Chloride-led Disruption of the Intestinal Mucous Layer Impedes *Salmonella* Invasion: Evidence for an ‘Enteric Tear’ Mechanism

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Key Words

Mucus • Ion transport • Bacterial adherence • Bacterial invasion • Enteric tear mechanism

Abstract

Background/Aims: Methods: The intestinal epithelial layer can switch from a net absorptive state to one of net secretion in the presence of luminal toxins and pathogens. This suggests an innate defence role for regulated secretion of mucus, electrolytes and water. We hypothesised that chloride-led fluid secretion across the mucus-covered intestinal epithelium alters barrier properties by influencing the overlying mucous-gel layer. Results: We demonstrated that chloride-led disruption of the epithelial-associated mucus-gel covering HT29-MTX-E12 (E12) human colonic epithelial monolayers, a goblet-cell like line derived from parental HT29 cells, resulted in reduction of associated mucus as well as a reduction in mucous-gel density and barrier properties. Changes in epithelial secretory state were accompanied by increased water transport, and the resulting loss of gel integrity reduced *Salmonella typhimurium* invasion of epithelia in both E12 monolayers and of isolated rat ileal mucosae. However, neither chloride secretion nor mucus disruption altered numbers of adhering bacteria.

Conclusion: These data suggest a role for chloride led fluid secretion in the shedding of the adherent mucous-gel layer, possibly as a rate-limiting innate defence mechanism, and offer evidence for “enteric tears” in intestinal host defence.

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Introduction

Although intestinal mucous-gels are composed mostly of water, and their hydration state regulates viscoelasticity [1, 2], the source of water for gel formation remains unexplored. There is some evidence that water secretion, goblet cell exocytosis and gel formation are functionally coupled. For example, pharmacological inhibition of fluid secretion reduces mucus formation in rats [3] and stimulation of chloride secretion accompanies the release of mucin granules from the apical cytoplasm of goblet cells [4, 5]. The coordinated release of intracellularly stored mucus and the flux of water from the serosa to the lumen across the epithelium may contribute to regulation of the mucous-gel layer which, contributes to a putative “enteric tear” mechanism. Coordinated fluid and mucin secretion, which is essential in ocular homeostasis and airway clearance [6, 7], may also protect the intestinal epithelium by flushing toxic or

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pathogenic components away from the epithelial surface [4]. Evidence of these pathways at the intestinal epithelium [8-12] suggests the presence of an “enteric tear mechanism” [13], mechanically flushing antigens away from the epithelium through an ion-led change in osmotic gradient and accompanying movement of water to hydrate and disrupt the mucous-gel layer [14-16].

Human epithelial cell monolayers derived from colonic carcinomas such as T84 and Caco-2 have provided insight into mechanisms of coupled electrolyte and fluid transport [17-20]. An additional approach using subclones of HT29-MTX cells, which form a mucous-gel layer overlying the epithelial cells, has been used to examine selected properties of mucus not found in T84 or Caco-2 [21]. One such clone, the HT29-MTX-E12 (E12) cell line, has been studied in the context of a system for screening drug transport, bioadhesive molecules [21-25] and functional parameters [26-29], including the role of mucus in host-pathogen interactions [30].

We hypothesised that chloride-led fluid secretion across the mucus-covered intestinal epithelium alters barrier properties by influencing the overlying mucus-gel layer. Employing pharmacological secretagogues, we have characterised, for the first time, the ion transport pathways present in E12 monolayers and assessed whether increased ion transport altered the barrier properties of the E12 mucous-gel layer. We investigated whether active ion transport altered the physiology and barrier properties of epithelia-associated mucous-gel, through mucus protein assays, histology and the flux of hydrophobic compounds through E12 monolayers. To investigate whether these changes in mucous-gel barrier function had a role in innate defence, we next examined whether changes in epithelial secretory state and mucus integrity influenced host-bacterial interactions in E12 monolayers and verified both these findings and the chloride-led transport of water in isolated rat ileal segments. Our data suggests that chloride led disruption of the mucous-gel layer reduces the ability of *Salmonella typhimurium* to invade the epithelium, offering a role in innate epithelial defence and providing further evidence for the “enteric tear mechanism”.

