Typhoid toxin provides a window into typhoid fever and the biology of *Salmonella* Typhi

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***Salmonella* Typhi is the cause of typhoid fever, a disease that has challenged humans throughout history and continues to be a major public health concern. Unlike infections with most other *Salmonellae*, which result in self-limiting gastroenteritis, typhoid fever is a life-threatening systemic disease. Furthermore, in contrast to most *Salmonellae*, which can infect a broad range of hosts, *S.* Typhi is a strict human pathogen. The unique features of *S.* Typhi pathogenesis and its stringent host specificity have been a long-standing puzzle. The discovery of typhoid toxin not only has provided major insight into these questions but also has offered unique opportunities to develop novel therapeutic and prevention strategies to combat typhoid fever.**

Salmonella Typhi | typhoid fever | bacterial pathogenesis | bacterial toxins | cell autonomous immunity

The bacterial pathogen *Salmonella enterica* serovar Typhi (*S.* Typhi) is the cause of typhoid fever, a systemic, life-threatening disease of humans (1–5). A related but evolutionarily distinct *Salmonella* serovar, *S.* Paratyphi serotype A, can also cause an enteric fever type of disease virtually indistinguishable from typhoid (6, 7). These *Salmonella* serovars are therefore commonly referred to as "typhoidal *Salmonellae*." Typhoid fever is one of the oldest human diseases about which we have written records in western literature. It is thought to have been the cause of the "plague of Athens," which ravaged the city of Athens during the Peloponnesian war of 430 B.C. In his "History of the Peloponnesian War," Thucydides, who himself suffered from the disease, described the epidemic and its devastating effect in great and dramatic detail (8). Although his eloquent description of the disease was not specific enough to implicate a particular etiological agent, a recent study of teeth recovered from an ancient Greek burial site dating back to that time detected DNA sequences suggesting that *S.* Typhi was the actual cause of this epidemic (9). In modern times, typhoid fever and *S.* Typhi are intimately linked to the tragic story of Mary Mallon ("Typhoid Mary"), an Irish cook who was chronically infected with *S.* Typhi and inadvertently spread the disease to many households in the New York city area in the early 1900s. Due to the lack of a treatment that would stop her from shedding *S.* Typhi and her refusal to abandon her much-loved profession, Mary Mallon went on to spend the rest of her life in confinement (10). Despite its long-standing presence in human history, typhoid fever is still today a major public health concern claiming the lives of ∼200,000 people, mostly children in developing countries (2, 11).

Unlike other *Salmonella enterica* ("nontyphoidal") serovars such as *S.* Typhimurium, which can infect a broad range of animal hosts generally causing self-limiting gastroenteritis (12, 13), *S.* Typhi is an exclusive human pathogen, where it causes severe systemic illness (1, 3, 4, 14). The genome sequence of *S.* Typhi has provided major insight into its evolutionary history, and phylogenetic analysis indicates that this bacterium is highly monomorphic and may have entered the human population relatively recently (15–17). The *S.* Typhi genome harbors a higher-than-expected number of pseudogenes, an indication that the process of human–host adaptation is resulting in the reduction of its genome. Traditionally, the mechanisms of *S.* Typhi pathogenesis have been inferred from studies of related *Salmonella* serovars such as *S.* Typhimurium in the mouse model of infection (12, 13). Although these studies have revealed major insight into core-common *Salmonella* virulence traits, they have been less informative about specific aspects of typhoid fever in humans. In particular, these studies have not provided information on the pathogenesis of typhoid fever's unique symptomatology nor have they provided insight into the mechanisms of *S.* Typhi's strict host specificity. Comparison of the genome sequences of *S.* Typhi with those of nontyphoidal *Salmonellae* reveals relatively few unique genes, most of which are associated with lysogenic bacteriophages or genomic islands, and fewer encode potential virulence factors (15, 16, 18, 19). This article will focus on typhoid toxin, an *S.* Typhi virulence factor recently discovered in my laboratory, which is also encoded by the related typhoidal serovar *Salmonella* Paratyphi A but it is largely absent from nontyphoidal *Salmonella enterica* serovars. The study of typhoid toxin has revealed unique insight not only into the pathogenesis of typhoid fever but also into the molecular bases for *S.* Typhi's remarkable host specificity.

Discovery of Typhoid Toxin

The discovery of typhoid toxin began when our laboratory identified a toxic activity associated with *S.* Typhi that exhibited many of the features seen in cells intoxicated with an exotoxin known as "cytolethal distending toxin" (CDT) (20–22). Similar to cells intoxicated with CDT, *S.* Typhi-infected cells seemed distended, with nuclei double the normal size (Fig. 1). Flow cytometric analysis of DNA content determined that, similar to cells intoxicated with CDT, these cells were arrested in the G2/M phase of the cell cycle (20) (Fig. 1). Intoxication required bacterial infection, and no toxic activity was detected in supernatants or cell lysates of bacteria grown in standard culture medium (20). In fact, toxicity was dependent on the ability of *S.* Typhi to invade cultured cells and to transit the endocytic pathway for at least 3 h to receive the environmental cues that result in the stimulation of toxin gene expression (20, 23). These observations explained why, despite the intense scrutiny of this pathogen for more than a century, this toxin activity had remained undetected. Toxicity was shown to be associated with a protein encoded within a genomic islet that exhibits very significant amino acid sequence similarity to CdtB, the active subunit of CDT (20). Similar

