsever's Solution (Sigma-Aldrich, Steinheim, Germany) at 4°C until analyzed.

RBCs from healthy anonymous donors expressing the P_1, P_2, p, P_1^k, or P_2^k phenotype [detected as described (21)] were washed and stored in stabilization solution for RBCs (ID-CellStab; Bio-Rad, Cressier, Switzerland) at 4°C until analyzed.

Microvesicles were isolated as previously described (11), with the following modifications. For microvesicle isolation from whole blood (RBC-pat or RBC-control), samples were centrifuged at $2600 \times g$ for 15 min at room temperature (RT) to obtain platelet-poor plasma and further centrifuged at $9900 \times g$ for 5 min. The microvesicle-containing supernatant was stored at −80°C until assayed. For microvesicles derived from washed RBCs (RBC-control or RBC-BC), supernatants were centrifuged twice at $1850 \times g$ for 20 min and stored at −80°C until analyzed. After thawing, samples containing microvesicles were centrifuged at $19{,}900 \times g$ for 10 min at RT to pellet the microvesicles. The pellet was washed in PBS and recentrifuged. All buffers and cell media were filtered (0.2 μm; Pall, Ann Arbor, MI) before use.

Identification of RBCs

RBCs were identified by flow cytometry using mouse anti-human CD235a (Glycophorin A):PE (1:800) or mouse IgG2b:PE (1:800) as the isotype control (both from BD Bioscience, Sparks, MD).

Detection and quantification of microvesicles

Microvesicle size (0.1–1 μm) was determined by comparison with FITC-labeled melamine resin particles of a defined size (1 μm; Sigma-Aldrich) mixed 1:1000 with PBS in a separate tube. The number of microvesicles/ml was calculated using a specified quantity of blank calibration particles (6–6.4 μm; BD Bioscience). These calibration particles were added prior to analysis to enable quantification of microvesicles. The number of counted calibration particles was 10,000/tube. The following formula was used for calculation of microvesicles/μl: n = (microvesicles/calibration particles counted) × (calibration particles added/μl platelet-poor plasma).

Detection of C3, C5b-9, and C9 on RBCs and RBC-derived microvesicles

For C3 detection, RBCs were incubated with chicken anti-human C3:FITC (1:800; detects the entire C3 protein) or chicken anti-human insulin:FITC (1:800) as the control Ab (both from Diapensia, Linköping, Sweden) for 20 min at RT in the dark. For C5b-9 detection, RBCs were incubated with mouse-anti human C5b-9 (1:100; Quidel, San Diego, CA) or mouse IgG1k (1:100; AbD Serotec, Düsseldorf, Germany) as the isotype control, for 30 min at RT. This incubation was followed by three washes in PBS, centrifugation (5 min, $830 \times g$), and incubation with goat anti-mouse IgG:FITC (1:500; Dako, Glostrup, Denmark) for 10 min at RT in the dark.

Microvesicles derived from RBCs were detected using mouse anti-human CD235a:PE incubated for 20 min at RT in the dark. Microvesicles also were incubated with chicken anti-human C3:FITC (1:1600) or mouse anti-human activated C3b (1:100; Hycult Biotech, Plymouth Meeting, PA) or, as control Abs, chicken anti-human insulin:FITC (1:1600) or mouse IgG2a (1:100; Dako) for 20 min. For C5b-9 or C9 detection, microvesicles were incubated with mouse anti-human C5b-9 (1:500) or mouse anti-human C9 neoantigen (1:100); mouse IgG1k (1:500) or mouse IgG1 (1:100; all from Hycult Biotech) was used as a control Ab. The C3 and C3b Abs, as well as the C5b-9 and C9 Abs, gave comparable results with regard to detection of C3 or C9, respectively, when tested in the same samples. These steps were followed by three washes in PBS, centrifugation ($19{,}900 \times g$ for 10 min), and incubation with goat anti-mouse IgG:FITC (1:1000) or rabbit anti-mouse IgG:FITC (1:500; both from Dako) for 10 min at RT in the dark. Factor H deposition on RBC-derived microvesicles was detected using mouse anti-human factor H:FITC (1:200) or mouse IgG:FITC (1:200; both from Life Span BioSciences, Seattle, WA). After incubation with the conjugated primary Abs, the samples were washed once in filtrated PBS to remove unbound Ab.

P1PK blood group phenotype and genotype

RBCs (RBC-pat and RBC-BC) were analyzed for levels of P^k/Gb3 and P1 by flow cytometry, as previously described (21). Washed RBCs (~0.5×10^6) were suspended in PBS and fixed with 0.07% glutaraldehyde for 10 min at RT, followed by incubation with rat anti-human CD77 (5 μl; Immunotech, Marseille, France) or anti-human P1 (5 μl; Immunocore, Oxon, U.K.) for 10 min at RT and 35 min at 4°C. The cells were washed in PBS, incubated with the secondary Abs goat anti-rat IgM:PE (1:5; Beckman Coulter, Brea, CA) or rabbit anti-human IgM:FITC (1:5; Dako) for 10 min at RT in the dark, and analyzed by flow cytometry.

To distinguish the P^1 and the P^2 blood group genotypes, allelic discrimination was performed, using a single nucleotide polymorphism genotyping assay, as previously described (21). DNA from whole blood was isolated using a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. For allelic discrimination, DNA (2 μl at 10 ng/μl) was used together with a custom-made TaqMan SNP Genotyping Assay and analyzed on the 7500 Sequence Detection System (both from Applied Biosystems, Carlsbad, CA). The assay detects a variation at position 42 (C/T) in the *A4GALT* exon 2a that determines the donor P_1 or P_2 phenotype: C/C = P_1, C/T = P_1, T/T = P_2.

The P^k and P1 levels on RBCs and allelic discrimination of P^1 and P^2 blood group genotypes in patients and controls are presented in Supplemental Table II.