Materials and Methods

Animals

Rats (male Wistar, 200-300 g) employed in electrophysiological and transport studies were obtained from the UCD Biomedical Facility. These animals were housed at constant temperature, with free access to food and water, and

with a 12 hour day/night cycle. All studies were carried out in accordance with the use of post-mortem animal tissue policy of the Animal Research Ethics Committee of University College Dublin. Rats were euthanized by cervical dislocation and exsanguination.

Cell culture

E12 monolayers were cultured and maintained as previously described [21, 23]. Briefly, cells were seeded at a density of 5×10^4 cells/well on 12 well Costar Transwell™ permeable inserts (0.4 µm pore polycarbonate membrane, catalogue #3401) or, for electrophysiological experiments on Corning Costar Snapwell™ permeable inserts (0.4 µm pore polycarbonate membrane, catalogue #3407). Routine testing of cultured cells confirmed lack of mycoplasma.

Water transport studies

The isolated ileum was flushed with oxygenated medium and everted on a glass rod as previously described [31, 32]. Sacs were weighed on a microbalance and placed in oxygenated, phenol free HEPES-buffered DMEM (Gibco, # 21063-029). The sacs were tied securely with silk suture and a small animal vascular catheter (Data Sciences International Physiocath™ 277-1-002) was tied into the other end. A 1ml syringe with a sterile 26 gauge micro lance was fixed to the catheter and the sac filled with a 500 µl solution of forskolin or vehicle in DMEM. Each sac was weighed on a microbalance and then placed in a separate sealed 50 ml flask containing 10 ml of oxygenated DMEM on a shaking water bath for 20 minutes at 37°C. Sacs were then removed from the bath and the internal contents recovered by aspiration using a fresh 1 ml volume syringe with 26 gauge micro lance. The recovered fluid was weighed on a microbalance for accuracy of volume. The fluid transport across the intestinal wall was measured by loss of liquid from the everted sac.

Electrophysiological studies

E12 monolayers or rat ileal sheets, stripped of the smooth muscle layer, were mounted in Ussing chambers, bathed both apically and basolaterally with phenol-free, HEPES-buffered DMEM (Gibco, # 21063-029) or Krebs-Henseleit physiological solution (pH 7.4, osmolarity 330 mOsmol) at 37°C and gassed continuously with 95% O₂/5% CO₂. After a 10 minute equilibration period, electrical parameters of the monolayers were recorded. Basal potential difference (PD) was recorded and clamped by an applied short circuit current (I_{sc}) throughout the experiment (DVC 4000, WPI, UK). Transepithelial electrical resistance (TEER), which is an indirect marker of epithelial barrier function, was calculated according to Ohm's law. Electrogenic changes in I_{sc} were measured in response to secretagogues in the presence and absence of ion channel or pump inhibitors. All chemicals and drugs were obtained from Sigma Chemical Company (Dorset, UK).

Western blot for mucin proteins

Confluent 1 cm² cell monolayers (approximately 1×10^6 cells) were treated with secretagogues or vehicle for 20 mins and washed 3 times with warm media to remove disrupted

mucins. Cells were then lysed in 2X Laemmli Buffer (100 μ L/cm²). Samples were denatured through boiling for 5 minutes. Protein was loaded into a 1% agarose gel submerged in a 40 mM TAE Buffer (pH 8.0) containing 0.1% SDS and separated by electrophoresis for 1.5 hrs at 65V. Gels were then washed for 20 minutes in a neutralising solution (0.6 M NaCl/60 mM sodium citrate) and transferred to pre-neutralised nitrocellulose membrane (pore size 0.45 μ m) by vacuum blotting for 2 hrs at 4.5 kPa using a Vacugene XL blotter. Nitrocellulose membranes were blocked and probed with primary antibodies Lum 2-3 for MUC2 [33]. After overnight incubation and washing, the membrane was probed with HRP-conjugated secondary antibodies (anti-mouse, Cell Signal Technologies), washed and developed with Supersignal (Pierce, #34081) on X-ray film. Blots were analysed semi-quantitatively by measuring pixel number using ImageJ (National Institutes of Health, Bethesda, MD; <http://rsb.info.nih.gov/ij/>) as previously described [34, 35].