Significance

***Salmonella* Typhi is the cause of typhoid fever, a disease that has challenged humans throughout history but that continues to be a major public health concern resulting in ∼200,000 deaths every year. The recent discovery of typhoid toxin has provided unique opportunities to develop much needed preventive and therapeutic strategies.**

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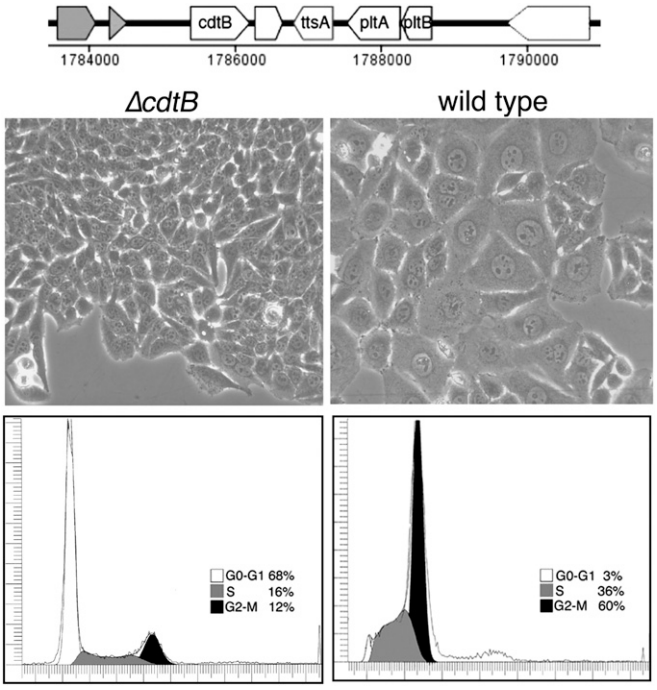

Fig. 1. Typhoid toxin induces cell cycle arrest in intoxicated cells. A diagram of the genetic locus that encodes the typhoid toxin genes is depicted (*Top*). Culture epithelial cells were infected with WT *S.* Typhi or a typhoid toxin-deficient mutant derivative (*ΔcdtB*), and, 72 h after infection, the infected cells were examined by phase contrast microscopy (*Middle*) or processed to measure DNA content by flow cytometry (*Bottom*). The peaks corresponding to cells in G0-G1, S, or G2 are indicated.

to CDT, the morphological changes and cell cycle arrest observed in *S.* Typhi-intoxicated cells were shown to be due to DNA damage caused by the DNase activity of this CdtB homolog (21). Within the same genomic islet encoding *cdtB,* ORFs encoding homologs of the ADP ribosyl transferase "A" subunit and one of the components of the heteromeric "B" subunit of pertussis toxin (thus named *pltA* and *pltB*, respectively, for pertussis-like toxin A and B) were also identified (23). Unexpectedly, mutations in either *pltA* or *pltB* resulted in total loss of the *cdtB*-dependent toxic activity functionally linking these three genes. Additional biochemical experiments went on to demonstrate that CdtB, PltA, and PltB form a complex, an observation that formalized the discovery of typhoid toxin (23). Consequently, typhoid toxin seemed to have evolved from the combination of the activities of two exotoxin ancestors, CDT and pertussis toxins.

Insights from the Atomic Structure of Typhoid Toxin: How Two Toxins Became One

The crystal structure of typhoid toxin showed a pyramid-shaped complex composed of five PltB molecules (located at the base) and one molecule each of PltA and CdtB (located at the vertex of the pyramid) (24) (Fig. 2). The A_2B_5 organization of two "A" subunits linked to the same "B" subunit observed in typhoid toxin is unprecedented among other known members of the AB_5 toxin family, such as cholera, shiga, or pertussis toxins, which have only one A subunit (25, 26). The PltB oligomer is arranged as a pentamer, with a central channel that is lined by five helixes, and exhibits structural similarity to B subunits of other AB toxins (27, 28). The PltB protomer shows a typical oligosaccharide-binding fold located on the side of the pentamer, a location similar to that of toxins that preferentially bind glycoproteins (25). As predicted by the amino acid sequence similarities, the PltA and CdtB subunits exhibit a very similar structure to the pertussis toxin S1 (and other ADP ribosyl transferases), and to the CdtB subunit of cytolethal distending toxin. The linear arrangement of the typhoid toxin complex determines that there are no interactions between CdtB and PltB, which explains why deleting *pltA* results in complete loss of CdtB-mediated toxicity (23). The association of PltA with the PltB oligomer is largely mediated by a short helix at the carboxyl terminal end of PltA, which inserts into the hydrophobic lumen of the PltB channel. In contrast, a disulfide bond between Cys214 of PltA and Cys269 of CdtB covalently links these two subunits (Fig. 2). The structure indicates that there is minimal interaction between CdtB and PltA other than the disulfide bond, which is corroborated by the observation that the complex disassembles upon reduction of this bond (24). Interestingly, despite the high amino acid sequence conservation between PltB and CdtB and its homologs in other toxins, the Cys residue that tethers these two subunits together is unique to the typhoid toxin subunits. Therefore, the evolution of uniquely positioned Cys residues in both PltA and CdtB allowed the tethering of CdtB to the PltA/PltB complex, thus giving rise to a powerful new toxin from the merger of components of other bacterial toxins.