Stx2, anti-Stx2, and LPS

Stx2 was obtained from C. Thorpe (Phoenix Lab, Tufts Medical Center, Boston, MA). LPS contamination (300 endotoxin units/ml, 25 ng/ml) was detected using Endochrome 140 (Charles River, L'Arbresle, France). Anti-Stx2 IgG1 (11E10) was a gift from T.G. Obrig (Department of Microbiology and Immunology, University of Maryland, Baltimore, MD). LPS from *E. coli* O157 (O157LPS) was a gift from R. Johnson (Public Health Agency, Guelph, ON, Canada).

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Stx2 stimulation of RBCs

Washed RBC-BC, RBC-control, or whole blood was stimulated with Stx2. Approximately 15×10^6 RBC-BC or 4.5×10^8 RBC-control were used per experiment. RBC-control were stimulated with Stx2 (0, 25, 50, 100, 200, and 400 ng/ml) to generate a dose-response curve. In all of the following experiments, described, RBCs were stimulated with 200 ng/ml Stx2, for RBC-BC, diluted in 100 μl ABO-matched citrated plasma, or for RBC-control suspended in 500 μl citrated autologous plasma diluted 1:2 in RPMI 1640 (Invitrogen, Paisley, U.K.) for 40 min at 37°C under gentle shaking. In certain experiments, Stx2 incubation occurred in the presence of EDTA (10 mM; MERCK, Darmstadt, Germany; binds Ca^{2+} and Mg^{2+}) or EGTA (10 mM; Complement Technology, Tyler, TX; binds Ca^{2+}) to differentiate activation of the alternative versus the classical pathway of complement. Samples were analyzed for microvesicle release and the presence of C3, C5b-9, and phosphatidylserine (described below) on microvesicles.

Whole blood diluted 1:2 in DMEM (Invitrogen) was stimulated with Stx2 for 40 min at 37°C under gentle shaking and used to assay hemoglobin release and lactate dehydrogenase (LDH) activity. In certain experiments, Stx2 incubation occurred in the absence of plasma (Table II). For heat-inactivation experiments, whole blood was centrifuged (2600 × *g* for 15 min at RT), and the plasma was removed. A portion of the plasma was heat inactivated at 56°C for 60 min. The plasma samples were then centrifuged at 4000 × *g* for 5 min, resuspended with the blood cells, and stimulated with Stx2, as above. In other experiments, whole blood diluted 1:2 in DMEM was preincubated with the humanized anti-C5 Ab eculizumab (25 μg/ml; Alexion, Cheshire, CT), the purinergic P2 receptor antagonist suramin (200 μM; Sigma-Aldrich), or EDTA (50 mM) for 20 min at 37°C under gentle shaking. The samples were then incubated with Stx2 at 37°C for an additional 40 min under gentle shaking and analyzed for release of hemoglobin and microvesicles.

Certain experiments were performed with factor B– or C2-depleted serum. Whole blood was centrifuged as above, and the plasma was removed and replaced by factor B– or C2-depleted serum (both from Complement Technology). Samples were then stimulated with Stx2, as above, and analyzed for microvesicle release.

Hemolytic assay

Release of hemoglobin was assayed using an ELISA for detection of human hemoglobin (Human Hemoglobin-GWB-DM374H; GenWay, San Diego, CA), according to the manufacturer's instructions.

LDH activity

LDH activity was measured using a colorimetric assay (Abcam, Cambridge, U.K.), according to the manufacturer's instructions.

Detection of Stx2 binding to human RBCs

For detection of Stx2 binding to RBC-BC, ~0.4×10^6 RBCs of defined P1PK blood group phenotypes were washed three times with PBS, followed by centrifugation (830 × *g* for 5 min). The cells were incubated with Stx2 for 30 min at 37°C under gentle shaking, followed by three washes. The cells were then incubated with mouse anti-Stx2 (200 ng/ml) or mouse IgG1k for 30 min at RT. This incubation was followed by three washes and incubation with goat anti-mouse IgG:FITC (1:500; Dako) for 10 min at RT in the dark. Samples were analyzed by flow cytometry.

Detection of phosphatidylserine on RBC-derived microvesicles

RBC-derived microvesicles were incubated with mouse anti-human CD235a:PE and annexin V:Cy5 in 1× annexin V binding buffer (1:200; BD Biosciences, Sparks, MD). Controls were mouse IgG2b:PE or assay without annexin V buffer. Detection was performed by flow cytometry.

Detection of membrane-bound complement regulators on RBCs

RBC-control samples were analyzed for expression of complement inhibitors on the cell surface by flow cytometry. Cells were incubated with mouse anti-human CD35:FITC (1:100), CD55:allophycocyanin (1:50), CD59:FITC (1:100) or with isotype controls mouse IgG:FITC (1:100) or mouse IgG:allophycocyanin (1:50; all from BD Biosciences). The cells also were analyzed for factor H deposition using mouse anti-human factor H:FITC (1:100) or mouse IgG:FITC (1:100) for 20 min at RT in the dark.

Inhibition of RBC purinergic receptors

Experiments were designed to investigate whether microvesicle release was mediated via purinergic receptors. Citrated whole blood from healthy volunteers was centrifuged at 180 × *g* for 10 min, the supernatant was removed, and the RBCs were washed three times in filtered Alsever's Solution at 180 × *g* for 10 min. Approximately 1.8×10^8 RBCs were resuspended in 1 ml DMEM with the P2 receptor antagonists suramin (200 μM) or PPADS (200 μM; Sigma-Aldrich) or with PBS for 20 min at 37°C under gentle shaking. The samples were then stimulated with Stx2 or calcium ionophore A23187 (10 μM; Sigma-Aldrich), as a positive control for microvesicle release, for 40 min at 37°C under gentle shaking, followed by centrifugation twice at 1850 × *g* for 20 min at RT to isolate microvesicles.