Visualisation of the mucous-gel layer

Monolayer-membranes were sandwiched between thin sections (approximately 200 μ m thick) of chicken liver [23]. The resulting 'sandwiches' were snap-frozen in liquid nitrogen, embedded in optimal cutting temperature (OCT) compound, cut into 20 μ m sections and transferred to glass slides for staining. To visualise the mucous-gel layer, sections were stained with Alcian blue (1%) in distilled water adjusted to pH 2.5 with glacial acetic acid (3%) for 10 min at room temperature and washed with distilled water. Sections were then counterstained with neutral fast red (1%) for 5 min at room temperature and washed under a flowing water source and then with distilled water. For the purpose of fluorescent imaging, no neutral fast red stain was employed, as this interfered with bacterial stains. Images were obtained with a Nikon Eclipse E400 Light/Fluorescent Microscope with Nikon Digital Camera (Model:DXM 1200) and analyzed using Nikon ACT-1 imaging software (version 2.0).

Transport of dexamethasone across E12 monolayers

Confluent, polarised E12 monolayers, with distinct apical and basolateral membranes [21], grown on Transwell™ inserts were incubated for 120 min in 10 mM HEPES-buffered DMEM medium, in the presence of apically-added dexamethasone (10⁻⁸ M). Dexamethasone is a moderately hydrophobic molecule with a log D of 1.95, the mucous gel presents a barrier to dexamethasone flux, with a 4-fold increase in flux in the absence of mucus [25]. Basolateral samples (100 μ l) were taken at 20-minute intervals and the medium replaced with fresh buffer. In order to investigate the contribution of the mucous-gel to dexamethasone transport, mucus was removed by incubating monolayers apically with the mucolytic, N-acetylcysteine (NAC, 10 mM), for 15 min at 37°C and washing the monolayers three times in buffer. Basolateral samples were assayed for dexamethasone by a competitive ELISA kit (BioX diagnostics). Apparent permeability (P_{app}) for monolayers and sacs was calculated from cumulative concentration vs. time profiles using the following equation:

$$P_{app} \text{ (cm/sec)} = (dQ/dt)/(A * C_o) \text{ [36]}$$

Where dQ/dt (μ g/s) represents the cumulative concentration increase in the receiver-side with respect to time, A (cm²) is the surface area of the monolayer and C_o (μ g/mL) is the initial concentration in the apical donor compartment.

Infection studies

Primary bacterial cultures of *Salmonella* (*S.*) *typhimurium* (isolated from food, Strain A) were streaked on Columbia blood agar plates and incubated overnight at 37°C. Colonies were isolated, cultured in LB broth, shaking at 37°C overnight. 50 μ l of the resulting culture was inoculated into 5ml of fresh LB broth and incubated static for 3 hours at 37°C resulting in a bacterial population of 1×10⁸ CFU/ml. The static culture was centrifuged at 3,000×g for 10 min. The resulting pellet was re-suspended in DMEM-HEPES buffer and diluted to the appropriate multiplicity of infection (MOI). For *in vitro* experiments, monolayers were pre-treated with either forskolin or bumetanide (5 min) and bacteria were fluorescently stained before apical challenge of E12 monolayers on Transwell® inserts at MOI 100 (10⁸ bacterial cells) for 10 min at 37°C. For *ex vivo* experiments, horizontally mounted rat ileal sheets, Peyer's patch associated villi (PPAV) or follicle-associated epithelium (FAE) were pre-treated with either forskolin or bumetanide (5 min) and challenged with *Salmonella* at MOI 100 (10⁸ bacterial cells) for 10 min at 37°C. E12 monolayer were either washed and collected for mucus visualization as described above or fixed in formalin and embedded in paraffin for immunostaining. Tissues were fixed in formalin and embedded in paraffin for immunostaining.

