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The Antibiotic Polymyxin B Impairs the Interactions between Shiga Toxins and Human Neutrophils

Domenica Carnicelli,* Valentina Arfilli,* Francesca Ricci,† Claudio Velati,† Pier Luigi Tazzari,‡ and Maurizio Brigotti*

Hemolytic uremic syndrome (HUS) is the life-threatening sequela of intestinal infections by Shiga toxin (Stx)–producing Escherichia coli (STEC) in children. Human neutrophils specifically bind Stx through TLR4, the receptor of LPS. The binding could be considered protective (Stx sequestration) or harmful (toxin delivery to target organs). The amount of Stx on neutrophils is in equilibrium with the amount of Stx present in the gut, and it is also related to renal and neurologic symptoms. The TLR4-mediated interaction of LPS with innate immune cells is hampered by the well-known antibiotic polymyxin B. In this study, we show that the same antibiotic impairs the binding of Stxs to neutrophils, also blocking their functional effects (release of CXCL8, formation of neutrophil/platelet aggregates) involved in HUS pathogenesis. Controls for contaminating LPS in Stx-induced neutrophil responses inhibited by polymyxin B were performed. Stx interact with human neutrophils through their A chain, since these leukocytes do not express globotriaosylceramide, the specific receptor for Stx B chains. Consistently, polymyxin B blocked the enzymatic activity of Stx1, Stx2, Stx1 A chain, and the analogous plant protein gelonin, whereas the antibiotic did not show any protective effect on Stx-induced cytotoxicity in globotriaosylceramide-expressing Raji cells. Antibiotic administration is not recommended in human STEC infections during the prodromal intestinal phase, and the toxicity of polymyxin B could further discourage its therapeutic use. However, nontoxic, nonbactericidal polymyxin derivatives have been developed and might be used in animal models of STEC infection to study their efficacy in preventing the onset of HUS during the systemic blood phase of Stx.

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The two main Stx variants, Stx1 and Stx2, have been well characterized both at the molecular and functional levels (5). They are composed of a B pentamer chain noncovalently linked to a single enzymatically active A chain. Epidemiological studies revealed that STEC strains producing only Stx2 were more frequently associated with HUS, and thus this variant emerged as a key virulence factor for the development of HUS (6). During intestinal human infections by the noninvasive STEC, the bacteria produce and release Stx that cross the intestinal mucosa, reach the bloodstream, and move to the target organs, thereby damaging the intestinal, renal, and cerebral endothelia (5, 7, 8). Endothelial cells from these body sites are endowed with specific glycolipid receptors, denominated globotriaosylceramide and globotetraosylceramide (Gb3Cer and Gb4Cer, respectively), which avidly interact with the B pentamer of Stx, hence allowing internalization (5, 9). The Stx A chain expresses the enzymatic activity by targeting ribosomal 28S RNA (10) and DNA (11, 12), hence inducing the related toxic and proinflammatory effects (13–15). Interestingly, the Stx A chain is specifically recognized by human neutrophils (16) through TLR4 (17). The consequence of this putative protective action is the activation of the leukocyte and the release of an array of proinflammatory mediators, including CXCL8 (17). Alternatively, Stx might exploit the binding to neutrophils to be “piggyback” transported to cerebral and renal endothelia, because Stx are not internalized by neutrophils (18). In the past, this topic was a matter of debate because not all laboratories have been able to demonstrate the interactions between human neutrophils and Stx. The history of this debate and possible explanations of the conflicting results have been reviewed elsewhere (19). Briefly, the seminal paper demonstrating for the first time such an interaction (18) was followed by a second paper (20) in which some of the authors reversed their position. However, we and other groups confirmed these findings in vitro and in patients (16, 21–25).
Moreover, a possible explanation of the discrepancies was suggested, because subtle conformational changes of Stx purified by multistep and laborious methods induce loss of neutrophil-binding activity without interfering with the toxic activity (19, 26). All the authors confirming the reliability of Stx/neutrophil interactions used highly purified holotoxins isolated by affinity chromatography with one- or few-step methods, whereas more complicated isolation procedures or commercially available toxins were preferred in all but one the papers obtaining negative results (19, 26).

