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Escherichia coli STb Enterotoxin Toxicity and Internalization Investigations: A Mini-Review

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Abstract: STb toxin is produced by strains of enterotoxigenic *Escherichia coli* isolated from various animals including man. The molecule comprises 48 amino acids (M.W. of 5200 Da and a pI of 9.6) with 4 cysteines involved in the formation of 2 disulfide bridges. Studies on STb toxin have resulted in the elucidation of the role of sulfatide as the receptor. This molecule was confirmed as a functional receptor as alteration or blocking strategies reduced or abrogated the toxicity as determined in the rat ligated loop assay. A study using a chemical cross-linker indicated that hexamers and heptamers of STb were formed. Oligomer formation could be observed with intact toxin but was abrogated in presence of β-mercaptoethanol. A structure-function study of STb using point mutations on residues, as determined by nuclear magnetic resonance, known to point to the solvent indicated that, electrostatic and hydrophobic interactions are important for enterotoxicity and binding to sulfatide, respectively. Mutants unable to form oligomer also showed an important reduction in toxicity. An electrophysiological study using planar lipid bilayers technology indicated that ions channels were formed when STb was added. Recently, we conducted experiments on pig jejunal brush border membrane vesicles and observed permeabilization of this vesicular model. With electron microscopy techniques, we could observe internalization of STb toxin after administration into rat intestinal loops. Using a cellular model (NIH-3T3 fibroblasts) confocal microscopy and cell markers, we observed endocytosis of STb. Contrarily to native toxin, reduced and alkylated STb toxin was not internalized. Using the NIH-3T3 fibroblasts cell model, STb endocytosis was shown to be mediated by two endocytic pathways, one clathrin-dependent and the other caveolae-dependent. These data together indicate that STb, after recognition of its receptor, is internalized into susceptible cells.

Key words: *Escherichia coli*, STb enterotoxin, toxicity, internalization, endocytosis

INTRODUCTION

Escherichia coli STb enterotoxin is found in association with bacterial isolates from various animals including man (Dubreuil, 1997). This heat-stable toxin (30 min at 100°C) acts in the small intestine of these animals and result in secretory diarrhea. Water, Na^+, Cl^- and HCO_3^- are the electrolytes that have been found to be involved in the secretion process. This toxin stimulates a cyclic

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nucleotide-independent secretion, not altering cGMP or cAMP levels, in intestinal mucosal cells. For now, although numerous signaling molecules were related to the toxic action of STb, the precise secretory mechanism involved is not clear (Dubreuil, 1997). To better understand the effects of STb toxin on the intestinal mucosa, *in vivo* structure-function studies were conducted in rat ligated intestinal loops using purified STb mutant molecules (Labrie *et al*., 2001a). In addition, cellular and membranar models were set up and used to focus on the internalization and endocytic processes of STb toxin.

STb Toxin and Receptor

STb toxin has been observed in *E. coli* strains from swine, cattle, horses, dogs, cats, chickens, ferrets and humans. The mature toxin comprises 48 amino acids including four cysteines residues involved in the formation of two disulfides bridges (Fig. 1). The two bridges are necessary for the toxicity and one mutation of any of the cysteine residues result in an atoxic molecule (Arriaga *et al*., 1995; Okamoto *et al*., 1995). STb has a molecular weight of 5200 Da and a pI of 9.6. The first 7 amino acids at the NH2-terminus are not involved either in the toxicity or the structure of the toxin (Sukumar *et al*., 1995). Only one antigenic type of STb was observed although recently Fekete *et al*. (2003) observed a STb molecule where two amino acids differed (His12-Asn and Lys23-Ile) from the already described toxin. The biological activity of this molecule was not determined. Nuclear magnetic resonance (NMR) studies have established a structure with two anti-parallel α-helices separated by a loop rich in glycines (Sukumar *et al*., 1995). An amphipathic helix between residues 10 and 23, exposes several polar side chains. Facing it is a hydrophobic helix extending from residues 38 to 44 (Fig. 1). The synthesized mature toxin is secreted in the culture supernatant and is not associated with the cellular fraction (Kupersztoch *et al*., 1990).

