A dietary non-human sialic acid may facilitate hemolytic-uremic syndrome

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Hemolytic-uremic syndrome (HUS) is a systemic disease characterized by microvascular endothelial damage, mainly in the gastrointestinal tract and the kidneys. A major cause of HUS is Shiga toxigenic Escherichia coli (STEC) infection. In addition to Shiga toxin, additional STEC virulence factors may contribute to HUS. One is the newly discovered subtilase cytotoxin (SubAB), which is highly toxic to eukaryotic cells, and when injected intraperitoneally into mice causes pathology resembling that associated with human HUS. Recent data show that SubAB exhibits a strong preference for glycans terminating in α2-3-linked N-glycolyneuraminic acid (Neu5Gc), a sialic acid that humans are unable to synthesize, because we genetically lack the necessary enzyme. However, Neu5Gc can still be found on human cells due to metabolic incorporation from the diet. Dietary incorporation happens to be highest in human endothelium and to a lesser extent in the intestinal epithelium, the two affected cell types in STEC-induced HUS. Mammalian-derived foods such as red meat and dairy products appear to be the primary source of dietary Neu5Gc. Ironically, these are also common sources of STEC contamination. Taken together, these findings suggest a ‘two-hit’ process in the pathogenesis of human SubAB-induced disease. First, humans eat Neu5Gc-rich food, leading to incorporation of Neu5Gc on the surfaces of endothelial and intestinal cells. Second, when exposed to a SubAB-producing STEC strain, the toxin produced would be able to bind to the intestinal epithelial cells, perhaps causing acute gastrointestinal symptoms, and eventually damaging endothelial cells in other organs like the kidney, thereby causing HUS.

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HEMOLYTIC-UREMIC SYNDROME

Hemolytic-uremic syndrome (HUS) is a systemic disease process characterized by microvascular endothelial damage that occurs mainly in the gastrointestinal tract and in the kidneys, and sometimes also in the brain and the liver. It typically occurs in children younger than 5 years of age, but may also occur in adults, and among the elderly. Non-immune ‘microangiopathic’ hemolytic anemia, thrombocytopenia, and acute renal failure caused by endothelial damage are the principal clinical manifestations of severe HUS, which sometimes follows an episode of bloody diarrhea because of hemorrhagic colitis.1–4 HUS is thus subcategorized into typical HUS (diarrhea-associated, or D+) and atypical (without diarrhea, or D−). In the United States, about 90% of the cases are classified as D+, and we will therefore focus on D+ HUS and refer to it as just HUS in this article.

SHIGA TOXIGENIC E. coli, SHIGA TOXIN, AND HUS

The leading cause of HUS in North America and Western Europe is considered to be gastrointestinal infection with Shiga toxigenic E. coli (STEC), sometimes referred to as enterohemorrhagic E. coli (EHEC). The capacity of STEC to cause HUS has long been thought to be directly attributable to the production of Shiga toxin (Stx), a member of the AB5 toxin family.4 The StxB subunits bind to the glycolipid receptor, globotriaosylceramide (Gb3), triggering the uptake of the holotoxin. The internalized StxA subunits have RNA-N-glycosidase activity and inhibit host cell protein synthesis.

There are two major classes of Shiga toxin (Stx1 and Stx2), which share ~60% homology in both the A and B subunits. STEC strains may produce either or both toxin types. Pathogenesis of the human disease initially involves colonization of the gut by STEC, without significant invasion of the epithelium. Locally produced Stx is translocated across the intestinal barrier and absorbed into the circulation. The toxin then targets specific tissues in accordance with their expression of Gb3. In humans, Gb3 is found in highest concentrations in the renal tubular epithelial cells and in microvascular endothelial cells (particularly in the kidneys, gut, pancreas, and brain), and damage at these sites is consistent with the pathology of HUS. Microvascular and concomitant ischemic damage to the intestinal wall also accounts for the severe bloody diarrhea associated with STEC infection.4
The STEC strains belong to over 200 E. coli O:H serotypes, which vary in their capacity to cause HUS. The majority of cases in Europe and North America are caused by O157:H7 strains, although other STEC serotypes may be underdetected because of deliberate targeting of diagnostic strategies at O157 strains. Differences in virulence between the STEC strains may be because of the amount or subtype of Stx produced (strains producing Stx2 are more virulent than Stx1 producers). However, many STEC associated with HUS (including O157:H7) also carry the LEE (locus of enteroctye effacement), which encodes the capacity to adhere intimately and form attaching and effacing lesions on the enterocytes (gut epithelial cells). Most STEC strains also carry large (90–170 kb) plasmids, which encode several additional putative accessory virulence factors.

