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Review

Cholera toxin structure, gene regulation and pathophysiological and immunological aspects

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Abstract. Many notions regarding the function, structure and regulation of cholera toxin expression have remained essentially unaltered in the last 15 years. At the same time, recent findings have generated additional perspectives. For example, the cholera toxin genes are now known to be carried by a non-lytic bacteriophage, a previously unsuspected condition. Understanding of how the expression of cholera toxin genes is controlled by the bacterium at the molecular level has advanced significantly and relationships with cell-density-associated (quorum-sensing) responses have recently been discovered. Regarding the cell intoxication process, the mode of entry and intracellular transport of cholera toxin are becoming clearer. In the immunological field, the strong oral immunogenicity of the non-toxic B subunit of cholera toxin (CTB) has been exploited in the development of a now widely licensed oral cholera vaccine. Additionally, CTB has been shown to induce tolerance against co-administered (linked) foreign antigens in some autoimmune and allergic diseases.

Keywords. Cholera toxin phage, GM1 binding site, virulence gene regulation, toxin secretion, intracellular toxin traffic, immunotolerance induction, allergy treatment, autoimmune disease, oral cholera vaccine.

Toxin structure

Cholera toxin (CT) is produced by *Vibrio cholerae*. This organism was originally discovered as the causal agent of cholera by the Italian Filippo Pacini in 1854 and then rediscovered about 30 years later by Robert Koch. Although CT is produced almost exclusively by *V. cholerae* of few serogroups, recent research has shown that in some instances, the toxin may be naturally produced by other organisms, for example by the opportunistic pathogen *V. mimicus* by virtue of the horizontal acquisition of the relevant genetic information.

The existence of CT was first postulated by Robert Koch in 1886, who proposed that the symptoms caused by *V. cholerae* could be due to some 'poison' produced by the organism. This insightful proposition was confirmed by S. N. De in Calcutta in 1959 [1], who proved that cell-free extracts from *V. cholerae* cultures could induce fluid accumulation in rabbits when instilled into ligated small intestinal 'loops'. Later, evidence was provided for the presence of a toxic protein product in *V. cholerae* cell-free supernatants [2]. This protein was eventually named CT. Soon after these reports, several groups initiated biochemical

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characterization of the toxin and in 1969 CT from the hypertoxigenic *V. cholerae* Classical 01 Inaba strain 569B was purified and shown to be an 84-kDa protein [3]. The toxin was initially thought to consist of only one type of subunit that could form aggregates of various sizes [4], but this picture was rapidly changed when Lönnroth and Holmgren [5] using SDS-PAGE convincingly demonstrated for the first time that CT was a heterogeneous protein made up of two types of subunit: a large one with an estimated MW of approximately 28 kDa and several small ones with estimated MWs of 8–10 kDa each and an aggregate size of ca 56 kDa. The two types of subunit, designated H (for heavy) and L (for light) [5], completely lacked toxic activity on cells when separated from each other by dissociation at low pH but regained such activity when re-associated by neutralization [5,6]. The binding of CT to ganglioside GM1, which was separately shown to be the receptor for CT [7,8], was also demonstrated and shown to be mediated by the so-called choleragenoid toxoid, which was made up of L subunits [5]. The L subunits were therefore deduced to be responsible for cell-binding and the H subunit for the toxic activity of CT. In addition, it was shown that upon reduction, the H subunit could be further separated into two components [9] i.e. the now designated CTA1 and CTA2 fragments of CTA (see below).

Cholera is caused by the action of CT, which is secreted by *V. cholerae*. There is, however, another similar diarrhea caused by the closely related heat-labile enterotoxin (LT) that is produced by enterotoxigenic *Escherichia coli* (ETEC). CT and LT not only have a high degree of amino acid and nucleotide sequence identity (of the order of 80%) but they also have a very similar three-dimensional structure. In fact, the crystal structure that first became available was that for LT and not for CT, and it was often assumed that many details of the LT structure should apply also to CT. With the crystal structure of CT now available, most of those assumptions have proven entirely valid; however, there are some relatively minor structural and functional differences between the two toxins.

The pathogenic effects of CT and LT are very similar in that both cause secretory diarrheas from the upper part of the small intestine; however, cholera is generally more severe than ETEC diarrhea. Other distinctive differences between CT and LT are: a) CT is encoded on a chromosomally located prophage, whereas LT is usually plasmid encoded and not phage associated; b) CT is highly efficiently secreted by *V. cholerae*, whereas LT is very poorly secreted by ETEC; c) the A subunit of CT (CTA) is proteolytically cleaved by a *V. cholerae* protease into CTA1 and CTA2 fragments while the A subunit of LT (LTA) is excised into the analogous LTA1 and LTA2 by intestinal trypsin; d) CT, via its B subunit (CTB), binds almost exclusively to the membrane-bound ganglioside GM1, while the B subunit of LT (LTB), besides binding to GM1, can also bind to GD1b and to other carbohydrate residues present in intestinal glycoproteins.

In the assembled CT (Fig. 1A), the single toxic-active A subunit (CTA, Fig. 1B) is embedded in a circular B subunit homopentamer (CTB pentamer, Fig. 1C) responsible for toxin binding to cells. CTA comprises 240 amino acids and has a molecular weight of 28 kDa, whereas the 11.6-kDa B subunit monomers each comprise 103 amino acids. Although CTA is synthesized as a single polypeptide chain, it is post-translationally modified through the action of a *V. cholerae* protease that generates two fragments, CTA1 and CTA2, which remain linked by a disulfide bond. The enzymatic ADP-ribosylating activity of CTA resides in CTA1, whereas CTA2 serves to insert CTA into the CTB pentamer.

