Interactions between Shiga toxins and human polymorphonuclear leukocytes

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Abstract: Human intestinal infections by Shiga toxin (Stx)-producing Escherichia coli cause hemorrhagic colitis and hemolytic uremic syndrome (HUS), which represents the main cause of acute renal failure in early childhood. In HUS, Stx released in the gut enter the bloodstream and are targeted to renal endothelium. The mechanism of toxin delivery is still a matter of debate, although the role of polymorphonuclear leukocytes (PMN) as a Stx carrier has been indicated. The aim of this paper was to better define the interactions between Stx and human PMN. Direct and indirect flow cytometric analysis and binding experiments with radio-labeled toxins demonstrated that Stx bind to the surface of human mature PMN but not to immature PMN from G-CSF-treated donors. The use of the human myeloid leukemia cell (HL-60) model for inducible cell differentiation confirmed that the toxin binding occurs only after granulocytic differentiation. Stx binding caused a delay of the spontaneous apoptosis of PMN, as shown by the delayed appearance of apoptotic nuclei and activation of caspase 3 and by the higher number of cells negative to the annexin V-binding assay after 48 h. Moreover, flow cytometric analysis of mixed Stx-positive and Stx-negative PMN populations showed that the toxins were transferred from positive to negative PMN. The delayed, spontaneous apoptosis and the passage of the toxic ligand from older PMN to new, mature cells entering the circulation from the bone marrow may explain the previously reported persistence of Stx in the blood of children with HUS. J. Leukoc. Biol. 84: 1019–1027; 2008.

Key Words: hemolytic uremic syndrome • neutrophils • HL-60 • apoptosis

INTRODUCTION

Shiga toxins (Stx)-producing Escherichia coli (STEC) infections represent a major public health concern, because of the severe illnesses that they can cause, such as hemorrhagic colitis and hemolytic uremic syndrome (HUS) [1], which is characterized by thrombocytopenia and microangiopathic hemolytic anemia and is the main cause of acute renal failure in early childhood [2, 3]. STEC produces two main types of bipartite toxins, Stx1 and Stx2, which are capable of binding to glycolipid receptors [globotriaosylceramide (Gb3)] on the surface of target cells through their B subunits [4]. After endocytosis, the A subunit damages ribosomes [5] and DNA [6], thus causing the arrest of protein synthesis and the formation of apurinic sites in the nucleus. Target cells show a broad spectrum of responses, including the production of proinflammatory cytokines involved in HUS pathogenesis [7, 8] and the triggering of the apoptotic program [9].

There is no doubt that most histopathological lesions observed in HUS are the consequence of the interaction of these toxins with the endothelial lining of intestine, brain, and kidney [4]. The pathogenetic process initially involves colonization of the gut [4] by STEC serogroups mainly associated with HUS, such as E. coli O157 and E. coli O26 [10, 11], which adhere to the intestinal mucosa with a characteristic “attaching and effacing” mechanism [12]. Afterwards, they release in the intestinal lumen large amounts of Stx, which damage villus epithelial cells and are absorbed into the circulation and targeted to the renal endothelium [4]. Although the mechanisms of endocytosis and intracellular sorting of the toxins are well known [13, 14], as well as the transcellular process that allows Stx to cross polarized intestinal epithelial cells [15], the entry process of the toxins into the circulation is still unclear.

Early studies [16, 17] have shown that no active Stx can be detected in the plasma of patients with HUS. As far as the cellular component of the blood is concerned, a pivotal study demonstrated that Stx were bound almost exclusively to circulating polymorphonuclear leukocytes (PMN) in human blood [18]. However, some of the authors of this study subsequently failed to reproduce those results [19], and another study was unable to demonstrate a direct interaction between Stx and PMN [20].
Conversely, the detection of Stx bound to circulating PMN by specific antibodies and flow cytometric techniques has been reported in other studies, and this opportunity has been exploited for diagnosis of STEC infection in HUS patients [21–23]. The binding of Stx1 and Stx2 to PMN in human blood has been confirmed recently by Griener et al. [24] using immunofluorescence.

In a previous paper, we evaluated the kinetic of Stx during the course of natural disease by detecting their presence in the feces and in the circulating PMN of children with HUS, by the Vero cell cytotoxicity assay, and by flow cytometric analysis, respectively [23]. A positive relationship between the amounts of toxins present in the intestinal lumen and in the bloodstream was observed. We also showed that the toxins were detectable on the PMN several days after the onset of prodromal diarrhea and for a median period of 5 days after they were no longer detectable in stools [23].