Typhoid Toxin Secretion and Export from Mammalian Cells

Once synthesized by intracellular bacteria, typhoid toxin is secreted from *S.* Typhi into the lumen of the bacteria-containing vacuole by a specific and unique protein secretion system (29) (Fig. 3). Although many mechanistic details of this system have yet to be uncovered, it seems that the secretion mechanism is a recent exaptation of the holin/endolysin system used by bacteriophages to exit from infected bacterial cells (30). A central component of the typhoid toxin secretion system is TtsA (for typhoid toxin secretion protein A), which is encoded immediately adjacent to the typhoid toxin genes (29). TtsA is a putative *N*-acetyl-β-D-muramidase that belongs to a distinct class of bacteriophage endolysins. During phage release, endolysins are transported through the inner membrane by holins, a family of small membrane proteins that assemble into a protein channel through which endolysins reach the periplasmic space and trigger bacterial lysis (31). Holins are most often encoded in the vicinity of their cognate endolysins. However, no proteins with features similar to those expected of a functional holin are encoded in the vicinity of TtsA, suggesting that it may function in conjunction with a holin encoded elsewhere in the *S.* Typhi chromosome. Indeed, cross-talk among different endolysins and holins has been previously reported (30). It is expected that the delivery and/or activity of TtsA must be locally restricted because the TtsA-dependent secretion of typhoid toxin is not accompanied by bacterial lysis (29). Consistent with this hypothesis, a detailed mutagenesis and domain-swapping analysis of TtsA identified specificity determinants for typhoid toxin-secretion functions that are located within its peptidoglycan-binding domain (29). In fact, a phage endolysin could be rendered suitable for functions related to typhoid toxin secretion by introducing just a limited number of amino acid changes within its peptidoglycan binding. These observations suggest that the exaptation of the phage endolysin to perform protein secretion functions must be a recent evolutionary event. Recently, the secretion of another toxin was shown to occur through a similar mechanism (32), indicating that, as originally proposed (29), the typhoid toxin secretion system defines a novel protein secretion mechanism used by bacteria to secrete toxins or large extracellular enzymes.

After its secretion into the lumen of the *Salmonella*-containing vacuole, typhoid toxin is packaged into vesicle-carrier intermediates, and it is subsequently transported to the extracellular space where it can reach its target cells (Fig. 3) (23). The typhoid toxin export pathway is incompletely understood, but it requires the activity of the Rab GTPases Rab29 and Rab31 (33). Importantly, typhoid toxin cannot intoxicate the cells in which it is produced without first becoming extracellular. Addition of an antibody to the extracellular space, for example, effectively protects *S.* Typhi-infected cells from intoxication, supporting the notion that typhoid toxin can intoxicate infected cells only through a paracrine/autocrine pathway (23).

