BACTERIAL PATHOGENICITY

A histotoxin produced by *Salmonella*

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*Salmonella* Typhimurium strain GM3, known to be histotoxic for explants of terminal rabbit ileum *in vitro*, produces similar lesions *in vitro* when sterile filtrates, obtained from live organisms after interaction with gut explants *in vitro*, are used and when rabbit ligated ileal loops are challenged with live organisms. Epithelial damage occurs rapidly, within 2 h of adding organisms or sterile filtrates. This evidence is construed in terms of a secreted salmonella histotoxin that causes epithelial damage, detaching enterocytes which rapidly degenerate into spheroid cells devoid of microvilli. *Typhimurium* strain GM3 invades ileal mucosa and bacteria are found in the subepithelial tissues. After 12 h, bacteria were seen to be expelled from infected villi in a manner similar to that seen in non-histotoxic infection with *Typhimurium* strain TML.

**Introduction**

Despite intensive study, the number, nature and role of *salmonella* toxins involved in disease causation are unclear. This situation probably prevails because of the lack of meaningful experimental systems with which to study critically certain aspects of virulence in *Salmonella*. To assign biological significance to a putative virulence determinant, it is necessary to demonstrate its expression, and sometimes its extracellular release, at the right time and in an appropriate environment, namely, under ecologically significant conditions.

Evidence from quantitative and electron microscopy studies suggested that a histotoxin, which cleaved inter-enterocyte tight junctions, was involved in the interaction of *Salmonella* serotype Typhimurium strain GM3 and serotype Dublin strain 3246 with explants of rabbit terminal ileum [1]. Evidence for the existence of such a toxin produced by *Typhimurium* strain GM3 is presented here.

Two approaches were used. First, gut tissue was exposed to live organisms *in vivo* with an improved version of the rabbit ligated ileal loop test [2], the rabbit ileal anastomosis test, and *in vitro* in an organ-culture system [1, 3]; the tissues were then examined by electron microscopy. Second, gut explants were challenged for 2 h *in vitro* with live bacteria [1, 3] and chamber fluids were removed, filter sterilised and added to fresh tissue from the same animal, mounted in a second organ-culture apparatus. The effect of sterile culture filtrates on gut epithelium was compared to that seen in experiments *in vivo* and *in vitro* with live bacteria. Previously, both scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to analyse gut epithelia [1]. In the present study, only TEM was used, because it provided a more detailed picture of the initial stages of *Typhimurium* strain GM3-induced damage.

**Materials and methods**

*Bacterial strains used, their storage, culture conditions, bacteriological media, animals, organ-culture apparatus and media and solutions, transmission electron microscopy*

These were as described previously [1]. *Typhimurium* strain TML is virulent for man and causes fluid secretion in monkeys and rabbit ileal loops (RILs); *Typhimurium* strain LT7 is a non-invasive [3], non-virulent strain that does not cause fluid secretion in RILs [4], and *Typhimurium* strain GM3 is histotoxic and virulent for man [1].

**In-vivo loop experiments**

Two kinds of experiments were performed in which a modified rabbit ileal loop test [2], termed the rabbit...
ileal anastomosis test (RILAT), was used. In the first, the procedure was terminated after 12 h and in the second after 3 h. Briefly, the modified procedure was as follows. The peritoneal cavity was opened, the washed intestine was clamped with intestinal clamps and resected 10 cm proximal to the ileo-caecal junction. A length of intestine, proximal to the resection, required for the construction of ligated loops was measured and the intestine was clamped and resected; the isolated segment of intestine was temporarily replaced into the peritoneal cavity. The clamped, resected ends of the intestine were anastomosed, joint integrity and blockage were assessed and the intact intestine was replaced into the peritoneal cavity. The resected intestinal segment was removed from the peritoneal cavity, the ends were closed with suture and resected intestinal segment was removed from the peritoneal cavity. The peritoneal cavity was opened, the washed intestine was replaced into the peritoneal cavity. The clamped, intact intestine was clamped with intestinal clamps and integrity and blockage were assessed and the intact intestine was replaced into the peritoneal cavity. The resected intestinal segment was removed from the peritoneal cavity, the ends were closed with suture and ligated sample loops and spacer loops were constructed and inoculated as described previously [2]. The samples inoculated into ligated loops were prepared as for rabbit ileal invasion assays [3] and injected into intestinal loops measuring 5 cm in length. Phosphate-buffered saline (PBS) and cholera toxin (CT) 0.5 µg were used as negative and positive controls, respectively. After closure of the laparotomy, the rabbit was allowed to recover; the procedure was terminated after 3 or 12 h. The volume of accumulated loop fluid, an indicator of gastro-enteritic virulence, was then measured and a small piece of tissue from each loop was removed and processed for TEM.