Flow cytometry acquisition and interpretation of data

Flow cytometry was performed using a FACSCanto II instrument with FACSDiva software (Becton Dickinson Immmunocytometry Systems, San Jose, CA). For RBCs and microvesicles, forward scatter and side scatter were set in a logarithmic scale. For the analysis of RBCs, 10,000 cells were counted, and one-color analysis was used for detection of Stx2, C3 (Supplemental Fig. 1), C5b-9, CD55, CD59, or factor H. Two-color analysis was used to detect microvesicles from RBCs (labeled with anti-CD235a) with C3, C5b-9, C9, or annexin V in a cytogram with PE-A versus FITC-A or PE-A versus Cy5-A for annexin V. Results are presented as positive microvesicles after subtraction of the control Ab.

Statistical analysis

Differences between patients and controls or differences between stimulated and unstimulated blood samples were assessed by the two-tailed Mann–Whitney *U* test, or by the Kruskal–Wallis multiple-comparison test when comparing more than two groups, followed by comparison between specific groups using the Dunn procedure. A p value ≤ 0.05 was considered significant. Statistical analysis was performed using Prism version 6.0 (GraphPad, La Jolla, CA).

Results

C3 deposits on RBCs from HUS patients

RBCs from HUS patients (n = 10, Table I; whole blood from the acute phase was not available from all patients) were examined for C3 and C5b-9 deposition and compared with healthy adult controls (n = 7) (Fig. 1A). Median C3 binding on patient RBCs was 6.9% of the total RBC population compared with 2.5% in the controls ($p < 0.001$). Median C5b-9 binding was 0.4% of the total RBC population in patients, and no difference was found compared with controls (0%, data not shown). However, Patient 16 differed in that HUS developed on the day that the patient's first sample was taken; in this sample, 9.6% of the RBC population was labeled for C5b-9, whereas 5 d later, during the course of HUS, labeling decreased to 1.3% of the RBC population.

Elevated RBC-derived microvesicles in plasma from HUS patients

RBC-derived microvesicle levels were measured in citrated patient and control plasma samples (blood samples described in Table II). Pediatric samples were available from both the acute phase (Patients 1–14) and after recovery (n = 12), whereas samples from the adult patients were only available from the acute phase. In pediatric samples, RBC-derived microvesicle levels during the acute phase of HUS were significantly higher than at remission and in comparison with controls (Table III). Microvesicle levels after recovery were comparable to controls. Adult samples were taken considerably later on in the course of disease, and microvesicle levels were higher compared with adult controls (Fig. 1B).

C3, C9, and C5b-9 on patient RBC-derived microvesicles

C3 and C9 deposits on RBC-derived microvesicles in pediatric HUS patients during the acute phase and after recovery are presented in Table III. The results show a significantly higher amount of RBC-derived microvesicles bearing C3 and C9 during the acute phase compared with recovery, although patients exhibited slightly

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Table I. Characteristics of HUS patients included in this study

Patient No.	Sex	Age (y)	Bloody Diarrhea	Neurological Symptoms	Treatment				Long-Term Complications	*E. coli* Serotype[a]
					Plasma	Dialysis	Eculizumab	Transfusion		
1	F	1	+	—	+	+	—	—	—	O157
2	M	1	+	Seizures	+	+	—	—	—	O153
3	M	3	+	—	—	—	—	—	—	O157
4	M	3	+	—	+	—	—	—	—	O157
5	M	7	—	—	+	+	—	—	—	O157
6	M	1	—	—	—	—	—	+	—	O26
7	F	3	+	—	+	+	—	—	—	O157
8	F	2	—	—	+	—	—	—	—	O157
9	M	2	+	—	—	—	—	+	—	O145
10	M	10	—	—	+	+	—	—	—	O157
11	F	2	+	—	+	—	—	—	—	O157
12	F	11	+	—	—	—	—	+	—	O157
13	M	10	+	Coma, hemiplegia, seizures	+	+	—	—	—	O157
14	M	5	+	—	+	+	—	—	—	O157
15	M	1	+	—	—	—	—	—	—	O121
16	M	2	+	—	—	—	—	+[b]	—	O26
17	F	67	+	—	—	—	—	—	—	O104
18	M	69	+	—	+	—	—	—	—	O104
19	F	63	+	Confusion, paralysis	—	+	—	—	—	O104
20	F	46	NA	—	NA	NA	NA	+[c]	NA	O104
21	F	47	NA	Coma, focal neurologic symptoms	NA	+	+[d]	NA	—	O104
22	F	29	—	—	—	—	—	—	—	O104
23	F	67	+	Generalized myoclonus, hyperreflexia	—	—	—	—	Uremia	O104
24	F	62	—	Confusion	—	+	+[d]	+	—	O104
25	F	45	+	Coma, spastic paralysis	+[c]	+	+[d]	+	Optical nerve atrophy, muscular weakness, reduced GFR	O104

Samples taken from the pediatric patients were obtained upon admission before administration of any treatment. Samples from the adult patients were obtained several days after admission and, in certain cases, after the initiation of treatment. All patients had symptoms at the time of sampling.

[a]All strains produced Stx2.

[b]The second sample was obtained after blood transfusion.

[c]The blood sample was obtained after administration of plasma and/or blood transfusion.

[d]Eculizumab was given before blood sampling.

F, female; GFR, glomerular filtration rate; M, male; NA, data not available; +, present/given; —, absent/not given.

higher levels of C9 than did controls even after recovery. Deposits of C3 and C5b-9 on RBC-derived microvesicles were detected in plasma from adult patients. C3 was detected on RBC-derived microvesicles, but there was no significant difference compared with controls (Fig. 1C), whereas C5b-9 deposition was elevated in patients compared with controls (Fig. 1D).

Table II. Whole blood and RBCs used in this study

Source of Whole Blood and RBCs	Subjects	Anticoagulant	Used for Assay						
			Flow Cytometry of RBCs	Flow Cytometry of Microvesicles	Determination of P1PK Blood Group		Inhibition of RBC Purinergic Receptors	Hemoglobin Assay[a]	LDH Assay
					Flow Cytometry	DNA			
Whole blood-control	Healthy volunteers	Citrate	—	+	—	—	—	+	+
		Lepirudin	—	—	—	—	—	—	+
RBC-pat	HUS patients	EDTA	+	—	+	+	—	—	—
		Citrate	—	+	—	—	—	—	—
RBC-control	Healthy volunteers	EDTA	+	—	—	—	—	—	—
		Citrate	+	+	—	—	+	—	—
RBC-BC	Anonymous donors	Citrate	+	+	+	+	—	—	—

[a]In certain experiments, whole blood was centrifuged at 2600 × *g* to remove platelet-rich plasma, and the plasma was removed and replaced by the same volume of PBS or the same plasma was reinstated to obtain whole blood.

