Human Serum Amyloid P Component Protects against *Escherichia coli* O157:H7 Shiga Toxin 2 In Vivo: Therapeutic Implications for Hemolytic-Uremic Syndrome

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Shiga toxin (Stx) 2 causes hemolytic-uremic syndrome (HUS), an intractable and often fatal complication of enterohemorrhagic *Escherichia coli* O157:H7 infection. Here, we show that serum amyloid P component (SAP), a normal human plasma protein, specifically protects mice against the lethal toxicity of Stx2, both when injected into wild-type mice and when expressed transgenically; in the presence of human SAP, there was greatly reduced in vivo localization of Stx2 to the kidneys, suggesting a possible mechanism of protection. In humans, circulating SAP concentrations did not differ between patients with suspected enterohemorrhagic *E. coli* infection with antibodies to *E. coli* O157:H7 lipopolysaccharide and those without antibodies or between patients with HUS and those without. However, the potent protection conferred by human SAP in the mouse model suggests that infusion of supplemental SAP may be a useful novel therapeutic approach to the treatment of this devastating condition.

Infection with enterohemorrhagic *Escherichia coli*, particularly with the O157:H7 serotype, causes hemorrhagic colitis and, occasionally, hemolytic-uremic syndrome (HUS), especially in infants and the elderly [1]. In the United States, an estimated 73,000 *E. coli* O157:H7 infections occur annually, with HUS developing in ~8%–12% of them [2]. There is no effective specific therapy for this important infection, which can be transmitted in undercooked ground beef (hence the term "hamburger disease"), contaminated water, unpasteurized fruit juice, and unpasteurized milk or milk products [3, 4]. Production of Shiga toxins (Stxs) by *E. coli* O157:H7, in particular of Stx2, is clearly implicated in the pathogenesis of severe hemorrhagic colitis and HUS, although the precise pathogenetic mechanisms are not known [5]. The recent finding that human serum amyloid P component (SAP) interacts specifically with Stx2 and neutralizes its cytotoxicity in vitro [6, 7] suggests that SAP may be involved in the pathogenesis.

SAP is a member of the highly conserved pentraxin family of plasma proteins; in humans, the other member is C-reactive protein (CRP) [8]. SAP and CRP have homologous structures and show calcium-dependent binding to an overlapping but distinct range of specific ligands. CRP is the classical human acute-phase protein and has a dynamic range of ∼0.05–500 mg/L, whereas human SAP circulates at a relatively stable concentration of ∼20–35 mg/L. Of serum from all animal species tested, only serum from humans has an innate capacity to neutralize Stx2 in vitro, and this is attributable specifically to human SAP [6]. We show here, for the first time, that the avid specific binding between SAP and Stx2 protects mice against the lethal toxicity of Stx2 in vivo, suggesting that the administration of SAP could be a therapeutic option for clinical HUS.

**Methods.** For solid-phase binding assays, microtiter plates (Costar; Fisher) were coated with purified Stx1 or Stx2 holotoxins [9, 10] at a concentration of 1 μg/mL in PBS (pH 7.2) and then thoroughly washed before blocking was done with 3% (wt/vol) bovine serum albumin (BSA) in PBS. Control plates were treated with PBS alone before blocking. Binding of isolated human SAP [11] from serial dilutions in 10 mmol/L Tris and 0.14 mol/L NaCl (pH 8.0) (TN buffer), or in 10 mmol/L Tris, 0.14 mol/L NaCl, and 2 mmol/L CaCl2 (pH 8.0) (TC buffer), each of which contained 4% (wt/vol) BSA, was detected by use of rabbit anti–human SAP, followed by horseradish peroxidase–conjugated goat anti–rabbit IgG and, finally, ABTS chromogenic substrate (Roche).