**In-vitro toxin experiments**

Two experiments were performed, each of which involved two organ-culture chamber apparatuses.

In the first of these, a length of terminal ileum was removed, sufficient to mount in two organ-culture apparatuses; a sample of tissue was immediately fixed and processed for TEM. The ileal tissue was halved; one (designated '0-h tissue') was used immediately in the first chamber apparatus and the other (designated '2-h tissue') was placed in mucosal medium and held on ice, and gassed, until it was mounted into the second chamber apparatus. The '0-h tissue' was stripped and mounted into the first chamber apparatus. Typhimurium strain LT7, Typhimurium strain GM3 and mucosal medium were added to three chambers each. After 2 h at 37°C, the fluid contents of the chambers containing strains LT7 and GM3 were removed, bulked appropriately and filtered sterilised; sterility was checked by plating on to MacConkey agar. Sterile chamber contents were added to two chambers each in the second apparatus in which stripped '2-h tissue' had been mounted; fresh mucosal medium was added to two other chambers. Tissues from the chambers in the first apparatus were also processed for TEM immediately at the end of the 2-h incubation period together with a sample of '2-h tissue' which had been held in mucosal medium on ice. The second apparatus with the filtered chamber contents was incubated for 2 h at 37°C, when the tissues were removed and processed for TEM.

In a variation of the above experiment, invasive, fluid-inducing Typhimurium strain TML replaced mucosal medium. It was not logistically possible to include this strain in the previous experiment together with the other controls. For the first incubation, Typhimurium strains TML, LT7 and GM3 were used in three chambers each. For the second incubation, filtered chamber contents from the first incubation were used.

**Results**

**In-vivo experiments**

In the 12-h RILAT, the greatest amount of fluid secretion was observed in the CT positive control loop (17 ml), followed by Typhimurium strain TML (3.5 ml) and Typhimurium strain GM3 (2.2 ml). The negative control loops with Typhimurium strain LT7 or PBS did not contain accumulated fluid. This indicated that the potential of Typhimurium strain GM3 for fluid secretion was less than that of Typhimurium strain TML.

TEM studies showed good preservation of epithelia in PBS control tissue (Fig. 1a). In tissues challenged with strain GM3, bacteria were generally more difficult to find than in those challenged with strain TML. However, the demonstration that they were present in subepithelial tissue confirmed their invasiveness. GM3 organisms were also seen in bacteria-laden enterocytes being shed from villus tips (Fig. 1b). In TML-challenged tissue (not shown), bacteria-laden enterocytes were also seen to be shed from villus tips, in a manner similar to that described in Fig. 1b, which is almost identical to that previously reported for strain TML [5]. As these tissues were taken from secreting loops, there were very few luminal cells present in the sections. It was clear that differences in degrees of invasiveness between strains TML and GM3 [1] could not readily be explained in terms of what was observed histologically at 12 h after in-vivo challenge.

