Serum Amyloid P Component Is the Shiga Toxin 2-neutralizing Factor in Human Blood*

It has been suggested that some factor present in human plasma binds to Shiga toxin 2 (Stx2) and neutralizes it *in vitro* (Bitzan, M., Klemt, M., Steffens, R., and Muller-Wiefel, D. E. (1993) *Infection* 21, 140–145). This factor does not exist in other species (Caprioli, A., Luzzi, I., Seganti, L., Marchetti, M., Karmali, M., Clarke, I., and Boyd, B. (1994) *Recent Adv. VTEC Infect.* 353–356). Because analysis of this factor is important to understanding the pathology induced by Shiga toxin-producing *Escherichia coli*, we purified this factor from human plasma and identified it. Purification was carried out by serially subjecting human plasma to Con A-Sepharose, DEAE-Sepharose, hydroxyapatite, and gel-filtration high performance liquid chromatography (HPLC), using Stx2-neutralizing activity as the indicator. The gel-filtration HPLC fraction yielded a single band on SDS-polyacrylamide gel electrophoresis. Twenty N-terminal amino acid residues of this fraction were analyzed and found to correspond perfectly to human serum amyloid P component (HuSAP). Because commercially available HuSAP also showed Stx2 binding and neutralizing activity, we identified this factor as HuSAP.

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) has been recognized as a pathogen that causes bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) mostly in developed countries (3). STEC produces Stxs, which can be divided broadly into two groups, Stx1 and Stx2. Stx2, but not Stx1, is thought to be a major cause of toxicity that induces the pathology induced by Shiga toxin-producing *Escherichia coli* (Caprioli, A., Luzzi, I., Seganti, L., Marchetti, M., Karmali, M., Clarke, I., and Boyd, B. (1994) *Recent Adv. VTEC Infect.* 353–356). It has been suggested that some factor present in human plasma binds to Stx2 and neutralizes it *in vitro* (Bitzan, M., Klemt, M., Steffens, R., and Muller-Wiefel, D. E. (1993) *Infection* 21, 140–145). This factor does not exist in other species (Caprioli, A., Luzzi, I., Seganti, L., Marchetti, M., Karmali, M., Clarke, I., and Boyd, B. (1994) *Recent Adv. VTEC Infect.* 353–356). Because analysis of this factor is important to understanding the pathology induced by Shiga toxin-producing *Escherichia coli*, we purified this factor from human plasma and identified it. Purification was carried out by serially subjecting human plasma to Con A-Sepharose, DEAE-Sepharose, hydroxyapatite, and gel-filtration high performance liquid chromatography (HPLC), using Stx2-neutralizing activity as the indicator. The gel-filtration HPLC fraction yielded a single band on SDS-polyacrylamide gel electrophoresis. Twenty N-terminal amino acid residues of this fraction were analyzed and found to correspond perfectly to human serum amyloid P component (HuSAP). Because commercially available HuSAP also showed Stx2 binding and neutralizing activity, we identified this factor as HuSAP.

Stx2 Neutralizing Assay

A 100-µl volume of Stxl or Stx2 in PBS was inoculated on MaxiSorp enzyme-linked immunosorbent assay plates (Nunc) and incubated at 4 °C for 1 day. The wells were then washed with 1% BSA-PBS and blocked with 5% BSA-PBS at 37 °C for 1 h. After removing the blocking buffer, HuSAP serially diluted with 3% BSA-PBS was added to the wells, and the plates were incubated at 37 °C for 1 h. After washing with 1% BSA-PBS, the wells were incubated at 37 °C for 1 h with rabbit anti-HuSAP serum (Biogenesis) diluted 4000-fold with 3% BSA-PBS. The wells were then washed with 1% BSA-PBS and incubated at 37 °C for 1 h with peroxidase-conjugated goat anti-rabbit immunoglobulin (Kirkegaard & Perry Laboratories) diluted 1:2000 with 3% BSA-PBS. After washing with 1% BSA-PBS, peroxidase activity was detected colorimetrically by adding TMB (Kirkegaard & Perry Laboratories).

Stxs Binding Assay

A 1 µg/ml volume of Stx1 or Stx2 in PBS was inoculated on MaxiSorp enzyme-linked immunosorbent assay plates (Nunc) and incubated at 4 °C for 1 day. The wells were then washed with 1% BSA-PBS and blocked with 5% BSA-PBS at 37 °C for 1 h. After removing the blocking buffer, HuSAP serially diluted with 3% BSA-PBS was added to the wells, and the plates were incubated at 37 °C for 1 h. After washing with 1% BSA-PBS, the wells were incubated at 37 °C for 1 h with rabbit anti-HuSAP serum (Biogenesis) diluted 4000-fold with 3% BSA-PBS. The wells were then washed with 1% BSA-PBS and incubated at 37 °C for 1 h with peroxidase-conjugated goat anti-rabbit immunoglobulin (Kirkegaard & Perry Laboratories) diluted 1:2000 with 3% BSA-PBS. After washing with 1% BSA-PBS, peroxidase activity was detected colorimetrically by adding TMB (Kirkegaard & Perry Laboratories).

