The nature and role of mucosal damage in relation to *Salmonella typhimurium*-induced fluid secretion in the rabbit ileum

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Summary. The time course and nature of mucosal damage induced in rabbit ileal loops by two strains of *Salmonella typhimurium* (TML and W118) isolated from human infections was assessed by immunofluorescence microscopy and by scanning and transmission electronmicroscopy. *Salmonella*-induced fluid secretion occurred in the presence or absence of gross mucosal architectural damage. Neither strain caused mucosal ulceration. When damage did occur, the villi were shortened by loss of their tip regions with concomitant reforming of an intact mucosal surface. Immediately preceding the onset of fluid secretion, marked infiltration of the mucosa with polymorphonuclear leukocytes and occasional macrophages was seen. This revives an earlier suggestion that interaction between invading salmonellae and acute inflammatory cells may be an important factor in initiation of fluid secretion. Brush-border invasion by salmonellae cannot *per se* be the immediate cause of fluid secretion, because the latter occurred several hours after initial invasion.

Introduction

Salmonella-induced diarrhoea is a complex phenomenon in which several mechanisms may play a part (Stephen *et al.*, 1985). In a previous paper (Wallis *et al.*, 1985) we described the existence of salmonella enterotoxin(s), the significance of which in the causation of fluid secretion in experimental animals is now being investigated. In this paper we describe experiments designed to define the nature of, and evaluate the possible role of mucosal damage in, *Salmonella*-induced fluid secretion.

Various animal models have been used to study fluid secretion, or histological changes, or both, induced by salmonellae—rabbits (Taylor and Wilkins, 1961; Giannella *et al.*, 1973), opiate-treated, starved guinea pigs (Takeuchi, 1967), rats (Powell *et al.*, 1971), Rhesus monkeys (Rout *et al.*, 1974) and chicks (Popiel and Turnbull, 1985). In these studies no systematic attempt was made to correlate the severity and nature of tissue damage with the onset of fluid secretion. Giannella and his coworkers did report qualitative differences in rabbit ileal-tissue damage induced by strains differing in virulence for

monkeys (Rout *et al.*, 1974) and rabbits (Giannella *et al.*, 1973). Based on limited histological observations they suggested that fluid secretion in the rabbit model preceded the onset of structural damage to the mucosa. No kinetic data were reported for monkeys (Rout *et al.*, 1974); observations were made only at the peak of clinical diarrhoea.

In this paper we describe the time course of fluid secretion in rabbit ileal loops (RILs) infected with virulent *S. typhimurium* (strains TML and W118; Giannella *et al.*, 1973) and relate this to mucosal damage as demonstrated by light and immunofluorescence microscopy and by scanning and transmission electronmicroscopy.

Materials and methods

Bacterial strains

S. typhimurium strains TML and W118, generously provided by Drs R. A. Giannella and J. W. Peterson, had previously been shown to invade and to induce fluid secretion in RIL tests (RILTs); this biotypical behaviour has been confirmed (Wallis *et al.*, 1985). Strains were stored at -70°C in 1-ml volumes of equal proportions of glycerol and Mueller-Hinton broth.

Rabbits

Dutch rabbits (1.5–2.0 kg) from Hyline Rabbits,

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Cheshire, were used for experiment 1 and New Zealand rabbits (2.5–3.0 kg) from Regal Rabbits, Surrey, were used for experiments 2 and 3.

Fluid secretion

S. typhimurium strains were tested for the ability to induce fluid secretion in RILTs as described by Wallis *et al.* (1985). Cholera toxin (CT; Sigma) 1.5 µg was used as a positive control. Fluid secretion was measured as volume of fluid accumulated (v)/length of loop (L)(ml/cm).

Invasiveness

Strains of *S. typhimurium* were assessed for invasiveness by immunofluorescence microscopy and by scanning and transmission electron microscopy.

