Effect of shigella enterotoxin 1 (ShET1) on rabbit intestine in vitro and in vivo

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Abstract

**Background**—Shigella enterotoxin 1 is a novel enterotoxin elaborated by *Shigella flexneri* 2a that causes fluid accumulation in rabbit ileal loops and a rise in short circuit current in Ussing chambers.

**Aims**—To gain insights into the mechanism of action of shigella enterotoxin 1.

**Methods**—Supernatants from genetically engineered clones either overexpressing shigella enterotoxin 1 or producing deletion mutants of the toxin were tested in rabbit ileum both in vitro and in vivo.

**Results**—In rabbit ileum shigella enterotoxin 1 induced an irreversible rise in short circuit current that was not mediated by any of the recognised intracellular mediators of secretion. Deletion of 90% of the A subunit of the holotoxin ablated its enterotoxicity. In the in vivo perfusion model, the toxin induced a time dependent decrease in water absorption, whereas no changes were detected in the segment perfused with supernatants obtained from the deletion mutant. Finally, partially purified toxin induced a dose dependent increment in short circuit current that reached its plateau at a toxin concentration of 4×10^{-4} M.

**Conclusions**—Shigella enterotoxin 1 induces a time and dose dependent intestinal secretion in the rabbit animal model, suggesting that it may be responsible for the watery phase of *Shigella flexneri* 2a infection.

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*Shigella dysenteriae* type I was first isolated in 1898 during an epidemic of severe dysentery in Japan. Over the subsequent century other shigella species were identified, including *Shigella flexneri*, *Shigella boydii*, and *Shigella sonnet*, and the mechanisms whereby they cause disease have been intensively investigated. A cardinal feature of shigella virulence is the capacity of the organism to invade mammalian epithelial cells, followed by cell death, spread to adjacent cells, and elicitation of an intense inflammatory response with infiltration by polymorphonuclear leukocytes. The classic dysenteric stool is scanty and consists of blood and mucus. However, most ill patients develop an initial phase of watery diarrhoea, that in mild cases may represent the only clinical presentation of the disease, without evolving to dysentery.

We have recently reported the elaboration by shigella of two novel iron-regulated enterotoxins, shigella enterotoxin 1 (ShET1) and 2 (ShET2), that alter electrolyte and water transport in rabbit small intestine both in vitro and in vivo. ShET1 is a chromosomally encoded, 55 kDa complex protein that is universally elaborated by *Shigella flexneri* 2a strains but only rarely by other serotypes. Sequencing analysis of the genes encoding this toxin disclosed the presence of two contiguous open reading frames (orfs) of 534 (ser1A) and 186 (ser1B) bp respectively, governed by the same promoter and separated by only 3 bp. ShET2 is a 62-8 kDa single protein that our group originally described in enteroinvasive *Escherichia coli* (EIEC) and referred to as EIEC enterotoxin (EIET).

The gene encoding this toxin is located on the 140 mDa invasiveness plasmid of shigella and seems to be present in more than 80% of a wide array of shigella serotypes examined. Gene sequence analysis showed that both ShET1 and ShET2 are genetically unrelated to shiga toxin elaborated by *Shigella dysenteriae*. Furthermore, the three toxins also seem to be immunologically unrelated.

In this paper we further characterise the mechanism of action of ShET1, both in vitro and in vivo. Our data indicate that ShET1 induces a time and dose dependent intestinal secretion in the rabbit animal model, suggesting that the toxin may have a role in the pathogenesis of the watery phase of dysenteric infection.