+, present/given; —, absent/not given.

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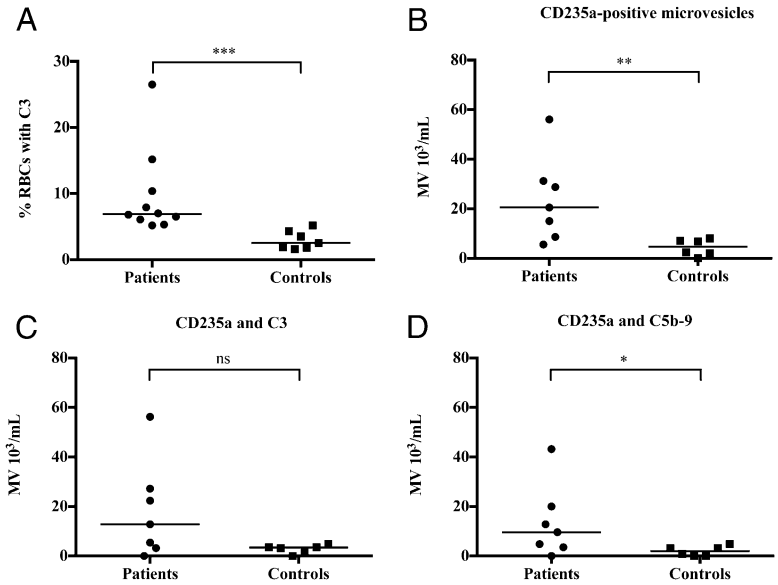

FIGURE 1. Complement on RBCs and RBC-derived microvesicles (MV) in STEC-HUS patients. RBCs from pediatric and adult patients (1, 2, 15, 16, 17, 18, 19, 21, 22, and 23 [Table I]) and controls were analyzed for C3 and C5b-9. (**A**) C3 was detected on the RBC surface during the acute phase of HUS and compared with controls. Data are presented as the percentage of the RBC population with C3 deposition with a median value (horizontal line) and individual values. RBC-derived MV were identified by labeling with an anti-CD235a Ab. (**B**) CD235a$^+$ MV. (**C**) C3 on RBC-derived MV. (**D**) C5b-9 on RBC-derived MV. In (B–D), MV (10^3/ml) are depicted as a median value (horizontal line) and individual values from adult Patients 19–25 with *E. coli* O104:H4 infection or controls. Individual results in patients and controls were reproducible. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. ns, not significant.

Stx2-induced hemolysis is blocked by complement and purine receptor inhibition

Hemolysis was detected as release of hemoglobin and LDH. Whole blood stimulated with Stx2 showed an increase in hemoglobin release compared with unstimulated samples. This effect was only noted in the presence of plasma and was inhibited in heat-inactivated plasma (Fig. 2A) or in the presence of eculizumab, suramin, or EDTA (Fig. 2B). An increase in LDH activity was found in Stx2-stimulated whole blood compared with unstimulated samples. This effect was blocked by heat inactivation (Fig. 2C) and by EDTA (Fig. 2D) and was not tested in the presence of eculizumab or suramin.

Stx2 binding and complement deposition on RBCs in vitro

Stx2 binding to RBC-BC (from donor waste buffy coats) expressing different P blood group phenotypes was assayed in samples from 16 donors representing all five P1PK and GLOB blood group system phenotypes. The most pronounced binding was demonstrated in donors expressing the P_1^k phenotype (Table IV).

Stx2 did not induce C3 or C5b-9 deposition on RBCs (data not shown); therefore, complement deposition on microvesicles released from RBCs was investigated.

Stx2-induced release of complement-labeled RBC-derived microvesicles

The dose-response curve showing Stx2 induction of RBC-derived microvesicle release is presented in Fig. 3. Stx2 induced the release of RBC-derived microvesicles from washed RBCs together with plasma for 40 min. The release was inhibited in the presence of EDTA, but not EGTA, indicating the role of the alternative pathway of complement (Fig. 4A). These microvesicles were C3 labeled, but a toxin-induced increase in C3 was not noted (Fig. 4B). C5b-9 labeling increased significantly after Stx2 stimulation (Fig. 4C). Furthermore, Stx2-induced RBC-derived microvesicles exposed phosphatidylserine (Fig. 4D). The effect of Stx2 on RBC-derived microvesicle release in whole blood from controls was inhibited in factor B–depleted, but not in C2-depleted, serum (Fig. 4E), which suggested a role for the alternative pathway of complement. However, incubation of whole blood with eculizumab did not inhibit the release of microvesicles from RBCs, suggesting that the terminal pathway of complement was not involved (Fig. 4F).

Stx2 induced RBC-derived microvesicle shedding from donors with the P_1 P_2, P_1^k, and P_2^k phenotypes, but not the p phenotype, as expected (Fig. 5). C3 and C5b-9 deposition was more prominent on microvesicles derived from P_1, P_1^k, and P_2^k RBCs than those from P_2 cells.

The Stx2 used was contaminated with a negligible amount of LPS (25 ng/ml). Stimulation experiments were performed with O157LPS alone at this concentration (concentration in final dilution 5 pg/ml), and no release of microvesicles was detected (data not shown).

Membrane-bound complement inhibitors on RBCs and RBC-derived microvesicles

The levels of complement inhibitors CD35, CD55, CD59, and factor H on unstimulated versus Stx2-stimulated RBC-controls were not significantly different. Likewise, the levels of factor H on RBC-derived microvesicles released after Stx2 stimulation were comparable to those on unstimulated microvesicles (Supplemental Fig. 2).