Visualisation of bacteria

Salmonella were labeled with 5- (and 6-) carboxytetramethylrhodamine, succinimidyl ester [5-(6)-TAMRA, SE] (catalogue no. C-1171; Molecular Probes). TAMRA stain (1 μ l of 10 mg/ml) was added to a 1 ml bacterial suspension to give a final concentration of 10 μ g/ml TAMRA stain. The bacteria were incubated static at 37°C in the dark for 30 min and washed repeatedly in sterile PBS, before resuspension in DMEM-HEPES. Immunostaining for *Salmonella* was carried out as previously described [37]. *Salmonella typhimurium* were labelled with mouse anti-*Salmonella typhimurium* (Abcam # ab13633) and visualised by HRP conjugated secondary antibody stain. Bacteria were counted manually under a light microscope. Invasion was classified as stained bacteria located within the cell cytoplasm. Adhesion was classified as stained, cell-associated bacteria located outside the epithelial cell cytoplasm. For each infected monolayer or ileal segment, bacteria were counted in 20 random fields, each 500 μ m in length, and the average number of bacteria calculated. Paraffin sections were cut to 5 μ m in width and based on this, bacterial adhesion per mm² area was calculated. This method of enumeration was compared to a gentamicin sulfate kill/CFU count method of bacterial enumeration [38] and no significant difference was found in results (data not shown).

Pharmacological Agent	Role	Polarity	Concentration	Vehicle
Carbachol	Secretagogue	Basolateral	10 ⁻⁵ M	H ₂ O/DMEM
Forskolin	Secretagogue	Basolateral	10 ⁻⁵ M	DMSO
Bumetanide	Transport inhibitor	Basolateral	10 ⁻⁴ M	DMSO
Amiloride	Transport inhibitor	Apical	10 ⁻⁴ M	DMSO
Barium Chloride	Transport inhibitor	Basolateral	10 ⁻³ M	H ₂ O/DMEM
Acetazolamide	Transport inhibitor	Basolateral	10 ⁻⁴ M	DMSO
4,4'-diisothiocyano-2,2'-stilbenedisulfonate	Transport inhibitor	Apical	10 ⁻⁴ M	DMSO
5-nitro-2-(3-phenyl-propylamino)-benzoate	Transport inhibitor	Apical	3x10 ⁻⁴ M	DMSO

Table 1. The secretagogues and ion transport inhibitors employed for the pharmacological examination of E12 electrophysiology. Agents were applied either apically or basolaterally depending on the polarization of the target molecule receptor. Concentrations shown are final concentrations. Solvent vehicles final concentration was 0.1% (v/v).

Results

Electrophysiological parameters in E12 monolayers

Initially, we sought to characterise the nature of electrogenic ion transport in E12 monolayers employing a range of secretagogues and ion transport inhibitors (Table 1). Basal short circuit current was stable ($6.1 \pm 0.9 \mu\text{A}\cdot\text{cm}^{-2}$). When challenged with the adenylate cyclase activator, forskolin, E12 monolayers exhibited a rapid onset and sustained response of ΔIsc $12.0 \pm 1.1 \mu\text{A}\cdot\text{cm}^{-2}$ (Fig. 1A). In contrast, the effects of the cholinergic agonist, carbachol (CCh) alone were modest (ΔIsc $3.3 \pm 0.7 \mu\text{A}\cdot\text{cm}^{-2}$) and transient (data not shown). In separate experiments, monolayers were pre-treated with selected agonists and antagonists prior to forskolin stimulation (Fig. 1B). Pre-treatment of E12 monolayers with CCh significantly increased ΔIsc responses to forskolin, likely through either activation of basolateral calcium-regulated potassium channels or priming of apical chloride channels. Neither acetazolamide (Azm) nor 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) altered forskolin response, suggesting that ΔIsc responses were not due to bicarbonate-chloride exchange or proton-linked sodium uptake. 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) and barium chloride reduced the ΔIsc responses stimulated forskolin, confirming that the ΔIsc response was due both to apical chloride secretion via the cystic fibrosis transmembrane regulator (CFTR) and dependent on basolateral barium-sensitive potassium channels. Finally, bumetanide significantly reduced basal ΔIsc response to forskolin, implying that the Na-K-Cl co-transporter (NKCC1) is an essential component. Localization of NKCC1 to the basolateral membrane was confirmed, as apical addition of bumetanide had no effect on ΔIsc response to forskolin (data not shown). Together, these data suggest that forskolin induces chloride-led ΔIsc