**Methods**

OVEREXPRESSION OF SHET1

The 1093 bp chromosomal fragment containing the ShET1 genes (ser1) was cloned in the multiple copy plasmid pBluscript SK as previously described. The plasmid so obtained, pser1, was transformed in DH5α (Fig 1) and overexpression of the ShET1 protein was obtained in iron depleted culture supernatants. More specifically, 100 ml Luria Bertani broth containing 25 μg/ml of the iron chelator ethylenediamine-N,N'-diacetic acid (EDDA) and 100 μg/ml ampicillin were inoculated with a single colony of DH5α (pser1) and incubated overnight at 37°C with shaking (200 rpm). Bacterial cells were removed by
Purification of ShET1

Overexpressed ShET1 was obtained as described above. The proteins contained in the DH5α (psetl) supernatant were then precipitated with saturated trichloroacetic acid (TCA) (20:80 v/v) and separated by SDS-PAGE electrophoresis. Resultant bands were transferred to a nitrocellulose membrane and cut into two strips to be either silver stained to visualize protein bands or reacted with rabbit antiserum against ShET1 for western immunoblotting. The single band corresponding to the 55 kDa ShET1 protein was carefully cut out of the gel and electroeluted using an electrophoresis chamber (Schleicher and Schuell, Keene, NH, USA). Purity of ShET1 was established by SDS-PAGE electrophoresis and by western immunoblotting. The amount of electroeluted ShET1 was determined by the Bradford method.\textsuperscript{13}

Construction of a setl deletion mutant

A Δsetl allele was obtained by deleting 90% of setlA gene (from AA residue 17 to AA residue 169), while the natural promoter and the whole B subunit were left intact (Fig 1). DNA segments were amplified from a library derived phagemid pF9-1-90\textsuperscript{9} containing the Δsetl allele and fused by polymerase chain reaction (PCR). Unique restriction sites were then introduced using external primers and were utilised to clone the Δsetl allele into Phlescript SK. The plasmid so obtained (named pΔsetl) was electroporated in DH5α and supernatants of this strain (DH5α (pΔsetl)) (Fig 1) were prepared as reported above and frozen until tested in the in vitro and in vivo assays described below. The amount of protein present in DH5α (pΔsetl) was determined by the method of Bradford\textsuperscript{13} and results were comparable with the total protein concentration present in the DH5α (psetl) supernatant (4.21 μg/ml v 4.41 μg/ml respectively).

Construction of maltose binding protein (MBP)-ShET1 (a subunit fusion protein) and preparation of anti-subunit polyclonal antibodies

The ShET1 A subunit gene was amplified by PCR using psetl as a template. Fidelity of PCR amplification was confirmed by DNA sequencing of the plasmid insert. setl A gene was then fused in frame with the maltose binding protein (MBP) gene, using vector pMalc\textsuperscript{21} to create a MBP-ShET1A fusion protein. The fusion product was expressed in Escherichia coli and obtained by disrupting the cells using a French press. The cell lysate was centrifuged for 30 minutes at 15000 g, and the supernatant was filtered, diluted five times in buffer A (20 mM Tris HCl pH 7.5, 1 mM EDTA, 0.2 M NaCl, 1 mM Na2SO4, and 1 mM 2-ME), and loaded on to an amylose column (New England Biolabs) at a flow rate of 1 ml/min. The column was washed with five volumes of buffer A and the fusion protein was eluted with 10 mM maltose in buffer A. Protein concentrations were assessed with a
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Bio-Rad protein assay, and purity was determined by SDS-PAGE followed by Comassie or silver staining.

To raise polyclonal antibodies against ShET1 A subunit, purified MBP-ShET1 A fusion protein was mixed with an equal volume of complete Freund’s adjuvant and injected into an adult male New Zealand white rabbit. Two booster doses were given with Freund’s incomplete adjuvant four and eight weeks later, and the animal was then bled.