The persistence of Stx-positive PMN is difficult to explain, as the blood half-life of PMN is considered to be 6–7 h [25]. The presence of Stx-positive PMN, when the toxins are no longer detectable in feces, could be explained by two different and possibly concurrent hypotheses: Stx on the PMN membrane might constitute a pool of molecules passing rapidly between different PMN with a transfer of the toxin from older PMN to new, mature neutrophils entering in circulation from the bone marrow; and Stx might induce a profound modification of the biology of PMN by inducing a prolongation of the functional lifespan of these cells.

The aim of this study was to better clarify the interactions between Stx and human PMN to further our understanding of the persistence of Stx-positive PMN in the blood of children suffering from HUS.

MATERIALS AND METHODS

Reagents

Na<sup>125</sup>l (2.125 Ci/μ atom iodide, 100 mCi/ml) was obtained from Amersham Pharmacia Biotech (Bucks, UK). Disposable plastics for laboratory use were obtained from Costar (Bwaydow, Cambridge, MA, USA). The reagents of analytical grade were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Stx purification

The prototypes Stx1 and Stx2 producers E. coli G600 (H19j) and E. coli G600 (933W) were kindly supplied by Dr. Alison O’Brien (Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD, USA). Stx1 was purified by receptor analog affinity chromatography [26] on globotriose-Fractogel (IsoSep AB, Lund, Sweden). Stx2 was obtained according to the method described by Downes et al. [27]. Stx preparations contained low amounts of LPS (2–3 ng/mg), as assayed by using the Limulus ameocyte lysate Pyrogen™ plus (Cambrex, Walkersville, MD, USA).

Fluorescent labeling of Stx1

Stx1 (100 μg) was conjugated with Alexa Fluor® 488 reactive fluorescent dye and was purified by spin column according to the directions of the manufacturer (Molecular Probes, Invitrogen, Eugene, OR, USA). The degree of labeling was ~1 mol Alexa Fluor 488 dye per mole toxin. The biological activity of the fluorescent toxin was assessed by testing the binding to immobilized Gal3 (globotriose-Fractogel), which paralleled that obtained with unlabeled Stx1, and by measuring the enzymatic activity of fluorescent toxin on [3H]DNA in vitro [28], which was similar (82%) to that obtained with native toxin.

Radiolabeling of Stx1

Two Iodo beads (Pierce, Rockford, IL, USA) were incubated 5 min at room temperature with 500 μCi Na<sup>125</sup>I in 500 μl 0.1 M sodium phosphate buffer, pH 7.2. Stx1 (100 μg) was then added, and the reaction was allowed to proceed for 10 min at room temperature. Labeled Stx1 was separated by unreacted iodine by gel filtration using a PD-10 column (Pharmacia, Piscataway, NJ, USA) equilibrated with PBS. The efficiency of labeling was checked by precipitating an aliquot of [125]Stx1 with 6% TCA in the presence of 0.01% sodium deoxycholate. The biological activity of the radiolabeled toxin (specific radioactivity ~100,000 cpm/pmole) was assessed by testing the binding to immobilized Gal3 (globotriose-Fractogel), which paralleled that obtained with unlabeled Stx1, and by comparing the IC<sub>50</sub> (concentration-inhibiting protein synthesis by 50%) of radiolabeled and native toxin on human endothelial cell translation after 16 h incubation, as described previously [6]. The calculated IC<sub>50</sub> were 0.7 pM and 1.1 pM, respectively.

Binding of fluorescent or native Stx1 to PMN in blood

Blood was obtained from three different healthy human donors and treated with EDTA. Small amounts of blood (100 μl) were incubated for 1 h at 37 °C with 5 pmol native Stx1 or Alexa Fluor® 488-Stx1. The samples were then lysed and fixed with the Multi Q Prep apparatus, washed twice with PBS, and evaluated by direct or indirect flow cytometric analysis (see below). Lysis was also performed with VitaLyse (BioE, St. Paul, MN, USA), as described previously [21], giving the same results. The blood samples from different donors showed no significant differences in toxin-binding activity.