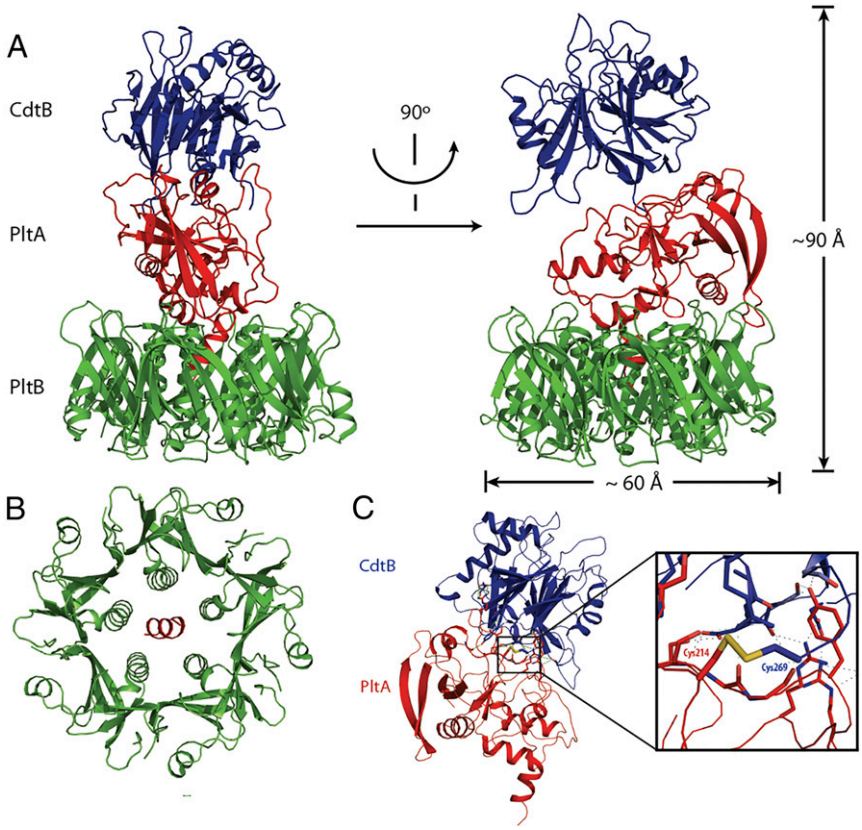

Fig. 2. Atomic structure of typhoid toxin. (*A*) Two views of the overall structure of the typhoid holotoxin complex shown as a ribbon diagram and related by 90° rotation about a vertical axis. CdtB, PltA, and PltB are shown in blue, red, and green, respectively. (*B*) Bottom view of the channel formed by the PltB pentamer (in green), depicting the PltA C-terminal α-helix (in red) within it. (*C*) Atomic interface between CdtB and PltA. (*Inset*) A detailed view of a critical disulphide bond between $PltA^{Cys\,214}$ and $CdtB^{Cys\,269}$ (adapted from ref. 24).

Consequently, cells lacking typhoid toxin receptors and harboring *S.* Typhi could serve as a toxin source while themselves not being a target of its activity, an aspect of the biology of typhoid toxin that may be central to its potential role during infection (see *Typhoid Toxin and Typhoid Fever*).

Typhoid Toxin and Typhoid Fever

The unique clinical presentation of typhoid fever contrasts the clinical presentation of infections by nontyphoidal *Salmonella* serovars, which most often result in a self-limiting gastroenteritis (e.g., "food poisoning") (12–14). The molecular bases for the differences in these clinical presentations have been a long-standing mystery in the field. Although the unique clinical presentation associated with typhoidal *Salmonellae* infections are likely to be multifactorial, typhoid toxin is likely to play a central role in the development of symptoms specific to typhoid fever. This hypothesis is supported by the observation that typhoid toxin is encoded by both typhoidal *Salmonella* serovars *S.* Typhi and *S.* Paratyphi whereas it is largely absent from nontyphoidal serovars. More importantly, administration of purified typhoid toxin into experimental animals was able to reproduce many of the pathognomonic symptoms of the acute phase of the disease (24). Inoculated animals showed signs of stupor and malaise, commonly observed during the acute phase of typhoid fever (1). However, these animals did not develop fever, suggesting that some of the typhoid fever-associated symptoms may not just be a consequence of the inflammatory response to infection, but, rather, they may be the result of the involvement of the central nervous system in the pathogenesis of typhoid fever. Animals that received active toxin also showed a markedly reduced number of circulating leukocytes (24), another pathognomonic symptom of the acute phase of typhoid fever (34). The development of symptoms was correlated with the catalytic activity of CdtB because a typhoid toxin preparation carrying a catalytic mutant of its CdtB subunit was unable to induce any symptomatology (24). In contrast, a typhoid toxin preparation carrying a catalytic mutant of its PltA triggered the same response as WT toxin, indicating that this subunit does not play a role in this activity. These observations, combined with observations in experimentally infected chimpanzees (see *Typhoid Toxin and* S. *Typhi Host Specificity*), suggest a direct role for typhoid toxin in the development of the acute phase symptoms of typhoid fever. It is also likely that typhoid toxin may play additional roles during *S.* Typhi infection. For example, its ability to cause leukopenia indicates that typhoid toxin may contribute to the infectivity of *S.* Typhi although human volunteer studies will be required to investigate this possibility. Finally, it is possible that typhoid toxin's main role is exerted during *S.* Typhi persistent infection. In fact, experiments in humanized mice suggest this possibility (35). *S.* Typhi is known to establish chronic, life-long infections in the gall bladder of convalescent individuals, likely a central attribute in its evolutionary success as a host-specific pathogen with no known reservoir. The general mechanisms that lead to the carrier state are very poorly understood, and the specific mechanisms by which *S.* Typhi avoids its clearance by the strong immune response mounted by persistent carriers are unknown. Given the well-known toxic effect of CdtB homologs on immune cells (36, 37), it is possible that typhoid toxin exported from infected cells to the immediately surrounding space may help to create a local environment in which this pathogen can avoid its detection by the immune system.