USSING CHAMBERS
Experiments were carried out as previously described. Briefly, adult male New Zealand white rabbits (body weight 2–3 kg) were killed by cervical dislocation. A 20 cm segment of ileum was removed, rinsed free of the intestinal content, opened along the mesenteric border, and stripped of muscular and serosal layers. Eight sheets of mucosa so prepared were then mounted in luce Ussing chambers (1.12 cm² opening), connected to a voltage clamp apparatus (EVC 4000 WPI, Saratosa, FL, USA), and bathed with freshly prepared buffer containing (in mM): NaCl, 53; KCl, 5; Na₂SO₄, 30.5; mannitol, 30.5; Na₂HPO₄, 1.69; NaH₂PO₄, 0.3; CaCl₂, 1.25; MgCl₂, 1.1; NaHCO₃ 25. The bathing solution was maintained at 37°C. A second cannula with water jacketed reservoirs connected to a constant temperature circulating pump and gassed with 95% O₂/5% CO₂. Potential difference (PD) and short circuit current (Isc) were measured under short circuit conditions and tissue resistance (Rt) was calculated as previously described. Filtered supernatants (300 μl) obtained from either DH5α (psetl) or DH5α (pΔsetl) were added to the mucosal surface and an identical amount was added to the serosal surface to preserve the osmotic balance. In selected experiments, partially purified ShET1 was added only to the mucosal side of the rabbit ileum. Variations in PD, Isc, and Rt were then recorded every 10 minutes. At the end of every experiment, 0.5 mM glucose was added to the mucosal side of each chamber. Only those tissues which showed an increase in Isc in response to glucose, indicating tissue viability (98% of the tissues tested), were included in the analysis.

IN VIVO PERFUSION ASSAY
Intestinal perfusion was carried out according to the method previously described by Sladen et al17 with minor modifications. More specifically, after a 24 hour fast, 2.5–3 kg adult male New Zealand white rabbits were anaesthetised with 50 mg ketamine/kg body weight, followed by intramuscular injection of 7.0 mg xylazine/kg body weight. Their body temperature was kept at 37°C by a lamp. The abdominal cavity was opened by a midline incision and two distinct segments of jejunum were cannulated. A second cannula was placed 10–15 cm below each proximal cannula. The segments were rinsed free of intestinal contents with 0.9% (w/v) NaCl warmed to 37°C. The proximal cannulae were connected by a polyvinyl tube to a peristaltic pump (model WPI SP220 I, and the two segments were perfused at a rate of 0.4 ml/min with a solution comprising 2.0 mmol/l glucose, 4.0 mmol/l KCl, 25 mmol/l NaHCO₃, 3.0 mmol/l PEG-4000, and 3.0 μCi/100 ml ¹⁴C-PEG-4000. The solution was made isotonic by adjustment with NaCl, and the pH fixed at 7.4 by gassing with 95% O₂/5% CO₂.

Eluates were collected in 20 minute aliquots from the distal cannulae. An initial equilibrium period of 30 minutes was allowed, followed by three consecutive 20 minute collection periods for baseline measurement of the net transport of water and electrolytes in each segment studied.

Subsequently, one of the two segments was perfused with the same solution described above containing 30 μl/ml DH5α (psetl) supernatant, while the second segment was perfused with a similar amount of DH5α (pΔsetl) supernatant. A second perfusion period (30 minute equilibration plus 6×20 minute collection) was then carried out. At the end of the experiment, the animal was killed and the segments perfused were isolated, measured, dried, and weighed. Water absorption was then calculated as previously described.19

Results

INTRACELLULAR MESSENGER(S) OF SHET1 ENTEROTOXIC ACTIVITY
To establish whether the enterotoxic effect of ShET1 was mediated by one of the described intracellular mediators of intestinal secretion (cAMP, cGMP, and Ca²⁺), 5 mM theophylline, 0.2 mM 8Br-cGMP, and 5×10⁻⁷ M Ca ionophore A23187 were tested in Ussing chambers in the presence and absence of DH5α (psetl) supernatant. Segments of rabbit ileum mounted in Ussing chambers were paired based on their Rt. Supernatant obtained from DH5α (psetl) (positive control) was added to one of the chambers, while the coupled chamber was exposed to DH5α (pBS) (negative control) supernatant. Once the increment of Isc induced by DH5α (psetl) reached a plateau, the second messenger to be tested was added to the serosal side of both chambers and the subsequent increment in Isc recorded. As shown in Fig 2, the Isc changes induced by the three intracellular messengers tested were similar in the ShET1 exposed and control exposed tissues, suggesting that none of them is involved in ShET1 secretory action.