Immunofluorescence microscopy. *S. typhimurium* antigen and antiserum to *S. typhimurium* were prepared as described by Roschka (1950). Samples for immunofluorescence microscopy were prepared as follows. Rabbits were anaesthetised at intervals after loop infection. Ileal loops were excised, and three full-thickness biopsies (c. 1 cm²) were removed from the loops and pinned flat on dental wax. Biopsies for examination by immunofluorescence microscopy were fixed in 95% ethanol overnight at 4 °C. They were then embedded and sectioned in paraffin wax (2-µm sections). Sections for immunofluorescence microscopy were incubated at 56 °C for 1 h, dewaxed and soaked in phosphate-buffered saline (PBS; NaCl 8.5 g/L, Na₂HPO₄ 1.07 g/L, NaH₂PO₄·2H₂O 0.39 g/L in distilled water, pH 7.1) for 20 min. The slides were then placed in a moist chamber and exposed to 0.05 ml of guinea pig anti-*Salmonella* antiserum (1 in 20 dilution) for 30 min. The slides were then washed in PBS for 10 min and treated with sheep anti-guinea pig-FITC conjugate (Wellcome Diagnostics; 1 in 20 dilution in PBS) containing rhodamine (Difco; 1 in 20 dilution) for 30 min in a moist chamber. The sections were finally washed in PBS for 10 min, mounted under a coverslip with alkaline-buffered glycerol (NaHCO₃ 0.175 g/L, Na₂CO₃ 0.16 g/L, glycerol 90% v/v in distilled water) and examined immediately by fluorescence microscopy with a Zeiss UV microscope (450–490 nm barrier filters). The presence of bright green-fluorescing, rod-shaped bacteria in the mucosal cells and lamina propria was taken as evidence of mucosal invasion.

Scanning electron microscopy (SEM). Biopsies were washed thoroughly with Hartley digest broth before fixation to remove debris that might obscure surface detail of the villi. Biopsies were immersed for 1 h in glutaraldehyde 2.5% then post-fixed for 1 h in OsO₄ 1%. Glutaraldehyde and OsO₄ were each made up in 0.05M phosphate buffer made up to a total molarity of 0.35M with sucrose. Biopsies were then immersed for 1 h in thiocarbonylhydrazide 1% at 50 °C, washed for 15 min in water at 50 °C, osmicated for 1 h in OsO₄ 2% in water at 50 °C (Ono and Takashio, 1978) and then dehydrated serially in acetone 25%, 50% and 100% before critical point drying with liquid CO₂. The specimens were fixed to copper plates with electrically conducting paint (RS 555–

156) and examined without coating in a JEOL 120CX II electron microscope fitted with a TEMSCAN attachment. Images were obtained via a secondary electron detector at accelerating voltages of 20 or 40 kV.

Transmission electron microscopy (TEM). Biopsies were fixed as for SEM. After fixation they were dehydrated in ethanol and 1 mm³ portions were embedded in an epon-araldite mixture (Mollenhauer, 1964). Sections were cut and stained in methanolic uranyl acetate and lead citrate, and examined in a JEOL 120CX II electron microscope at accelerating voltages of 60 or 80 kV.

Results

Time course of fluid secretion

The time courses for fluid secretion induced by *S. typhimurium* strains TML, W118 and by CT in RILs are shown in fig 1. Fluid secretion induced by CT was established by 8 h (V/L=0.5 ml/cm) and continued until at least 16 h after inoculation. *S. typhimurium* strains TML (two experiments) and W118 each induced net fluid secretion between 6 and 8 h which increased until at least 14 h after inoculation.

Immunofluorescence microscopy

S. typhimurium strain TML (experiment 1). Immunofluorescence microscopy of samples taken 2 h after inoculation showed individual *Salmonella* organisms invading the ileal mucosa (fig. 2a); this was confirmed by TEM (fig. 2b). Most organisms were still adherent to the brush border or free in the lumen (not shown in this field). By 4 h, microcolonies of bacteria could be seen within enterocytes. The degree of invasion thereafter increased with time, though within each ileal loop the degree of villus invasion was not uniform and sections of some villi were entirely free of organisms. By 8 h, a massive influx of infiltrating polymorphonuclear leukocytes (PMNLs) was seen. By 12 h, in villi with heavily invaded tips, tip enterocytes containing bacteria were rounding up and were being extruded (fig. 2c). This extrusion process, followed by resealing of the damaged mucosal layer, resulted in villus shortening. By 18 h, organisms could be seen towards the bases of villi (fig. 2d).

S. typhimurium strains TML (experiment 2) and W118 (experiment 3). The patterns of bacterial invasion were similar to that seen in experiment 1.

Scanning electron microscopy

In all three experiments the mucosal surfaces of uninfected control loops were covered with con-

Fig. 1. Time courses of fluid secretion in rabbit ileal loops inoculated with *S. typhimurium*, strains TML (experiment 1, fig. 1a; experiment 2, fig. 1b) and W118 (fig. 1c). Cholera toxin was used as a positive control. Data for each time point were obtained from individual rabbits, and are means derived from paired loops. Δ - - - Δ Cholera toxin; $\blacktriangle$ — $\blacktriangle$ *S. typhimurium*.

siderably more mucus and entrapped material than were time-matched infected secreting loops from the same animals.