All procedures for the mouse Stx challenges conformed to...
Table 1. Univariable relationships between *Escherichia coli* O157:H7 lipopolysaccharide antibody (Ab) and hemolytic-uremic syndrome (HUS) status and sex, age, circulating serum amyloid P component (SAP) concentration, and circulating C-reactive protein (CRP) concentration.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sex</th>
<th>Age Median (range) [no. of patients], months</th>
<th>SAP Median (range) [no. of patients], mg/L</th>
<th>CRP Median (range) [no. of patients], mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab-negative patients without HUS (n = 100)</td>
<td>Male, no. (%)</td>
<td>44/97 (45.4)</td>
<td>38 (4–122) [99]</td>
<td>20 (0.1–643) [100]</td>
</tr>
<tr>
<td></td>
<td>Female, no. (%)</td>
<td>56/95 (57.4)</td>
<td>34 (4–120) [97]</td>
<td>18 (0.1–638) [100]</td>
</tr>
<tr>
<td>Ab-positive patients with HUS (n = 100)</td>
<td>Male, no. (%)</td>
<td>36/92 (39.1)</td>
<td>52 (0–932) [83]</td>
<td>24 (3–92) [81]</td>
</tr>
<tr>
<td></td>
<td>Female, no. (%)</td>
<td>64/98 (65.9)</td>
<td>50 (0–932) [83]</td>
<td>24 (3–92) [81]</td>
</tr>
<tr>
<td>Ab-positive patients without HUS (n = 50)</td>
<td>Male, no. (%)</td>
<td>17/47 (36.2)</td>
<td>113 (7–1034) [36]</td>
<td>26 (4–90) [44]</td>
</tr>
<tr>
<td></td>
<td>Female, no. (%)</td>
<td>33/43 (77.0)</td>
<td>116 (7–1034) [36]</td>
<td>26 (4–90) [44]</td>
</tr>
<tr>
<td>Total (n = 250)</td>
<td>Male, no. (%)</td>
<td>97/236 (41.1)</td>
<td>89.5 (0–1034) [188]</td>
<td>28 (3–122) [224]</td>
</tr>
<tr>
<td></td>
<td>Female, no. (%)</td>
<td>153/214 (72.1)</td>
<td>99.9 (0–1034) [188]</td>
<td>28 (3–122) [224]</td>
</tr>
</tbody>
</table>

NOTE. Data on sex and age were not available for all of the patients, and a few samples did not yield reliable results, for technical reasons.

* For comparison of the 3 groups, calculated using the χ² test or analysis of variance, as appropriate.

** After age differences between the 3 groups were controlled for, there were no significant differences between groups: comparison between the Ab-negative patients without HUS and the Ab-positive patients with HUS gave \( P = .78 \), and comparison between the Ab-negative patients without HUS and the Ab-positive patients without HUS gave \( P = .50 \).

The requirements of the National Councils on Animal Care (Canada) and the United Kingdom Home Office. Three groups of 8 BALB/c mice (age, 6 weeks; weight, 20 g) received, respectively, an intraperitoneal (ip) injection of 50 mg/kg isolated human SAP in TN buffer, 50 mg/kg isolated human CRP [12] in TC buffer, or TC buffer alone. All mice then immediately received a dorsal subcutaneous (sc) injection of either 20 μg/kg Stx1 (twice the LD₅₀) or 0.2 μg/kg Stx2 (also twice the LD₅₀) mixed with 7.5 μg of Quil-A (saponin; Cedarlane Labs) in 100 μL of PBS. All mice subsequently received repeat ip injections of the same doses of SAP, CRP, or TN or TC buffer alone, at 12-h intervals. Beginning on day 3, all mice were monitored every 4 h and were killed in a CO₂ chamber as soon as clear signs of Shigatoxemia (lethargic disposition and/or hunched posture and ruffled fur) were observed. In a separate experiment, groups of between 11 and 19 human SAP–transgenic pure-line C57BL/6 mice, along with their wild-type littermates as control mice, received a dorsal sc injection of Stx1 or Stx2 at the doses described above (twice the LD₅₀). These mice, which did not receive ip injections of SAP or CRP, were then observed and treated as described above from 18 h onward.