Although the Stx-dependent mechanisms responsible for the transition from hemorrhagic colitis to HUS are not fully understood, the involvement of human neutrophils in HUS has been widely accepted. Various arguments have been put forward as a causative role: 1) free Stx have never been found in sera of patients with HUS (27–29), and, conversely, the toxins have been detected on the surface of their neutrophils (19, 27, 30); 2) patients’ neutrophils are activated, degranulated, and hyporesponsive to other stimuli (19, 31); 3) neutrophilia is a common finding in patients with HUS and is considered a poor prognostic factor (32–34); 4) the greater the amount of toxins in patients’ feces, the greater the saturation of neutrophil receptors by Stx (35); 5) the transfer of the toxins from neutrophils to target endothelial cells has been demonstrated in vitro (18) during leukocyte transmigration (22); 6) HUS patients with lower amounts of toxins in their neutrophils had severe renal involvement (19, 27, 30); 7) HUS with only mild renal involvement developed in patients with a high detectable amount of toxins on neutrophil membrane, although they were at risk for neurologic complications (19, 27, 30); and 8) soluble Stx2 has been found on leukocytes (monocyte or neutrophil/platelet aggregates circulating in patients with HUS (24); and 9) leukocyte- or platelet-derived microvesicles containing Stx2 have been found in the blood patients with HUS (24, 36) and in their renal cortex (37).

Polymyxin B is an old antibiotic (discovered in 1947) elaborated by Bacillus polymyxus that has been shown to be active against Gram-negative bacteria (38). The drug is composed of a pentacationic cyclic peptide linked to a hydrophobic acyl residue (38). The amphoteric drug by strongly interacting with lipid A portion of LPS (39, 40) produces perturbation of outer bacterial membrane, thus eliciting antimicrobial effects (38). A well-known action of the antibiotic is the block of the functional response of human neutrophils to LPS (41, 42). Because Stx1, Stx2, and LPS are sensed by human neutrophils block of the functional response of human neutrophils to LPS (41, 42). Neutrophils (5 × 10⁶/ml) were incubated 90 min at 37°C with Stx1 or Stx2 (60 nM) in 250 μl of the same buffer or with 10 μg LPS in 250 μl PBS. After incubation, the cells were spun down at 200 × g for 5 min and washed three times with 100 μl incubation buffer at 37°C. The extent of Stx binding to neutrophils was assessed by flow cytometry as described below. Alternatively, neutrophils carrying Stx or LPS on their membrane were resuspended in RPMI 1640 containing 10% low-endotoxin FBS (≤ 0.5 endotoxin unit/ml; Bio-Whittaker, Walkersville, MD) at 37°C and incubated up to 2 h for cytokerine determinations on culture supernatants (see below).

Detection of Stx bound to neutrophils
Stx1 or Stx2 bound on neutrophils were detected by flow cytometry as previously described (25). Neutrophils carrying Stx1 or Stx2 (see above) were incubated with a mouse mAb against Stx1 or Stx2 in the presence of human serum to saturate FcRs on polymorphonuclear leukocytes (PMN). After incubation with FITC goat anti-mouse IgG, flow cytometric analysis was performed to reveal the neutrophil-bound fraction of Stx1 or Stx2 visualized by a cytogram that combined forward scatter versus 90° side scatter, and fluorescence was analyzed by a cytogram that combined 90° side scatter and fluorescence and by a single-fluorescence histogram. Neutrophils were checked by staining with mAb to Ags associated to granulocytes (FITC-CD16 and FITC-CD65, Beckman Coulter, Miami, FL). The mean channel value of fluorescence (MCV) of the single histogram was chosen to measure the extent of binding of Stx to PMN (25). The single values were calculated by subtracting the control MCV (range, 0.4–0.6), that is, the MCV of neutrophils from the same donor incubated with primary and secondary Abs. The assay has been validated previously by comparing control subjects and HUS patients in a double-blind fashion (25) and by challenging Stx* PMN with a negative control Ab (35).

Detection of CXCL8
CXCL8 present in culture supernatants from control and Stx1-, Stx2-, or LPS-treated neutrophils was quantified by a specific ELISA kit (Quantikine human CXCL8 immunoassay; R&D Systems, Minneapolis, MN).

