Lack of specific binding of Shiga-like toxin (verocytotoxin) and non-specific interaction of Shiga-like toxin 2 antibody with human polymorphonuclear leucocytes

Joyce M. Geelen¹, Thea J. A. M. van der Velden¹, D. Maroëska W. M. te Loo¹, Otto C. Boerman², Lambertus P. W. J. van den Heuvel¹ and Leo A. H. Monnens¹

¹Department of Paediatric Nephrology and ²Department of Nuclear Medicine, Radboud University Nijmegen Medical Centre, PO Box 9101, 6500 HB Nijmegen, The Netherlands

Abstract

Background. After gastrointestinal infection with Shiga-like toxin (Stx) producing Escherichia coli, the toxin is transported from the intestine to the renal microvascular endothelium. This is the main target for Stx in humans. Previous studies indicated that polymorphonuclear leucocytes (PMN) could serve as carriers for Stx in the systemic circulation. As at a later stage we could not confirm these data, we performed new studies.

Methods. The binding of Stx1 to PMN was determined in vitro (isolated human PMN and whole blood) and in vivo (injection in mice). The specificity of binding of an antibody against Stx2 to PMN from patients with haemolytic uraemic syndrome (HUS) was determined. This was compared with binding to PMN from healthy controls, and patients after haemodialysis (HD) or on peritoneal dialysis (PD). Furthermore, PMN were incubated with Stx to study possible activation.

Results. No specific binding of Stx1 to PMN could be detected. After intravenous injection of the toxin in mice, it was not associated with PMN. The binding of an antibody against Stx2 to PMN was detected in both patients with HUS and patients after HD, but not in patients on PD. Stx was not able to activate PMN.

Conclusions. PMN are not acting as transporter for Stx in the pathogenesis of HUS. The interaction of a Stx antibody with PMN from HUS patients is not specific as it can also be observed in patients after HD (possibly due to activation of the PMN). Therefore, binding of Stx antibody to PMN is not reliable as a diagnostic tool for HUS.

Keywords: binding experiments; haemolytic uraemic syndrome; polymorphonuclear leucocytes; Shiga-like toxin

Background

Shiga-like toxin (Stx, also called verocytotoxin) is an important mediator in the pathogenesis of the diarrhoea-associated haemolytic uraemic syndrome (HUS). Stx is produced by the pathogen Escherichia coli, of which O157:H7 is the serotype most commonly involved in HUS [1]. In an earlier study of 50 isolates of E. coli associated with HUS in The Netherlands, 45 were positive for the Stx2 gene and five for both Stx1 and Stx2 [2].

As the renal microvascular endothelium is the most important target of the toxin, a transport route from the intestine to the kidney is postulated. Stx was never found in sera from patients with HUS, but Stx2 could be detected in kidneys from HUS patients [3]. Furthermore, antibodies against Stx2 are found in serum from patients with HUS [4]. After reaching the renal endothelium, Stx will initiate cell damage with the formation of thrombi in the glomerular arterioles and capillaries [1]. Acute renal failure with thrombocytopenia and haemolytic anaemia will occur, which are the hallmark features of HUS.

Earlier work of te Loo et al. [5] suggested that circulating polymorphonuclear leucocytes (PMN) transported Stx2 in patients with HUS [5]. However, with a new batch of the same antibody, these results could not be reproduced. Therefore, binding studies with Stx to PMN in vitro were repeated. Also, additional binding experiments were performed in vivo in mice. The interaction of an antibody against Stx2 with PMN collected from blood of HUS patients was re-evaluated. It was hypothesized that the antibody could bind aspecifically to activated PMN in
these patients. Consequently, the capacity of PMN to bind the antibody against Stx2 was compared between healthy controls, HUS patients, patients treated by haemodialysis (HD) and peritoneal dialysis (PD). It is known that PMN originating from patients on HD are highly activated [6].

In addition, if no binding of Stx to PMN occurs, it can be proposed that Stx has no effect on PMN function. For this reason, the expression of degranulation markers and cell adhesion molecules (CD66b, CD63, L-selectin and CD11/CD18) on PMN were studied after incubation with Stx2.