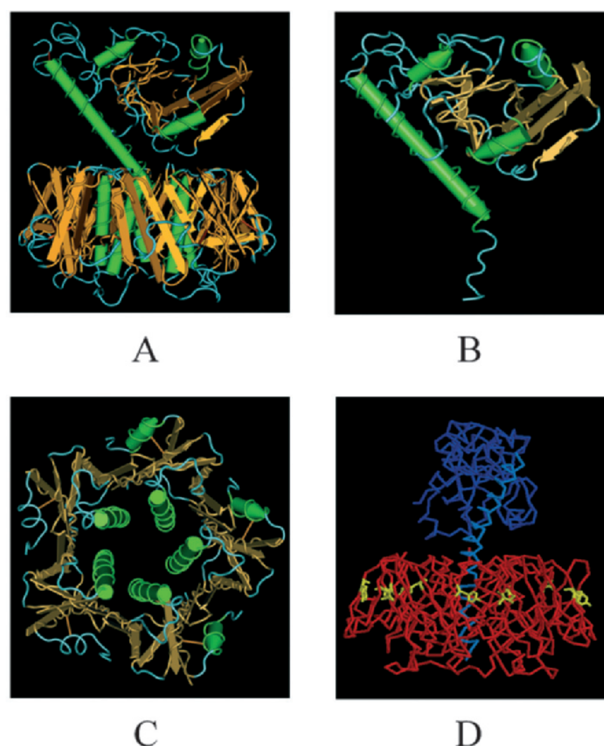

Figure 1. Crystalographic structure of cholera toxin and its A and B subunit. See text for details.

The CTB pentamer is held together both by hydrogen bonds and by salt bridges. In the refined crystal structure of LT [10], the total number of hydrogen bonds between neighboring monomers could be 26,

while the number of salt bridges could be four. Thus, considering the high sequence and structural similarity between LT and CT, the B subunits in the CTB pentamer likely are held together by a similar number of interactions, i.e. around 130 hydrogen bonds and 20 salt bridges. All these polar bonds together with tight packing of subunits via hydrophobic interactions would be responsible for the outstanding stability of pentameric CTB, a quaternary protein complex that unless it is also being boiled will remain associated during electrophoresis despite the presence of SDS and reducing agents. Extensive interactions between monomers could also explain the high resistance of the CTB pentamer to momomerization by acidification, a process that usually requires lowering the pH to values below 3. Very high stability during purification and other *in vitro* manipulations of CTB could in addition be due to pentamer-pentamer interactions. It has been demonstrated that in the absence of GM1, the imidazol group of histidine 13 in CTB establishes a reciprocal interaction between pairs of monomers in adjacent pentamers [11]. Although this histidine-mediated contact between pentamers was determined in two mutant CTBs, and thus has not been formally demonstrated in wild-type CTB, it is possible that an analogous interaction occurs in regular CTB pentamers and is perhaps related to the affinity of CT and CTB for nickel and other ions [12].

In vivo, the CTB pentamer attaches CT to the intestinal epithelial cell through its high-affinity binding to cell surface receptors identified as the monosialoganglioside GM1 [6, 13]. GM1 is present in many cell types, and CT can be demonstrated to bind to (and intoxicate) different types of cells experimentally. It should be noticed, however, that in non-synchronized cultures, not all cells will bind and internalize CT because GM1 expression on the cell surface is a cell-cycle-dependent process with preferential binding in G0/G1 [14].

The interactions between CTA (specifically CTA2) and the CTB pentamer are non-covalent, and the last four amino acids (lysine-aspartate-glutamate-leucine; KDEL) at the carboxy terminal of CTA2 protrude from the associated toxin and are basically not engaged in interactions with the pentamer. Taking the LT crystal structure as a reference [10], in the CTB pentamer, many of the amino acid residues that point toward the interior of the pore would be charged, some negatively and others positively. Charge neutralization calculations identify an excess of positive charges inside the pore. Some of these 'free' positive charges in the CTB pentamer pore are believed to interact with negatively charged residues in CTA2.

Genetics and regulation

There are more than 140 *V. cholerae* serogroups and among them only a few produce cholera toxin and cause disease. In fact, the overwhelming majority of clinical cases have been found to be due to infection by organisms belonging to only two serogroups: serogroup 01, and serogroup O139. Based on biological properties, members of serogroup 01 can be further sub-divided into the so-called El Tor and Classical biotypes. Typically the El Tor biotype is characterized by a positive Voges-Proskauer reaction (acetoin production), agglutination of chicken erythrocytes, resistance to polymixin B (50 U/ml) and production of toxin only under specific culturing conditions. The Classical biotype is characterized by a negative Voges-Proskauer test, no chicken erythrocyte hemagglutination, sensitivity to polymixin B and production of toxin under much less stringent *in vitro* culturing conditions. Interestingly, the El Tor and Classical biotypes also differ in the type of CT produced. Although the A subunits of the El Tor and Classical CT are identical in amino acid sequence, their B subunits have remarkably consistent biotype-specific amino acid substitutions at positions 18 and 47. Therefore, Tyr18 and Ile47 are typical of the El Tor biotype while His18 and Thr47 are typical of the Classical biotype. In Figure 1D, these two positions have been highlighted to show that both amino acids have their side chains exposed; these residues can be presumed to be part of the epitopes that determine the specificity of the recently described biotype-specific anti-cholera toxin monoclonal antibodies (mAbs) [15]. It should be noted that these residues do not take part in binding to GM1 and thus are unlikely to influence affinity for the receptor. This would agree with the known similar toxic activity of the El Tor and Classical toxins.

Whether vibrios produce CT (or, more precisely CTB) of the El Tor or Classical type has recently been found to relate to the presence of a phage that differs between the two *V. cholerae* biotypes [15]. For years the presence of this cholera phage was not obvious; however, constant genetic regions upstream and downstream of the CT-encoding operon (*ctxAB*) had been noticed early on and for a time the entire genetic unit was known as the 'cholera toxin cassette.' This 'cassette' was later identified as a prophage that could form filamentous non-lytic particles; this filamentous phage was denominated CTXΦ [16]. CTXΦ may be a special kind of filamentous phage because besides being able to produce viral particles it can either integrate into the *V. cholerae* chromosome(s) or replicate as a plasmid, while no other filamentous phage is known to form plasmids. Furthermore, there

seems to be a biotype-specific expression of phage particles, with El Tor strains being able to produce it while the Classical strains do not [17].

CTXΦ, as is the case with other bacteriophages, requires a receptor for attachment and transmission, and the receptor on *V. cholerae* for CTXΦ has been identified as the toxin co-regulated pilus (TCP), a pilus of approximately 8 nm in diameter and 1–4 mm in length, which is composed of some 1000 interwoven TcpA subunits forming a three-stranded braid [18]. Interestingly, other forms of ctxAB transmission by a different O139 *V. cholerae* filamentous phage, designated VGJΦ, have been proposed [19]. The VGJΦ phage apparently uses a different pilus, the mannose-sensitive hemagglutinin (MSHA), as a receptor, which might transmit CTXΦ, or its satellite phage RS1 (see below), between *V. cholerae* hosts by an efficient TCP-independent mechanism. Therefore the possibility remains that strains may not have to express TCP in order to acquire CTXΦ containing *ctxAB* to become toxigenic.