LACK OF REVERSIBILITY OF SHET1 ENTEROTOXIC EFFECT
To establish whether the ShET1 enterotoxic effect was reversible, rabbit ileal mucosa was first exposed to DH5α (psetl) supernatant. When the enterotoxic effect of supernatant containing ShET1 reached its plateau, the reservoir was emptied, rinsed twice with Ringer’s solution, and refilled with Ringer’s alone. No substantial changes in Isc were seen after ShET1 withdrawal (Fig 3), suggesting either an irreversible binding of the toxin with its receptor.
sequence of the set1 gene features a putative signal sequence. As these experiments were performed under denaturing conditions, we repeated the western blotting experiment using non-denaturing conditions. The results showed in Fig 4B confirmed that ShET1 is present in DH5α (pset1) supernatant, but is lacking in both DH5α (pBS) and DH5α (pΔset1) supernatants. Finally, to establish whether DH5α (pΔset1) expresses the ShET1 B subunit, the western blotting was repeated using polyclonal antibodies raised against the ShET1 A subunit and compared with western immunoblots performed using polyclonal antibodies raised against the entire holotoxin. As shown in Fig 4C, the polyclonal antibodies against the holotoxin visualised a 35 kDa band present in both DH5α (pset1) and DH5α (pΔset1) supernatants, but not in DH5α (pBS) negative control supernatant. This band was not visualised by the polyclonal antibodies raised against the ShET1 A subunit, suggesting that the 35 kDa band may represent a B, aggregate and, therefore, that the B subunit is still expressed by the deletion mutant.

WESTERN IMMUNOBLOTS OF DH5α (pset1) AND ITS DELETION MUTANT DH5α (pΔset1)
Western blotting performed with the rabbit antiserum raised against ShET1 showed the 55 kDa ShET1 band in DH5α (pset1) supernatant, but not in DH5α (pΔset1) supernatant (Fig 4A). Interestingly, the band was present only in the supernatant but not in the cell lysate of DH5α (pset1), suggesting that ShET1 is a secreted protein. These results are in keeping with the fact that the predicted amino acid or a continuous activation of the intracellular signalling that mediates the enterotoxin effect.

Figure 2: Peak increments in Isc after addition of 5 nmol/l theophylline (cAMP), 0.2 nmol/l 8-bromo-cGMP (cGMP), or 5 x 10⁻⁷ mol/l calcium ionophore A23187 (Ca²⁺) to tissues pretreated with either DH5α (pset1) (ShET1 positive) (■) or DH5α (pBS) (ShET1 negative) (●) supernatants. The second messengers were added to the serosal side once the Ic increment induced by ShET1 reached the plateau (110–120 min) (n=4 animals). Bars represent SEM.

Figure 3: Lack of reversibility of ShET1 enterotoxic effect. Supernatants obtained from both DH5α (pset1) (ShET1 positive) (▲) and DH5α (pBS) (ShET1 negative) (●) were added to rabbit ileal mucosa mounted in Ussing chambers and changes in Isc were followed over time. ShET1 induced a time dependent increase in Isc compared with the negative control that reached its plateau after about 100 minutes. Once the toxin was withdrawn from the reservoir (arrow), no modifications in Isc were observed, suggesting an irreversible binding of ShET1 to its receptor. Addition of 0.5 mM glucose to the mucosal side of the rabbit ileum induced a similar rise in Isc in both the ShET1 and negative control exposed tissues, proving that ShET1 did not affect the tissue viability (n=4 animals).