Fig. 1. Elucidation of active ion transport pathways in voltage clamped E12 monolayers. (A) Forskolin (10^{-5} M) evoked an inward Isc . Arrow denotes addition of forskolin. (B) Pre-treatment with various secretagogues or transport inhibitors prior to forskolin treatment. Veh (vehicle), CCh (carbachol), Azm (acetazolamide), DIDS (4,4'-diisothiocyano-2,2'-stilbenedisulfonate), NPPB (5-nitro-2-(3-phenyl-propylamino)-benzoate, Ba^{2+} (barium chloride) and Bumet (bumetanide). *** $p < 0.005$, ** $p < 0.01$ ($N=6$). Mann-Whitney, two tailed t-test, compared to Veh.

response that is dependent on basolateral potassium channels, NKCC1 transporter and CFTR.

Disruption of the E12 mucous-gel layer

Mucin proteins were identified by Western blotting (Fig. 2A). Because of their large molecular size (~ 900 KDa, measured with respect to laminin, which was employed as a comparative size marker), mucin protein bands were characteristically diffuse and did not separate or migrate through the gel [39]. Monolayers treated with forskolin for 10 min and washed, showed significantly less cell-associated Muc-2 as measured by mucin western blot densitometry (Fig. 2B). Carbachol treatment caused

Fig. 2. Forskolin induced chloride secretion promotes dissociation of mucus from E12 monolayer. (A) Whole cell Western blot for MUC2 shows that forskolin (Fsk), but not CCh reduced cell mucin content compared to vehicle control (Veh) as measured by (B) densitometry. (C) Forskolin-treated E12 monolayers show disruption of the mucous-gel by light microscopy, (D) untreated vehicle, stained with Alcian blue for mucus and nuclear fast red for epithelium. (E) Disruption of the mucous-gel layer promotes through chloride secretion increased the the apparent permeability coefficient (P_{app}) of dexamethasone. Removal of the mucous-gel by N-acetyl cysteine (NAC; 10 mM, 15 min) suggests that the increase in the P_{app} induced by forskolin is mucus-dependent. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus Veh, N= 6-9. Mann-Whitney, two tailed t-test.

Fig. 3. Chloride-led mucus disruption impedes E12 infection by *Salmonella typhimurium*. *Salmonella* adherence and invasion was enumerated by immunohistochemistry of infected E12 monolayers with H&E counterstain and analysing by computer image analysis. (A) A representative image of *Salmonella* (arrows) adherence to E12 monolayer (E12) grown on a permeable membrane (PM). (B) There was no significant difference in bacterial adherence to quiescent, bumetanide (0.1mM) -treated E12 monolayers [Q] over secretory, forskolin (10 μ M)-treated E12 monolayers [S]. (C) Chloride secretion and subsequent mucous-gel disruption significantly impedes invasion by *Salmonella typhimurium*. (D) Merged light (mucus - blue) fluorescent (*Salmonella* - red) image shows bacteria penetrating E12 mucous-gel during challenge. (E) Bacterial penetration of the mucous-gel is impeded upon treatment with forskolin. N=6, * $p < 0.05$, ** $p < 0.01$, Mann-Whitney, two tailed t-test.

no significant change in cell-associated mucin. In contrast, forskolin-treated E12 monolayers (Fig. 2C) displayed a reduced mucous-gel layer compared to untreated monolayers (Fig. 2D), as indicated by the reduced Alcian blue staining for mucus (These results were reproducible over five separate experiments). When E12 monolayers were treated with 10 mM NAC to remove the gel layer, only cytoplasmic and membrane-bound mucins remained visible (data not shown).