The *tcp* operon encoding TCP resides separately from the CTXΦ in the large *V. cholerae* chromosome (see below) in the so-called vibrio pathogenicity island (VPI). VPI might be a horizontally acquired foreign genetic element because its G+C content is significantly lower (35.6%) than for the rest of the *V. cholerae* genome (47.3%). Moreover, VPI has been proposed to also be a filamentous bacteriophage [20]; however, it has been difficult to confirm the existence of VPI-containing phages [21]. Notably, VPI codes for a regulatory protein (ToxT) that can directly activate transcription of both the *ctxAB* (Fig. 2) and the *tcp* operons in a coordinated manner. Because *toxT* is located within the VPI, once the ToxT protein is produced, it can activate its own expression through a positive-feedback loop [22]. ToxT is an AraC/XylS-type of transcriptional regulator and its expression is extensively regulated in *V. cholerae.* The membrane-localized transcriptional activators, ToxR/ToxS and TcpP/TcpH, are required to activate transcription of the *toxT* gene by binding to a region upstream of the *toxT* promoter. The role of TcpH, the companion of TcpP, is to prevent the degradation of TcpP [23]. Part of the regulation mediated by ToxT depends on the regulator ToxR, and this protein is encoded in the large chromosome outside the VPI and CTXΦ. Like TcpP, ToxR also exists as a complex with a companion protein, ToxS, but ToxS might operate differently to TcpH and serve only to stabilize ToxR in an active, dimeric form.

The ArcA protein, which controls the expression of a large number of anoxia-responsive bacterial genes, has been proposed to function as a positive regulator of *toxT* expression under both aerobic and anaerobic conditions, but in a ToxR- and TcpP-independent manner [24]. Besides ArcA other regulators that may modulate expression of *ctxAB* during infection have been proposed, for example, the *vieSAB* three-component signal transduction system, whose role may be to enhance CT expression via ToxT and, as the ArcA regulator, also in a ToxR- and TcpP-independent manner [25]. The VieSAB system apparently operates by means of di-cyclic-GMP with the VieA protein acting as a cyclic diguanylate phosphodiesterase [26]. Expression of the *tcpPH* operon is subject to regulation by a pair of transcriptional activators (Fig. 2), namely AphA and AphB. AphA has been shown to be a winged-helix-type [27] transcription activator that cooperates with the LysR-type regulator AphB at the *tcpPH* promoter [28]. There may be negative regulation of *tcpPH* expression by the cyclic AMP receptor protein, CRP [29], and by the protein PepA that is involved in responses to external pH [30].

Expression of AphA is regulated by the HapR protein, which is controlled by the *V. cholerae* quorum-sensing system [31]. At a high cell density, the quorum-sensing system decreases intracellular AphA levels and this lowers CT synthesis, while at low cell densities, AphA levels increase and there is expression of CT. At low cell density, the quorum-sensing signals CAI-1 and AI-2 trigger a phosphorelay that results in phosphorylation of the regulator LuxO. Phosphorylated LuxO activates the expression of several small regulatory RNAs that in conjunction with the RNA-binding protein Hfq destabilize the HapR message. Since HapR represses *aphA* expression, destabilization of its message allows AphA levels to remain high and this results in expression of CT (Fig. 2). At higher cell densities, LuxO becomes dephosphorylated and fails to activate the expression of the small RNAs. The absence of the destabilizing regulatory RNAs allows the *hapR* message to accumulate and HapR is produced, which then represses *aphA* to cause a reduction in CT expression (Fig. 2). The regulatory system described above may allow *V. cholerae* bacteria to express CT (and TCP) in a tightly controlled spatio-temporal manner within the gut, a suggestion supported both by *in vivo* experiments in mice [32] and by our previous findings of a time-related transcriptional activation of *toxT* during *in vitro* culture [33]. Part of this response might be a result of exposure to an oxygen-poor environment in the intestine. Microarray experiments of gene expression patterns in cholera stool-derived vibrios have shown increased expression of an assortment of genes, consistent with bacterial growth in an oxygen- and iron-limited host compartment, and at the same time both the *tcp* and *ctxAB* operons have been found to be either repressed [34] or expressed at low levels [35].