**SUBTILASE CYTOTOXIN, A SECOND TOXIN IN STEC STRAINS**

A more recently discovered and potentially important STEC virulence factor is subtilase cytotoxin (SubAB). SubAB is the prototype of a new and different AB5 toxin subfamily, initially detected in a LEE-negative O113:H21 STEC strain responsible for an outbreak of HUS in Australia. Despite the absence of LEE, O113:H21 STEC are highly virulent and such strains were, in fact, among the first STEC serotypes to be causally associated with HUS. SubAB is highly toxic for eukaryotic cells and its mechanism of action involves highly specific A-subunit-mediated proteolytic cleavage of the essential endoplasmic reticulum chaperone, Bip/GRP78. This triggers a massive endoplasmic reticulum stress response, ultimately leading to apoptosis.

Intraperitoneal injection of purified SubAB in mice causes microangiopathic hemolytic anemia, thrombocytopenia, and renal impairment, characteristics typical of Stx-induced human HUS. Histological examination of organs removed from SubAB-treated mice revealed extensive microvascular thrombosis and other histological damage in the brain, kidneys, and liver, as well as dramatic splenic atrophy. Peripheral blood leukocytes were increased at 24 h, and there was also significant neutrophil infiltration in the liver, kidneys, and spleen, and toxin-induced apoptosis was seen at these sites. These findings indicate that SubAB also likely contributes to pathology in humans.

The SubAB is known to be produced by a wide range of STEC strains belonging to diverse O-serogroups, and there is a strong association with strains that produce Stx2, but are LEE-negative. It has been found in STEC isolates from Australia, Europe, Japan, and America. At least in O113:H21 strains, its operon (SubAB) is carried on a self-transmissible megaplasmid, thereby underscoring the potential for further horizontal transfer to other E. coli serogroups, or indeed to other Enterobacteriaceae.

**SubAB GLYCAN RECEPTOR SPECIFICITY IS DIFFERENT FROM Stx**

Recent studies using glycan array, Biacore, and crystallographic analyses indicate that the pentameric binding subunit, SubB, exhibits strong specificity for glycan chains terminating in α2-3-linked N-glycolyneuraminic acid (Neu5Gc), a sialic acid (Sia) that humans are unable to synthesize (see below). Interactions with otherwise identical glycans terminating in N-acetyleneuraminic acid (Neu5Ac), which differs by a single OH group, were more than 10-fold weaker. The location of the glycan-binding pocket on the sides of the SubB pentamer, rather than at the base, as is the case for StxB, is also consistent with recognition of glycans displayed on host cell glycoproteins rather than glycolipids. Indeed, many glycoproteins express terminal α2-3-linked Sias on their N- and O-linked glycans.

**EVOLUTIONARY LOSS OF Neu5Gc EXPRESSION IN HUMANS**

The Sias are a family of nine-carbon backbone monosaccharides that display wide diversity in nature, with more than 50 different variants presented in a variety of linkages, which are often expressed in a cell-type-specific manner. Sias are typically located at the outermost end of the so-called ‘glycocalyx’, a layer of glycoconjugates covering every cell. Given this location, they are often used as key components of receptors for pathogens and for some of their toxins. Of note, Sias exist not only on the cell surface, but also on many secreted soluble proteins, such as gut mucins and plasma proteins, in which they could potentially function as competitive inhibitors of pathogen or toxin binding to cell surface Sias.