The basal P_{app} of dexamethasone across E12 monolayers was $5.6 \pm 0.6 \times 10^{-6}$ cm/sec, a flux rate comparable to previous studies of dexamethasone permeability in the Caco-2 monolayers [40]. Removal of

the mucous-gel layer with NAC significantly enhanced dexamethasone flux across E12 monolayers ($P < 0.0001$). Basolateral treatment with forskolin prior to dexamethasone addition also significantly increased the permeability ($P = 0.0125$), although not as dramatically as was the case with NAC pre-incubation. Combination of NAC and forskolin significantly increased the P_{app} of dexamethasone, but the increase was not significantly different from NAC treatment without forskolin, suggesting perhaps that the flux was maximal with NAC alone (Fig. 2E). Importantly, there was no significant change in the P_{app} of the hydrophilic paracellular flux marker, FITC-dextran 4400 (1.86×10^{-7} cm/sec, FD-4)

across E12 monolayers in the absence or presence of mucus ($\Delta P_{app} -0.08 \pm 0.06 \times 10^{-7}$ cm/sec), indicating that the monolayer was intact and that the increases in dexamethasone flux were not due to non-specific artefact. These results suggest that forskolin-induced chloride secretion disrupts the mucous-gel overlying E12 monolayers.

Secretion and its role in pathogen-epithelium interaction

We next sought to examine the relevance of chloride-led mucus disruption during exposure to bacteria. The secretory status and/or mucus integrity of E12 monolayers were pharmacologically manipulated to determine whether adhesion and/or adherence of pathogenic bacteria to epithelial monolayers might be affected (Fig. 3A). There was no significant difference in the numbers of epithelial-adherent *Salmonella* due to secretory status (Fig. 3B). However, removal of the mucus-gel layer significantly increased *Salmonella* invasion in both secreting and quiescent monolayers. Secreting monolayers showed significantly less invading bacteria than quiescent cells (Fig. 3C), while again, removal of the mucous-gel increased levels of invasion overall. Merged light-fluorescent images depicting fluorescent TAMRA-stained *Salmonella* in the mucous-gel layer suggest greater and more uniform penetration of the invading bacteria in quiescent cell mucus (Fig. 3D) while there is evidence of clumping and aggregation within the mucus of secreting cells (Fig. 3E).

Isolated rat ileal sheets responded with a sustained inward current I_{sc} responses to forskolin ($\Delta I_{sc} = 69.8 \pm 6.8 \mu A \cdot cm^{-2}$, Fig. 4A). Further, measuring water loss from an everted rat ileal sac, we demonstrated a significant serosal to mucosal movement (secretion) of water likely due to chloride secretion since it was stimulated over basal by forskolin (Fig. 4B). To investigate how these factors influence *Salmonella* invasion, we mounted rat ileal, PPAV and FAE mucosa into horizontal chambers and induced a secretory or quiescent state before *Salmonella* challenge. Again, *Salmonella* was identified on H&E section by immunohistochemistry and enumerated by image analysis (Fig. 5A&B). There was no difference in bacterial adherence to or invasion of PPAV or FAE mucosa due to secretory state. As with E12 monolayers, there was no difference in bacterial adherence to rat ileum due to secreting state (Fig. 5C). However there were significantly less invasion of *Salmonella* in tissue undergoing secretion compared to tissue in a quiescent state (Fig. 5D).

Fig. 4. Induction of chloride secretion in rat ileum correlates with water transport across rat ileal wall of everted sacs. (A) Forskolin (10^{-5} M) induced a sustained inward I_{sc} response in rat ileum in Ussing chambers. Arrow denotes addition of forskolin (B) Forskolin applied to the serosal side of sacs promoted water transport from the basolateral to the mucosal side, $N=4$, ** $p<0.01$, Mann-Whitney, two tailed t-test.

Fig. 5. Forskolin-stimulated water secretion impedes bacterial invasion into isolated rat ileal tissue. (A) & (B) Bacterial adhesion (bacteria indicated by a) and invasion (bacteria indicated by i) were enumerated by immunohistochemical staining of *Salmonella typhimurium* (arrows) on H&E stained rat ileal tissue with subsequent computer image analysis. (C) Forskolin ($10 \mu M$)-treated rat ileal segments in a secretory state [S] showed no difference in the level of bacterial adherence compared to bumetanide-treated quiescent tissue [Q]. (D) *Salmonella* invasion was significant decreased in ileal segments undergoing secretion compared to quiescent ileal tissue. $N=6$, ** $p<0.01$, Mann-Whitney, two tailed t-test.