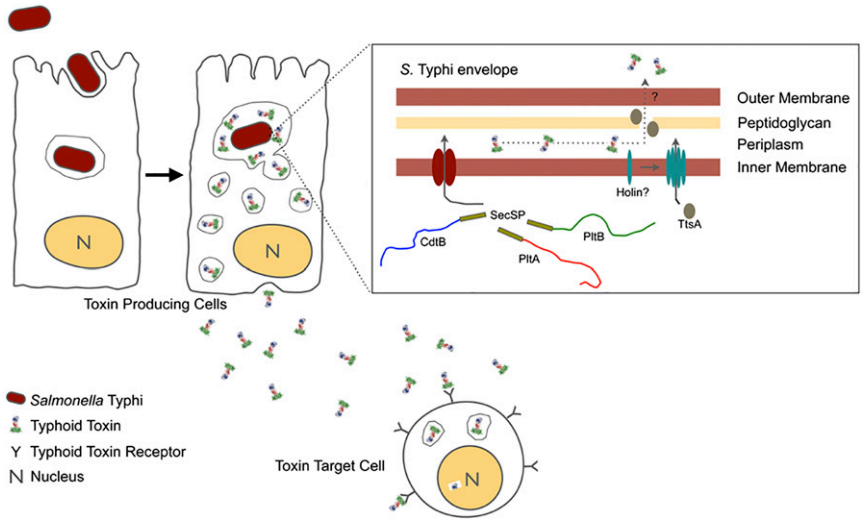

Fig. 3. Model for typhoid toxin secretion and export. Typhoid toxin expression is stimulated after *S.* Typhi enters into host cells, where it receives the specific cues to initiate its synthesis. The toxin subunits are secreted into the bacteria periplasm (*Inset*) via the *sec* machinery, which recognizes canonical secretion signals (secSP) in each one of the polypeptides. After assembly within the bacterial periplasm, the typhoid holotoxin is translocated through the peptidoglycan layer with the help of TtsA, an *N*-acetyl-β-D-muramidase that shares amino acid sequence similarity to a novel class of bacteriophage endolysins. Like its homologs TtsA lacks a typical secretion signal therefore it is expected that it reaches the periplasm aided by an as yet unidentified holin, a family of small membrane proteins that share the property of forming a protein channel through which endolysins reach the periplasmic space. After secretion from the bacterial cell into the *Salmonella*-containing vacuole, typhoid toxin is packaged into vesicle carrier intermediates, which transport the toxin to the plasma membrane for its delivery to the extracellular space. The toxin can then reach its cellular targets via paracrine or autocrine pathways.

Typhoid Toxin and *S.* Typhi Host Specificity

The remarkable host specificity for humans exhibited by *S.* Typhi and *S.* Paratyphi are incompletely understood and are most likely multifactorial. A major factor is clearly its inability to infect nonhuman hosts (see Salmonella *Typhi Host Restriction: Insights from the Study of Typhoid Toxin*). However, chimpanzees can be efficiently infected with *S.* Typhi and yet they do not develop typhoid fever (38, 39). In fact, *S.* Typhi reaches significantly higher numbers in the blood stream of experimentally infected chimpanzees than those observed in infected humans. Despite efficient infection, it has been previously observed that the symptoms in the experimentally infected chimpanzee are significantly different from those observed in humans. For example, one study described the clinical course of the disease in chimpanzees as "mild and brief" with "the hypertoxicity, typhoid facies, stupor, extreme lethargy, so generally associated with the disease in man, not discernible in the infected chimpanzees" (38). In other words, the *S.* Typhi-infected animals exhibited symptoms more in line with those of a relatively mild "nontyphoidal *Salmonella*" infection than those of typhoid fever. The identification of typhoid toxin's host receptors has provided an explanation for the discrepancies between the clinical presentations of *S.* Typhi infection in humans and chimpanzees and has led to major insights into the molecular bases of *S.* Typhi host specificity. In epithelial cells, the typhoid toxin receptor is podocalyxin-like protein 1 (PODXL) (24), a polarly localized member of the CD34 sialomucin protein family that is also expressed in vascular endothelial cells (40, 41). In contrast, in macrophages and T and B cells, the receptor is CD45 (24), which is ubiquitously expressed in all hematopoietic cells other than erythrocytes and platelets (42). Typhoid toxin recognizes common glycan moieties on these heavily glycosylated surface proteins (24). A detailed glycoarray analysis determined that typhoid toxin binds a diverse group of sialylated glycans with preferential binding to termini with the consensus sequence Neu5Ac2-3Galβ1-3/β1-4Glc/GlcNAc (24, 43). This broad glycan-binding specificity resembles the binding properties of pertussis toxin. However, in contrast to pertussis toxin, which broadens its binding specificity through heterogeneity in each of its four heteromeric B-subunit components, typhoid toxin achieves broad binding specificity with a single polypeptide, PltB, which forms its homomeric B subunit (24, 43). Remarkably, however, typhoid toxin does not bind to otherwise identical glycans terminated in Neu5Gc (i.e., differing by a single oxygen atom) (43). This observation provided major insight into the molecular bases for *S.* Typhi human-host specificity because sialoglycans on human cells are unusual in that they are primarily terminated in *N*-acetylneuraminic acid (Neu5Ac) (44). In other primates (e.g., chimpanzees) and most other mammals, glycans are largely terminated in *N*-glycolylneuraminic acid (Neu5Gc). The reason for this difference is the presence of a mutation in the human gene that encodes the enzyme CMP-*N*-acetylneuraminic acid hydroxylase (CMAH), which converts Neu5Ac to Neu5Gc. This mutation is thought to have emerged after hominids separated from other primates. Consistent with its inability to bind Neu5Gc-terminated glycans, typhoid toxin does not bind to chimpanzee cells and tissues, thus explaining why, despite significant replication, *S.* Typhi does not cause typhoid fever in chimpanzees (38, 39). Mice, like most mammals, express a functional CMAH. However, in the mouse, the expression pattern of *CMAH* is variable, and therefore mouse tissues display sialoglycans terminated in both Neu5Ac and Neu5Gc (45). This observation explains why, in the mouse, typhoid toxin is capable of inducing typhoid fever symptoms (24). However, a mouse engineered to constitutively express the *CMAH* gene and thus displaying only Neu5Gc-terminated glycans was shown to be totally refractory to typhoid toxin (43). Therefore, the exquisite binding selectivity for glycans that are predominantly expressed in human cells provides an explanation for the inability of *S.* Typhi to cause typhoid fever in nonpermissive species that, like chimpanzees, allow significant bacterial replication. These observations also further support the role of typhoid toxin as a central factor in the development of clinical typhoid fever.