Indirect flow cytometric analysis of Stx bound to cells

Stx bound to leukocytes, to human myeloid leukemia cells (HL-60), or to isolated PMN (see below) were detected by flow cytometry as described previously [21, 23]. Briefly, white blood cells isolated after erythrocytic lysis from 100 μl blood, HL-60, or isolated PMN were incubated with equal amounts of an appropriate dilution of mouse mAb against Stx1 or Stx2 in the presence of human serum to saturate FcRs on PMN. After incubation with FITC-goat anti-mouse IgG, flow cytometric analysis was used to reveal the cell-bound fluorescence. A flow cytometer (FC500, Beckman Coulter, Miami, FL, USA) was set to acquire and gate events by forward-scatter versus 90° side-scatter and by green fluorescence versus 90° side-scatter. This set resulted in a prompt analysis of morphology and fluorescence, allowing a clear evaluation of control and positive samples and a highly sensitive detection of both Stx. The mean channel value of fluorescence (MCF) of the cells was chosen as an objective parameter to measure the extent of binding of Stx to cells [21]. The single values were calculated by subtracting the control MCF (range 0.3–0.5), i.e., the MCF of cells incubated with primary and secondary antibodies in the absence of the toxins or with secondary antibody plus Stx. The assay has been validated previously by comparing control subjects and HUS patients in a blind manner [21] and by challenging Stx-positive PMN with a negative control antibody [23].

Culture of HL-60

The HL-60 cell line used in this study was described previously [29, 30] and generously provided by Prof. Alberto M. Martelli (University of Bologna, Italy). The cells were cultivated in RPMI-1640 medium containing 10% FCS with penicillin/streptomycin and glutamine added. All cultures were grown at 37°C in a humid incubator purged with 5% CO<sub>2</sub>/95% air. Previous studies with HL-60 have demonstrated that nuclear segmentation (granulocytic differentiation) in response to all-trans-retinoic acid (RA; Sigma Chemical Co.) treatment reaches its peak in ~4 days, after which apoptosis begins to predominate [29, 30]. Nuclear shape was determined from the 4′-6-diamidino-2-phenylindole (DAPI) staining. After washing in PBS and distilled water, the coverslips were air-dried and mounted in a solution containing 0.2 mg/ml DAPI (Sigma Chemical Co.) in water, diluted 1:500 in 1,4 diazobicyclo[2.2.2]octane (Sigma Chemical Co.) in water, and was purified by spin column according to the directions of the manufacturer (Molecular Probes, Invitrogen, Eugene, OR, USA). The degree of labeling was ~1 mol Alexa Fluor 488 dye per mole toxin. The biological activity of the fluorescent toxin was assessed by testing the binding to immobilized Gal3 (globotriose-Fractogel), which paralleled that obtained with unlabeled Stx1, and by measuring the enzymatic activity of fluorescent toxin on [3H]DNA in vitro [28], which was similar (82%) to that obtained with native toxin.
Isolation of PMN

To obtain endotoxin-free PMN from healthy donors, the solutions used throughout the method [31] were sterile and prepared with clinical water. Theuffy coat obtained from 40 ml EDTA-treated blood was stratified on Ficoll Paque (Pharmacia; Ficoll/buffy coat ratio=0.75) and centrifuged at 400 g for 30 min at 20°C. After removal of monocytes and lymphocytes, the granulocytes stratified on the packed red cells were collected and diluted to 10 ml with PBS. Erythrocytes were sedimented by adding 2.5 ml 4% (w/v) dextran 500 (Pharmacia), and the PMN was spun down from the supernatant at 250 g for 5 min at room temperature. Contaminating red cells were removed with an osmotic lysis (60 s in 1.5 ml 0.2% NaCl followed by the addition of 3.5 ml 1.2% NaCl). PMN was pelleted at 200 g for 5 min, washed twice with PBS containing 1% (w/v) BSA, and resuspended in 1 ml of the same buffer. The total number of granulocytes obtained was 25–30 × 10⁶.

Binding of native or radiolabeled Stx to PMN or HL-60

Epipodoflur tubes were precoated with PBS containing 1% BSA to avoid nonspecific loss of toxins [32]. Endotoxin-free neutrophils (2×10⁶/ml) or HL-60 were incubated with different concentrations of unlabeled or radiolabeled Stx (0.5–75 nM) in PBS containing 1% BSA for 90 min at 37°C, with occasional stirring by gentle inversion of the tube. The cells were spun down at 200 g for 5 min and washed three times with the same buffer containing BSA, maintained at 37°C. The extent of binding of native Stx to neutrophils or HL-60 was measured by flow cytometry as described above, whereas the binding of [125I]Stx1 to neutrophils was quantified by counting the cell-associated radioactivity with a γ-counter after extensive washing. The binding of radiolabeled Stx1 in the presence of a 50-fold excess of native, unlabeled Stx1 (nonspecific binding) was equal to the background value obtained in the absence of cells and was subtracted in each experiment.