S. typhimurium strain TML (experiment 1). Little evidence of gross change in villus architecture was seen in loops infected with strain TML by 4–6 h (fig. 3a). By 8 h, increased surface irregularity was apparent (fig. 3b). At 12 h, extrusion of villus-tip material was obvious (fig. 3c). Mucosal damage was maximal at 16 h; villi were shortened and blunted and there was extensive shedding of villus-tip

material (fig. 3d). There was evidence of recovery of villus surface structure at 18 h when compared with an 18-h negative control loop from the same animal (fig. 4a, b).

S. typhimurium strain TML (experiment 2). No obvious difference between infected and control mucosae was observed until 8 h when villi from infected loops appeared comparable with those in fig. 3b. Significant extrusion of villus-tip material was observed only at 18 h, when extrusion was much less than is indicated by fig. 3c in experiment 1;

Fig. 2. Invasion of *S. typhimurium*, strain TML, experiment 1 (see fig. 1*a*). *a*. Individual organisms, visualised by immunofluorescence microscopy, invading the lateral region of a villus, 2 h after inoculation; →: brush border; bar = 10 µm. *b*. A similar preparation to that in 2*a*, taken 2 h after inoculation and examined by TEM. Note only local disturbance of brush border and invasion of enterocytes. Organisms were not seen penetrating through tight cell-cell junctions; → tight junction; bar = 1 µm. *c*. Fluorescent-antibody-stained section of *S. typhimurium*-infected loop, taken 12 h after inoculation. Note villus-tip enterocytes, laden with bacteria, rounded-up and being extruded. The mucosa appears to become rapidly resealed (→); bar = 1 µm. *d*. A similar preparation to that in 2*c* taken at 18 h. Note increased numbers of bacteria in the region of the bases of villi; bar = 10 µm.

Fig. 3. SEM analysis of *S. typhimurium*-induced changes in villus architecture; experiment 1 (see fig. 1*a*). Biopsies were taken from separate rabbits 4, 8, 12 and 16 h after inoculation of RILs (*a*, *b*, *c* and *d* respectively), processed and analysed by SEM. The appearances of villi ranged from normal (*a*) through surface wrinkling (*b*) to progressive shedding of tip cells (*c*, *d*). Note the SEM appearance of 3*c* and compare this with 2*c*. Fig. 3 (and also fig. 4) comprises representative regions of mucosa selected from a larger field at 400 $\times$ magnification and containing around 50 villi. Bar = 100 μ m.

Fig. 4. SEM analysis of *S. typhimurium*-induced changes in villus architecture (experiment 1; see fig. 1*a*). Biopsies were taken from the same rabbits at 18 h: (*a*) after inoculation of RIL with *S. typhimurium*, and (*b*) from a control loop that received sterile medium. Note: (i) evidence of recovery of villi, which, though shortened, show less tip derangement and a return to more normal surface appearances ($\rightarrow$). (ii) Ligation for 18 h *per se* does not induce changes in villus surface structure. The picture shown was virtually indistinguishable from control biopsies taken at 0 h. Fig. 4 (and also fig. 3) comprises representative regions of mucosa selected from a large field at $400\times$ magnification and containing around 50 villi. Bar = $100\ \mu\text{m}$.

gross villus-tip destruction, as seen in experiment 1 (fig. 3*d*), was not observed. No clear indication of shortening of villi was observed.

S. typhimurium strain W118 (experiment 3). The maximal change observed was seen at 10 h; this was not progressive, as in experiment 1. Villi appeared

to exhibit surface irregularity (similar to fig 3*b*) but subsequent mucosal samples from animals at different time points were not significantly different from corresponding time-matched controls. There was less evidence of significant villus shortening than in experiment 1.

Transmission electronmicroscopy

S. typhimurium strain TML (experiment 1). Invasion of ileal mucosa by strain TML was observed by 2 h after inoculation (fig. 2b), but with

mainly local derangement of the brush border during invasion of ileal enterocytes. By 4 h, microcolonies of TML were seen in intra-cytoplasmic vacuoles containing remnants of microvilli and also in the cytoplasm; these findings were similar to

Fig. 5. TEM analysis of tissues infected with *S. typhimurium* (experiment 1; see fig. 1a). Biopsies were taken 6, 8, 8, and 14 h (a, b, c and d respectively) after inoculation of RILs, from three different rabbits. a. PMNL interacting with bacteria (→) at surface of enterocyte layer; bar = 1 μm. b. Massive infiltration of PMNLs into mucosa between adjacent enterocytes; bar = 10 μm. c. PMNLs apparently being extruded into the lumen; bar = 10 μm. d. Extrusion of rounded-up, bacteria-containing enterocyte (→) (to be compared with figs. 2c and 3c,d); bar = 10 μm. Note: the cell-cell junctions appear to remain intact until the final expulsion of the infected enterocyte.