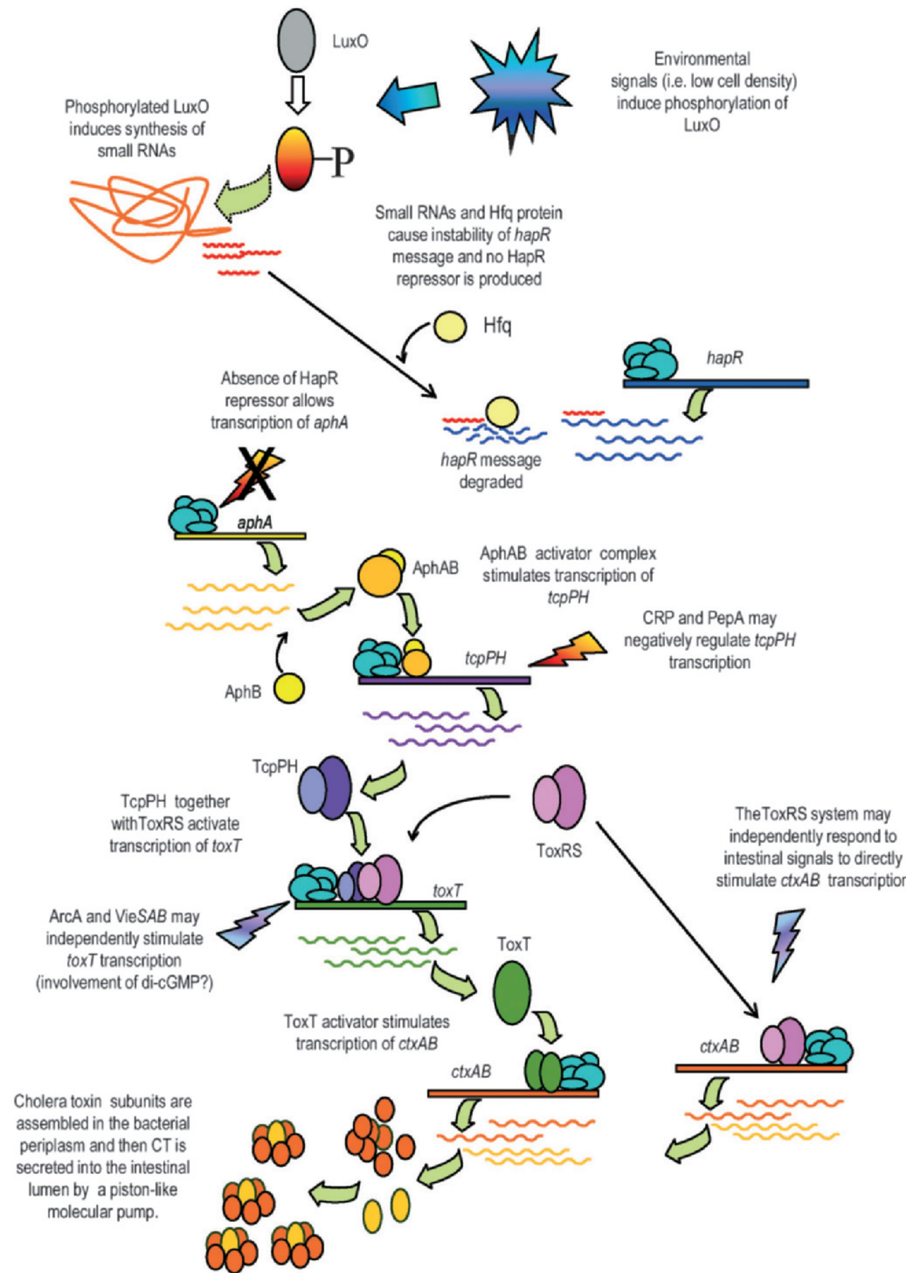

Figure 2. Diagrammatic representation of cholera toxin gene regulation. The figure is self-explanatory, please refer to the text for complementary information.

However, these results are not against the notion that TCP and CT are produced during infection; rather, they indicate that at the high (presumably maximal) bacterial densities that exist at the time of fluid purging, the LuxO-HapR system will have been triggered, resulting in a down-regulation of *ctxAB* and *tcpA*. Suppression of this type would be coherent with our earlier experimental evidence showing a lack of CT induction upon growth of vibrios in sterile cholera-stool-derived fluid, which suggested that repression of *ctxAB* expression had occurred in the intestine [36]. Prior to the described down-regulation, the intestinal environment might instead stimulate virulence gene expression. Thus, it has been reported that bile acids can induce a ToxT-independent but ToxR-driven transcriptional activation of *ctxAB* [37]. Taken together, the referred experiments demonstrate that despite with inherent limitations, *in vitro* models may be useful to identify specific host signals inducing or repressing virulence expression in *V. cholerae.* In keeping with this notion, a novel culturing system based on shallow static cultures has been proposed to study virulence regulation *in vitro* [38]. The proposed method uses identical growth conditions for both El Tor and Classical strains to induce expression of virulence genes at 37 °C and thus it

avoids the need to grow Classical strains at lower temperatures (commonly 30 °C) or the use of bi-phasic cultures for El Tor strains; hence, the method seems just right for biotype-specific *V. cholerae* gene expression studies [39].

As mentioned above, the *ctxAB* genes encoding CT are contained in the CTXΦ phage. However, toxigenic *V. cholerae* strains that hold the bacteriophage in their genome are lysogens, that is, they do not produce infectious CTXΦ particles. The prototype *V. cholerae* genome is made up of two chromosomes: a large one of approximately 3 Mb (2961 kb in the El Tor strain N16961) and a small one of around 1 Mb (1072 kb in strain N16961). The CTXΦ prophage usually inserts at a specific site (*att)* near the replication terminus of the large chromosome; however, alternative integration into the small chromosome has been documented [40].

The CTXΦ genome consists of a core region (4.5 kb) and an RS2 region (2.4 kb); the core region encodes CT and proteins that are required for viral morphogenesis, while the RS2 region encodes the regulation (RstR), replication (RstA) and integration (RstB) functions of the CTXΦ genome [41]. The CTXΦ prophage is often flanked by a genetic element known as RS1. Remarkably, the RS1 DNA can also be packaged into filamentous phage particles (designated RS1Φ) by using the CTXΦ-encoded morphogenesis proteins [42, 43]. RS1Φ is a satellite phage that can control expression and dissemination of CTXΦ. This is so because RS1 encodes RstC, an anti-repressor that controls CTXΦ lysogeny and thus the production of CTXΦ particles. RstC might also increase production of CT by read-through transcription, as transcripts initiating at a derepressed *rstA* promoter could extend through *ctxAB*, which lies downstream [42].

When RS1, RS2 and the core region of El Tor and Classical *V. cholerae* strains are compared one finds that sequences are not identical. Therefore, El Tor and Classical strains carry different CTXΦ phages and these two types of CTXΦ are often discerned through the sequence of the phage regulatory protein RstR [44]. In addition, the El Tor and Classical phages have amino acid substitutions at positions 18 and 47 in their CTB subunits. Therefore, when vibrios hold a Classical CTXΦ, they produce Classical-type CTB whereas if they hold the El Tor CTXΦ they produce El Tor-type CTB.

The genes encoding CT are likely a dispensable 'passenger' in CTXΦ because closely related organisms such as *V. mimicus* may hold an 'empty' CTXΦ lacking the *ctxAB* genes [45]. Similarly, there are the so-called pre-CTX prophages in environmental *V. cholerae* strains that do not hold *ctxAB* [46]. This invites the suggestion that other passenger gene(s), whether involved in pathogenesis or not, could take the place of *ctxAB* and be similarly integrated into the bacterial genome through CTXΦ infection.