It is unclear why Stx2 induces HUS in humans despite the presence of this factor. To assess the role and function of this factor in STEC infection in humans, we purified this factor from human plasma and identified it.

**EXPERIMENTAL PROCEDURES**

**Materials**

Con A-Sepharose, DEAE-Sepharose, PD-10, NAP-5, and gel-filtration HPLC (Superose 6 HR 10/30) were from Amersham Pharmacia Biotech. Hydroxyapatite (Gigaprep grade K-1008) was from Seikagaku Corp. D-Mannose was from Wako Pure Chemicals. 2-Methyl-D-mannoside was from Nakalai Tesque. Polyvinylidene difluoride membrane (ProSpin) was from Applied Biosystems. Human serum amyloid P component (HuSAP) was from Alexis.

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The abbreviations used are: Stx, Shiga toxin; HPLC, high-performance liquid chromatography; HuSAP, human serum amyloid P component; STEC, Shiga toxin-producing *Escherichia coli*; HUS, hemolytic uremic syndrome; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

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were incubated at 37 °C with 5% CO₂ for 4 days. A 100-μl volume of 0.028% (w/v) neutral red (Merck) in growth medium was added, and incubation at 37 °C with 5% CO₂ was continued for 1 h to stain the living cells. After washing the wells with PBS, 100 μl of 50% ethanol, 1% acetic acid solution was added to extract the dye, and the absorbance at 550 nm was measured.

Method 2—After incubation at 37 °C with 5% CO₂ for 4 days, living cells were stained with neutral red and quantified as described above. Neutralizing activity was calculated according to the following formula:

\[
\text{neutralization (\%)} = \left( \frac{A_{550\text{, Stx2}}^{\text{test sample}} - A_{550\text{, Stx2}}^{\text{medium}}}{A_{550\text{, Stx2}}} \right) \times 100.
\]

Other Procedures

SDS-PAGE was performed according to Laemmli, using 4–20% polyacrylamide gradient slab gel (Daiichi Pure Chemicals), and the separated proteins were silver-stained (Wako Pure Chemicals). Throughout the purification procedures, the protein concentration was estimated by BCA protein assay or micro BCA protein assay (Pierce) with BSA as a standard. Spectrophotometric determinations were made in a Shimadzu UV-160A spectrophotometer. Protein sequencing was performed using an Applied Biosystems 494 protein sequencer.

RESULTS

Purification of Stx2-neutralizing Factor from Human Blood

Bitzan et al. (1) have reported the presence of a non-immunoglobulin factor in human plasma that neutralizes Stx2 in vitro. Caprioli et al. (2) have shown that this activity does not exist in other species and that the human high-density lipoprotein fraction neutralizes Stx2 in vitro. However, we were unable to detect any Stx2-neutralizing activity by human HDL (data not shown). Because analysis of this Stx2-neutralizing factor is important to understanding the pathology of STEC, we purified this factor from human plasma and identified it. Plasma obtained from one healthy male donor was used as the starting material. Purification was serially carried out by Con...
A-Sepharose, DEAE-Sepharose, hydroxyapatite, and gel-filtration HPLC.

**Step 1**—Chromatography on Con A-Sepharose—The human plasma was applied to a Con A-Sepharose column (column bed volume, 40 ml) equilibrated with PBS, and the column was thoroughly washed with PBS and then serially eluted with 0.1 M δ-mannose in PBS and 0.5 M α-methyl-δ-mannoside in PBS. As shown in Fig. 1a, Stx2-neutralizing activity was observed in the eluate from 0.1 M δ-mannose in PBS. This eluate was applied to a PD-10 column equilibrated with 10 mM Tris-HCl (pH 8.0) to equilibrate the same buffer.

**Step 2**—Chromatography on DEAE-Sepharose—The eluate from the PD-10 column was applied to a DEAE-Sepharose column (column bed volume, 5 ml) equilibrated with 10 mM Tris-HCl (pH 8.0), and the column was serially washed with 10 mM Tris-HCl (pH 8.0) and 0.1 M NaCl in 10 mM Tris-HCl (pH 8.0), then developed with a linear salt gradient from 0.1 to 0.3 M NaCl in 10 mM Tris-HCl (pH 8.0), and finally washed with 2 M NaCl in 10 mM Tris-HCl (pH 8.0). As shown in Fig. 1b, Stx2-neutralizing activity was observed in the eluate at about 0.2–0.24 M NaCl in 10 mM Tris-HCl (pH 8.0). These fractions were collected and applied to a PD-10 column equilibrated with 50 mM sodium phosphate (pH 6.8) to equilibrate the same buffer.

