Production of Nitric Oxide Is Lower in Shiga Toxin-Stimulated Neutrophils of Infants Compared to Those of Children or Adults

Shoji Tsuji,1 Anna Iharada,1 Takahisa Kimata,1 Tomohiko Shimo,1 Masato Hirabayashi1 and Kazunari Kaneko1

1Department of Pediatrics, Kansai Medical University, Osaka, Japan

Hemolytic uremic syndrome (HUS) is defined by the simultaneous occurrence of microangiopathic hemolytic anemia, thrombocytopenia, and acute renal injury (Noris and Remuzzi 2005). HUS occurs in about 3 to 4% of patients suffering from enterohemorrhagic Escherichia coli O157:H7 (O-157) infection (Akashi et al. 1994; Bell et al. 1994). About 90% of HUS cases in infants are due to O-157 infections that generate Shiga toxin (Stx). Seventy percent or more of the patients with HUS are children under 15 years of age and those ranging from 0 to 4 years of age account for close to half. Furthermore, the incidence of HUS due to O-157 infections in infants is about 7 times higher than that of adults (Kamioka et al. 2008), although its precise reason is unknown.

It has been reported that reactive oxygen species (ROS) have an antibacterial action (Cohen 1994) and contribute to the development of HUS (King et al. 1999; Liu et al. 2002; Gomez et al. 2005; Holle et al. 2005). However, the role of nitric oxide (NO), another pivotal biological reactive species generated by vascular endothelial cells or white blood cells, has not been extensively studied.

NO has cytoprotective effects (Elrod et al. 2008) and plays some role in the pathophysiology in HUS (Dran et al. 2002); namely, administration of arginine, a NO synthesis promoting substance, to the HUS model mice generated with Stx reduced their mortality. Therefore, NO may be involved in the pathophysiology of HUS. However, no studies on NO generated from neutrophils have been reported.

In the current study, we hypothesized that NO plays an important role in the cellular defense mechanisms in HUS caused by Stx from O-157, and thus measured the NO productivity of neutrophils stimulated with Stx to explore the reason for the higher incidence of HUS in infants.

Materials and Methods

Study population

Healthy volunteers (institutional staffs and staffs’ children) were enrolled into this study. They were classified into three groups according to their ages: Adults, ten healthy adults (25-47 years of age, median 34.3 years of age); Infants, ten healthy infants (6-27 months of age, median 9 months of age); and Children, ten healthy children.
Cell cultures were centrifuged, and each cell pellet was resuspended in 500 µl of 3 mM EDTA in PBS and was applied to flow cytometry. Two-colors fluorescence staining was analyzed by a cytofluorometer (Epics® XL II, Coulter Co., Hialeah, Florida, USA) with System II Software. Data from 10,000 events per sample were acquired. Excitation and emission maxima of DAF-FM DA were 495 and 515 nm, respectively. The mean fluorescence intensity (MFI) was determined after gating for neutrophils by their forward and side scatter characteristics.

Reverse transcription-polymerase chain reaction (RT-PCR) to examine mRNA expression of inducible NO synthase (iNOS)

A whole blood sample (100 µl) was transferred to a sterile 6-ml centrifuge tube, and Stx-1 (partially purified at least 10^4 Verocytotoxic units/mg; Toxin Technology, Inc., USA) was added to a final concentration of 0.5 µg/ml. The possibility of the contamination of Stx-1 with lipopolysaccharide (LPS) was low. Each sample was incubated for 90 min at 37°C after addition of 4,5-diaminofluorescein-FM diacetate (DAF-FM DA; Daiichi Pure Chemical Co., LTD, Tokyo, Japan) to a final concentration of 10 µM, and 1,000 µl of 3 mM EDTA (Wako Pure Chemical Industries, Ltd, Osaka, Japan) in phosphate buffered saline (PBS: Takara Shuzo Co. Shiga, Japan) was added. Erythrocytes were hypotonically lysed for 30 sec. The mixture was centrifuged, and each cell pellet was resuspended in 500 µl of 3 mM EDTA in PBS and was applied to flow cytometry. Two-colors fluorescence staining was analyzed by a cytofluorometer (Epics® XL II, Coulter Co., Hialeah, Florida, USA) with System II Software. Data from 10,000 events per sample were acquired. Excitation and emission maxima of DAF-FM DA were 495 and 515 nm, respectively. The mean fluorescence intensity (MFI) was determined after gating for neutrophils by their forward and side scatter characteristics.

RT-PCR analysis for expression of iNOS mRNA

The expression of iNOS mRNA in neutrophils was analyzed by RT-PCR (Fig. 3a). The RT-PCR analysis of GAPDH mRNA was performed as an internal control. Although the expression of iNOS mRNA was detected in neutrophils after stimulation with Stx-1 in all three groups, its amount was significant lower in infants than in adults and children (P < 0.05, Fig. 2). In contrast, there was no significant difference in the amounts of NO produced by neutrophils between adults and children (Fig. 2).