EFFECT OF SHET1 DELETION MUTANT IN USSING CHAMBERS
The apparent molecular size of ShET1 by both gene sequencing and western blot analysis suggests a holotoxin stoichiometry (A₅-B₂) similar to that of other well established enterotoxins. To establish whether the ShET1 A subunit plays a pivotal role in ShET1 enterotoxicity, supernatants obtained from DH5α (pΔset1) lacking 90% of ShET1 A subunit were tested in Ussing chambers. When added to rabbit ileal mucosa, this supernatant failed to induce the increase in Isc typically seen with DH5α (pset1) supernatant (Fig 5).

IN VIVO PERFUSION EXPERIMENTS
We have previously shown that ShET1 containing supernatants induce a mild, but significant fluid accumulation in rabbit ileal loops in vivo. To better define the mechanism of secretion of ShET1 and to establish the in vivo effect of the deletion mutant of ShET1 protein, two contiguous segments of jejunum were simultaneously perfused in the same animal with a saline solution (period 1), followed by a second period during which solutions containing either DH5α (pset1) or DH5α (pΔset1) supernatants were perfused. The first period of perfusion was characterised by the active absorption of water and electrolytes in both segments (Fig 6). After 60 minutes the addition of ShET1 to the perfusion solution produced a significant decrease in water absorption, whereas no changes were detected in the segment perfused with ShET1 protein deleted of its A subunit (Fig 6).

DOSE-RESPONSE CURVE OF SHET1 ENTEROTOXICITY
To establish whether the enterotoxic effect of ShET1 was dose dependent, increasing
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Figure 4: Western immunoblots of DH5α (pset1) and its deletion mutant DH5α (pΔset1). (A) Denaturing gel. Supernatants and cell lysates obtained from DH5α (pBS), DH5α (pset1), and DH5α (pΔset1) were separated by SDS-PAGE under denaturing conditions, transferred to nitrocellulose, and developed using rabbit antiserum to the active fraction obtained from *Shigella flexneri* 2a strain M4243acrv. Lane A: DH5α (pBS) cell lysate; lane B: DH5α (pBS) supernatant; lane C: DH5α (pset1) cell lysate; lane D: DH5α (pset1) supernatant; lane E: DH5α (pΔset1) cell lysate; lane F: DH5α (pΔset1) supernatant. Arrow shows 55 kDa S/ETI band present in D, but not in the other lanes. (B) Non-denaturing gel. Supernatants obtained from DH5α (pBS), DH5α (pset1), and DH5α (pΔset1) were separated by SDS-PAGE under non-denaturing conditions, transferred to nitrocellulose, and developed using rabbit antiserum to the active fraction obtained from *Shigella flexneri* 2a strain M4243acrv. Lane A: DH5α (pBS); lane B: DH5α (pset1); lane C: DH5α (pΔset1). Note the presence of S/ETI band in B, but not A and C. (C) Western immunoblot developed with anti-S/ETI A subunit polyclonal antibodies. Supernatants obtained from DH5α (pBS), DH5α (pset1), and DH5α (pΔset1) were separated by SDS-PAGE under denaturing conditions, transferred to nitrocellulose, and developed using either rabbit antiserum to S/ETI holotoxin (left) or S/ETI A subunit (right). Lane A: DH5α (pBS); lane B: DH5α (pset1); lane C: DH5α (pΔset1). Note the presence of a 35 kDa band (arrow) in lanes B and C when antibodies against the holotoxin were used, whereas this band was not detected with antibodies against the S/ETI A subunit.

Figure 5: Enterotoxic effect of S/ETI deletion mutant in Ussing chambers. Peak increments in Isc after addition of supernatants obtained from DH5α (pset1) (S/ETI positive), its mutant DH5α (pΔset1) deleted of the S/ETI A subunit, or the negative control DH5α (pBS). The S/ETI deletion mutant failed to induce the rise in Isc found with S/ETI containing supernatants. *p<0.01 (n=5 animals).