**Step 3**—Chromatography on Hydroxyapatite—The eluate was applied to a hydroxyapatite column (column bed volume, 3 ml) equilibrated with 50 mM sodium phosphate (pH 6.8). The column was washed with 50 mM sodium phosphate (pH 6.8), then developed with a linear gradient of 50–250 mM sodium phosphate (pH 6.8), and finally washed with 250 mM sodium phosphate (pH 6.8). As shown in Fig. 1c, Stx2-neutralizing activity was observed mainly in the eluate at ~125–250 mM sodium phosphate (pH 6.8). Fractions of the eluate at ~200–250 mM sodium phosphate (pH 6.8) were collected, frozen, and dried in a rotary evaporator. The dried sample was dissolved in distilled water.

**Step 4**—Chromatography by Gel-filtration HPLC—The pool of the eluate from Step 3 was applied to a gel-filtration HPLC (Superose 6 HR 10/30) using PBS as the elution buffer. As shown in Fig. 1d, Stx2-neutralizing activity was observed in the peak fractions, and the peak fractions (fractions 27–29) were pooled.

SDS-PAGE analysis of the peak fractions obtained by gel-filtration HPLC indicated a single staining band of 25 kDa (Fig. 2). This Stx2-neutralizing factor was purified about 1270-fold and the yield of the purified protein was 2.5% (Table I).

**Identification of the Purified Protein**

The purified protein was applied to a polyvinylidene difluoride membrane, and the N-terminal amino acid sequence of the protein was analyzed with an Applied Biosystems 494 protein sequencer. Twenty N-terminal amino acid residues could be analyzed, and the sequence obtained (HTDLSGKVFVFPRES- VTDHV) corresponded completely to the human serum amyloid P component, which is a member of the human plasma pentraxin family consisting of a complex of 10 identical 25-kDa subunits non-covalently associated in two pentameric rings interacting face-to-face (15).

**Characterization of HuSAP**

To confirm that the Stx2-neutralizing factor in human blood is HuSAP, we tested the Stx2-binding activity and the Stx2-neutralizing activity of commercially available HuSAP in vitro. The binding capacity of HuSAP to Stxs was estimated by enzyme-linked immunosorbent assay. As shown in Fig. 3, HuSAP bound to Stx2 but not to Stx1. The Stx2-neutralizing activity of HuSAP was also tested by a cell cytotoxicity assay. As shown in Fig. 4, HuSAP was found to neutralize Stx2 cytotoxicity.
Because commercial HuSAP showed Stx2 binding and neutralizing activity, we ultimately concluded that the Stx2-neutralizing factor in human blood was HuSAP.

**DISCUSSION**

HuSAP is synthesized by the liver and circulates in human blood at 30–45 µg/ml (16). It is found in all types of amyloid deposits (17), including in plaque from Alzheimer’s disease (18), in glomerular basement membrane, and in elastic fibers in blood vessels (19, 20). It also binds in a calcium-dependent manner to a variety of ligands, including DNA and chromatin (21, 22), fibronectin (23), C4-binding protein (23, 24), glycosaminoglycans (25, 26), collagen (27), and laminin (28). HuSAP shows no polymorphism or heterogeneity, and no deficiency of HuSAP has been reported, suggesting that it has important functions. However, there has been no report on the function of HuSAP in STEC infections.

Surveillance studies have shown a strong statistical association between the Stx2 gene and severity of disease (8, 9). In addition, when mice were challenged with STEC co-producing Stx1 and Stx2, anti-Stx2 monoclonal antibodies rescued the mice from death. On the other hand, anti-Stx1 monoclonal antibodies could not rescue them (10). These studies suggest that Stx2 is a more significant factor than Stx1 in the progression of the disease in STEC infections. It is unclear why only some STEC-infected patients develop HUS despite the presence of Stx2-neutralizing factor (HuSAP) in their blood. This phenomenon can be explained by hypothesizing that HuSAP acts as a carrier protein of Stx2 as well as a Stx2-neutralizing factor in humans as described below.

Caprioli et al. (2) have reported that the Stx2-neutralizing activity in serum is unique to humans. We tested mouse, rat, hamster, guinea pig, rabbit, chicken, dog, pig, sheep, deer, goat, horse, bovine, and monkey sera and confirmed that they were negative for in vitro Stx2-neutralizing activity (data not shown). Mice challenged with STEC had a higher mortality rate (29, 30), whereas the rate of occurrence of HUS in STEC-negative for Stx1, but not Stx2, has been detected in intravenous immunoglobulin (34, 35). Third, studies on serum amyloid P component knockout mice have suggested that serum amyloid P component has an important physiological role in inhibiting the formation of pathogenic autoantibodies against chromatin and DNA (36).

In summary, this study has confirmed that HuSAP is the factor in human plasma that binds to Stx2 and neutralizes it in vitro. HuSAP may function not only as a Stx2-neutralizing factor in STEC infections but as a carrier of Stx2 as well. However, further studies will be necessary to clarify more precisely the physiological role of HuSAP in STEC pathology.

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