The atomic structure of PltB bound to its glycan receptor provided major insight into the structural bases for typhoid toxin's remarkable binding specificity (43). The structure showed the canonical glycan-binding site on the side of the PltB pentameric ring making contact with Neu5Ac through multiple direct and water-mediated hydrogen bonds with Tyr33, Ser35, Lys59, Thr65, and Arg100, and hydrophobic contacts with the aromatic rings of Tyr33

and Tyr34 (Fig. 4). The oligosaccharide-binding fold of PltB shares significant structural similarities with SubB, the B subunits of subtilase toxin (24, 27). However, SubB exhibits the opposite specificity, strongly favoring binding to Neu5Gc-terminated glycans (27). Comparison of the atomic structures of PltB and SubB bound to their glycan receptors provided important insight into the structural bases for typhoid toxin's binding specificity (Fig. 4). The arrangement of the main chain of Neu5Ac relative to the binding pocket of PltB is very similar to that of Neu5Gc bound to SubB (27), and many of the critical interactions between the glycans and specific residues of PltB and SubB are conserved. However, a residue equivalent to Tyr78 in SubB, which forms a critical hydrogen bond with the extra hydroxyl group in Neu5Gc, is missing from PltB (Fig. 4). Instead, at this position, PltB has the nonpolar residue Val103 and thus is unable to interact with Neu5Gc. These observations not only provide a structural explanation for typhoid toxin's inability to bind Neu5Gc-terminated glycans but also suggest an evolutionary pathway by which this toxin may have restricted its binding to human-specific glycans.

Salmonella Typhi Host Restriction: Insights from the Study of Typhoid Toxin

In contrast to most *Salmonella enterica* serovars, which can infect a broad range of hosts, *S.* Typhi and *S.* Paratyphi can infect only humans (1, 3, 12). The strict human host specificity not only has been a major impediment for *S.* Typhi research, but also has had a significant influence in its discovery. Karl Joseph Eberth, a pathologist at the University of Würzburg, is credited with having observed for the first time the "typhoid bacilli" in samples from infected individuals (46, 47). However, he was unable to culture the organism because he lacked the necessary expertise, which at that time was available at only a limited number of laboratories. It was Georg Theodor August Gafky, a Robert Koch disciple at the University of Berlin, who finally cultured the typhoid bacilli, which was named *Eberthella typhi* (48). However, *S.* Typhi's strict host specificity prevented Gafky from fulfilling the postulates enunciated by his influential mentor, Robert Koch. Gafky tirelessly tried infecting a variety of animals, including Java monkeys, mice, and guinea pigs, but (predictably) failed to reproduce the disease, thus failing to fulfill one of the criteria established by his mentor to identify the causative agent of an infectious disease. He would go on to die without the certainty that he had indeed cultured the typhoid bacilli, and one can imagine unable to convince his mentor of his most important discovery.