Detection of apoptosis

Apoptosis was evaluated by the observation of changes in nuclear shape of PMN (from lobulated to shrunken, ovoid, and pyknotic), determined from the absence of cells and was subtracted in each experiment.

Transfer of Stx between PMN

The passage of Stx between PMN from the same donor was measured by indirect flow cytometric analysis as described above. The interleukocytic passage was also evaluated with PMN from donors differing in the isoforms NA1 and NA2 of the membrane glycoprotein FcyRIIIb (CD16b) and easily distinguishable with the FITC-conjugated mAb 1D3 to CD16b (Beckman Coulter). The binding of Stx1 to PMN and their reactivity to mAb anti-CD16b were assayed by flow cytometry as described above with the following modifications: Cells were first incubated with anti-Stx1 mouse mAbs, and after two washes with PBS, goat anti-mouse IgG conjugated with APC (Beckman Coulter) was added. After two washes, samples were incubated with normal mouse IgG to saturate residual anti-mouse IgG sites, and FITC anti-CD16b (strongly reacting with NA1 granulocytes) was added. After two washes, samples were analyzed by flow cytometry, with a FC500 cytometer equipped with two lasers. Granulocytes were gated selectively by morphology.

Statistical analyses

Continuous variables were described through mean and SD. Data analysis was performed with SPSS Version 14.0. We assessed differences between groups using the Student's t-test for continuous variables after controlling normality and homoscedasticity assumptions.

RESULTS

Stx-binding experiments with mature and immature PMN

The binding of Stx1 to blood components was evaluated by using native and labeled Stx1. As shown in Figure 1A, the addition of Stx1 conjugated with Alexa Fluor 488 dye to blood samples resulted in the fluorescent staining of PMN as assessed by direct flow cytometric analysis. Although monocytes were evaluated by flow cytometry, with a FC500 cytometer equipped with two lasers. Granulocytes were gated selectively by morphology.

Fig. 1. Detection of Stx1 bound to PMN in human blood by direct and indirect flow cytometric analysis. Cytograms combining 90° side-scatter (SS) versus fluorescence [three-region cytogram: lymphocytes, monocytes (M), and granulocytes (PMN)]. (A) Overlapped cytograms analyzing control and Alexa Fluor 488-Stx1-treated blood. (B) Overlapped cytograms analyzing control and Stx1-treated blood.
were also partially positive, most of the fluorescent toxin was clearly detected on PMN membranes. When native Stx1 was added to blood, PMN captured the added toxin (Fig. 1B). Parallel experiments with Stx2 gave similar results (data not shown). In these experiments, the Stx associated to PMN were detected by flow cytometry after incubation with anti-Stx1 and anti-Stx2 mouse mAb and secondary FITC-goat anti-mouse IgG [21, 23]. In a second set of experiments, the binding of radioiodinated Stx1 to endotoxin-free PMN [31] isolated from healthy donors was investigated by counting the radioactivity associated to PMN after incubation and extensive washing. We observed reproducible and quantitative binding of labeled Stx1 to PMN. The number of binding sites on PMN calculated at saturation (50 nM [125I]Stx1, 90 min at 37°C) was 195,000 per cell and resulted similarly to previously reported values [18]. It should be noted that the binding of labeled Stx1 occurred in the presence of large amounts of trivial proteins (BSA 1%), and it was almost completely inhibited by a 50-fold excess of native, unlabeled Stx1. Thus, the interaction of Stx1 to mature PMN was confirmed with two alternative and independent techniques. In contrast, immature human granulocytes (1% lobulated nuclei, Fig. 2B), isolated from different donors treated with G-CSF [34], did not bind native Stx1 (MCV=0.04±0.05) compared with mature PMN (98% lobulated nuclei, Fig. 2D) from healthy donors (MCV=3.2±0.3, P<0.001), as shown by the representative flow cytometric analysis depicted in Figure 2, A and C. To confirm that Stx binding occurs with mature PMN only, the human myeloid leukemia cell HL-60 model for inducible cell differentiation was used [29, 35]. Treatment with all-trans-RA induces granulocytic differentiation of these cells, which exhibit many similarities with normal PMN: nuclear lobulation, nitroblue tetrazolium reduction by superoxide anions, enhanced expression of the cell-surface antigen CD11b and of phagocytotic capability, and, finally, death by apoptosis within a few days [29, 36, 37]. HL-60 were exposed to 1 μM RA for 3 days, and nuclear shape changes were quantified on DAPI-stained cyto- spun preparation and compared with undifferentiated cells. Only 8% of untreated cells exhibited multilobulated nuclear shapes (Fig. 3B), and nearly 60% of RA-treated cells were lobulated (Fig. 3D). Thus, after RA treatment, HL-60 exhibited the typical nuclear shape change as evidence of granulocytic differentiation. Binding experiments performed with native Stx1 showed that the toxin did not bind to HL-60 (MCV=0.01±0.02), whereas a significant binding was observed with RA-treated HL-60 (0.53±0.1, P<0.05), as shown by the representative histograms of Figure 3, A and C. Taken together, these observations indicate that only mature PMN bind Stx.