Materials and Methods
Toxin purification

The Stx1 producer E. coli C600 (H19J) and the Stx2 producer C600 (933W) were supplied by Dr. Alison O’Brien (Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD). Stx1 and Stx2 were purified by receptor analog affinity chromatography, the former on globotriose-Fractogel (IsoSep, Lund, Sweden) (43) the latter on Galb1,4Galα1,3Galb1,4GlcNAc2-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) (15) following in both cases by a passage through ActiClean Etox columns (Sterogene Bioseparations, Carlsbad, CA) to remove trace endotoxin contaminants. Stx1 and Stx2 preparations contained low amounts of LPS (< 7.5 ng/ml), as assayed by using the Limulus amebocyte lysate Pyrogen Test Plus (Cambrex, Walkersville, MD). LPS from E. coli serotype O111:B4 (TLR grade) was obtained by Alexis Biochemicals. E. coli SY327 (pSC25, ampicillin resistance) containing full-length A chain of Stx1 was a gift of Dr. Carolyn Hovde Bohack (University of Idaho, School of Food Science, Moscow, ID). Stx1 A chain was purified according to Zollman et al. (44) by substituting the affinity chromatography on Blue Sepharose CL-6B (Sigma-Aldrich, St. Louis, MO) for Matrex Gel Green A dye-agarose gel. Gelonin (Sigma-Aldrich) was used because this plant protein displays the same mechanism of action of Stx and is composed of a single enzymatically active A chain analogous to the A chain of Stx. Isolation of human neutrophils and binding assays with toxins
Highly purified neutrophils were isolated under endotoxin-low conditions from buffy coats of several healthy donors after centrifugation over Ficoll-Paque, followed by dextran sedimentation and hypotonic lysis of contaminating erythrocytes, as described previously (17, 21, 45). Neutrophils were further enriched by positively removing any eventual contaminating cells to reach 99.7% purity using the EasySep human neutrophil enrichment kit (Stemcell Technologies, Vancouver, BC, Canada). For binding experiments with Stx, Eppendorf tubes were precoated with PBS containing 1% endotoxin-low (≤ 1 endotoxin unit/mg) BSA (Sigma-Aldrich) to avoid nonspecific loss of the toxins (46). Neutrophils (5 × 10⁶/ml) were incubated 90 min at 37°C with Stx1 or Stx2 (60 nM) in 250 μl of the same buffer or with 10 μg LPS in 250 μl PBS. After incubation, the cells were spun down at 200 × g for 5 min and washed three times with 100 μl incubation buffer at 37°C. The extent of Stx binding to neutrophils was assessed by flow cytometry as described below. Alternatively, neutrophils carrying Stx or LPS on their membrane were resuspended in RPMI 1640 containing 10% low-endotoxin FBS (≤ 0.5 endotoxin unit/ml; Bio-Whittaker, Walkersville, MD) at 37°C and incubated up to 2 h for cytokine determinations on culture supernatants (see below).

Detection of CXCL8
CXCL8 present in culture supernatants from control and Stx1-, Stx2-, or LPS-treated neutrophils was quantified by a specific ELISA kit (Quantikine human CXCL8 immunoassay; R&D Systems, Minneapolis, MN).

Raji cells cultures and protein synthesis

Raji cells were cultured in RPMI 1640 medium (Lonza, Walkersville, MD), containing antibiotics (60 μg/ml penicillin, 60 μg/ml streptomycin, Cambrex) and supplemented with 4 mM 1-glutamine (Sigma-Aldrich) and 10% FBS (Lonza). Protein synthesis was measured, after 3 h incubation with Stx, as the rate of incorporation of [3H]leucine into proteins during 60 min incubation in complete medium as described previously (16).

Adenine release from DNA in vitro

Adenine release from DNA was measured by using as substrate the 2251-bp [3H]DNA labeled in the purine ring of adenine obtained by PCR amplification of the 731–2981 region of the pBR322 plasmid (12). Enzymatic reactions were performed in 250 μl 50 mM sodium acetate buffer (pH 4) containing 30 mM succrose and 0.3 μg substrate corresponding to 205.5 pmol adenine having a specific radioactivity of 3000 dpm/pmol. After 40 min at 45°C, DNA molecules were removed by passing the samples through Bond Elut NH2 columns as previously described (12) and the combined HCl flow-through and washing were measured by liquid scintillation counting.

Detection of leukocyte/platelet aggregates by direct flow cytometry
Whole-blood samples (1 ml) from various donors were incubated for 4 h at 37°C with Stx2 (1 nM) in the presence and in the absence of polymyxin B (5 μg/ml) purchased from Sigma-Aldrich. After erythrocytic lysis, cells were incubated with anti-CD41 PE (Beckman Coulter) to reveal platelets and anti-CD14 FITC (Beckman Coulter) for monocyte detection or anti-CD16 PCS (Beckman Coulter) to identify neutrophils on gated granular cells. Appropriate controls with isotype Abs have been performed to ensure proper staining of cells with the specific Abs. CD14/CD41 double-
positive cell populations or CD16/CD41 granular double-positive cell populations were identified as monocyte/platelet or neutrophil/platelet aggregates, respectively.

Mice
Female FVB/NCr1 mice were handled in the local animal facility according to European directive 2010/63/UE and Italian law (DL 26/2014). Experimental protocols were approved by the Institutional Review Board. Blood samples were collected for Stx2/neutrophil binding assays in vitro.