In mammals, the most prevalent Sias are Neu5Ac and Neu5Gc. The latter is formed by hydroxylation of CMP-Neu5Ac in a complex enzymatic mechanism catalyzed by the product of the Cmah gene. Although chimpanzees and all other non-human hominids synthesize Neu5Gc, humans cannot do so, because of a specific and human-universal inactivating mutation in the Cmah gene.

**HUMAN CELLS CAN INCORPORATE DIETARY Neu5Gc**

Human cells fed with free Sias can metabolically incorporate and present them on their surfaces, as if they were made in the same cell. Apparently, for this reason, small amounts of Neu5Gc can still be found in humans because of metabolic incorporation into the cells from the diet. Mammalian-derived foods, such as red meats (lamb, pork, and beef) and dairy products, seem to be the primary source of dietary Neu5Gc. Neu5Gc incorporation is highest in the human endothelium and to a lesser extent in the intestinal epithelium, the two affected cell types in STEC-induced HUS. It is thought that the endothelial incorporation occurs over a long period of exposure to dietary Neu5Gc. However, incorporation must happen over shorter periods of time in the epithelial cells of the intestine, as these cells have a turnover rate of just a few days. Regardless of the kinetics, the Neu5Gc expression enhances the binding of SubAB to target tissues expressing them (see examples in Figure 1). Further evidence was obtained by showing that SubAB toxin binding to human tissues was...
blocked by a monospecific Chicken IgY antibody directed against Neu5Gc and not by a control IgY antibody (see Figure 1).

Neu5Gc SENSITIZES HUMAN CELLS TO SubAB, IN VITRO AND IN VIVO

As SubAB binds to Neu5Gc with much higher affinity than to Neu5Ac, we asked whether this could affect cell killing in vitro. Indeed, when human cell lines were fed with Neu5Gc or Neu5Ac, those incorporating Neu5Gc were killed at a much lower toxin dose. Thus, dietary consumption of Neu5Gc has the potential to similarly sensitize human tissues to this toxin. In keeping with this, SubAB binds directly to Neu5Gc-enriched cells on colon epithelial cells and kidney glomerular endothelial cells in human tissue sections. This binding was shown to be Neu5Gc-dependent, as it was blocked by pre-incubation of the slides with chicken anti-Neu5Gc IgY antibodies, but not with control IgY preparations (Figure 1). Moreover, binding was markedly lower when cells were treated with a SubAB derivative with a single amino acid substitution in its B subunit that abrogates binding to the OH group present in Neu5Gc, but not in Neu5Ac.

Neu5Gc AND IN VIVO SENSITIVITY TO SubAB

On the basis of our in vitro findings, we initially predicted that wild-type C57Bl/6 mice would be more susceptible to intraperitoneal challenge with purified SubAB than Cmah-null mice. Surprisingly, these Neu5Gc-deficient mice died somewhat earlier than the wild-type mice, with the toxicity presumably being mediated by the weaker binding to endogenous Neu5Ac-containing targets. The likely explanation for this paradox is that Neu5Gc also exists in high amounts on red blood cells and soluble serum proteins in non-human mammals, such as chimpanzees and mice, that have a functional Cmah gene product. These can act as a ‘sink’, soaking up the toxin and thereby protecting the Neu5Gc-containing endothelial surfaces of the organism. Further experiments substantiated this explanation, showing that SubAB binding to Neu5Gc-containing glycans in gut and kidney sections was blocked well by the serum from chimpanzee and wild-type C57Bl/6 mice, but not by the human or Cmah-null mouse serum. Thus, humans who consume Neu5Gc-containing food, such as red meat, could be particularly vulnerable to SubAB, not only because they load Neu5Gc into gut epithelium and vascular endothelium generating high-affinity receptors for SubAB, but also because...
they do not have any Neu5Gc-containing serum proteins that could provide neutralizing protection against SubAB. 20