Discussion

The intestinal mucous-gel layer is the outermost line of defence of the intestinal barrier, offering protection through physical steric hindrance, charge and hydrophobic gradients and antimicrobial peptides [41–44]. Despite this, the mucus-gel layer is often overlooked in the study of intestinal disease and infection. This may be in part, due to the lack of suitable models for studying mucous-gel biology in real time [23]. E12 cells display a goblet-cell like morphology and contain apical mucin granules [45–47] that are secreted to form an overlying mucous-gel layer that is adherent to the cell monolayer [21]. As such, E12 monolayers offer an ideal platform for examining the role of the mucous-gel at mucosal interactions, such as drug delivery [21–24] and immune-pathogen responses [30, 48].

The coordinated effort of the mucous-gel layer and ion secretion pathways in clearance of luminal antigens is reinforced by the role of CFTR in airway disease [6, 7]. These pathways are also evident at the intestinal epithelium [8–12] acting as an “enteric tear mechanism” [13]. The secretion of ions across the intestinal epithelium changes the osmotic gradient and leads to a passive transport of water, hydrating the mucosa [14–16]. We hypothesised, that such an event would directly influence epithelial-pathogen interactions, given the established role of mucus gel as a staging point for pathogenic infection [48–51]. Initially we sought to characterise chloride transport in E12 monolayers through examination of *I*_{sc} in response to pharmacological stimulation. The addition of forskolin to E12 monolayers mounted in Ussing chambers led to a rapid significant rise in *I*_{sc}. The magnitude of *I*_{sc} response was relatively low when compared to that in T84 and HT29 monolayers [20, 52]. We speculate that the presence of goblet cells (~60% of the heterogeneous population [21]) may account for the relatively lower capacity of remaining crypt-like epithelial cells to secrete chloride ions. Goblet cells do not appear to contribute significantly to electrogenic ion transport [53]. Pre-treatment with CCh led to a synergistic increase in *I*_{sc} responses when forskolin was added compared to forskolin alone, while pre-treatment of cells with bumetanide, NPPB, and barium resulted in significant inhibition of the response to forskolin suggesting that the NKCC, apical chloride channels and basolateral barium-sensitive K⁺ channels are involved in E12 electrogenic chloride secretion transport responses. Similar findings have been reported in other goblet cell-like HT29 subclones [45]. The emptying of cytoplasmic mucins from goblet cells in

response to fluid secretion has been previously observed in cell and tissue models [4, 54–56] and *in vivo* this event may disrupt the mucous-gel layer overlying the epithelium.

E12 monolayers expressed MUC2 which contributes to gel formation in the colon [57] in a manner which results in a stratified distribution of layers of mucus [58] and, of these, the inner of the two MUC2 containing layers is free of bacteria [59]. Disruption of the mucous-gel layer increased permeability of lipophilic drugs across the epithelial monolayer. These data suggest that fluid secretion has a dual role in i) establishment and ii) sacrificial shedding of a mucous-gel layer, possibly contributing to containment of luminal microorganisms, thus providing evidence for “enteric tears” in colonic defence [13] in a manner which involves coordinated secretion of water and mucus, the importance of which has recently been demonstrated in a mouse model of colitis [60].

We next examined the consequence of stimulated chloride secretion on the stability of the mucous-gel overlying E12 monolayers. Routine fixation processes for intestinal tissue disrupt the mucous-gel and typically result in the capture of only cytoplasmic and residual surfaces mucins [61]. Here we used a technique which minimised the direct disruption and dissociation of the mucous-gel layer or possible formation of ice crystals within the gel. Upon challenge with forskolin and induction of chloride secretion, apical mucin granules were secreted and visible disruption to the mucous-gel occurred. Immunoblotting showed a reduction of MUC2 protein in lysates of E12 monolayers, treated with forskolin. We used the apparent permeability of dexamethasone as a surrogate marker of changes in density to the E12 monolayer mucous-gel layer. The mechanism for this enhanced absorption is unknown, but dexamethasone is unlikely to exert an effect on cell protein production in the short course of the flux experiments. Indicated by a lack of reduction of the 4kDa FITC-dextran marker flux (data not shown), tight junctions were not affected in that time frame. We propose that the lowered mucus density overlying E12 monolayers may underlie the enhanced absorption, causing an increase in the flux of hydrophobic molecules physiologically hindered by the presence of mucus. These findings are in keeping with other reports [21, 27] and support the hypothesis that mucus can impede the transport of hydrophobic drugs across epithelium [21, 62].