Recent work in our laboratory aimed at characterizing the cellular pathway that transports typhoid toxin from its site of production within infected cells to the extracellular space unexpectedly led to major insight into the mechanisms that restrict *S.* Typhi replication in nonhuman hosts (33, 49, 50). By extension, these studies shed light on mechanisms used by broad host *Salmonellae* to overcome this restriction and thus be able to explore multiple hosts. Studies aimed at identifying Rab GTPases potentially involved in toxin transport led to the serendipitous identification of three highly related Rab GTPases (Rab29, Rab32, and Rab380) that are efficiently recruited to *S.* Typhi-containing vacuoles but are not recruited to vacuoles containing *S.* Typhimurium (33, 49). These studies also led to the identification of an *S.* Typhimurium gene (*gtgE*) that, when expressed in *S.* Typhi, was able to prevent the recruitment of the Rab GTPases to the *S.* Typhi-containing vacuole. More importantly, expression of *gtgE* allowed *S.* Typhi replication in mouse macrophages and mouse tissues, thus overcoming to a significant extent the host-restriction barrier that prevents these bacteria from replicating in this nonpermissive host. GtgE turned out to be an effector of one of the *S.* Typhimurium type III secretion systems, which are specialized nanomachines that deliver bacterial proteins into mammalian cells to modulate cellular functions for the pathogen's benefit (51, 52). GtgE is a specific protease for Rab29, Rab32, and Rab38, which explains its ability to block the recruitment of these GTPases to the *Salmonella*-containing vacuole (33, 49). Further studies demonstrated that, of the three GTPases targeted by GtgE, Rab32 plays the most important role in the pathogen-restriction pathway because *S.* Typhi was able to efficiently replicate in cells and tissues of mice deficient in Rab32 or in its exchange factor BLOC3 (50). Therefore, a Rab32-dependent host-defense mechanism is what prevents *S.* Typhi replication in hosts other than humans (Fig. 5). Immune cells are generally viewed as the central element in host defense mechanisms. However, the first line of defense against microbial infection, and evolutionarily the oldest, is composed of cell-autonomous mechanisms that protect individual cells against pathogen invasion (53, 54). These mechanisms synergize with the immune system to confer whole-body protection against pathogens. Rab32 therefore defines a novel cell-autonomous defense mechanism that restricts *S.* Typhi replication in nonhuman hosts most likely by orchestrating the delivery of an antimicrobial factor to the *S.* Typhi-containing vacuole (Fig. 5). It is well-established that, in specialized cells, Rab32, in conjunction with other Rab GTPases and multiprotein assemblies known as BLOC 1, 2, and 3, coordinates the delivery of specific cargo to lysosome-related compartments such as melanosomes and T-cell granules (55–58). This cargo includes antimicrobial compounds as well as enzymes required for the synthesis of melanin and its phenyl

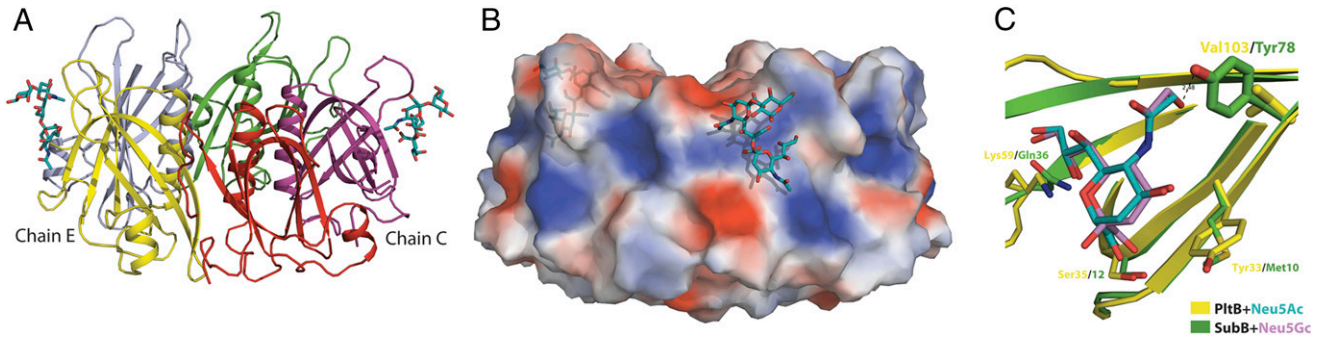

Fig. 4. Atomic structure of typhoid toxin B subunit bound to its glycan receptor. (*A*) The atomic structure of the PltB pentamer in complex with the GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glc oligosaccharide is shown as a ribbon diagram with each protomer depicted in a different color. Cyan, blue, and red sticks represent the sugar carbon, nitrogen, and oxygen atoms, respectively. (*B*) Surface charge distribution of the PltB pentamer structure and sugar-binding pockets. (*C*) Comparison of the sugar-binding sites of PltB and SubB bound to Neu5Ac and Neu5Gc, respectively. Critical residues that differ between SubB (Tyr78) and PltB (Val103) are highlighted as sticks. Other interacting amino acids and sugars are shown in lines. PltB is shown in yellow, Neu5Ac in Cyan, SubB in Green, and Neu5Gc in light purple (adapted from ref. 43 with permission from Elsevier; www.sciencedirect.com/science/journal/00928674).