Toxin secretion

CT is secreted by *V. cholerae* and is transported across the outer membrane by the so-called type II secretion system. The type II secretion system serves to export toxin and other proteins such as extracellular enzymes and it may comprise 15 gene products, 12 of which are required for translocation of specific substrates, including CT and hemagglutinin, across the outer membrane [47]. In *V. cholerae*, the CT secretion system (named Eps, for extracellular protein secretion) contains pseudopilins that may form a pilus to extrude substrates to the extracellular space via a pore in the outer membrane (EpsD) using a mechanism analogous to a piston [47]. Energy for secretion likely comes from EpsE, a cytoplasmic ATPase. The activity of these secretory proteins may be diverse; for example, the EpsD secretin from *V. cholerae* is required both for type II secretion and for extrusion of CTXΦ [48]. The ATPase EpsE forms a trimolecular complex together with other proteins of the secretion system (EpsL and EpsM) at the cytoplasmic membrane, and this complex could serve for the secretion apparatus to transduce energy across the periplasmic compartment through protein contacts [48]. It has been suggested that EpsE could acquire two conformational states; a monomeric one with low catalytic activity and an oligomeric status with higher ATPase activity. The conversion between these two states may be directed via the interaction between domains in EpsL and EpsE and may be involved in reponses to the local membrane environment [48].

CT is secreted from *V. cholerae* after its assembly in the periplasm [49, 50] and it has been shown that both whole CT and the pentameric CTB can be secreted; however, there could be a mechanism to ensure exit of fully assembled toxin with A subunit incorporated into the pentamer and no wasteful secretion of empty pentamer given that *in vivo* pentamer formation is aided by the presence of CTA subunits [50], suggesting that the A subunit may act as a nucleation center for holotoxin assembly in the periplasm. Enhanced assembly mediated by the A subunit would favor secretion of holotoxin. Moreover, A subunits in the absence of CTB are not transported across the *V. cholerae* external membrane [49] and unincorporated A subunits could be prone to degradation, in analogy to protein hybrids derived from LTA in *E. coli* [51].

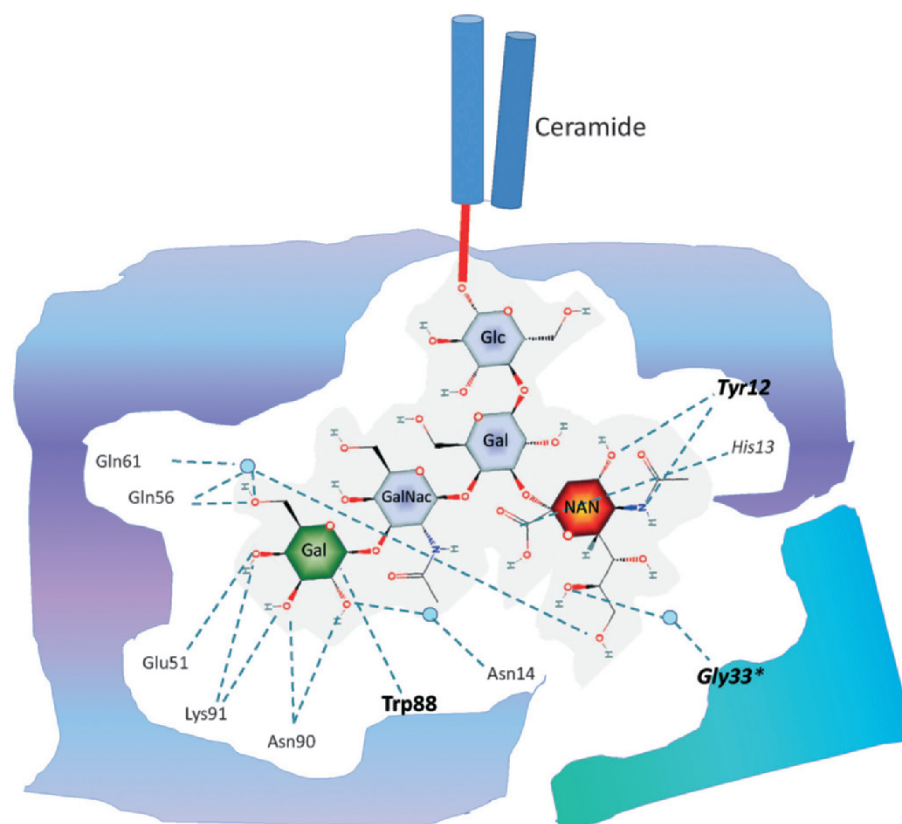

Figure 3. Cholera toxin B subunit GM1 receptor-binding pocket. The pentasaccharide structure of the GM1 receptor is shown and residues in green and red are those that establish direct interactions with the B subunit, either directly or via the solvent (small spheres). Interactions, mostly hydrogen bonds, are depicted by a broken line. Indispensable residues for binding are in bold and larger font. The asterisk denotes the amino acid residue that comes from the adjacent subunit (see text). All indicated interactions involve side chains of amino acids except for those shown in italics. Gal, galactose; GalNac, N-acetylglucosamine; NAN, N-acetylneuraminic acid; Glc, Glucose. Longitude of broken lines is not meant to depict real atomic distances and the relative locations of amino acids are merely diagrammatic.

Pathogenic events: toxin binding, intracellular transport, ADP ribosylation and diarrhea

CT is released from *V. cholerae* cells in a very efficient manner, and more than 90% of the toxin is usually found extracellularly and in a soluble form [49]. Once in the intestinal lumen, CT initiates its toxic action on cells by binding with high affinity and exquisite specificity to cell membrane receptors, which were identified more than 30 years ago as the monosialoganglioside GM1: [Gal(β1–3)GalNac(β1–4)(NeuAc(α2–3)Gal(β1–4)Glc]→ceramide. Both the specific sugar residues in GM1 and the amino acid residues in CTB that interact with each other have been defined and based on data in Merritt et al. [52, 53] we diagrammatically represent those interactions (Fig. 3). Although there is one GM1-binding site in each B subunit monomer, a single amino acid (Gly33* in Fig. 3) from the neighboring CTB monomer also has a role in the binding [52], explaining the dramatically higher binding strength of the CTB pentamer compared with that of individual B subunit monomers. Critical residues for interaction with GM1 binding have been defined as Trp88, Gly33 (from adjacent monomer) and Tyr12 [54].
After binding to GM1, which appears to be localized mainly in lipid rafts on the cell surface, CT is endocytosed by the cell. For cell intoxication to occur, the A subunit (or, more specifically, CTA1) needs to be transported to the cytosol to induce the activity of adenylate cyclase (AC). A schematic summary for intracellular toxin transport is presented in Figure 4. The precise mode by which CTA1 reaches the cytosol is still not fully resolved. However, in the current model, CT or pentameric CTB may be endocytosed, depending on cell type, either through caveolin-coated vesicles, clathrin-coated vesicles, by the so-called Arf6 endocytic pathway and perhaps via a still-undefined fourth pathway [54–56]. After endocytosis, CT or the CTB pentamer travels to the endoplasmic reticulum (ER) via a retrograde transport pathway. This pathway was earlier reported to be Golgi dependent by Majoul et al. [57] but others have suggested that it may also exist in the absence of a functional Golgi system in Exo2-treated cells [58]. There is association of the CT-GM1 complex with the actin cytoskeleton via lipid rafts, and it is therefore thought that the actin cytoskeleton has a role in CT trafficking from the plasma membrane to the Golgi-ER [59]. After CT has reached the ER, CTA dissociates from CTB [57, 58].
The KDEL carboxy terminal of CTA2 is a classical eukaryotic signal for retention in the ER lumen and this sequence was initially thought to be crucial for