Table III. C3 and C9 deposition on RBC-derived microvesicles from pediatric HUS patients

	CD235a$^+$ Microvesicles [10^3/ml; median (range)]					
	Total	*p* Value	C3$^+$	*p* Value	C9$^+$	*p* Value
Acute phase (*n* = 14)	540 (30–1491)	<0.01[a] <0.05[b]	284 (12–837)	<0.01[a] <0.05[b]	245 (54–570)	<0.01[a] <0.01[b]
Recovery (*n* = 12)	110 (25–546)	NS[b]	83 (20–293)	NS[b]	57 (22–212)	<0.05[b]
Pediatric controls (*n* = 4)	174 (71–276)		50 (32–144)		25 (15–36)	

[a]Versus recovery.
[b]Versus pediatric controls.

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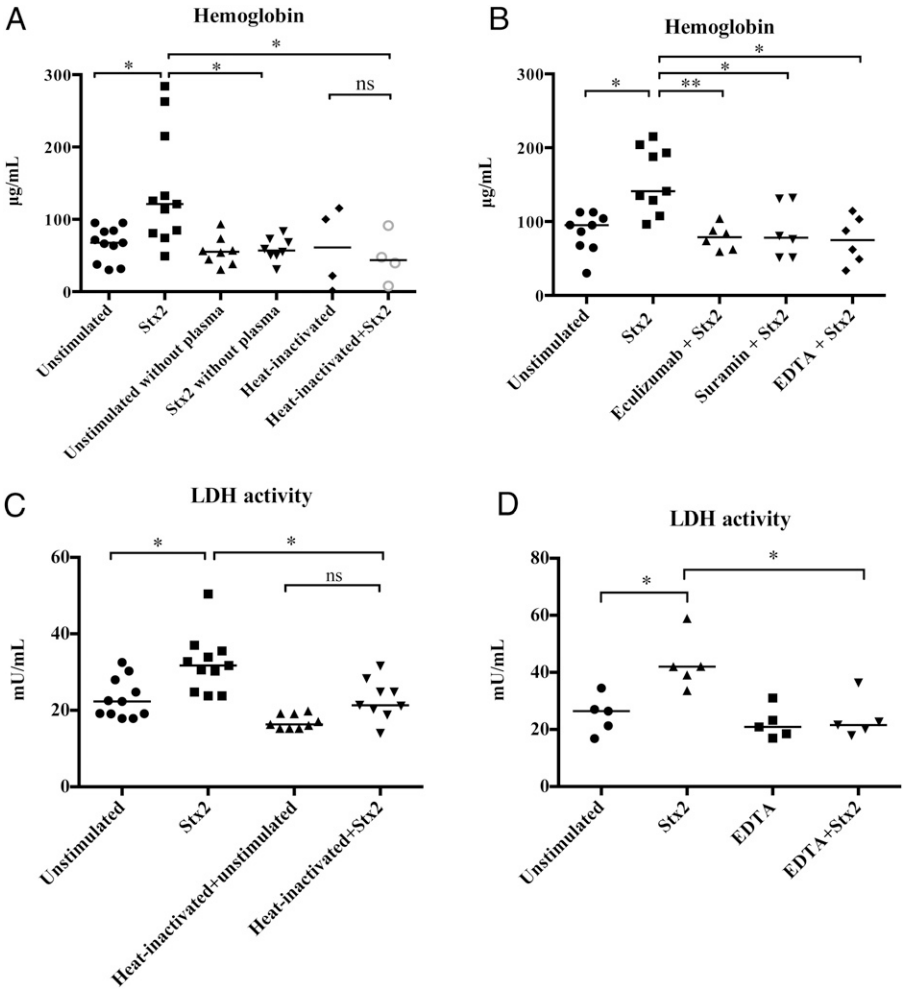

FIGURE 2. The effect of Stx2 on hemoglobin release and LDH activity. (**A**) Hemoglobin release was only demonstrated in Stx2-stimulated blood samples containing normal plasma (samples in which whole blood was centrifuged and the plasma was removed and reinstated). The release was inhibited without plasma and in heat-inactivated plasma. Multigroup comparison, $p < 0.05$. (**B**) Whole blood stimulated with Stx2 induced the release of hemoglobin, which was inhibited by eculizumab (anti-C5 Ab), suramin (purinergic P2 receptor antagonist), and EDTA. Multigroup comparison, $p < 0.01$. (**C**) Whole blood stimulated with Stx2 induced the release of LDH, which was inhibited by heat inactivation. Multigroup comparison, $p < 0.001$. (**D**) Whole blood stimulated with Stx2 induced the release of LDH, which was inhibited by EDTA (50 mM). Multigroup comparison, $p < 0.05$. Data are depicted as median (horizontal line) and individual values. Results shown were reproducible and summarized from five separate experiments, using various donors $*p < 0.05$, $**p < 0.01$. ns, not significant.

Purinergic receptor inhibition blocks RBC-derived microvesicle release

RBC-control ($n = 7$, RBCs isolated from healthy volunteers) were left unstimulated or were stimulated with Stx2 alone or together with the P2 receptor antagonists suramin (nonselective) or PPADS (blocks mainly the P2X receptors) (22). An increase in RBC-derived microvesicles was noted when comparing unstimulated and Stx2-stimulated RBCs, but no increase was detected when Stx2-stimulated RBCs were preincubated with suramin or PPADS (Fig. 6A). The same tendency was seen when RBCs were stimulated with the calcium ionophore A23187 (data not shown). Focusing specifically on C3-labeled RBC-derived microvesicles released from whole blood stimulated with Stx2, results showed that coincubation with Stx2 and suramin lowered the amounts of complement-coated RBC-derived microvesicles compared with incubation with Stx2 alone (Fig. 6B). Taken together, these results indicate that microvesicle release from RBCs can be abrogated by purinogenic receptor inhibitors.