Methods

Materials

Purified Stx1, used for the binding experiments, was kindly provided by Dr M. A. Karmali (Public Health Agency of Canada, Ontario, Canada). The 125I-Stx1 B-subunit was a gift from Dr L. Johannes (Institut Curie, Paris, France). The unlabelled Stx1 B-subunit was a gift from Dr L. Brunton (University of Toronto, Canada). For the stimulation of PMN, Stx2 was purchased from Toxin Technology (Eschwege, Germany). Ethylenediaminetetraacetic acid (EDTA) tubes were ordered from BD Vacutainer (Alphen a/d Rijn, The Netherlands). Ficoll-Paque™ PLUS was purchased from Amersham Biosciences (Uppsala, Sweden). Human serum albumin (HSA; Cealb) from Sanquin (Amsterdam, The Netherlands) was used. Hanks’ balanced salt solution (HBSS) was ordered from MP Biomedicals (Eschwege, Germany).

Monoclonal antibodies against CD66b [fluorescein isothiocyanate (FITC)-labelled] and CD63 (PE-labelled) were obtained from Coulter/Immunotech (Marseille, France). Antibodies against L-selectin, CD11b/CD18 (both FITC-labelled) and CD13 (PE-labelled) were purchased from DAKO (Heverlee, Belgium), as was the secondary goat-anti-mouse FITC and goat serum. HBSS without phenol red was obtained from Life Technologies (Paisley, Scotland), luminol and 12-myristate 13-acetate and horseradish peroxidase were purchased from Sigma (St Louis, USA).

The antibody against Stx2 was a kindly gift from Dr A. O’Brien (Uniformed Services University of Health Sciences, Bethesda, USA). This is a well-characterized, humanized antibody as described previously [7]. The secondary antibody (goat-anti-human FITC) for this antibody was purchased from Jackson Immunoresearch (Cambridgeshire, UK). The Fc-receptor blocker was ordered from Miltenyi Biotec (Utrecht, The Netherlands). Mouse serum was obtained from the Central Animal Laboratory (Nijmegen, The Netherlands). Lipopolysaccharide (LPS) was collected from Sigma (St Louis, USA).

Isolation of polymorphonuclear leucocytes

Fresh venous blood was drawn and collected in EDTA tubes. Blood was diluted two times with phosphate-buffered saline (PBS) and centrifuged (30 min, 400 g at 4°C) over Ficoll-Paque™ Plus. After centrifugation, the pellet containing PMN and erythrocytes was collected. Erythrocytes were lysed using ammonium chloride. This procedure routinely revealed a PMN population of over 95%.

Binding of Shiga-like toxin to isolated human polymorphonuclear leucocytes

After isolation of PMN from healthy donors (n = 7), cells were washed with PBS and re-suspended in HBSS containing 1% HSA at 0°C. During a period of 3 h, 5 × 10^5 PMN were incubated with 125I-Stx1. Incubations were performed with different concentrations of Stx1 ranging from 0.3 up till 70 nmol/l. Stx1 was radio-iiodinated according to the iodogen method. To determine non-specific binding, the same incubation was carried out in the presence of a 25-fold molar excess of unlabelled Stx1. After the incubation, free 125I-Stx1 was separated from the PMN using Ficoll-Paque PLUS, and cells were washed. Cell-associated 125I-Stx1 was determined in a gamma counter. The binding of 125I-Stx1 to monocytes showed an adequate Kd value in the Scatchard plot analysis. In the glycolipids extracted from human monocytes, 125I-Stx is bound to Gb3.

Binding of Shiga-like toxin in whole blood

EDTA-blood from seven healthy human donors was obtained. Whole blood (2–2.5 ml) was incubated with 125I-Stx1 B-subunit (0.75 µg, 3 µCi) during 2.5 h at 4°C.

In order to up-regulate a possible receptor, blood from four donors was pre-incubated with LPS (1 µg/ml) during 2 or 16 h at 37°C, before adding the 125I-Stx1 B-subunit.