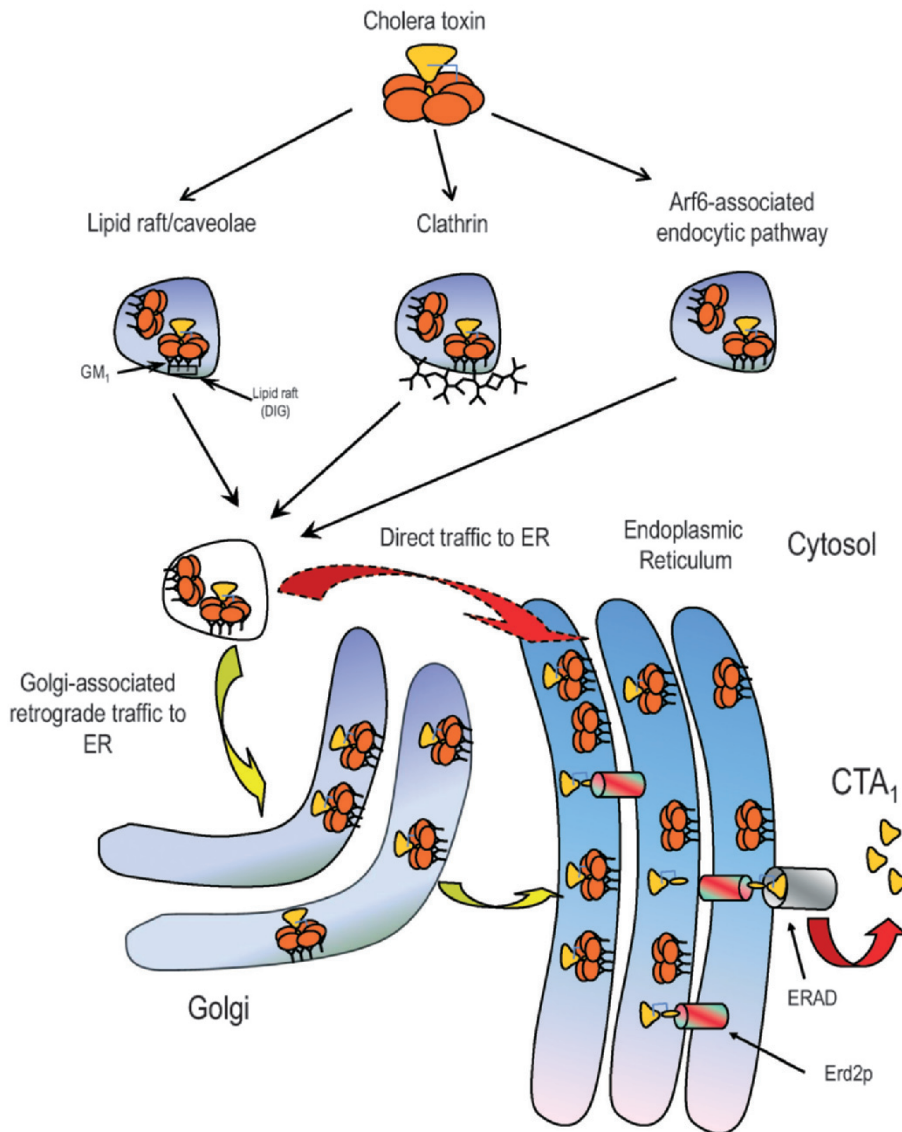

Figure 4. Cholera toxin intracellular traffic. The figure is self-explanatory. Please refer to the text for complementary information.

localization of the entire toxin to the ER but its mutagenesis or blocking does not prevent localization of CT to the ER. Moreover, CTB, which does not contain a KDEL sequence, is also transported in a retrograde manner to the ER [60]. It has therefore been concluded that the KDEL sequence serves to enhance retrieval of dissociated CTA from the Golgi apparatus to the ER, instead of being essential for retrograde transport. Similar to the endocytosis and retrograde transport of CT to the ER, the translocation of CTA1 to the cytosol also involves a natural recycling cellular process; this is the ER-associated degradation pathway, or degradasome, which retrieves misfolded proteins from the ER for their degradation in the cytosol [60, 61]. However, although transported by the degradasome, CTA1 apparently escapes proteolysis, presumably because of a low lysine content, which is the target for ubiquitinylation [61, 62]. To pass through the degradasome, CTA1 would have to undergo unfolding and refolding, a process possibly involving reduction by protein disulfide isomerase (PDI) followed by reoxidation by Ero1 [63].

The entry of CTA1 to the cell cytosol is the key step for intoxication because CTA1 catalyzes the ADP ribosylation of the trimeric Gsα component of AC. This enzymatic reaction is allosterically activated by the so-called ADP-ribosylation factors (ARFs), a family of essential and ubiquitous G proteins. Crystal structures of a CTA1/ARF6-GTP complex reveal that binding of the ARF activator elicits striking changes in CTA1 loop regions that allow the nicotinamide adenine dinucleotide (NAD+) substrate to bind to the active site [64]. Within CTA1, the A1–3 subdomain has been

shown to be important both for interaction with ARF6 and for full expression of enzymatic activity *in vivo*. The A1–3 subdomain was, however, not essential for degradasome-mediated passage of CTA1 from the ER to the cytosol [65].