Discussion

Hemolysis is one of the main manifestations of HUS. This study demonstrates that Stx2 induces hemolysis, as shown by the release of hemoglobin and LDH. This effect was only demonstrated in the presence of plasma, suggesting that a component of plasma contributed to its induction. The plasma-mediated effect was inhibited by heat inactivation, indicating that a heat-labile factor contributed to hemolysis. The role of complement in Stx2-mediated hemolysis was more conclusively demonstrated by inhibition with the terminal complement pathway Ab eculizumab and with EDTA.

In various hemolytic conditions complement deposition on RBCs contributes to the hemolysis. Complement activation occurs on blood cells and endothelial cells during Stx2-mediated infection (11, 23), but the role of complement in hemolysis during HUS has not been addressed. We demonstrated deposition of C3 on RBCs from patients with acute HUS but no C5b-9, with the exception of one patient whose sample was taken early on in the course of

Table IV. Stx2 binding to RBCs expressing different blood group phenotypes

Phenotype	Stx Binding [% of Positive Cells; Median (range)]	$P^k/Gb3^+$ Cells [MFI; Median (range)]
P_1 ($n = 5$)	0.6 (0.2–1)	14.7 (8.9–27.6)
P_2 ($n = 3$)	0.4 (0.3–0.6)	6.12 (4.2–12.2)
p ($n = 3$)	0.7 (0.6–0.8)	4.9 (3.8–5.7)
P_1^k ($n = 3$)	42.7 (22–69.9)	1301 (1168–1466)
P_2^k ($n = 2$)	15.5 (15.2–15.7)	678 (661–695)

MFI, mean fluorescent intensity.

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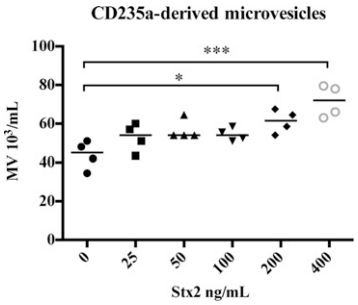

FIGURE 3. Stx2 induced a dose-dependent release of RBC-derived microvesicles (MV). RBC-derived MV from RBC-controls (n = 4) were stimulated with increasing Stx2 concentrations. MV levels were measured by flow cytometry using CD235a for detection of RBC origin. Data are presented as median (horizontal lines) and individual values. Results shown were reproducible and summarized from two separate experiments, using various donors. Multigroup comparison, $p < 0.01$. $*p < 0.05$, $***p < 0.001$.

disease and C5b-9 decreased 5 d later. RBCs with C5b-9 deposits may undergo hemolysis; thus, they may not be detectable in samples taken after HUS develops. For this reason, microvesicles released from RBCs were studied. In patient plasma, high levels of C5b-9–labeled RBC-derived microvesicles were detected, indicating that complement activation on RBCs had proceeded to the terminal complement pathway. Levels were considerably higher in the pediatric patients from whom samples were taken earlier on in the course of disease compared with adult patients. Thus, complement deposition on RBCs occurs during HUS, presumably more so in the earlier stages of disease, and the presence of the complement lytic complex C5b-9 on the RBC membranes suggests that complement might play a role in the hemolytic process.

In addition to inducing complement deposition on RBCs, as well as the release of hemoglobin and LDH, a marker for intravascular hemolysis (24), Stx2 induced the release of complement-coated RBC-derived microvesicles in vitro. The release of microvesicles may be a secondary phenomenon occurring during the hemolytic process; however, it also may be an important event contributing to lysis of the RBCs. Stx2 induced RBC-derived microvesicle shedding from donors with the P_1, P_2, P_1^k, and P_2^k phenotypes, but not the p phenotype, and C3 and C5b-9 deposition was more prominent on microvesicles derived from P_1, P_1^k, and P_2^k RBCs, suggesting that complement activation and microvesicle release from RBCs are related to toxin receptor expression. The release of microvesicles appeared to be mediated by the alternative pathway of complement because it was inhibited in the absence of factor B, but it was not inhibited in the absence of C2 or by eculizumab, suggesting that microvesicle release may primarily involve the earlier stages of the alternative pathway. However, Stx2-induced hemoglobin release was abrogated by heat inactivation, eculizumab, and EDTA, indicating that hemolysis was associated with complement activation via the alternative and terminal pathways.

RBCs are known to release microvesicles when stored for transfusion, a process that is related to aging or stress (25). Interestingly, in the context of HUS, microvesicles, in general, and those from RBCs, in particular, are procoagulant as a result of the