To investigate the amount of binding of Stx1 B-subunit to PMN in the circulation, nine normal SE mice (female, age 10 weeks) were injected intravenously with 125I-Stx1 B-subunit. A total amount of 3.5 µg protein (1 µCi) was administered intravenously through the tail vein. In five mice, all blood was collected after 2.5 min. Two of these mice were co-injected with an excess of unlabelled Stx1 B-subunit (100 µg/mouse), to determine the specificity of the binding. The amount of unlabelled Stx1 B-subunit will saturate the specific Stx1 B-subunit binding sites in vivo. In four mice, blood was collected after 15 min. One of these mice received 100 µg unlabelled B-subunit. Blood was collected from the orbital plexus.

Blood from both mice and men, was centrifuged over Ficoll-Paque PLUS (30 min, 400 g at 20°C). The different layers (plasma, interphase, Ficoll-Paque PLUS and the pellet containing PMN and erythrocytes) were separated. The interphase was centrifuged twice to remove platelets (10 min, 200g). Supernatants were also collected. From each layer, a fraction was counted in a gamma counter for the presence of 125I-Stx1 B-subunit. Subsequently, the fraction containing erythrocytes and PMN was lysed with ammonium chloride and washed. By this means, the presence of 125I-Stx1 B-subunit on the PMN could be measured. The 125I activity in each fraction was calculated.

Analysis of binding antibody Shiga-like toxin to polymorphonuclear leucocytes

Blood was collected from healthy controls (n = 6), patients with HUS (Stx present in faeces) (n = 3), patients on PD (n = 3) and patients on HD (n = 14). After isolation of PMN, cells were incubated with Fc-receptor blocker (15 µl for 15 min) and blocked with 10% goat- and mouse serum.
Subsequently, PMN were incubated with the antibody against Stx2 followed by goat-anti-human FITC. After the incubations, the cells were suspended in paraformaldehyde 0.5% and analysed with FACS analyser.

Three other antibodies against Stx2 (two commercial and one non-commercial) reacted in a variable degree with normal PMN.

**Stimulation of human polymorphonuclear leucocytes with Shiga-like toxin**

Whole blood (100 μl) from four healthy volunteers was incubated with either LPS 1 μg/ml, Stx2 0.1 nM, Stx2 10 nM alone or LPS in combination with Stx2 (0.1 or 10 nM) for 2 h at room temperature. Subsequently, erythrocytes were lysed using ammonium chloride. PMN were identified by flow cytometry (Coulter® Epics® XL-MCL) using their morphological criteria and using CD13-PE as specific marker. Monoclonal antibodies against CD63 and CD66b were used as markers of degranulation. Also, monoclonal antibodies against t-selectin and CD11b/CD18 were used to study the expression of adhesion molecules. Antigen expression was measured as mean fluorescence intensity of 5000 cells by flow cytometry. Data acquisition and analysis were performed using XL-2 software (Coulter).

Furthermore, the superoxide production in PMN of eight healthy donors was measured after stimulation with Stx2 0.1 nM, Stx2 10 nM, LPS 1 μM alone or LPS in combination with Stx2 (0.1 or 10 nM) for 10 min, 1 h and 4 h, respectively. Phorbol 12-Myristate 13-Acetate (PMA) was used as a positive control. Immediately after the incubation, luminol-enhanced chemiluminescence of proteose-peptone-elicited PMN was measured on a Victor 1420 counter (Wallac, Turku, Finland) at 37 °C. The measurements were performed in 96-well microplates as described previously [8]. Each well contained 2 x 10^5 PMN, 50 μM luminal and 4.5 U/ml horse-radish peroxidase in 200 μl of HBSS without phenol red, supplemented with 0.25% HSA. Wilcoxon signed rank test was used to test significance of within-group differences. Differences were considered significant at \( P < 0.05 \).

### Results

**Binding of Shiga-like toxin to isolated polymorphonuclear leucocytes**

PMN from healthy donors were isolated and incubated with ^125^I-Stx1. To test the specificity of the binding, simultaneous incubations were done in the presence of an excess of unlabelled Stx1. In none of the performed experiments \( (n = 7) \) was specific interaction of ^125^I-Stx1 with PMN found.