Rousset *et al*. (1998a) using a semi-quantitative assay based on fluorescent microscopy determined the chemical nature of STb receptor on pig jejunum as being a glycosphingolipid.

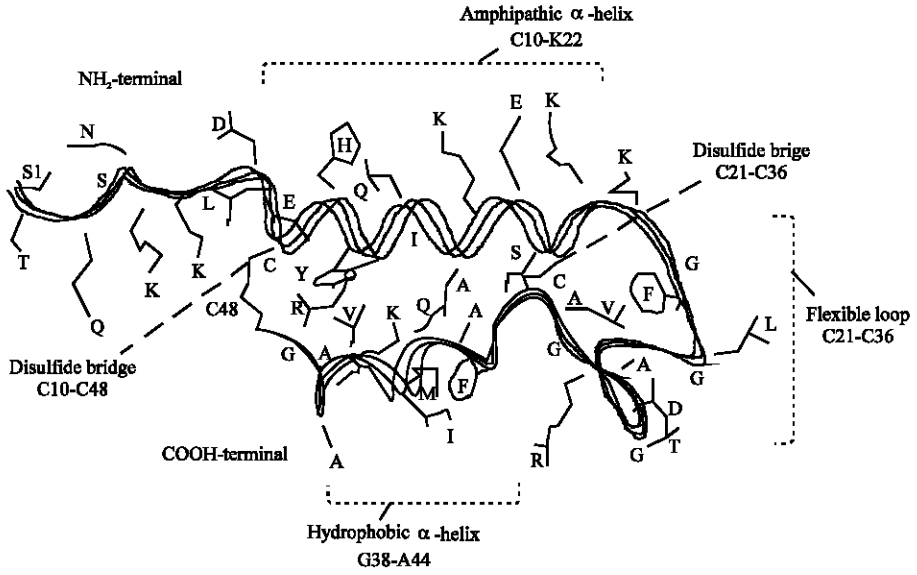

Fig. 1: Nuclear magnetic resonance-derived three dimensional structure of STb toxin adapted from Sukumar *et al*. (1995)

This result was obtained by treating the jejunum with various enzymes and chemical treatments and reacting the treated tissue with FITC-labelled STb to observe the binding or absence of binding. In another study, the same authors tested commercially available molecules of lipidic nature after immobilization on polyvinyl difluoride membranes for binding to STb (Rousset *et al*., 1998b). Sulfatide (3'SO_4-galactosyl-ceramide) was the molecule that showed the highest binding to STb toxin. The reaction was dose-dependent and saturable (Rousset *et al*., 1998b). Sulfatide was then extracted from pig jejunum brush border and its chemical nature confirmed by thin layer chromatography (TLC) following the observed Rf compare to reference sulfatide and reaction with a monoclonal antibody. The functionality of this molecule was determined in vivo using the rat loop assay. Following treatments with laminin (a compound known to bind specifically to sulfated glycolipids) and a sulfatase decreased markedly the biological activity observed. A scatchard analysis of STb binding to sulfatide indicated a Kd in the μM range (Beausoleil *et al*., 2001). A mass spectrometry analysis of the extracted sulfatide recuperated from TLC plates showed that it was composed of hydroxylated molecules comprising 16, 22 and 24 carbons and molecules saturated with 16 carbons. The hydroxylated sulfatide with a ceramide of 16 carbons was the main molecule in the extract (Beausoleil *et al*., 2002b).