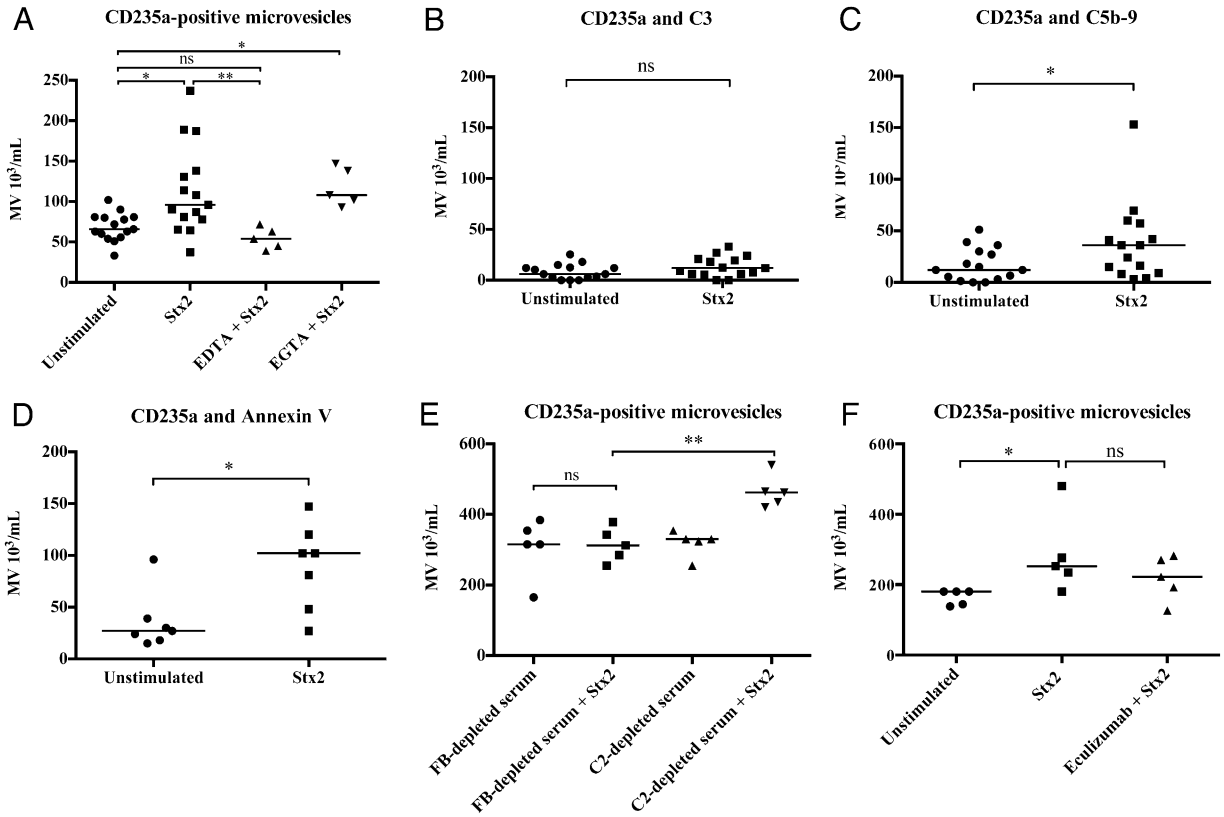

FIGURE 4. The effect of Stx2 on microvesicle (MV) release. Release of RBC MV from RBC-control stimulated with Stx2. (**A**) MV labeled with CD235a were of RBC origin. Stx2 induced the release of RBC-derived MV, the effect was decreased by EDTA but not by EGTA. Multigroup comparison, $p < 0.001$. (**B**) C3 deposition on RBC-derived MV. (**C**) C5b-9 deposition on RBC-derived MV. (**D**) Phosphatidylserine (annexin V) labeling of RBC-derived MV after stimulation with Stx2. (**E**) Stx2-induced release of RBC-derived MV from whole blood (from controls) was blocked in factor B (FB)-depleted, but not C2-depleted, serum. Multigroup comparison, $p < 0.05$. (**F**) Stx2-induced release of RBC-derived MV was not inhibited by eculizumab. Multigroup comparison, $p < 0.05$. The results are depicted as median (horizontal lines) and individual values. Results shown were reproducible and summarized from three separate experiments, using various donors. $*p < 0.05$, $**p < 0.01$. ns, not significant.

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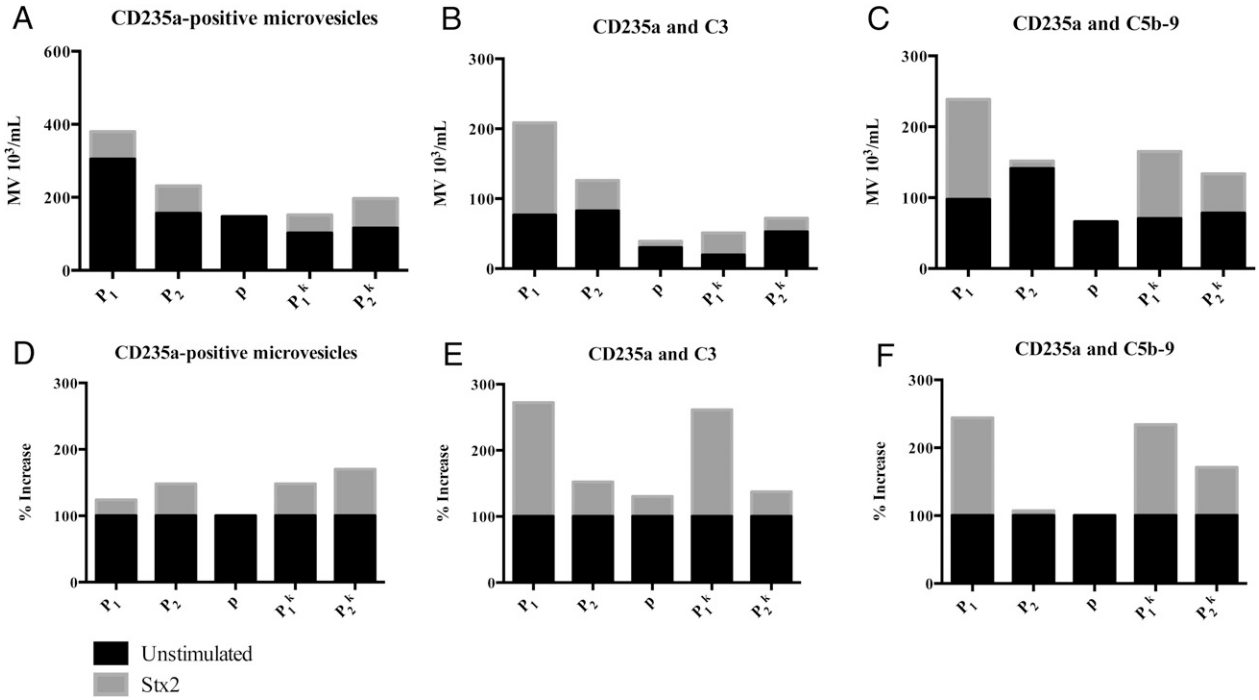

FIGURE 5. Stx2-induced release of microvesicles (MV) from RBCs expressing different P phenotypes. RBCs from 11 donors and five P phenotypes [P_1 ($n = 2$), P_2 ($n = 2$), p ($n = 3$), P_1^k ($n = 2$), and P_2^k ($n = 2$)] were stimulated with Stx2 and analyzed by flow cytometry for CD235a$^+$ MV. (**A**–**C**) Median MV in unstimulated and Stx2-stimulated samples. (**D**–**F**) Percentage increase in MV release in unstimulated (100%) and Stx2-stimulated samples.

expression of negatively charged phospholipids and tissue factor on their surface (26–28), which may enhance the prothrombotic state. In this study, we show that the RBC-derived microvesicles released after stimulation with Stx2 expressed phosphatidylserine, which is known to induce activation of coagulation factors V and X (29, 30) and subsequently promote thrombosis.