Accordingly, a RILAT experiment – terminated at 3 h after challenge – was performed, in which the differences were dramatic. Tissue inoculated with PBS showed intact junctional complexes (Fig. 1c) and generally intact epithelium (Fig. 1d). A similar picture was observed with tissue challenged with strain TML (not shown); very few enterocytes were observed in the lumen of these loops, none of which contained fluid at 3 h. In contrast, Typhimurium strain GM3 produced a dramatically different picture. Individual detached enterocytes, still bearing microvilli, were seen in many fields in the absence of bacteria (Fig. 1e). In other fields extracellular bacteria were observed in the lumen surrounding detached epithelial cells; the most dramatic of the many examples observed is shown in Fig. 1f.
**In-vivo experiments.** (a) PBS control tissue showing good preservation of epithelium 12 h after challenge in vivo; bar, 4 μm. (b) Bacteria-laden cell (arrow) being shed from Typhimurium GM3-challenged epithelium 12 h after infection and bacteria (arrowhead) inside epithelial cell; bar, 10 μm. (c) PBS control tissue after 12 h showing good preservation of tight junction (arrow), zonula adherens (arrowhead) and desmosome (curly arrow); bar, 0.4 μm. (d) PBS control tissue after 12 h showing generally intact epithelium; bar, 10 μm. (e) A field showing enterocytes detached from Typhimurium GM3-infected tissue 3 h after infection - note the absence of both extracellular and intracellular bacteria; bar, 4 μm. (f) In contrast to (e) other fields showed extracellular organisms in the lumen surrounding detached epithelial cells of which (f) is a dramatic untouched example; bar, 4 μm.

**In-vitro experiments**

Having established that the epithelial disruption mediated by Typhimurium strain GM3 was not an in-vitro artefact, further experiments were designed to test the hypothesis that the early epithelial disruption was toxin-mediated.

In organ-culture experiments with bacteria and chamber culture filtrates, it was important to demonstrate that control tissues remained intact throughout the period from their initial removal from the animal to the end of the experiment, because they involved treatments and use of gut explants in ways not hitherto studied in this laboratory. Fresh '0-h tissue' is shown in Fig. 2a and b; '2-h tissue' held in mucosal medium on ice for 2 h showed no significant structural changes when compared with '0-h tissue' (not shown). The most important control was that of the effect of sterile chamber contents from Typhimurium strain LT7 on tissue in the second incubation: this was a control on 'spent' medium, namely, mucosal medium modified by bacterial growth and by the presence of any secreted 'gut' factors. No damage to the epithelium was observed (Fig. 2c). The appearance of the tissue was almost identical to that observed after incubation of '2-h tissue' in mucosal medium (Fig. 2d) and to that caused by Typhimurium LT7 bacteria themselves (not shown but similar in appearance to that shown in Fig. 2c and d). Whilst incubation in mucosal medium at 37°C in the chamber of both '0-h tissue' and '2-h tissue' showed no disruption of the epithelium, a slight oedema in the regions of the villous tips was observed (Fig. 2c and d).
Fig. 2. In-vitro experiments. (a) Fresh '0-h tissue', straight from the animal, showing intact villus (bar, 50 μm) and (b) intact epithelium; bar, 10 μm. (c) 2-h supernate from chambers challenged with Typhimurium strain LT7, the control on 'spent' mucosal medium modified by bacterial growth and by the presence of any secreted 'gut' factors, did not disrupt gut epithelia in the second incubation and caused only slight oedema in the tip regions of villi; bar, 10 μm. (d) '2-h tissue' after incubation in mucosal medium at 37°C in the chamber showing only slight oedema in the tip regions of villi but no disruption of the epithelium (see text for explanation of oedema in (c) and (d)); bar, 10 μm. (e) Typhimurium strain GM3 caused the release of large numbers of cells possessing microvilli, or remnants of microvilli, from epithelia. Also shown are degenerate vacuolated cells almost devoid of microvillus elements, severely damaged or dead cells, cell debris and organisms mainly outside cells or entrapped in cell debris. VT, intact villus tip; bar, 10 μm. (f) Intermediate stage of enterocyte degeneration; bar, 10 μm. Sterile supernates from chambers challenged with Typhimurium strain GM3 induced epithelial disintegration: (g) a single cell (arrow) detaching from the epithelium, bar, 4 μm; (h) a detached group of several microvillus-bearing enterocytes in varying degrees of association, bar, 10 μm; (i) detached single cells, one of which still possesses microvilli; bar, 4 μm.
Typhimurium GM3 bacteria caused large numbers of cells to be released from the epithelium (Fig. 2e and f) and most of them were identifiable as detached enterocytes because they possessed microvilli or remnants of microvilli. However, many cells were almost spherical, devoid of microvillous elements, vacuolated or severely damaged. The appearance shown in Fig. 2f may be interpreted as an intermediate stage in the degeneration of detached enterocytes; the cell is about to shed its microvillous membrane and assume a degenerate spherical morphology. Bacteria were rarely seen inside healthy cells, but were present outside cells or entrapped in cell debris (Fig. 2e). Sterile supernates from chambers challenged with Typhimurium strain GM3 induced an almost identical picture of epithelial disintegration; examples of an individual enterocyte in the process of detachment from the epithelium (Fig. 2g), and completely detached groups of cells (Fig. 2h) or individual cells (Fig. 2i) still bearing microvilli are shown. The fields shown were typical.