Structure-function Studies in Relation to Receptor Binding and Toxicity

Numerous mutants of STb toxin (in one or two amino acids) were realized on exposed residues based on the result of an NMR study using mainly the alanine scanning technique (Labrie *et al*., 2001a). Residues pointing to the solvent, found in the α-helices and in the loop, were targeted. Using HPLC-purified toxins, the attachment to sulfatide in ELISA and toxicity in the rat loop model were tested. Thus, when a mutant had a decreased binding to sulfatide in ELISA a corresponding or higher decrease in toxicity was observed. This indicated that binding to sulfatide is a pre-requisite for toxicity. Residues Lys22, Lys23 and Arg29 were directly implicated in the binding to sulfatide and the subsequent toxicity expression.

Labrie *et al*. (2001b) using a cross-linker (bis (sulfosuccinimidyl) suberate) observed, in solution, the formation of STb oligomers. Hexamers and heptamers were seen in SDS-Page and their formation was prevented by addition of β-mercaptoethanol. Using mutagenesis, it was determined that the hydrophobic α-helix is responsible for the oligomerization of STb molecules. Interfering with oligomerization prevented the expression of toxicity in the rat loop assay as oligomer formation is probably required for interaction with susceptible cells. In particular, Phe-37, Ile-41 and Met-42 were directly involved in the formation of oligomers (Labrie *et al*., 2001a; Labrie *et al*., 2001b).

Artificial Membrane Model Experiments Including Planar Lipid Bilayers and Brush Border Membrane Vesicules

An electrophysiological study using planar lipid bilayers demonstrated the capacity of STb to form ionic pores in artificial lipid membranes constituted of phosphatidylethanolamine, phosphatidylcholine and cholesterol in a 7:2:1 ratio. This was observed in presence or absence of sulfatide in the membrane. The observed ionic pores were voltage-dependent (Labrie *et al*., 2001c).

Brush border membrane vesicules (BBMVs) were produced from pig jejunal tissue and these were incubated with STb toxin (Zhang *et al*., 1997). The BBMVs were loaded in KCl and a membrane potential sensitive fluorescent probe was used (3,3' dipropylthiadicarbocyanine iodine) to reveal pore formation (Kirouac *et al*., 2003). Valinomycin, a potassium ionophore, was used to create a membrane

potential. Using this system, STb was observed to produce pores as revealed in different ionic solutions and the pores created were cations specific. The formation of pores was pH-independent.

STb Internalization and Endocytosis

Using Chinese Hamster Ovary cells (CHO) it was previously shown that STb could allow the adsorption of Trypan blue stain revealing a certain degree of cellular membrane alteration produced by the toxin (Beausoleil *et al*., 2002a). An electron microscopy study was done by injecting and incubating STb for 3 h in intestinal rat loops. The intestinal tissue was fixed and dehydrated and thin sections were done and incubated with anti-STb gold conjugate. In transmission electron microscopy, STb toxin was internalized into rat intestinal epithelial cells (Labrie *et al*., 2002). For comparison, a STb double mutant molecule (Ile41Glu-Met42Arg) that was shown not to oligomerize was not observed inside the cells. This mutant when tested *in vivo* was not toxic indicating that internalization of STb is necessary for expression of the biological activity.

Endocytocis of STb was studied in a cellular model (NIH-3T3 mouse fibroblasts). STb toxin was directly labelled with FITC and various markers of cell compartments and known endocytic pathways were used. STb toxin was, on the cellular model used, rapidly endocytosed (within a few minutes) and the toxin was associated with many cellular structures. Under the same conditions, reduced and alkylated toxin that is inactive in vivo was not endocytosed but the altered toxin was associated with the cell surface. Confocal microscopy revealed colocalization between STb and AMFR (autocrine motility factor receptor) a marker for the smooth endoplasmic reticulum. Treatment with methyl-β-cyclodextrin, a cholesterol extracting reagent, disrupting caveolae expression, prevented the colocalization of STb with AMFR but STb was still endocytosed under this condition. Internalization of STb through caveolae was found to colocalize with smooth endoplasmic reticulum and associated with mitochondria (observed using a mitochondrial heat-shock protein 70 marker). When an acid treatment of the cells was done to block the clathrin-dependent pathway, internalization was still observed. Endocytosis was also observed to occur through vesicular structures and found colocalized with fibronectin fibers. Sulfatide, as the STb receptor, colocalized intracellularly with STb and also with the tubules of the smooth ER. Using this cellular model, STb endocytosis was observed to proceed through two distinct pathways, one clathrin-dependent and one caveolae-dependent.