LINKING HUS WITH SubAB AND Neu5Gc

Taken together, our findings are suggestive of a ‘two-hit’ process in the pathogenesis of SubAB-induced disease in humans (Figure 2). The scenario is that humans first eat Neu5Gc-rich foods, such as red meats and dairy products, which leads to metabolic incorporation and presentation of Neu5Gc on glycoconjugates on the microvascular endothelial cells in the kidney glomeruli and elsewhere, as well as on the surface of the gut epithelial cells. This process can generate high-affinity receptors for SubAB on the cell surface. The very same foods happen to be the commonest source of Shiga toxigenic E. coli (STEC) bacteria, including those strains capable of producing SubAB. STEC infection caused by consumption of contaminated food will result in toxin production in the gastrointestinal tract. The toxin could then bind to the Neu5Gc-containing receptors on the gut epithelium and cause gastrointestinal symptoms. This may also allow the toxin to be better absorbed systemically, and the lack of competing Neu5Gc-glycoproteins in human serum would then allow maximal binding of SubAB to high-affinity receptors on the endothelium in organs, such as the kidney, thereby triggering hemolytic-uremic syndrome (HUS) (based on Supplementary Figure 1 from Byres et al.12).

Figure 2 | Humans who eat foods rich in the non-human sialic acid, N-glycolyNeraminic acid (Neu5Gc), can become susceptible to food poisoning by the Neu5Gc-prefering subtilase cytopotoxin (SubAB) toxin. Dietary intake of Neu5Gc-rich foods, such as red meats and dairy products, can result in metabolic incorporation and display of Neu5Gc on glycoconjugates on the microvascular endothelial cells in the kidney glomeruli and elsewhere, as well as on the surface of the gut epithelial cells. This process can generate high-affinity receptors for SubAB on the cell surface. The very same foods happen to be the commonest source of Shiga toxigenic E. coli (STEC) bacteria, including those strains capable of producing SubAB. STEC infection caused by consumption of contaminated food will result in toxin production in the gastrointestinal tract. The toxin could then bind to the Neu5Gc-containing receptors on the gut epithelium and cause gastrointestinal symptoms. This may also allow the toxin to be better absorbed systemically, and the lack of competing Neu5Gc-glycoproteins in human serum would then allow maximal binding of SubAB to high-affinity receptors on the endothelium in organs, such as the kidney, thereby triggering hemolytic-uremic syndrome (HUS) (based on Supplementary Figure 1 from Byres et al.12).

POETIC JUSTICE FOR RED MEAT EATERS?

Assuming that the ‘two-hit’ scenario shown in Figure 2 is indeed true, it would be ironic that consumption of

epithelial and endothelial cells, and thereafter challenge these mice with SubAB. However, because of yet unknown metabolic differences between mice and humans, we have so far been unable to get a human-like level of Neu5Gc incorporation into the Cmah-null mice.19 This interesting difference could be related to the fact that humans lost the ability to synthesize Neu5Gc more than 2 million years ago. Thus, it is possible that unlike mice, humans have lost the ability to degrade Neu5Gc, once it is incorporated into cells.

Furthermore, directly addressing the role of SubAB and dietary Neu5Gc in human disease epidemiologically would, of course, be very complicated. Human challenge studies with either purified SubAB or with SubAB-producing STEC are out of the question. Examination of the incidence and/or severity of HUS in vegans versus meat-eaters would also be difficult because of the low overall incidence of STEC infection, the fact that not all STEC produce SubAB, and that as it is a newly discovered toxin, few clinical laboratories have the capacity to detect its presence in STEC isolates. Hence, more studies on how to further humanize the mouse model are needed.
Neu5Gc-rich foods not only sensitizes human tissues to SubAB, but also simultaneously increases the likelihood of infection with SubAB-expressing STEC. These pathogens are usually found in the intestines of livestock, particularly cattle, and most commonly enter the human food chain by fecal contamination of red meat and dairy products during processing. Thus, those who consume large amounts of these types of foods may be unwittingly preparing their bodies for damage by a SubAB-producing organism that contaminates their next meal of the same food types.

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