Furthermore, culture filtrates of Typhimurium strain TML did not disrupt epithelia (not shown), but a slight oedematous reaction was sometimes seen in villous tips, similar to that shown in Fig. 2c and d. Evidence of tight junction cleavage was not observed, even in areas where inter-enterocyte fluid accumulation had occurred.

**Discussion**

This study provides strong evidence that Typhimurium strain GM3 induces toxin-mediated damage in distal rabbit ileal epithelia in the early stages of the infection process. When sterile culture filtrates, from gut challenged with bacteria in vitro, were added to fresh tissue in vitro, they reproduced the same lesion as induced by bacteria themselves. The finding that the key features of epithelial damage induced by sterile filtrates were the same as those produced by bacteria in vivo confirmed that the effects seen in vitro were not artefactual. Although this study has only presented direct evidence for an epithelium-disrupting toxin for Typhimurium strain GM3, the similarity of the lesions produced by Typhimurium strain GM3 and Dublin strain 3246 [1] suggests that a similar toxin might also be produced by Dublin strains.

Previous studies had shown that Typhimurium strain GM3 was relatively non-invasive [1], giving rise to the possibility that the original culture sent from the 'local laboratory' to the Central Public Health Laboratory, Colindale, London (the source of the strain used in this study), had been derived from a colony of non-pathogenic bacteria. The first experiment proved that this was not the case: Typhimurium strain GM3 was capable of producing experimental fluid secretion. Although not statistically analysed, the comparative fluid secretion data for strains TML and LT7, and CT were typical of those obtained from previous large-scale studies in this laboratory. It is of interest that the amount of fluid secretion induced by strain GM3 was less than that by strain TML, although strain GM3 produced more epithelial damage than strain TML. Previous observations had shown that massive structural damage to gut epithelia could be induced when pre-treated with nitrogen mustard then challenged with Typhimurium strain TML without ensuing fluid secretion [6]. Fluid secretion does not appear to be a direct function of structural epithelial damage. It is clear that several factors are involved in the pathophysiology of diarrheal fluid secretion and that one factor may be the nature, rather than the extent, of the damage caused by invading salmonellae. For Typhimurium strain TML it has been shown that massive shedding of bacteria-laden cells occurred with immediate rescaling of the epithelia, resulting in shortening of villi followed by their rapid reconstruction [5].