CONCLUSIONS

Recent studies have shed new light on the way STb act on susceptible cells. The observation that STb is internalized put this toxin in another category from *E. coli* STa toxin a surface acting molecule that acts via signal transduction. We can now propose a model for the interaction of STb with the cellular surface (Fig. 2). STb toxin, in solution, can adopt an oligomeric conformation with hexamers and/or heptamers as the final structure. These oligomers would bind to sulfatide present at the surface of the intestinal epithelial cells. Then, as observed in EM on rat intestine and also with NIH-3T3 cells, STb can cross the eukaryotic cell membrane. On the other hand, as observed with membranar models (PLB and BBMVs), STb is able to form ionic pores. The internalized toxin could then act on the intracellular target that remains, for now, unknown. The endocytosis study indicated that STb is endocytosed through two distinct pathways. One pathway is clathrin-dependent and STb is internalized into vesicular structures and is then found associated with fibronectin. The other pathway is caveolae-dependent and the toxin colocalizes with the smooth endoplasmic reticulum and is possibly associated with mitochondria (Fig. 3).

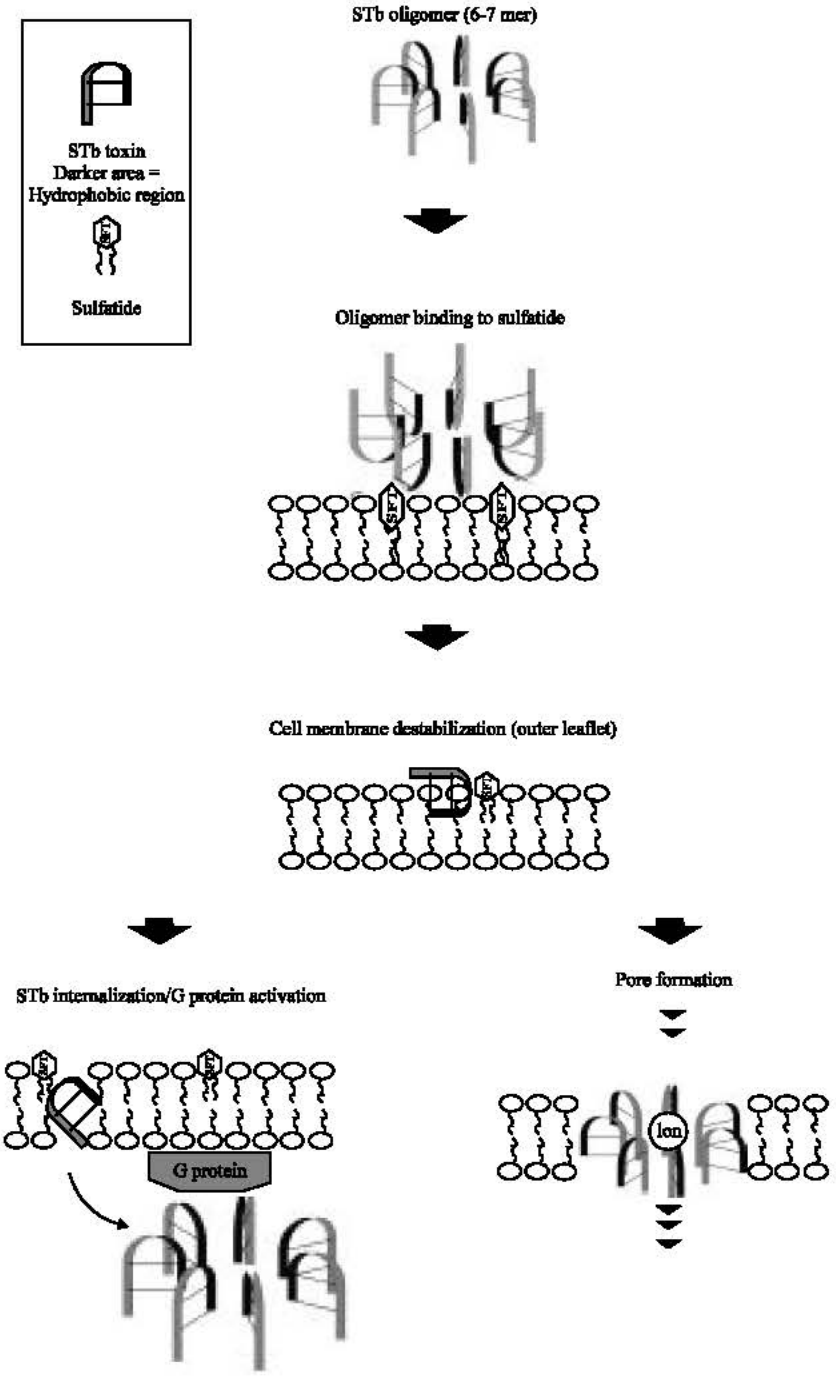

Fig. 2: Hypothetical interaction of STb oligomers with cell membrane after interaction with sulfatide (SFT). STb was shown to be internalized using the intestinal rat loop intestinal assay and the NIH-3T3 cellular model (Left panel). On artificial membrane models (PLB and BBMVs) STb was also observed to form ionic pores (Right panel)

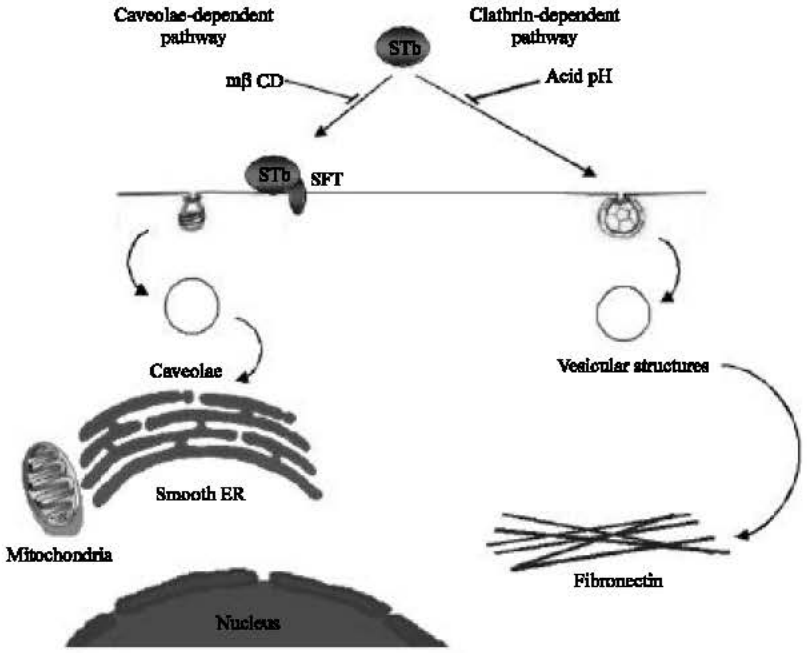

Fig. 3: Using NIH-3T3 cell line, STb was endocytosed through two distinct pathways. One caveolae-dependent that is directed to the smooth endoplasmic reticulum (smooth ER) and colocalizes with mitochondria. The other pathway is clathrin-dependent and STb is internalized through vesicular structures and colocalizes with fibronectin filaments. (mβCD: methyl β-cyclodextrin; SFT: sulfatide)

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