Delayed apoptosis in Stx-treated PMN

A possible explanation of the long persistence (~5 days) of Stx on PMN in the blood of HUS patients [23] is that Stx might modify the biology of PMN by inducing a prolongation of the functional lifespan of these cells. Besides their functional properties, PMN undergoing apoptosis lose the multilobulated shape of the nucleus that became ovoid, shrunken, and pyknotic, and this is considered one of the morphological characteristics of the apoptotic PMN [38]. We found that the appearance of apoptotic nuclei after 10 h incubation of freshly isolated PMN was reduced significantly by the presence of low concentrations (1 nM) of Stx1 and Stx2 (Fig. 4).

At 24 h, a higher concentration of Stx1, leading to full saturation of PMN receptors, was required to obtain a significant reduction of the nuclear shape changes, which represent middle-to-late events in the apoptotic program (Fig. 4). As the activation of executioner caspases is considered an early apoptotic event, we measured the cleavage of a specific colorimet-

Fig. 2. Representative single histogram analysis illustrating binding of Stx1 to mature human PMN assessed by indirect flow cytometric analysis. (A) Overlapped histograms of control (white) and (50 nM) Stx1-treated (gray), immature PMN from G-CSF treated donors (samples obtained after informed consent following institutional guidelines) [34]. (B) DAPI-stained cyto- spun preparation of freshly isolated, immature PMN (Time 0). (C) Overlapped histograms of control (white) and (50 nM) Stx1-treated (gray), mature human PMN from healthy donors. (D) DAPI-stained cytospin preparation of freshly isolated, mature PMN (Time 0).
ric substrate for caspase 3 in lysates from cultured PMN. An approximate threefold increase in caspase 3 activity was observed in control cells after 5 h of incubation, when only negligible levels of caspase 3 activity were detectable in Stx1- or Stx2-treated cells (Fig. 5). At 20 h, a fivefold increase in caspase activity was measured in each condition. Thus, a transient impairment in caspase 3 activation (5 h) preceded the decrease in the number of apoptotic cells observed in Stx-treated PMN (10 h).

Freshly isolated PMN must be considered a mixture of mature cells that have entered the bloodstream from the bone marrow at different times, rather than a homogeneous population of leukocytes. Therefore, PMN preparations include cells differing in age and differently engaged in the apoptotic program. To explain the long persistence of Stx-positive PMN in patients, we attempted to identify the younger PMN population and to evaluate its maximal lifespan. After 48 h of culture (total PMN number $1 \times 10^6$), most of treated (1 nM both toxins) and untreated cells were apoptotic at morphological examination. We identified by flow cytometry the PMN that had not yet acquired the typical apoptotic morphology. These PMN were analyzed for binding to annexin V and simultaneously with a PI dye exclusion test. The annexin V assay is based on the binding of FITC-conjugated annexin V to phosphatidylserine exposed on the outer leaflet of the plasma membrane lipid bilayer of apoptotic cells, followed by flow cytometric detection. Annexin V-positive cells were considered early apoptotic cells (Fig. 6, quadrants B4), and annexin V and PI double-positive cells were classified as late apoptotic cells (Fig. 6, quadrants B2). The results depicted in the representative cytotograms of Figure 6 clearly show that the number of live PMN that are not yet initiated in the apoptotic program (quadrants...
Transfer of Stx between PMN

The transient block of spontaneous apoptosis induced by Stx treatment is not sufficient per se to explain the persistence (5 days) of Stx-positive PMN observed in HUS patients [23], as it is likely that long-living, Stx-positive PMN leave the blood well before.