**Binding of Shiga-like toxin in whole blood**

To establish whether PMN in whole blood can bind Stx, we incubated blood from healthy donors \( (n = 7) \) with ^125^I-Stx1 B-subunit. The B-subunit of Stx is the binding part of the toxin, whereas the A-subunit will only stimulate the uptake of the toxin and does not affect binding [9].

The experiment was performed with and without previous stimulation of the blood with LPS. The major part of the B-subunit can be found in the plasma. Seventy-one percent of total activity was detected in plasma, whereas 0.09% was found in the fraction containing the PMN \( (n = 3) \). Pre-incubation with LPS did not change the amount of bound toxin [0.09 and 0.02% of total activity after, respectively, 2 and 16 h pre-incubation \( (n = 4) \)].

To investigate the binding of Stx to PMN in vivo, the ^125^I-labelled Stx1 B-subunit was injected in mice \( (n = 9) \). After 2.5 and 15 min, blood from the mice was collected and the distribution of the B-subunit in the various blood fractions was determined. Stx1 B-subunit rapidly cleared from the blood as is shown in Figure 1. Subsequently, the different cell populations were separated and the ^125^I-Stx1 B-subunit activity in each fraction was determined. The major part of the ^125^I-Stx1 B-subunit was found in the plasma. Only a minute fraction of the activity was found in the PMN fraction. The addition of an excess of unlabelled Stx1 B-subunit did not reduce the activity (Table 1).

**Table 1. Distribution of ^125^I-Stx1 B-subunit in blood of mice**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Unlabelled Stx1-B</th>
<th>Number of mice</th>
<th>Plasma (% of total)</th>
<th>Ficoll (% of total)</th>
<th>Erythrocyte (% of total)</th>
<th>PMN (% of total)</th>
<th>Monocyte + lymphocyte (% of total)</th>
<th>Platelets (% of total)</th>
</tr>
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<tr>
<td>2.5</td>
<td>–</td>
<td>3</td>
<td>94.871</td>
<td>3.926</td>
<td>1.568</td>
<td>0.003</td>
<td>0.040</td>
<td>0.373</td>
</tr>
<tr>
<td>2.5</td>
<td>+</td>
<td>2</td>
<td>90.566</td>
<td>6.912</td>
<td>2.942</td>
<td>0.006</td>
<td>0.016</td>
<td>0.478</td>
</tr>
<tr>
<td>15</td>
<td>–</td>
<td>3</td>
<td>93.302</td>
<td>4.107</td>
<td>1.645</td>
<td>0.006</td>
<td>0.072</td>
<td>0.708</td>
</tr>
<tr>
<td>15</td>
<td>+</td>
<td>1</td>
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<td>3.566</td>
<td>1.327</td>
<td>0.003</td>
<td>0.025</td>
<td>0.660</td>
</tr>
</tbody>
</table>

*Fig. 1. The clearance-curve of Stx in mice. ^125^I-Stx1 B-subunit was rapidly cleared from the blood of mice. After 5 min, ~20% only was still present.*
Binding of antibody Shiga-like toxin to polymorphonuclear leucocytes

The specificity of attachment of the antibody against Stx2 was tested with PMN of patients with HUS, patients on HD or PD and healthy controls.

In two out of three blood samples from HUS patients in active disease, the antibody did not show any staining of the PMN (Figure 2). Only the PMN of one patient showed an attachment of the antibody to the PMN during active disease, which disappeared during remission.

The healthy controls were negative (n = 6). In patients on PD, no attachment of the Stx2 antibody to PMN was found. However, if the antibody was added to PMN from patients on HD, in 11 out of 16 experiments, positive staining of the PMN with the antibody was observed (Figure 3). In these patients, no symptoms of HUS could be detected.

Stimulation of polymorphonuclear leucocytes with Shiga-like toxin

Incubation of PMN with different concentrations of Stx2 did not cause any change in the expression of degranulation markers (CD66b and CD63) on PMN (Figure 4). Similar results were obtained with the adhesion molecules. Stx2 did not induce significant changes in the expression of CD11b/CD18 and shedding of L-selectin (Figure 4).

Incubation of PMN from healthy donors did not show an increase of superoxide production whereas LPS induced a small increase (data not shown).