To assay for tissue distribution, Stx1 and Stx2 were purified and iodinated as described elsewhere [9, 10], and 40 ng of biologically active \(^{125}\)I-labeled Stx1 \((1.6 \times 10^7 \text{ cpm/μg})\) or \(^{125}\)I-labeled Stx2 \((0.5 \times 10^7 \text{ cpm/μg})\) in 100 μL of PBS containing 7.5 μg of Quil-A were injected sc into the backs of male human SAP–transgenic C57BL/6 mice or the control mice. Forty-eight hours later, blood samples were obtained from these mice, and their livers, kidneys, spleens, hearts, lungs, brains, and turbinates were removed and weighed. The amount of radioactivity (counts per minute per gram) present in these samples was then determined using an LKB Wallac 1270 Rackgamma II gamma counter.

To ascertain SAP and CRP concentrations in patients with suspected *E. coli* infections, serum samples sent (with informed consent) to the Health Protection Agency (London, United Kingdom) for detection of antibodies to *E. coli* O157:H7 lipopolysaccharide (LPS) were used. SAP and CRP concentrations were measured in 100 patients with enteric infections who were antibody positive but did not have HUS, in 100 patients who were culture and serologically positive for *E. coli* O157:H7 but did not have HUS, and in 50 patients with HUS who were culture and antibody positive. Data on sex and age were not available for all.

Figure 1. Binding of human serum amyloid P component (SAP) to immobilized Shiga toxin (Stx) 1 (□), to Stx2 (●), and in uncoated control plates (▲) in the presence (A) and absence (B) of calcium. Each point represents the mean ± SD of triplicate observations.
of the patients, and a few samples did not yield reliable results, for technical reasons.

Mann-Whitney $U$ tests were used to compare values between groups in the $^{125}$I tissue-distribution experiments. Differences between the groups shown in table 1 were tested for significance using $\chi^2$ tests and analysis of variance, as appropriate. Multiple linear regression methods were then used to determine whether any differences between groups remained after adjustment for age differences.

Results. We have previously demonstrated that a specific binding exists between human SAP and Stx2 that requires both the A subunit and the B pentamer of Stx2 [7]; however, we report here, for the first time, that, in contrast to all of the known specific ligand-binding interactions of SAP, the interaction with Stx2 does not require calcium (figure 1). We observed no binding between SAP and Stx1 in either the presence or the absence of calcium (figure 1).

BALB/c mice receiving 50-mg/kg injections of pure human SAP twice daily were completely protected against the lethality of Stx2 administered at a dose that was twice the LD$_{50}$ (figure 2A) but remained fully susceptible to Stx1 (figure 2B), which is consistent with the binding specificity of SAP in vitro (figure 1). Human CRP, which is very closely related to SAP but binds to different ligands, conferred no protection (figure 2A and 2B). The peak and trough values of circulating human SAP concentrations were $\sim$400 mg/L and $\sim$25 mg/L, respectively, and were therefore considerably greater than the physiological human concentrations for most of the time. In contrast, circulating human SAP concentrations in the transgenic mice were $\sim$20–60 mg/L, which is close to physiological human concentrations. After challenge with Stx2, these mice survived significantly longer than did the control mice (their wild-type littermates), although they did eventually succumb to the high dose (figure 3). However, the transgenic mice were as susceptible to Stx1 as the control mice (figure 3).

The tissue-distribution experiments revealed that, 48 h after injection, the amount of residual Stx2 in those organs and tissues examined was almost 50% less in the transgenic mice than in the control mice. There was also significantly less Stx2 in each of the target tissues except for the heart, but there was significantly more in the blood (figure 4A). In contrast, we detected no difference between the transgenic mice and the control mice in the persistence or distribution of Stx1 (figure 4B).
Figure 4. Tissue distribution of the 125I-labeled Shiga toxin (Stx) 2 (A) or 125I-labeled Stx1 (B) that remained 48 h after injection into wild-type control mice (black bars) and mice transgenic for human SAP (white bars). Results shown are mean ± SD counts per minute per gram. For Stx2, 13 transgenic mice and 8 control mice were used; for Stx1, 5 transgenic mice and 6 control mice were used. There were no significant differences in Stx1 localization between the wild-type mice and the transgenic mice for any tissue (P > .13 to P = .93). For Stx2, there was significantly more in the blood of the transgenic mice (P < .02) and significantly less in all organs (P < .001 to P < .005), except for the heart.