Concentrations of purified toxin obtained by electroelution were added to the mucosal side of rabbit ileum mounted in Ussing chambers and changes in Isc recorded. S/ETI induced a dose dependent increment in Isc that reached its plateau at a toxin concentration of 4×10⁻⁵ M and an ED₅₀ of 9×10⁻⁷ M (Fig 7).

**Discussion**

*Shigella flexneri* 2a is one of the most common serotypes causing bacillary dysentery worldwide. The ability to invade and spread within the epithelial cells and thereby to cause dysentery has always been considered the cardinal mechanism of pathogenicity of *Shigella flexneri*. Nevertheless, the clinical presentation of shigellosis, with an initial phase of watery diarrhea that in mild cases may represent the only gastrointestinal symptom, would predict the existence of enterotoxins. Further evidence of the involvement of other pathogenic factors (other than associated with shigella invasion and multiplication within the gut system) is needed.
antigen of S. flexneri 2a to Escherichia coli K-12.29 This vaccine also induced adverse reactions in 31% of vaccinees; among the side effects, watery diarrhoea was reported.

We have recently reported the elaboration of two distinct enterotoxins by Shigella flexneri 2a,30 that may be responsible for the watery phase of shigellosis. We have shown that, when grown in iron depleted medium, enteroinvasive Escherichia coli (EIEC) elaborate an enterotoxin (EIET) that causes fluid accumulation in isolated ileal loops and an electrical response in Ussing chambers.12 Based on the similarities known to exist between EIEC and shigella,30 we investigated the possibility that Shigella flexneri 2a elaborates a similar toxin. Our group has recently cloned and sequenced the EIET gene.30 A virtually identical gene (99% homology) was found on the 140 mDa invasiveness plasmid of Shigella flexneri 2a and gene probe studies showed that this gene is widely present in all shigella serotypes.10 Surprisingly, plasmid cured derivatives of Shigella flexneri 2a still retain enterotoxic activity, both in vivo and in vitro.8 The combination of genetic analysis and intestinal electrophysiology allowed us to localise the chromosomal genes responsible for this residual enterotoxicity. Analysis of the sequence of the cloned genes responsible for ShETI enterotoxic activity disclosed two distinct, yet contiguous orfs, encoding for two proteins of predicted MW of 7 kDa and 20 kDa, respectively. The common A:B active binding subunit motif often found among bacterial enterotoxins, including cholera toxin (CT),18 heat labile enterotoxin (LT) of enterotoxigenic E. coli,19 and shiga toxin of S. dysenteriae 1,20 may be reflected in these data.

The apparent molecular sizes of active material as predicted by both the gene sequencing and the western blot experiments,9 are consistent with such stoichiometries based on the sizes of the A (20–32 kDa) and B (7–11 kDa) subunits of these recognised enterotoxins. By extension, a holotoxin consistent with a size of 55 kDa and an A:B structure would be predicted by these conventions. These tentative configurations also satisfy the usual requirements for both a binding and an active domain that allow the enterotoxin to attach and gain entrance to enterocytes and to initiate events that culminate in intestinal secretion. The data presented in this paper suggest that, as for other enterotoxins, the ShETI A subunit is responsible for the secretory activity, whereas the B subunits may be involved in the irreversible binding of the toxin to the enterocyte receptor. With regular discoveries of additional members of a family of CT-LT-like toxins among salmonellae, pseudomonas, campylobacter, aeromonas, and other genera, new combinations of A:B structures seem likely to emerge.

It is intriguing that none of the currently identified intracellular messengers of intestinal secretion is involved in ShETI enterotoxicity. However, the possibility that intestinal secretagogues may act via new intracellular modulators is emerging both for bacterial enterotoxins (DDEC enterotoxin, F Raimondi and A Fasano, unpublished data) and inflam-
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