After ADP-ribosylation by CT, the AC remains in its GTP-bound state, resulting in enhanced AC activity and an increased intracellular cAMP concentration. Higher levels of cAMP produce an imbalance in electrolyte movement in the epithelial cell, namely a decrease in sodium uptake together with an increase in anion extrusion, mostly chloride, by the cystic fibrosis trans-membrane conductance regulator (CFTR), which is seen as an attractive target to inhibit CT-induced diarrhea [66]. Decreased sodium uptake reduces water intake by the enterocyte, and, at the same time, augmented chloride and bicarbonate extrusion gives rise to sodium outflow, and thus water secretion; the combined effect produces vast fluid loss from the intestine in the order of 500–1000 ml/h [67], but in extreme cases fluid loss can be a colossal 30–40 l per day.

Besides the direct effect of CT on AC activity and cAMP production in enterocytes, it has been proposed that the diarrheal response to CT might have a significant (perhaps up to 50%) neurological component [68].

Experimental evidence for the involvement of the enteric nervous system in the pathophysiology of cholera has been obtained mainly *in vivo* and on extrinsically denervated pharmacologically nerve-blocked intestinal segments of cats and rats. It is thought that CT stimulates enterochromaffin cells to release serotonin; in turn, serotonin would promote the release of the secretagogue vasointestinal peptide from intestinal neural networks [68].

CT and immunomodulation

In recent years, the immunological properties of CT and LT have attracted a great deal of attention. Both CT and LT are exceptionally potent oral-mucosal immunogens and they have also been found to be strong adjuvants for many coadministered antigens. These properties may be explained by three main characteristics of the CT and LT molecules. First, consistent with their functions as potent enterotoxins, these proteins are remarkably stable to proteases, bile salts and other compounds in the intestine. Secondly, as discussed above, both CT and LT also bind with high affinity via their B subunits to GM1 ganglioside receptors, which are present on most mammalian cells including not only epithelial cells, such as the 'M cells' covering the Peyer's patches, but also all known antigen-presenting cells (APCs); this facilitates the uptake and presentation of the toxins to the gut mucosal immune system. Thirdly, CT and LT have strong inherent adjuvant and immunomodulating activities, properties that depend both on their cell-binding and, residing in the A subunit, their enzymic ADP-ribosylating activity.

The CTB whole-cell oral cholera vaccine

The toxicity of CT has precluded its use for human vaccination. Instead, non-toxic CTB has been extensively used without any side effects as a mucosal immunogen in humans. Indeed, recombinantly produced CTB [69] is an important component of an oral cholera vaccine for human use. In addition to CTB, this vaccine also contains inactivated whole-cell cholera vibrios and is now being registered (Dukoral®) in more than 50 countries worldwide [70]. The vaccine has proved to be very safe and efficiently immunogenic in both adults and children. Excellent safety with only few and very mild adverse reactions has been documented both in many clinical phase 1, phase 2 and phase 3 studies and in post-license follow-up analyses in countries with well-functioning systems for monitoring and reporting adverse reactions where more than 10 million doses have been given. The efficient immunogenicity of the oral CTB whole-cell cholera vaccine has also been manifested in many clinical studies in different populations and age groups. When given orally in two or three doses, the vaccine has been found to stimulate the same levels of intestinal IgA anti-toxin and anti-bacterial (mainly anti-lipopolysaccharide) antibodies as seen in convalescents from severe clinical cholera disease as well as to induce very long lasting (more than 5 years) immunologic memory in the intestinal mucosa. A high protective efficacy of the vaccine has been demonstrated in three large phase 3 field trials in Bangladesh, Peru and Mozambique, being 85–90% for the first 6 months after vaccination in both endemic (Bangladesh and Mozambique) and non-endemic (Peru at the time of the study) populations, and remaining at or above 60% for another 2–3 years in adults and children above age 5 years. In children below age 5, the short-term efficacy, which is significantly mediated by locally produced IgA anti-toxic antibodies, was 100% for the first 6 months, but waned more rapidly than in older children and adults to be only 30% in the second year of follow-up. A large effectiveness trial undertaken in a high-endemic area of Mozambique showed that the oral CTB whole-cell cholera vaccine was safe and highly effective (80–90% protection) also when used as a public health intervention tool in a population with a high frequency of HIV-infected individuals [71].

Because of the close immunological relationship between CTB and LTB, the CTB whole-cell cholera vaccine in addition to protecting against cholera also has been found in several placebo-controlled trials to provide 60–80% short-term protection against diarrhea caused by LT-producing *E. coli* causing cholera-like diarrheal disease (ETEC diarrhea). ETEC diarrhea is the most common bacterial enteric infection in most developing countries and is also a common illness affecting 20–30% of all travelers to these countries, so the CTB-mediated protection against ETEC diarrhea mediated by the cross-reacting CTB component of the cholera vaccine is a significant extra benefit of cholera vaccination.

Based on its excellent safety and immunogenicity in humans when given by the oral route, the CTB-containing cholera vaccines as well as the isolated CTB component have often been used as model immunogens for studies of mucosal immune responses in humans, also after other mucosal routes of immunization. Indeed, much of our current knowledge of the localization of the mucosal immune responses after different routes of immunization and of the links between mucosal inductive and expression sites in humans has emerged from studies in volunteers using CTB as immunogen [reviewed in ref. 72].

CT and LT as mucosal adjuvants

Besides being strong mucosal immunogens, both CT and LT are powerful mucosal adjuvants. They strongly potentiate the immunogenicity of most other antigens, whether these are linked to or simply admixed with the toxins, provided that the other antigen is given at the same time and at the same mucosal surface as the toxins.

CT and LT can affect several steps in the induction of a mucosal immune response, which alone or in combination might explain their strong adjuvant action after oral immunization. Thus, CT has been found to a) induce increased permeability of the intestinal epithelium leading to enhanced uptake of coadministered antigens; b) induce enhanced antigen presentation by various APCs; c) promote isotype differentiation in B cells leading to increased IgA formation; d) exert complex stimulatory as well as inhibitory effects on T cell proliferation and cytokine production. Related to this, in addition, both CT and LT have been shown to not only avoid inducing oral tolerance but also to abrogate otherwise efficient regimens for tolerance induction by oral antigen administration.

Among these many effects, those leading to enhanced antigen presentation by various APCs are probably of the greatest importance for the adjuvant activity. CT or LT markedly increase antigen presentation by dendritic cells, macrophages and B cells [73]. They have also been found, at least *in vitro*, to stimulate intestinal epithelial cells to become effective APCs. Consistent with this activity, CT/LT upregulates the expression of MHC/HLA-DR molecules, CD80/B7.1 and CD86/B7.2 costimulatory molecules, as well as chemokine receptors such as CCR7 and CXCR4 on both murine and human dendritic cells and other APCs. Importantly, CT/LT also induces the secretion of interleukin (IL)-1b from both dendritic cells and macrophages. IL-1 not only induces the maturation of dendritic cells, but is also by itself an efficient mucosal adjuvant when coadministered with protein antigens and might mediate a significant part of the adjuvant activity of CT [74].