Statistical analysis
Statistical analysis was performed with GraphPad Prism 5. Continuous variables were described through means and SD. Differences in continuous variables were tested with a t test after controlling the normality of their distribution. A p value <0.05 was considered statistically significant. Correlation between variables was assessed using a Pearson correlation coefficient.

Results
Inhibition of the binding of Stx to neutrophils by polymyxin B
The binding of Stx1 and Stx2 to human neutrophils was measured by indirect flow cytometric analysis with mAbs to Stx and a secondary fluorescent Ab. As depicted in the representative experiments shown in Fig. 1A, polymyxin B was very effective in inhibiting toxin-binding activity under conditions allowing full saturation of the neutrophil receptors (16, 26) by 60 nM Stx, as observed in HUS patients (27, 35). The antibiotic (10 μg/ml; ~7 μM) significantly impaired the interaction of Stx with neutrophils measured with five different endotoxin-low cell preparations (Fig. 1B). Dose response experiments are shown in Fig. 2. The mean values obtained using five neutrophil preparations with different antibiotic concentrations were used to calculate the IC_{50} of polymyxin B on Stx1 (Fig. 2A) and Stx2 neutrophil-binding activity (Fig. 2B). The values were very similar and ranged from 2.45 to 3.5 μg/ml, corresponding to 1.77–2.53 μM polymyxin B.

Polymyxin B inhibits the Stx-induced release of CXCL8 by neutrophils
Further evidence of such an antibiotic-induced inhibitory effect was obtained by measuring the release of the chemokine CXCL8 by neutrophils challenged with Stx1, Stx2, and LPS, in the absence and in the presence of pretreatment with polymyxin B (10 μg/ml). Fig. 3 shows the significant inhibitions obtained in the presence of the antibiotic with the tested bacterial exo- and endotoxins after 20 h incubation of neutrophils from a representative donor (absolute values in Fig. 3A) and from three different preparations (expressed as percentages in Fig. 3B). The sensitivity of LPS and Stx to polymyxin B constitutes a further convergence between these biochemically different toxins in interacting with neutrophils.

Inhibition of the enzymatic activity of Stx by polymyxin B
Stx/neutrophil interactions are mediated mainly by the enzymatically active A chain of the bacterial toxins (16, 23, 26), also because human neutrophils lack the glycolipid Gb3Cer (47), which is specifically recognized by the B chains in target cells (5, 9). Thus, we hypothesized that a direct interaction between the A chain of Stx and the antibiotic occurred, as in the case of lipid A (39, 40), the toxic moiety of LPS, and that this prevented the binding of the toxins to TLR4. To validate this hypothesis, we directly evaluated the effect of the antibiotic on the enzymatic activity of Stx and related plant proteins on nucleic acids (removal of adenine bases) in vitro by means of a very simplified assay containing only purified [3H]DNA, labeled in the purine ring of adenine, and the toxins (12). The antibiotic strongly inhibited the release of adenine from DNA catalyzed by Stx after 40 min incubation (Fig. 4A). The IC_{50} values of polymyxin B in protecting DNA from depurination by Stx1 and Stx2 (Table I) are in line with those obtained for the inhibition of the binding of the holotoxins to neutrophils. Note that the enzymatic activity of the isolated Stx1 A chain was strongly inhibited by the antibiotic (Fig. 4A, Table I). Accordingly, the DNA deacylating activity of the related plant protein gelonin (from Gelonium multiflorum seeds), which consists only of a single chain (A chain) containing the catalytic site, is inhibited by polymyxin B, although to a lower extent (Fig. 4A, Table I). Finally, to exclude effects of polymyxin B on the substrate rather than on the enzymes, we separately treated Stx1 and DNA with the antibiotic (10 μg/ml, 10 min on ice) before performing shorter 20-min enzymatic reactions. Preincubation of Stx1 with polymyxin B greatly enhanced the inhibitory effect of the antibiotic, whereas preincubation of DNA with polymyxin B had no effect (Fig. 4B). Taken together, the results strongly indicate a direct interaction of polymyxin B with the A chain of Stx.

Stx-induced cytotoxicity in Gb3Cer-bearing cells is not hampered by polymyxin B
We also measured the effect of polymyxin B on the cytotoxicity elicited by Stx1 and Stx2 in human cells endowed with Gb3Cer, such as Raji cells. As shown in Fig. 5, the presence of polymyxin B (~7 μM) did not change the inhibitory power of both Stx on whole-cell protein synthesis, as measured by the IC_{50} values obtained with Stx1 and Stx2 in the absence (1.09 pM, r = −0.995; 0.97 pM, r = −0.994; respectively) and in the presence (1.10 pM, r = −0.992; 0.86 pM, r = −0.999; respectively) of polymyxin B. Preincubation of the antibiotic with Stx (30 min at 37°C) before their addition to cells did not significantly modify the results. It could be argued that polymyxin did not directly target B chains and that the interactions of the antibiotic with the A chain, although not interfering with the binding of the holotoxin to Gb3Cer, are probably lost during the complex internalization process.