Discussion

In a previous study, we have demonstrated specific binding of $^{125}$I-Stx1 to freshly isolated, non-stimulated human PMN. Also, a rapid binding of FITC-labelled Stx1 to PMN was detected when incubated in whole
blood [5]. In the current study we were unable to reproduce these results. Neither in isolated PMN nor in whole blood was a specific binding of Stx1 to PMN observed. Fernandez et al. [10] also studied the binding of Stx to PMN. PMN from healthy adults and children were incubated with different concentrations of Stx1 or Stx2 for different time intervals. The binding of Stx to PMN was determined by flow cytometry using specific anti-Stx antibodies, and by quantification of the remnant Stx, evaluating the cytotoxicity of the supernatants on vero cells. Neither of the two approaches revealed a positive binding of Stx to PMN surface in any of the conditions assayed. We also evaluated a possible binding of 125I-Stx1 B-subunit after intravenous injection in mice. In vivo, no specific interaction of Stx1 with PMN could be detected. Some caveats should be noted. It is not allowed to extrapolate data obtained in mice to the human. It is also possible that labelled toxin is detached from cells during the experimental procedure (centrifugation on Ficoll). We have no experience with erythrocytes, but labelled Stx on the surface of monocytes at a constant temperature is not released by centrifugation.

A lack of specific binding of Stx to PMN suggests the absence of a direct effect of Stx on PMN function. Stx2 did not stimulate superoxide production in PMN and was unable to induce the expression of degranulation and activation markers CD63, CD66b and CD11b/CD18. Similar results were reported by Aoki et al. and Holle et al. [11,12].

However, Stx2 also has an inhibitory effect on spontaneous neutrophil apoptosis. Surprisingly, this effect is only partially inhibited by anti-Stx2 antibody [13]. Could Stx2 interfere with triggering factors for spontaneous apoptosis?

How to reconcile these data with the flow cytometric detection of Stx on PMN from HUS patients by us and recently by Brigotti et al. [14]? Using flow cytometry, in 13 out of 20 children with HUS, they showed binding of anti-Stx mouse monoclonal antibody to PMN. In our last three investigated patients with a proven HUS, PMN from only one patient were clearly positive for the antibody against Stx2 and became negative at remission. A possible explanation is the exposure of altered plasma membrane structures on activated PMN. In D+HUS children, PMN have been found to adhere more avidly to the endothelium and induce endothelial injury, assessed morphologically by degradation of endothelial cell fibronectin [15]. Alpha-1 antitrypsin complexed elastase is raised in HUS patients reflecting PMN activation and degranulation [16]. Fernandez et al. showed a reduced degranulatory capacity in response to cytokines and a decreased intracellular granule content indicating a preceding process of activation [10]. To underpin our hypothesis, we have tested the interaction of the antibody against Stx2 with PMN activated by HD and compared it with PMN obtained from patients treated by chronic PD. Degranulation of PMN appears to be an ongoing process during HD, starting directly after the initiation [17]. In the majority of patients on HD, the activated PMN collected after an HD session showed a binding of the antibody against Stx2. This was not seen in PMN from patients treated by chronic PD.

**Fig. 3.** Flow cytometric analysis for detection of antibody against Stx2 on PMN of patients on dialysis. PMN of patients on dialysis were incubated with a antibody against Stx2. (A) PMN from a patient on HD. (B) PMN from a patient on PD. Binding of the antibody can be observed on PMN originating from patients on HD. No binding can be found on PMN from patients on PD.
From these experiments we hypothesize that in activated PMN, membrane changes take place that cause binding of the Stx2-antibody. The characteristics of these binding sites are still unknown. Therefore, a reliable diagnosis of HUS patients demonstrating the presence of Stx on the surface of PMN by anti-Stx2 is doubtful.

It remains an unsolved issue how Stx is transported in the circulation. Recently, the acute phase protein human serum amyloid P has been suggested by Kimura et al. [18] as a possible neutralizing factor. But whether it can also target Stx to the glomerular endothelium needs to be elucidated.

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**Conflict of interest statement.** The results presented in this article have not been published previously in whole or part, except in abstract form.

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