Fig. 6. TEM analysis of *S. typhimurium*-infected villus (experiment 2; fig. 1*b*). Biopsy was taken 8 h after inoculation of a RIL. Note: (i) highly invaginated surface of enterocyte layer (*a*) (compare with fig. 3*b*); (ii) large numbers of invading (*b*) and dividing (*c*) bacteria; (iii) evidence for infiltrating cells of the immune system (IC→); bar = 10 μ m.

those recorded below for experiment 2 (figs. 6a–c). By 6 h, phagocytosis of invading bacteria by PMNLs was observed (fig. 5a), and by 8 h, large numbers of PMNLs were infiltrating the mucosa (fig. 5b) and extruding on to the mucosal surface (fig. 5c). At 12, 14, 16 and 18 h, many bacteria could be seen within severely damaged enterocytes with disrupted brush borders; some enterocytes were being extruded into the lumen from the tip region (fig. 5d).

S. typhimurium strain TML (experiment 2). The general patterns of bacterial invasion and PMNL infiltration already described for experiment 1 were again observed. Several features may be highlighted from micrographs of biopsies taken 8 h after inoculation. The highly invaginated outline of the longitudinal section (fig. 6a) corresponded with an SEM appearance similar to that in fig. 3b. Large numbers of intramucosal bacteria (figs. 6b,c) some of which were clearly dividing (fig. 6c) were observed; the latter were dividing in a cell with an intact brush border. It is not possible to be certain whether these organisms were being translocated into the lamina propria or were about to be expelled in rounded-up enterocytes. There was clear evidence in this and in many other sections of resident intra-epithelial lymphocytes and other infiltrating cells of the immune system.

S. typhimurium strain W118 (experiment 3). TEM appearances broadly resembled those seen with strain TML in experiment 2.

In all three experiments, sections through crypt regions revealed crypt cells with intact morphology without evidence of bacterial invasion.

Discussion

The onset of fluid secretion took place in all three experiments before (experiment 1), or in the absence of (experiment 2 and 3), the appearance of gross architectural damage to villi. The major difference between experiments 1 and 2, in which the same strain (TML) of *S. typhimurium* was used, was a different source and breed of rabbits. Thus, gross mucosal damage is not a prerequisite for *Salmonella*-induced fluid secretion and, when it occurs, may be host-dependent.

The methods used to analyse tissue damage can profoundly affect the perception of the nature and extent of morphological changes. For example, SEM, which in any one field gives an overview of around 50 villi at a magnification of $\times 400$, readily permits assessment of the proportion of damaged villi as judged by surface appearance (figs. 3 and 4). Importantly, the technique permits a more accurate

evaluation of the frequency of damaged villus tips and hence the weight that should be given to the frequency of individual damaged enterocytes—seen in transmission electronmicrographs—in relation to pathophysiological changes. Indeed, without SEM, it would have been possible, depending on the plane of sectioning of biopsies taken from early time points in experiment 1, to have misinterpreted the extent of architectural derangement observed by TEM of villi from infected loops.

The notion that gross structural damage is not an essential prerequisite for fluid secretion in salmonella infections is in keeping with the key findings of Rout *et al.* (1974) who showed that *Salmonella*-induced tissue damage depended on the site of infection. Jejunal secretion occurred in *S. typhimurium*-infected Rhesus monkeys without jejunal inflammation, histological damage or bacterial colonisation and invasion, albeit at one fixed time point during the diarrhoeal illness, although these processes did take place in the distal intestine. The data presented here also help to explain the earlier work of Fromm *et al.* (1974) with Ussing Chambers, which first led to the suggestion that *Salmonella*-induced diarrhoea is a secretory diarrhoea. The intestinal mucosa was taken from RILs 18 h after infection with strain TML. By this time the mucosa—had it been damaged as indicated by figs. 3a–d—would have been sufficiently re-formed and intact to allow successful stripping of mucosa for mounting in Ussing chambers and detection of intestinal secretion (see below).