Inhibition of the formation of Stx2-induced neutrophil/platelet aggregates by polymyxin B
It has been shown that Stx2 added to human blood induced the formation of neutrophil/platelet or monocyte/platelet aggregates with activated thrombocytes and leukocytes, leading to the release of microvesicles (24, 36). Complement activation on these complexes and on microvesicles should play a definite role in the development of HUS, because patients show higher platelet/leukocyte aggregates in blood with respect to healthy subjects (24, 36). The effect of polymyxin B on the formation of platelet/leukocyte aggregates after treatment of human blood with Stx2 (1 nM) is shown in the representative experiment in Fig. 6A (selected cytograms in Supplemental Fig. 1) and by analyzing the results obtained with four different neutrophil preparations (Fig. 6B). Polymyxin B (5 μg/ml) approximately halved the amount of Stx2-induced neutrophil/platelet aggregates, whereas a lower nonsignificant inhibition was observed with monocyte/platelet aggregates (Fig. 6, Supplemental Fig. 1). This is in keeping with the notion that the antibiotic challenges the A chain of Stx, hence impairing the interaction with cells that express TLR4 but lack Gb3Cer (human neutrophils); conversely, the antibiotic did not target B chains, thus allowing the effect of the toxins on Gb3Cer-bearing cells (human platelets and monocytes), even in the presence of TLR4 on their surface. This is consistent with kinetic data showing that the affinity of Stx for Gb3Cer is 10-fold higher with respect to TLR4 (18).

Controls for contaminating LPS in Stx preparations
Stx are bacterial products that are unavoidably contaminated by LPS, which, in turn, is targeted by polymyxin B. Thus, an important point to address in interpreting some of the results described above...
is the possible role of an eventual contamination by LPS on the functional responses of neutrophils inhibited by polymyxin B. The Stx batches used for these studies showed trace amounts of endotoxin (<7.5 pg/μg protein, as measured by Limulus amebocyte lysate), thereby accounting for the presence of ~0.5 or 30 pg/ml LPS in the leukocyte/platelet aggregate formation or CXCL8 as-

FIGURE 1. Inhibition by polymyxin B of the binding of Stx to human neutrophils. The binding of Stx1 and Stx2 to human neutrophils was assessed by indirect flow cytometric analysis as described in Materials and Methods. Neutrophils were pretreated 30 min with 10 μg/ml polymyxin B before the addition of 60 nM toxins. (A) Representative single histogram analysis showing the inhibition induced by polymyxin B on the binding of Stx1 and Stx2 to human neutrophils. (B) Percentage of Stx bound to neutrophils (mean ± SD) obtained in five different neutrophil preparations. Significant inhibitions (**p < 0.0001) have been obtained in the presence of polymyxin B. Under these conditions, the MCVs of Stx1-treated or Stx2-treated neutrophils was 4.35 ± 0.70 and 5.65 ± 0.97 (means ± SD; n = 5), respectively.
However, blood stimulated with 1 pg/ml LPS showed no significant formation of leukocyte/platelet complexes with respect to control blood samples (Fig. 6), and neutrophils stimulated with 30 pg/ml LPS constantly showed no significant release of CXCL8 with respect to controls (Fig. 3A). Moreover, heat treatment (30 min at 95˚C) of Stx totally blocked the Stx1-induced CXCL8 release and substantially blocked the stimulation induced by Stx2 (Fig. 3), known to be more heat-resistant than Stx1 (48). Furthermore, the same heat treatment similarly impaired the formation of neutrophil/platelet or monocyte/platelet aggregates by Stx2 (Fig. 6). However, because synergy of Stx with LPS has been reported (49), the heating experiments do not exclude the involvement of contaminant LPS in the functional effects triggered by Stx on neutrophils and monocytes. To address this issue, we compared the functional activities (CXCL8 release and formation of leukocyte/platelet aggregates) induced by Stx on neutrophils with those elicited by Stx spiked with contaminant LPS at the above-mentioned concentrations. The experiments shown in Figs. 3 and 6 excluded synergistic interactions because only slight nonsignificant variations (stimulation or inhibition, respectively) have been obtained. Furthermore, we have previously reported (17, 26) that partially unfolded Stx1 showed negligible neutrophil-binding activity and did not elicit the release of CXCL8 by human neutrophils, even though it was contaminated by amounts of LPS similar to those found in our Stx preparations. Interestingly, the change in conformation is partial and specific because unfolded Stx1 expressed enzymatic and Gb3Cer-binding activities (17, 26). Taken together, the involvement of contaminating LPS in the Stx-induced functional responses of neutrophils inhibited by polymyxin B appears unlikely.