The main difference between strains TML and GM3 in their interaction with gut tissue was observed in the early stages of infection. In-vivo expression of epithelium-damaging toxin was rapid, the effects being seen within 3 h; thus, Typhimurium strain GM3 is both histotoxic and invasive. Destruction of the epithelium concomitantly alters the nature of the tissue being invaded, exposing new cells and routes of tissue invasion. The observations in this and the accompanying paper [1] can explain some earlier, often-quoted results [7] that *Salmonella* penetrated the gut of guinea-pigs via basolateral membranes and not through the brush borders of enterocytes. We, and others, have repeatedly demonstrated that salmonellae do invade the gut via the brush borders of enterocytes. However, it is not difficult to see how earlier deductions [7] might have been made if the strain used expressed the type of toxin reported here.

The main feature of the early damage to epithelia is detachment of enterocytes which is almost certainly preceded by cleavage of tight junctions. This leads to the release of microvilli-bearing cells which degenerate rapidly into spherical highly vacuolated entities. The toxin could be an internalised cytoxin that causes disruption of tight junctions and subsequent intracellular disorganisation from the inside. Alternatively, it could act externally on tight junctions with subsequent changes occurring as a result of highly differentiated enterocytes being removed from their normal environment. The bacteria-laden cells that are being shed (Fig. 1b) do not appear to be extensively vacuolated, indicating that perhaps the toxin is expressed intraluminally rather than intracellularly. The observations from TEM reported in this paper correlate well with those from SEM reported previously [1]. Depending on the plane of sectioning, sections of detaching balloon-like enterocytes ([1], Fig. 4b and c) would give rise to the kind of TEM sections exemplified in Fig. 2f. While
no change in epithelial integrity was observed in control tissues, slight oedema was observed (Fig. 2c and d). This is to be expected, based on the work which established the organ-culture system [3]: substitution of choline for most of the Na⁺ retards (but does not stop) the inward flow of fluid into the tissue, where it accumulates in the absence of an intact blood supply and lacteal drainage [3].

This work highlights the need to conduct experiments on toxins of salmonellae under ecologically significant conditions, something which has not been done satisfactorily with the much studied salmonella cholera-like enterotoxin encoded by the *stn* gene [8, 9]. An earlier study [4] showed no correlation between the abilities of bacterial extracts and of live organisms from which these extracts were derived, to cause fluid secretion in rabbit ligated loops. Also, more recent studies have shown that *stn* knock-out mutants of bovine strains of Typhimurium and Dublin were as enteropathogenic as the respective wild-type strains from which they were derived [10]. Despite the fact that the role of enterotoxin in salmonellosis remains unresolved, research on *stn* continues unabated with respect to: optimising production *in vitro* [11–13], purification [14–16], molecular characterisation [17] and the species distribution of *stn* [13] or *stn* epitopes [18]. Studies on the production, release and extraction of salmonella enterotoxin(s) active on cell lines — CHO, Y1 adrenal, Vero and HeLa cells [14] — continue to be carried out without attempts to evaluate biological relevance in terms of disease causation.

The relatedness of the toxin described here to various toxic activities (other than *stn*) described by others and the relevance of those latter toxins as virulence determinants, need to be addressed. Cell-free extracts of Typhimurium from chicks were reported to destroy the epithelia of isolated intestinal segments of 1-day-old chicks [19]; complete shedding of the epithelia occurred with both bacterial cultures and cell-free extracts. However, that work can be criticised for several reasons. First, in that and several other studies, cell-free extracts were presumably used because culture filtrates were inactive; but unless ‘toxin(s)’ can be shown to be released *in vivo* at appropriate sites and times, it is difficult to attribute relevance in virulence to them. Another criticism relates to the nature of the gut explant systems used. We have developed a system whereby the insult to the epithelium, whether from organisms or their products, is exclusively from the luminal side of the tissue; cut segments bathed in medium allow the possibility of access to the tissue via cut surfaces and the serosal side of the tissue. Again, these workers do not mention whether live bacteria induced the same lesions *in vivo* to the same degree and with comparable rapidity. Finally, the authors themselves emphasise that the physiological immaturity of the 1-day-old chick may result in an exaggerated response to both bacterial and toxic challenge.