A possible explanation could be the passage of Stx from old neutrophils to new, mature cells entering the circulation from the bone marrow, as in the case of a relay race. To test this hypothesis, a single, Stx1-positive PMN population (MCV = 3.6 ± 0.3) was obtained after incubation with Stx1 at saturating conditions, assessed by indirect immunofluorescence, as depicted in the representative histogram (Fig. 7B). This PMN population was then mixed with a Stx1-negative leukocyte population (MCV = 0.4 ± 0.1; Fig. 7A). After 6 h incubation at 37°C, flow cytometric analysis revealed that the two original populations (Stx1-positive and Stx1-negative) of PMN were substituted by a new, single PMN population having an intermediate value of fluorescence (MCV = 2.5 ± 0.3; Fig. 7, C and D), thus indicating that a redistribution of the toxic ligand among the PMN present in the assay had occurred. Parallel experiments with Stx2 gave similar results (data not shown).

To confirm the interleukocytic passage, we repeated the experiment by using PMN from donors differing in the isoforms (NA1 and NA2) of the granulocyte membrane glycoprotein FcγRIIIb. These antigens are easily distinguishable with the ID3 mAb anti-CD16b, which strongly reacts with PMN from NA1/NA2 heterozygote donors (NA1 PMN, Fig. 8A), whereas it shows low reactivity to PMN belonging to NA2 homozygote donors (NA2 PMN, Fig. 8B), irrespective of the presence of Stx1 on their membrane (Fig. 8C). The latter NA2 PMN population bearing Stx1 was mixed with the control NA1 PMN shown in Figure 8A. After 6 h incubation at 37°C, in the upper-right quadrant of D, a new PMN population, positive to anti-Stx1 and anti-CD16b mAbs, appeared. As the glycoprotein FcγRIIIb is a constitutive, unexchangeable component of a neutrophil membrane, this new cellular population can be generated only by the passage of the toxin from NA2 to NA1 PMN. This behavior was confirmed by the concomitant presence in the lower quadrants of Figure 8D of NA2 cells having lower amounts of Stx1 on their membrane. In conclusion, the data clearly demonstrated the transfer of the toxic ligand between different neutrophils.

DISCUSSION

Our study provided additional elements to understand the interactions between Stx and PMN and showed that the toxins only bind to mature cells. We also documented that Stx binding causes a delay of the spontaneous apoptosis of PMN, which are also able to transfer the toxins from positive to negative PMN.

Although contrary reports have been published by some of the authors who had first described the phenomenon [19] and by others [20], the binding of Stx1 and Stx2 to PMN has been reported in many patients with HUS [21–23]. Moreover, a recent paper provided evidence that Stx1 and Stx2 bind specifically to PMN in human whole blood [24]. We have confirmed these findings by demonstrating with two independent techniques (direct and indirect flow cytometric analysis, binding experiments with radiolabeled toxins) that Stx1 binds to mature PMN. It is important to note that the above-mentioned, contrary studies [19, 20] reported the lack of binding of Stx1 to neutrophils after incubation with the toxin and isolation of the cells on Ficoll layers or Mono-Poly resolving media. Under these conditions, the toxins may detach from the PMN mem-
brane, as the dissociation constant of the interaction Stx1/ neutrophil receptor is fairly elevated as compared with the dissociation constant calculated with the Gb3 receptor. Conversely, the flow cytofluorimetric analysis of leukocytes performed after incubation with labeled and unlabeled toxins in the present study and the immunofluorescent techniques used in the paper from Griener et al. [24] assured the minimal perturbation of the samples giving positive results. Moreover, we have to underline that we did observe specific interaction with isolated, endotoxin-free PMN under physiological conditions (37°C) and short incubation times (90 min).