**Discussion**

In the present study, we show that the well-known antibiotic polymyxin B is capable of blocking the interaction of Stx1 and Stx2 with human neutrophils, also impairing their response to toxin challenge, such as the release of CXCL8 and the formation of neutrophil/platelet aggregates. The antibiotic seems to interact with the A chain of Stx, as indirectly demonstrated by the lack of any antibiotic-induced protective effects in cytotoxicity experiments with cells expressing Gb3Cer (the molecule recognized by the B chains), and directly proven by the polymyxin B–induced inhibition of the enzymatic activity expressed by Stx, the A chain of Stx1, and the related single-chain plant protein gelonin. Note that the A chains of Stx are mainly responsible for the specific binding of holo-Stx to human neutrophils (16, 23, 26).

**FIGURE 2.** IC50 of polymyxin B in inhibiting the neutrophil-binding activity of Stx. The binding of Stx1 and Stx2 to human neutrophils was assessed by indirect flow cytometric analysis as described in Materials and Methods. IC50 of polymyxin B inhibiting the binding of Stx1 (A) or Stx2 (B) to neutrophils was calculated by the linear regression between mean percentage of neutrophil-bound toxins obtained in five different cell preparations and the log of polymyxin B concentrations.

**FIGURE 3.** Polymyxin B impairs the release of CXCL8 by neutrophils challenged with LPS, Stx1, and Stx2. CXCL8 present in culture supernatants from control and Stx1-, Stx2-, or LPS-treated neutrophils after 20 h incubation was quantified by a specific ELISA. (A) Absolute values obtained in a representative experiment; data are means ± SD values (n = 2). (B) Percentage of CXCL8 released by neutrophils (three different preparations); data are means ± SD values (n = 3). In the presence of 60 nM Stx1 the release ranged from 264 to 1353 pg/ml, in the presence of 60 nM Stx2 it ranged from 536 to 859 pg/ml, and in the presence of 40 μg/ml LPS it ranged from 536 to 859 pg/ml, and in the presence of 40 μg/ml LPS it ranged from 184 to 800 pg/ml. Controls for contaminating LPS are also shown (dashed bars). *p < 0.05, **p < 0.01, ***p < 0.001.
HUS represents a relevant public health concern because of the dramatic triad (thrombocytopenia, hemolytic anemia, and acute renal failure) affecting children and of the lack of any specific treatment (2, 4). Most HUS cases arise from infection by E. coli strains that produce Stx, whose prominent pathogenetic role is widely accepted. Moreover, neutrophils have been suggested to play a role in the development of HUS in STEC-infected children. In this light, the administration of polymyxin B might interfere with a crucial point in the pathogenesis of HUS (i.e., Stx binding to neutrophils, formation of neutrophil/platelet aggregates). Note that the IC₅₀ values of polymyxin B in Stx-binding assays with human neutrophils fit within the range of plasma concentrations measured in pharmacokinetic experiments in treated patients after i.v. administration of the antibiotic (50).

It is well known that monocyte/LPS interactions induce up-regulation and release of proinflammatory cytokines, for example TNF-α and IL-1β. In turn, these mediators, by enhancing Gb3Cer expression (51, 52), render renal endothelial cells more susceptible to toxin action. The same protective effect afforded by polymyxin B on neutrophils challenged with Stx might occur with LPS targeting monocytes in blood or platelets. Thus, the same antibiotic could impair the interaction of two potent bacterial virulence factors with target cells involved in the pathogenesis of HUS. This could be particularly important in preventing the generation of tissue factor–bearing leukocyte/platelet-derived microvesicles, also containing Stx2, which are involved in HUS pathogenesis (24, 36, 37). Microvesicles have been shown to constitute a novel mechanism of transfer of Stx2 to glomerular endothelial cells in vitro and in patients with HUS (37). They are produced in vitro upon incubation with LPS and Stx2 both interacting with TLR4 present on the membrane of the different target cells, that is, neutrophils, monocytes, and platelets (24). The impairment of LPS binding via TLR4 to all the involved actors and the selective inhibition of Stx2 binding to neutrophils through the same receptor would probably reduce the formation of leukocyte/platelet-derived microvesicles, eventually hampering toxin transfer to target endothelia.