The nature of the structural damage to villi is worthy of further comment. It may be seen (fig. 2c) that when villus-tip cells are shed there is an apparent re-sealing of the mucosa. At no time was there evidence of massive stripping of enterocytes which would lead to mucosal ulceration. This interpretation is strengthened by scanning electronmicrographs (figs. 3 and 4) in which it can be seen that, at the height of fluid secretion, there is an apparent return towards normal surface appearances. It is likely that much of the tissue debris and mucus seen in these micrographs would be flushed away by intestinal secretion and peristalsis in the unligated intestine. Koo *et al.* (1984) suggested that *Salmonella*-induced damage was due to the production of a Shiga-like cytotoxin (*i.e.*, a toxin which inhibits protein synthesis). They posed the question, unanswered as yet, as to why the nature and extent of mucosal damage is greater in shigellosis than in salmonellosis. There are several reasons why we believe that such a cytotoxin need not be involved. It is possible to re-interpret their data (table 1 and fig. 5; Koo *et al.*, 1984) to show that salmonella

infection did not inhibit protein synthesis in enterocytes infected *in vivo* and analysed *in vitro*. These authors comment on, but do not explain, the fact that their infected tip cell-fractions contained more protein than comparable control preparations. The simplest explanation for this is that the protein concentration reflects the number of cells present in the fractions. At 18 h there would be a massive number of tissue PMNLs, which would not synthesize much protein. The decrease in enterocyte alkaline phosphatase activity reported by Koo *et al.* (1984) is more likely to be associated with loss of villus tip cells, a possibility they acknowledged (see also fig. 3, above), rather than being due to inhibition of protein synthesis, the explanation they preferred. Although the RIL is a good model for salmonella ileitis (Giannella *et al.*, 1973) in that virulence in this model correlated with virulence in monkeys, and by extension man, it is not a good model for shigellosis (Gemski *et al.*, 1972; Levine *et al.*, 1973). Much more marked changes occurred in villus architecture in *Shigella dysenteriae*-infected loops (Levine *et al.*, 1973) than we have observed in *S. typhimurium*-infected loops. It is, therefore, unwise to compare salmonella and shigella infections in this model. Any perturbation of the integrity of enterocytes in the villus tip may signal accelerated enterocyte expulsion. We have observed a similar phenomena in an unrelated system—rotavirus-infected villi in neonatal mice (unpublished observations).

The rounding-up and extrusion of villus-tip cells followed by resealing of the damaged villus tips described above, resulted in villus shortening. Villus-tip enterocytes are thought to be the site of solute and electrolyte absorption (Field, 1979) and crypt cells the site of secretion of electrolytes and water. Hence damage to villus tips during experimental *S. typhimurium* infection could result in decreased absorption, thereby unmasking physiological crypt secretion. This is clearly not the initiating factor in fluid secretion, but the fluid secretory response could thus be amplified or prolonged if, or when, this process of villus shortening occurs.

In all experiments there was a massive influx of inflammatory cells. PMNLs have previously been suggested as the source of endogenous prostaglandin which, when released as a result of bacterium-

PMNL interaction (Gots *et al.*, 1974; Giannella, 1979), act as mediators of fluid secretion. Our results support a possible role for PMNLs in the induction of fluid secretion in that the inflammatory response occurred in all three experiments and at times preceding the onset of overt fluid secretion. These earlier observations are being repeated, extended and quantified to assess the possible significance of the influx of inflammatory cells in the causation of fluid secretion.

Initial brush border damage or penetration cannot be the cause of fluid secretion because the latter begins several hours after the primary invasion of organisms. However, since as far as is known all virulent salmonellae so far described are invasive, it is clear that the process of invasion and tissue translocation is an important preliminary step towards the expression of the virulent phenotype. It is not possible to be sure whether the organisms shown in fig. 6 are in the process of penetrating deeper, or are about to be expelled. If the latter, the organisms that eventually reach the *lamina propria* (fig. 2) may be those that invade the basal regions of villus epithelium, because there was little evidence, even in experiment 1, of expulsion of infected enterocytes from the basal regions. The somewhat more dramatic pictures reported by Takeuchi (1967) of "swelling and elongation of microvilli" coincident with the approach or adherence of *Salmonella* organisms were also seen in the present study but should not be regarded as typical, because the appearances seen in fig. 2*b* were observed much more frequently.

Finally, the present study strongly suggests that damage of ileal villus architecture by *S. typhimurium* is confined to the tip regions and may be a non-specific response to infection which expels invading pathogens. This damage is not essential for the induction of intestinal fluid secretion but the villus shortening which follows could give rise to a "physiological diarrhoea" which might be important in prolonging, though not initiating, fluid secretion.

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