Human PMN do not possess a Gb3 receptor or Gb3-related glycolipids [24, 39]. Moreover, undifferentiated HL-60 cells possess only trace levels of Gb3 [40] and did not bind Stx. Conversely, we did observe detectable binding after granulocytic differentiation of HL-60 cells, a condition that does not induce up-regulation of Gb3 expression [41, 42]. Our results suggest that Stx interact directly with human PMN by a Gb3-independent mechanism through surface molecules expressed in mature cells. Theoretically, it seems unnecessary that toxins enter the circulation and must find a binding molecule on PMN, rather than going directly to the high-affinity receptors on the endothelial lining of the kidney. However, to our knowledge, Stx have never been detected in the plasma of HUS patients. In the pivotal study from Karmali and colleagues [16], which first demonstrated the association between the syndrome and STEC infections, sera of 27 STEC-infected patients with HUS were tested for Stx activity by the Vero cell assay with negative results. It should be noted that free fecal Stx were found in more than 50% of those patients and that the sampling times for feces and serum were basically the same. A subsequent study with similar sampling times confirmed these results on the sera from 37 HUS patients with STEC infection tested for neutralizing antibodies against Stx by the Vero cell assay [17]. A possible explanation of the lack of detection of free Stx in blood might be that PMN encounter the toxins far from circulation—in the lamina propria and/or in the crypts of the gut during the inflammation that follows the Stx-induced injury of the intestinal endothelial lining. Several arguments could sustain this hypothesis: The influx of neutrophils in the intestinal lamina propria and in the crypts of patients with HUS has been reported [43–46]; it has been demonstrated that the inflammatory transmigration of PMN enhanced the translocation of Stx across intestinal epithelial cells [47]. Thus, PMN are present close to the basal surface of intestinal epithelial cells during the passage of toxins from the intestinal lumen. As Stx-treated PMN show a delayed triggering of the apoptotic program, as demonstrated in the present paper, it is possible to postulate that transmigrated neutrophils loaded with Stx survive in the exudates and are then removed by lymphatic drainage, finally reaching the bloodstream. Whatever the mechanism of Stx entry in the blood, the absence of detectable, free plasmatic Stx in patients makes the long-lasting, Stx-positive PMN a suitable target for the diagnosis of STEC infection in HUS patients.

Our data also showed a delay in the execution of the spontaneous apoptotic program in Stx-treated PMN examined at different times (5–48 h) and by using three different techniques (morphological, biochemical, and cytofluorimetric assays). This Stx-in-

![Fig. 7. Representative single histogram cytofluorimetric analysis of the passage of Stx1 between different PMN belonging to the same donor. White blood cells were isolated by centrifugation after the lysis of erythrocytes as described in ref. [21], incubated with Stx1 (50 nM), and washed as described in Materials and Methods. (A) Stx1-negative PMN. (B) Stx1-positive PMN. (C) PMN deriving from the incubation (6 h at 37°C) of Stx1-negative (6×10⁵ leukocytes) and Stx1-positive (6×10⁵ leukocytes) cells. (D) Overlapped histograms. The binding of Stx1 to PMN was assessed by flow cytometry, as described in Materials and Methods, and expressed as single histogram analysis.](image-url)
duced delay was not observed by Flagler et al. [20], who recently reported the lack of influence of Stx on neutrophil apoptosis with the same cytofluorimetric technique, performed at a single time (20 h). We have repeated the experiment described in ref. [20], obtaining similar results (data not shown). A reasonable explanation of this apparent discrepancy could be the different effect of Stx on PMN, differing in age and differently engaged in the apoptotic program. We hypothesize that the Stx-induced delay in the neutrophil apoptosis mainly occurs in the youngest fraction of the entire population of PMN. The youngest cells may progressively prevail over older PMN and become detectable by cytofluorimetric analysis after 48 h of incubation according to ref. [48].

Our experiments on Stx-treated and untreated PMN populations also showed that the toxins were transferred from positive to negative PMN. This observation suggests that in HUS patients, Stx on the PMN membrane might pass from circulating neutrophils to new, mature cells entering into the circulation from the bone marrow. The passage of the toxic ligand from positive to negative PMN might involve serum-binding proteins, as it is known that human serum amyloid P specifically interacts with Stx2 in vitro [49, 50] and enhances the experimental binding of the toxin to neutrophils [24]. Finally, it should be stressed that the passage of Stx from PMN to sensitive Gh3-positive cells has been demonstrated in vitro by te Loo et al. [18] and recently confirmed [24], suggesting a role of PMN in the transfer of Stx to renal glomerular endothelial cells in HUS. Further studies are needed to improve our understanding of the persistence of Stx in the blood of children with HUS and its clinical impact.

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