**FIGURE 4.** Polymyxin B inhibits the enzymatic activity of Stx and related plant proteins. (A) Effect of polymyxin B on Stx1-, Stx2-, gelonin-, and Stx1 A chain–dependent adenine release from [³²H]DNA in vitro measured as described in Materials and Methods. Data are expressed as percentage of adenine release; the SD values (n = 3) of single points are indicated. The [³²H]adenine release by 0.4 nM toxins and related proteins after 40 min at 45°C was 11,644 ± 4,019 dpm with Stx1, 18,396 ± 1,772 dpm with Stx2, 43,222 ± 9,392 dpm with Stx1 A chain, and 6,043 ± 1,240 dpm with gelonin. All the tested proteins were more effective in releasing adenine from DNA at 45°C than at 37°C. The Pearson correlation coefficients of the different straight lines are shown in Table I. (B) Effect of preincubation with polymyxin B (10 min on ice) of DNA or Stx1 on the rate of [³²H]adenine release after 20 min at 45°C. Significant differences (*p < 0.05, **p < 0.01) have been obtained.

<p>| Table I. Polymyxin B inhibits the DNA depurinating activity of Shiga toxins and related proteins |
|--------------------------------------------------|--------------------------------------------------|</p>
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<tr>
<th>IC₅₀ Polymyxin B (µg/ml)</th>
<th>Pearson Correlation Coefficient (r)</th>
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<tr>
<td>Stx1</td>
<td>8.72</td>
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<tr>
<td>Stx2</td>
<td>6.58</td>
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<tr>
<td>Stx1 A chain</td>
<td>0.91</td>
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<tr>
<td>Gelonin</td>
<td>83.60</td>
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Adenine released from [³²H]DNA by Stx1, Stx2, Stx1 A chain, and gelonin is shown in Fig. 4 and measured as described in Materials and Methods. IC₅₀ was calculated by the linear regression between percentage of activity and the log of polymyxin B concentrations.

**FIGURE 5.** Effect of polymyxin B on the IC₅₀ of Stx on protein synthesis by Raji cells. Raji cells were pretreated 30 min with polymyxin B (10 µg/ml) or vehicle before the 3 h incubation with toxins. Protein synthesis was measured as described in Materials and Methods. Polymyxin B addition to controls did not induce any inhibitory effect. IC₅₀ was calculated by the linear regression between percentage of activity and the log of Stx concentrations. (A) The [³¹H]leucine incorporated in control cells was 35,376 ± 5,816 dpm (mean ± SD, n = 3). (B) The [³¹H]leucine incorporated in control cells was 30,545 ± 3,251 dpm (mean ± SD, n = 3).
Recently, however, several novel derivatives of polymyxins have been developed and patented (38, 54). This was made possible by the knowledge of the contribution of each amino acid residue in polymyxin B to antimicrobial and LPS-binding activity (55). Because renal toxicity has been shown to be related to the number of cationic charges in polymyxins, novel derivatives such as NAB739 (developed and patented by Northern Antibiotics) with substitution of some of the positively charged amino acids showed reduced toxicity while maintaining similar or stronger antimicrobial activity. Other polymyxin derivatives (NAB7061 and NAB741, also developed and patented by Northern Antibiotics) are devoid of potent bactericidal action, even though they are capable of perturbing outer bacterial membrane, thus increasing permeability to other antibiotics (54). The latter phenomenon is mediated by the direct interactions of the antibiotics with LPS resident in the outer bacterial membrane. For this reason, the latter derivatives seem to be promising because their interactions with LPS, and arguably with Stx, could be preserved in the absence of a direct bactericidal action, which has been shown to be dangerous during the prodromal intestinal phase of STEC infections. This is in keeping with the notion that hydrophobic interactions are the driving force for the association of polymyxin B with LPS and lipid A (40). Consistently, preliminary results obtained in our laboratory showed that preincubation at physiological pH of Stx2 (500 nM) with polymyxin B (100 μg/ml) under conditions (0.5 M NaCl) that enhance the formation of hydrophobic/van der Waals interactions, also increased the inhibitory action of the antibiotic on the binding of the toxin to neutrophils, whereas protonation (pH 4, 0.5 M NaCl) induced poor effects.