After adjustment for subject age, there was no significant difference in SAP concentration between the patients who were positive for antibody to E. coli O157:H7 LPS and those who were negative and between the patients with HUS and those without HUS (table 1). CRP values were also similar (table 1), with frequent high concentrations (as would be expected in response to a serious bacterial infection). There was the usual significant overall correlation between SAP and CRP concentrations, even though SAP is not an acute-phase reactant [13]. Although circulating SAP concentrations are known to be lower in infants than in older children and adults [13], there was no evidence that the patients with HUS had significantly lower SAP concentrations, even though they were younger than the patients in the other groups (table 1).

Discussion. We show here, for the first time, that the interaction between human SAP and Stx2—2 well-characterized ligand-binding proteins—has novel features and important functional effects in vivo and may have clinical therapeutic relevance. All previously reported ligand-binding interactions of SAP under physiological conditions of pH and ionic strength have been strictly calcium dependent [14], but we found avid binding between human SAP and Stx2 to be unaffected by the absence of calcium. Stx2 has previously been reported to bind only to particular cellular glycan or glycolipid receptors and their synthetic analogues [10, 15, 16]. However, we have reported previously [7] that the binding between SAP and Stx2 is not inhibited by the polyvalent globotriasosyl-ceramide receptor analogue Daisy, which blocks the binding of Stx2 to its cell surface receptor; thus, Stx2 does not bind to SAP via recognition of the SAP glycan moiety. Elucidation of the molecular basis of the biologically potent interaction between SAP and Stx2 will require further investigation, and crystallization of the SAP-Stx2 complex for x-ray analysis is currently in progress.

Kimura et al. [17] recently reported that intravenous injection of a single dose of 2.5–5.0 mg/kg human SAP did not protect mice against a lethal dose of Stx2 that was 10 times the LD50, probably because the 3-h circulating half-life of human SAP in mice means that this trace amount of human SAP was present for only the first few hours of exposure to this massive lethal dose. In contrast, we show here that mice receiving regular 50-mg/kg doses of human SAP twice daily were completely protected against twice the LD50 of Stx2. Transgenic mice expressing human SAP at close to physiological human concentrations also showed substantially delayed lethality. These findings suggest that human SAP may play a role in innate immunity against Stx2, especially given that SAP is known to be less abundant during infancy, when HUS is most common. However, we found no association between clinical HUS and circulating SAP concentration.

Our finding that human SAP markedly reduces the accumulation of Stx2 in the primary target organs in vivo, including the kidneys and possibly the brain, suggests a mechanism by which SAP may confer protection in the mouse model. More importantly, the present observations indicate that administration of exogenous SAP in patients suffering from an enterohemorrhagic E. coli infection and HUS could potentially be of therapeutic benefit. To provide good manufacturing practice–grade clinical SAP for the radiolabeled-SAP scintigraphy service at the United Kingdom National Health Service National Amyloidosis Centre [18], we recently developed, in collaboration with Bio Products Laboratory (Elstree, United Kingdom), an efficient method for large-scale isolation of SAP from donor plasma. Clinical trials in which substantial doses of SAP are used to treat enterohemorrhagic E. coli infection and HUS may, thus, become feasible. Although a phase 1 trial would obviously be essential, there is nothing to suggest that short-term major increases in circulating SAP concentration would be harmful, and we have seen no adverse effects after repeated daily
administration of up to 40 mg/kg isolated human SAP in mice and rats.

References