Rapidly induced changes have been observed in intercellular junctions in MDCK cell monolayers challenged with Typhimurium strain SL1344 [20, 21], but again no evidence was presented as to the relevance of this phenomenon *in vivo*. A zonula occludens toxin (ZOT) of *Vibrio cholerae* has been described which alters the permeability of rabbit epithelia [22, 23]. It has been suggested that ZOT might be responsible for the alteration in apical junctional complexes observed in human duodenal biopsies from cholera patients. However, it is the zonula adherens in human tissue and not the tight (occludens) junction that is affected by *V. cholerae* [24], emphasising the care needed in extrapolation from model systems to actual disease situations. The relationship of these ‘junction-altering’ activities with the histotoxin reported here is not known.

An extracellular salmonella protease that degrades collagens (types I and III), fibronectin, immunoglobulins (IgG and IgM), lysozyme and haemoglobin and is toxic to HEp-2 cells has been described [25, 26]; these properties make this protease a putative virulence candidate. It is suggested that the protease is potentially important in invasion and might contribute to the complications seen in systemic and chronic infections [25].

Currently there is renewed interest in salmonella cytotoxins. However, the precise relevance of such work to pathogenic mechanisms in salmonellosis in general — or to our work in particular — is unclear, because experiments are usually carried out on cell lines (Vero cells [27], MDBK and Vero cells [28], CHO, Y1 adrenal, Vero and HeLa cells [14] and Vero cells [29]) without any correlative in-vivo work and (mostly although not entirely) with bacterial extracts, the uncritical use of which has already been alluded to above. While it is true that, in our experiments detached enterocytes undergo rapid degeneration, this is probably a sequel to detachment rather than a primary cytotoxin-mediated effect.

The evidence for a lytic macrophage toxin in *Salmonella* that is expressed intracellularly is more convincing. Lysis of murine macrophages following infection of macrophage monolayers *in vitro* with serotypes Typhimurium, Dublin and Choleraesuis has been described [30]. Lysis was enhanced by the presence of the virulence plasmid, but was not mediated by the *spv* genes, and occurred after internalisation because cytochalasin D abolished the lytic effect. Fewer and less healthy peritoneal and splenic macrophages were recovered from mice infected by wild-type Dublin strains compared with plasmid-cured derivatives [30]. It is claimed that invasive, but not non-invasive, strains of Typhimurium and Typhi induce apoptotic death in macrophages [31]. In contrast, it has been claimed that *Salmonella*-induced apoptotic death of macrophages is mediated...
by a toxin secreted by a functional type III system and does not require bacterial invasion for expression of lytic activity [32].

Finally, in addition to the cytotoxic effect on macrophages, the selective destruction by Typhi of differentiated Caco-2 cells grown in monolayers, resulting in more efficient transmigration of Typhi than, for example, Typhimurium has been described [33]. However, we have shown that the histotoxic Dublin strain used in our work did not cleave tight junctions in Caco-2 cell monolayers [1]. The initial penetration and destruction of murine M cells by invasive strains of Typhimurium followed by secondary invasion of adjacent enterocytes [34] has been attributed to the ‘balanced production’ by Typhimurium of haemolysin [35], the toxin which had earlier been described as necessary for intra-macrophagic survival of Salmonella [36]. However, while the relevance of M cells as an important, perhaps primary, portal of salmonella entry into gut mucosa may have been established in the mouse for Typhimurium [34, 37, 38], this is not generally true for all Salmonella-host interactions. Work in this laboratory has shown conclusively that, in rabbits, Typhimurium can also penetrate the gut by direct entry into enterocytes [1, 5]. Similar findings have been made for Typhimurium and Dublin in calves [39, 40] and pigs (Bolton et al., unpublished observations).

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