Carefully conducted Stx–neutrophil binding functional assays in vitro would reveal the best derivatives to be used as inhibitors of such an interaction. Importantly, the polymyxin derivatives should be validated in appropriate animal models before concluding that their administration as drug in STEC-infected children is safe. In this regard, the type of animal model is of paramount importance because it is necessary to recapitulate the whole pathogenetic process of human HUS, including STEC intestinal infection, toxin delivery in blood, and the onset of the triad of HUS. Several mouse model of HUS have been described; for example, TLR4-deficient mice have been compared with wild-type mice following exposure to Stx (56). However, these models are not useful in our experimental settings because the Stx receptor profile found in human neutrophils is quite different in mice (23). The former possess uniquely TLR4 as Stx receptor (17), as human neutrophils do not possess the complete set of enzymes necessary for the synthesis of Gb3Cer and Gb4Cer (47). On the contrary, mouse neutrophils showed the expression of both TLR4 and Gb3Cer. Supplemental Fig. 2 illustrates that the binding of Stx2 to mouse neutrophils, measured by indirect flow cytometric analysis, is paradoxically nephrotoxicity and, to a lower extent, neurotoxicity. Moreover, caution must be used in proposing polymyxin B administration in STEC-infected children, because severe adverse reactions are associated with systemic polymyxin therapy in humans (53). The most serious adverse effects are paradoxically nephrotoxicity and, to a lower extent, neurotoxicity.

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**FIGURE 6.** Inhibition by polymyxin B of the formation of platelet/neutrophil aggregates by Stx2. Whole blood was treated with 1 nM Stx2 in the presence and in the absence of 5 μg/ml polymyxin B. Then, the formation of neutrophil/platelet or monocyte/platelet aggregates was assessed by direct flow cytometric analysis as described in Materials and Methods. (A) Values obtained in a representative experiment expressed as percentage of aggregates on the neutrophil or monocyte population; data are means ± SD (n = 2). (B) Values from four independent experiments expressed as percentage of total aggregates formed by Stx2; data are means ± SD (n = 4). After incubation of human blood with Stx2, in the absence of the antibiotic, the percentages of neutrophil/platelet aggregates and of monocyte/platelet aggregates on the whole population of neutrophils or monocytes were 40.8 ± 18.9% and 30.9 ± 31.7% (mean ± SD, n = 4), respectively. Controls for contaminating LPS are also shown (dashed bars). *p < 0.05, **p < 0.01, ***p < 0.0001.

However, antibiotic administration is not recommended in human STEC infections and should even be strongly discouraged during the prodromal intestinal phase because the antimicrobial treatment may cause 1) phage induction and consequent enhanced Stx expression in bacteria, and 2) bacterial lysis with subsequent toxin release (4). Moreover, caution must be used in proposing polymyxin B administration in STEC-infected children, because severe adverse reactions are associated with systemic polymyxin therapy in humans (53). The most serious adverse effects are...
and Stx1 A chain, respectively. We thank Prof. Pier-Luigi Lollini and Dr. Giordano Nicoletti (Dipartimento di Medicina Specialistica, Diagnostica e Sperimentale, Università di Bologna) for help in experiments with blood from mice.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


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SUPPLEMENTAL FIGURE 1. Selected cytograms of the representative experiments shown in Figure 6A. The effect of polymyxin B (5 μg/ml) on the formation of blood leukocyte/platelet aggregates by 1 nM Stx2 was assessed by flow cytometric analysis as described under Material and Methods. Quadrants 2 (upper right quadrants) identify CD16/CD41 granular double positive cell populations corresponding to neutrophil/platelet aggregates (left panels) or CD14/CD41 double positive cell populations corresponding to monocyte/platelet aggregates (right panels).
SUPPLEMENTAL FIGURE 2. Inhibition by monoclonal antibodies to Gb3Cer of the binding of Stx2 to mouse neutrophils. In each experiment blood samples from different FVB/NCrl mice were collected and immediately processed after pooling. Stx2 (50 nM) was added to 100 μl blood samples and, after 1 h incubation at 37°C, leukocytes were isolated after erythrocytic lysis. The binding of Stx2 to neutrophils was assessed by indirect flow cytometric analysis as described under Material and Methods. Mouse neutrophils were gated by morphology and checked by staining with Alexa Fluor® 647 rat anti-mouse Ly-6G (BioLegend Inc.). (A) Representative experiment showing the absolute value (MCV) of the binding of Stx2 to mouse neutrophils in the absence or in the presence of antiGb3Cer mAb (monoclonal rat IgM antiCD77, Beckman Coulter) or its isotype antibody (5 μg). Data are means ± SD (n = 2). (B) Percentage of Stx2 bound to mouse neutrophils obtained in two different experiments, the SD values of single points are indicated (n = 2). Significant inhibitions (***p < 0.001, **p < 0.01) have been obtained in the presence of antiGb3Cer mAb.