**Table 1. Primers used for analysis of iNOS and GAPDH mRNA.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Size (bp)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS sense</td>
<td>250</td>
<td>5′-TCTG TTG CAG CAG AGA CAG GA-3′</td>
</tr>
<tr>
<td>iNOS antisense</td>
<td></td>
<td>5′-CCA AAC ACA GCG TAC CTG AA-3′</td>
</tr>
<tr>
<td>GADPH sense</td>
<td>294</td>
<td>5′-CAC CCA GAA GAC TGT GGA-3′</td>
</tr>
<tr>
<td>GADPH antisense</td>
<td></td>
<td>5′-ACC TGG TGC TCA GTG TAG-3′</td>
</tr>
</tbody>
</table>
Decreased NO Production by Stx-Stimulated Neutrophils in Infants

In addition, cytoprotective effects of NO have been recently postulated (Dran et al. 2002). The current study hypothesized that the suppressed neutrophil NO production is associated with the defective defense mechanisms against Stx leading to HUS, because NO is generated from not only vascular endothelial cells but also circulating neutrophils (Wright et al. 1989; Evans et al. 1996).

This study demonstrated that the amount of NO synthesized by iNOS of neutrophils stimulated by Stx-1 was...
significantly lower in infants in comparison to older children and adults. This finding might partly explain the higher incidence of HUS induced by Stx-1 in infants with O-157 infection. Prior to the present study, we also analyzed the effect of Stx-2 on the production of NO from neutrophils. However, there was no significant difference in the amounts of NO production between Stx-1 and Stx-2 (data not shown).

LPS contamination in the Stx-1 cannot be ruled out, although we used partially purified Stx-1 in the present study. In this connection, our previous study (Tsuji et al. 2002) showed that the amount of neutrophil NO produced

Fig. 3. RT-PCR analysis for neutrophil mRNAs.
(a) Expression of iNOS mRNA.
The expression of iNOS mRNA in neutrophils was analyzed by RT-PCR. The expression of iNOS mRNA was detected in neutrophils after stimulated with Stx-1, but expression of iNOS in infants was less than in adults and children. GAPDH was performed as an internal control. The gel images are representative of 4 separate experiments.

(b) Densitometry analysis of semi-quantitative RT-PCR results.
Densitometry of PCR bands for adults, infants, and children based on the % of GAPDH control bands using the Image J Program software package (Version 1.45). The amount of iNOS transcript was significant lower in infants (median 52.1: 30.0-89.5%) than in adults (median 106.5: 94.3-111.5%) and children (median 103.4: 87.2-124.0%; n = 4, P < 0.05).

The central horizontal line in the box represents the median value, and the bottom and top edges of the box are located at lower quartile and upper quartile, respectively. The central vertical lines extend from the box to the maximum value or minimum value.

*P < 0.05
Decreased NO Production by Stx-Stimulated Neutrophils in Infants

with lipopolysaccharide was increased by about 1.3 times in comparison to non-stimulation. On the other hand, the amount of neutrophil NO was increased about 8 times upon Stx-1 stimulation. It is therefore conceivable that the contribution of lipopolysaccharide contamination may be negligible.

The current study together with the previous reports (Dran et al. 2002; Elrod et al. 2008) suggests a cellular defense mechanism against Stx in O-157 infection (Fig. 4). Stx released from O-157 binds to an unknown receptor different from globotriaosyl ceramide (Gb3/CD77), which is the known receptor of Stx (Griener et al. 2007). The binding of Stx to the receptor leads to the increase in the synthesis of iNOS, which then increases the neutrophil NO production. On the other hand, ROS is also generated by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is present on the surface membrane of a neutrophil. The generated ROS reacts with NO to produce peroxynitrite (ONOO⁻), which has a potent antibacterial action. Both NO and ONOO⁻ exhibit cytoprotective effects and anti-bacterial activity, respectively. Thus, the suppressed neutrophil NO production in infants causes defective immune responses against Stx, resulting in its augmented toxicity leading to HUS.

However, there are several limitations associated with the current study. First, ROS were not measured under the experimental conditions. ROS production should be evaluated in combination with NO to verify the hypothesis, although numerous studies have already confirmed increased production of ROS in HUS (King et al. 1999; Liu et al. 2002; Gomez et al. 2005; Holle et al. 2005). Secondly, patients with HUS caused by Stx were not included. Comparison of NO production between infants with O-157 infection complicated by HUS and those uncomplicated by HUS is worthwhile to determine the role of NO in its pathophysiology.

In conclusion, the Stx released from O-157 stimulated the neutrophil NO production probably via iNOS, and the degree of this increase was lower in neutrophils of infants compared to those of children or adults. These results suggest that NO plays a role in the cellular defense mechanisms against Stx and may explain the higher incidence of HUS in infants, although further study is clearly needed.

Acknowledgments

This work was supported by a Grant-in-Aid from the Mami Mizutani Foundation, the Morinaga Hoshikai and the Osaka Kidney Foundation (OKF10-0005).

Conflict of Interest

The authors declare no conflict of interest.

References