The complement inhibitors CR1, CD55, and CD59 are present on RBC membranes (31). Incubation with Stx2 did not affect the expression of these cell-bound inhibitors or factor H deposition on RBCs. Under conditions in which overwhelming complement activation takes place, these complement regulators may not suffice to fully prevent complement activation from occurring. Cells may shed microvesicles to eliminate membrane-bound complement as a protective mechanism against complement-mediated damage. Thus, the presence of elevated amounts of circulating complement-labeled microvesicles may be a protective mechanism, because complement-labeled microvesicles are opsonized and prone to undergo phagocytosis (11). Stx2 stimulation induced factor H deposition on RBC-derived microvesicles. Binding of factor H to the cell/microvesicle membrane is most probably protective in nature, aimed at inhibiting complement activation via the alternative pathway; however, the presence of factor H could enhance C3 detection, because C3 binds to factor H. Nonetheless, the results indicate that the small amount of factor H detected on microvesicles did not prevent complement activation and the deposition of C5b-9.

A novel finding in this study was that Stx2-induced complement-mediated hemolysis and microvesicle release from RBCs could be blocked by inhibition of purinergic receptors. Complement-mediated hemolysis was shown to be mediated via purinergic receptors and amplified through ATP release; thus, inhibition of P2 receptors by suramin and PPADS blocked hemolysis and ion influx (16). The finding that purinergic receptor antagonists inhibited both complement-mediated hemolysis [this study and (16)] and the release of RBC-derived microvesicles suggests that both of these biological processes may be dependent on purinergic signaling. P2 receptor activation was proposed to cause hemolysis by an influx in Ca^{2+}, triggering KCl efflux and RBC shrinkage (16). Suramin was shown to reversibly inhibit complement in the 1970s (32). The investigators postulated that suramin could compete with complement components for enzymatic sites. More recent studies showed that suramin is a potent inhibitor of P2 receptor

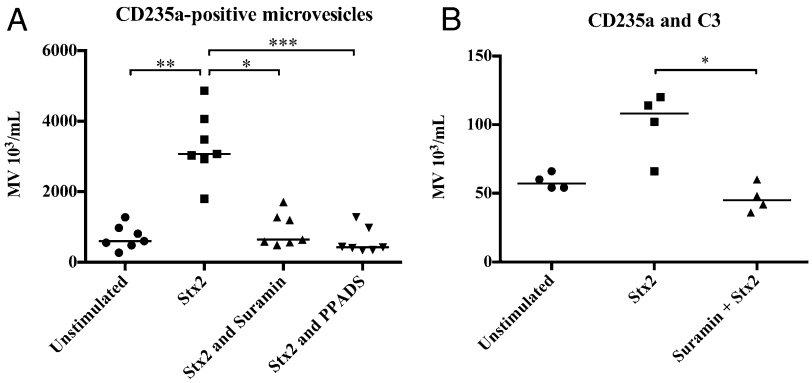

FIGURE 6. Suramin and PPADS inhibit Stx2-induced RBC-derived microvesicle (MV) release. (**A**) Stx2 induced the release of RBC-derived MV from RBC-control ($n = 7$). MV release was completely inhibited by the purinergic receptor inhibitors suramin and PPADS. Multigroup comparison, $p < 0.001$. (**B**) RBC-derived MV were measured in whole blood from healthy volunteers ($n = 4$) stimulated with Stx2 alone or together with suramin. The results are depicted as median (horizontal lines) and individual values. Results shown were reproducible and summarized from three separate experiments, using various donors. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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activation (16, 17); in this study, the effect of suramin on the inhibition of Stx2-induced hemolysis and the release of RBC-derived microvesicles was also confirmed using an alternative P2 antagonist, PPADS.

In addition to Stx2, other bacterial toxins were shown to induce hemolysis by purinergic P2 receptor activation (33–35). P2 receptors have not been studied with regard to RBC microvesicle release, but stimulation of the P2X7 receptor was shown to induce blebbing of microvesicles from microglia and dendritic cells (36–38). Because microvesicles might be prothrombotic and harmful in the context of HUS, the finding that P2 receptor antagonists can block their release may have therapeutic value. However, this requires further investigation because membrane blebbing also may be beneficial by eliminating harmful substances from the cell, such as complement.

The concentration of Stx2 used in the in vitro experiments does not necessarily reflect the in vivo setting because only negligible amounts of Stx2 are detected in the circulation during HUS (39). Stx2 was found to circulate bound to HUS patient blood cells, including platelets (26), neutrophils (26, 40, 41), and monocytes (26). Thus, the precise amounts circulating are hard to estimate. In this study, we used a concentration chosen after establishing a dose-response curve and in accordance with previous studies by our group (11) in which in vitro experiments using Stx2 mimicked the in vivo setting.

Data from the large German STEC outbreak did not support the use of the anti-C5 Ab eculizumab in patients with STEC-HUS (42, 43). Eculizumab was used in patients who already developed severe HUS. The results of the current study provide evidence that Stx promotes complement-mediated hemolysis. Presumably, this phenomenon would occur a few days before the patient is admitted and would not be fully amenable to treatment after admission, because full-blown hemolysis already occurred.

This study demonstrated that patients with STEC-associated HUS, regardless of the *E. coli* serotype, exhibited complement deposition on RBCs and RBC-derived microvesicles. In vitro studies demonstrated that Stx2 induced complement-mediated hemolysis, as well as the release of complement-coated RBC microvesicles, thus mimicking the situation in patient circulation. Furthermore, RBC-derived microvesicles may contribute to the prothrombotic state occurring during HUS.

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Disclosures

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