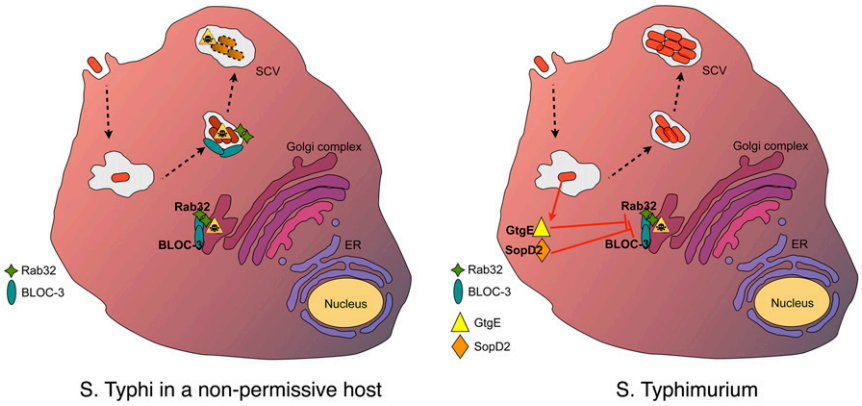

Fig. 5. Model for the Rab32/BLOC-3–dependent cell-autonomous defense pathway that restricts the growth of *S.* Typhi in nonhuman hosts and the mechanisms evolved by the broad-host *S.* Typhimurium to counter it. In nonpermissive hosts, Rab32 (and its exchange factor BLOC3) coordinate the delivery of an antimicrobial factor to the *Salmonella* Typhi-containing vacuole. The broad host *S.* Typhimurium counters this host-defense pathway by delivering two type III secretion effector proteins, GtgE and SopD2, which exert their function as specific protease and GAP for Rab32, respectively (adapted from ref. 50 with permission from Elsevier; www.sciencedirect.com/science/journal/19313128).

oxidase intermediates, many of which have potent antimicrobial activity (56, 58–61). The nature of the antimicrobial factor or factors that limit *Salmonella* replication in nonspecialized cells, however, is still unknown, and, although cells other than melanocytes do not make melanine, the machinery that orchestrates its synthesis is largely present in most cells. It should be noted that, in insects, this pathway has been shown to play a central role in the defense response against microbial pathogens (62, 63).

In addition to GtgE, broad host *Salmonellae* such as *S.* Typhimurium use a second effector protein, SopD2, that also counters the Rab32-dependent host defense pathway (50). SopD2, however, does not have protease activity, but it inactivates Rab32 functioning as GAP for this GTPase. An *S.* Typhimurium mutant strain simultaneously lacking *gtgE* and *sopD2* has a drastic virulence phenotype. C57BL/6 mice receiving ∼1,000 WT LD50 of the *ΔgtgE ΔsopD2 S.* Typhimurium mutant strain were completely refractory to infection, showing no symptoms and rapidly clearing the infection. The virulence defect of the *ΔgtgE ΔsopD2 S.* Typhimurium mutant strain was completely reversed in C57BL/6 mice deficient for BLOC3 or Rab32, demonstrating that these two effector proteins specifically target this host defense pathway (50). Taken together, these observations highlight the importance of the Rab32-dependent host defense pathway in the control of an intracellular vacuolar pathogen. Furthermore, because both *gtgE* and *sopD2* are either absent (*gtgE*) or a pseudogene (*sopD2*) in *S.* Typhi (15), these observations also explain the inability of this human-specific pathogen to explore other hosts. The absence of these effectors from *S.* Typhi would suggest that the Rab32-dependent restriction pathway is not functional in humans. However, the components of this pathogen-restriction pathway are highly conserved, making this hypothesis highly unlikely. Instead, these findings suggest that, in humans, *S.* Typhi must be located within cells different from those occupied by *S.* Typhimurium in mice and that, in those cells, this pathway may not be operational. In fact, a recent genome-association study has identified a polymorphism within Rab32 that correlates with increased susceptibility to the human-specific pathogen *Mycobacterium leprae* (64), suggesting that this pathway is operational in humans and is also important for the control of other intracellular vacuolar pathogens.

Opportunities for Novel Therapeutic and Prevention Strategies

The discovery of typhoid toxin and its role in the pathogenesis of typhoid fever has provided unique opportunities for the development of novel and sorely needed diagnostic, therapeutic, and prevention strategies to combat typhoid fever. The rather unique distribution of typhoid toxin largely restricted to *S.* Typhi and *S.* Paratyphi, coupled to its strong immunogenicity, suggests that it could serve as the basis for the development of much needed, cost-effective serological tests that could help not only in the diagnosis of typhoid fever but also in the more reliable study of its incidence and epidemiology. The potential link of typhoid toxin to the life-threatening symptomatology of the acute phase of typhoid fever raises the possibility of developing antitoxin therapeutic strategies that, in combination with standard antimicrobials, could significantly improve the outcome of typhoid fever treatment. Finally, given the good track record of other bacterial toxoids as immunogens (e.g., tetanus and diphtheria toxoids) and the demonstrated immunogenicity of typhoid toxin, its incorporation into vaccine formulations may lead to a much-needed effective vaccine not only against typhoid fever caused by *S.* Typhi but also against *S.* Paratyphi A, against which no vaccines are currently available. It is yet unclear whether typhoid toxin contributes to the infectivity of *S.* Typhi, and human volunteer studies will be necessary to address this issue. However, the linkage of typhoid toxin to the development of typhoid fever symptoms suggests that, at the very least, a strong antitoxin immunity should be able to protect against the disease.

Concluding Remarks

For over a century, scientists have wondered why *S.* Typhi and *S.* Paratyphi infections had such unique symptomatology and pathogenesis. They have also wondered why these pathogens can cause disease only in humans. The discovery of typhoid toxin has provided major insight into both of these fundamental questions. Furthermore, it may have provided the long sought "Achilles heel" of *Salmonella* Typhi, which might lead to the eventual eradication of typhoid fever. Studies that started as a detour from the main focus of my laboratory have provided exciting and productive years of research. I hope that these discoveries will one day fulfill their promise and contribute to the eradication of typhoid fever.

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