To avoid the toxicity problems with whole CT or LT, the recombinantly produced CTB and LTB proteins have been explored for their ability to increase immune responses against co-administered antigens. However, their capacity as mucosal adjuvants has proved to be much less than that of the holotoxins. Indeed, both CTB and LTB are poor adjuvants when given to animals together with non-coupled antigens by the oral route, although they display a more significant adjuvant activity when administered via the nasal route. Adjuvanticity of CTB or LTB is much improved when coupled to antigens. This is due both to the increased uptake of the coupled antigen across the mucosal barrier and to the more efficient GM1-receptor-mediated uptake and presentation of the coupled antigen by APCs including dendritic cells and macrophages as well as naive B cells. Recently, various molecular engineering approaches have permitted the generation of various LT and CT A subunit mutants (Table 1), that are substantially reduced in, or in some cases practically devoid of, enterotoxic activity, but which retain detectable adjuvanticity when given to animals by a mucosal route.

Analogous site-directed mutagenesis has more recently been carried out in the functionally related LTII enterotoxin [84]. A different approach has been taken by Lycke [85], who instead of attenuating the A subunit made a gene fusion protein between fully active CTA1 and a *Staphylococcus aureus* protein A derivative named DD. The CTA1-DD fusion protein binds specifically to immunoglobulins on antigen-presenting B cells via the DD protein and induces ADP ribosylation by the CTA1 moiety. When given intranasally together with protein antigens, CTA1-DD substantially increases both mucosal and systemic immune responses. Yet another type of promising adjuvant protein was recently described by Adamsson et al. [86]. They coupled the well-known CpG oligonucleotide adjuvant to CTB and showed that the CpG-CTB conjugate had markedly increased activity in activating different APCs *in vitro* and in

Table 1. Mutant adjuvant-active enterotoxins with decreased enterotoxicity due to site-specific mutations in the A subunit.

Mutant name	Type of mutation in A subunit	LT/CT	Reference
LT-K63	change of Ser at position 63 for Lys	LT	75
E112K/KDEV	change of Glu at position 112 for Lys, and Leu at position 240 for Val	CT	76
E112K/KDGL	change of Glu at position 112 for Lys, and Asp at position 239 for Gly	CT	76
LT-R72	change of Ala at position 72 for Arg	LT	77
LT-G192	change of Arg at position 192 for Gly	LT	78, 79
6-CTA	Addition of 6 amino acids at position 1	CT	80
16-CTA	Addition of 16 amino acids at position 1	CT	80
23-CTA	Addition of 23 amino acids at position 1	CT	80
E112K	change of Glu at position 112 by Lys	CT	81
S61F	change of Ser at position 61 by Phe	CT	81
E29H	change of Glu at position 29 by His	CT	82
S63Y	change of Ser at position 63 by Tyr	LT	83
D110–112	Deletion of amino acids 110, 111 and 112	LT	83

stimulating both T cell and antibody responses *in vivo* [86]. It is notable, however, that the adjuvant activity of most of these different proteins is greater when given together with antigens by the nasal route than by the oral-mucosal route. It probably remains to be shown that a fully non-toxic LT or CT mutant molecule can serve as a useful adjuvant for increasing the gastrointestinal or other IgA antibody response to an orally or intragastrically coadministered protein antigen.

CTB for mucosal immunotherapy

Mucosal tolerance is a mechanism whereby the immune system upon encounter with harmless antigens through a mucosal surface develops means to avoid reacting in a deleterious manner to the same antigen even if the antigen is encountered by a systemic route. This permits mammals to coexist with their normal flora and to eat large amounts of foreign food proteins without inducing harmful systemic immune responses. Since induction of mucosal tolerance is antigen specific but can be expressed in a non-specific manner ('bystander suppression') via the production of suppressive cytokines by regulatory T cells in the inflamed microenvironment of the target organ; this approach has been utilized to suppress immune responses against self-antigens. It has been possible to prevent or to delay onset of experimental autoimmune diseases in a variety of animal systems by feeding selected autoantigens or peptide derivatives [reviewed in ref. 72].

While mucosal tolerance is usually effective in animal models for preventing inducible autoimmune diseases, its efficacy has been more variable and limited when utilized as an intervention strategy in animals in which the disease has already been induced or has developed spontaneously. This may explain in part the disappointing results of recent clinical trials of oral tolerance in patients with type I diabetes [87], multiple sclerosis [88], and rheumatoid arthritis [89], diseases in which there may be multiple target autoantigens that remain largely unknown. A significant improvement has been achieved by coadministering CTB as an immunomodulating agent to enhance the tolerogenic activity of autoantigens as well as allergens given orally or nasally. The use of antigen coupled to CTB has been found to minimize by several hundredfold the amount of antigen/tolerogen needed and also to reduce the number of doses that would otherwise be required by reported protocols of orally induced tolerization [90]. Furthermore, and most important, unlike the use of free antigen, CTB-linked antigens have been shown to work also in an already sensitized individual. In experimental systems this has resulted in effective suppression of various pathological immune responses associated with experimental autoimmune diseases [91–94], type I allergies [95, 96], and allograft rejection [97, 98], also when the CTB-antigen conjugate was administered as therapy rather than for prevention.

While there are many studies documenting the efficacy of mainly CTB but also LTB in inducing peripheral tolerance to coadministered cell antigens or allergens in animal systems, only recently was initial proof of principle demonstrated in humans. Thus, based on previous encouraging results in a rat model of heat-shock-protein-induced uveitis [99], a small phase 1/2 trial in patients with Behcet's disease (BD) was undertaken with very encouraging results [100]. BD is an autoimmune eye disease often associated with extraocular manifestations and abnormal T cell reactivity to a specific peptide ('BD peptide') within

the human 60-kD heat shock protein. Oral administration of CTB-BD peptide conjugate, three times weekly, had no adverse effects and enabled gradual withdrawal, without any relapse of uveitis, of existing treatment with immunosuppressive drugs in the majority of patients with BD.

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