Hydration-induced swelling is necessary for formation of a functional mucous-gel [54]. Since mucins are negatively charged, it has been proposed that luminal cations (Na⁺ and K⁺) are involved in maintaining a stable gel. In this regard, since our experiments were performed

with standard physiological solutions, the only mechanism for altering cation levels is via absorptive or secretory processes of the epithelium. It is generally accepted that cation flux into the lumen of intestine follows active transport of anions and that the consequent osmotic gradient pulls water across the monolayers of epithelial cells. The role of anions in mucous-gel formation remains controversial although the dependence of water flux upon electrogenic chloride secretion is generally accepted [17, 40]. While a role for bicarbonate ions in mucus formation has been recently demonstrated using an airway-derived cell line [63], whether it plays a role in E12 mucous-gel formation has not been ascertained.

Major changes in intestinal epithelial secretory function are a hallmark of invasion by enteric pathogens [64-68]. Such secretory states, invariably lead to luminal water influx [15, 16] and consequently increasing intestinal mucus thickness [69], distancing the epithelium from the luminal contents and affording the epithelium better protection. This suggests that secretory events are an innate protective response to pathogens. One such pathogen, *Salmonella typhimurium* is a ubiquitous mammalian pathogen that must adhere to epithelial surfaces before invading epithelial cells and causing secretory diarrhoea [38, 64]. Further, *S. typhimurium* may bind directly to mucins before invasion of epithelia [49, 70]. Considering the changes in mucous-gel observed in E12 monolayers after forskolin treatment, we hypothesised that a secreting state may impede and flush away potentially invading bacteria. Active chloride secretion resulted in a significant reduction in numbers of invaded *S. typhimurium* in E12 monolayers. However, there was no significant reduction in adherent bacteria. Mucus-gel histology suggests that water secreted during robust chloride secretion may over-hydrate and disrupt the mucous-gel. To further expand our observations, we examined the effect of epithelial chloride secretion on *S. typhimurium* infection in an *ex vivo* rat intestinal model. *Salmonella* species are known to initiate infection of mammalian hosts by penetrating the intestinal epithelium of the small bowel [71], and as such ileal tissue was the most physiologically relevant tissue for our studies. Forskolin induced chloride secretion resulted in a 3.5-fold increase in water transport into the lumen of rat ileal sacs. The difference in secretory status did not affect the

number of bacteria adhering to rat intestinal epithelium but did reduce *Salmonella* invasion in actively secreting rat ileum by 60% over quiescent tissue ($P < 0.05$). Surprisingly, removal of mucus with NAC did not abolish the capacity of chloride secretion to reduce bacterial invasion, although with NAC treatment there was an overall significant increase in bacterial adherence and invasion. This may suggest that although bacteria may reach the glycocalyx of the epithelial cell membrane, membrane-bound mucins, which are not removed by NAC play the key role in preventing bacterial invasion of the cells. We were unable to distinguish features of the two distinct mucus layers [72, 73] which may be important if only the outer, less dense layer is a principal site of bacterial attachment [59]. Significant to these findings, there was no difference in *S. typhimurium* adherence or invasion in either FAE or PPAV, suggesting that antigen sampling is not affected by the enteric tear mechanism. This may be due to the reported findings that the Peyer's patch have an absent or reduced mucous-gel layer [74]. Other endogenous protective elements may also be important to consider with respect to a holistic understanding of how the selective barriers of the gut physiologically function [75].

In summary, we have demonstrated *in vitro* evidence of a functional coupling of water transport and mucus secretion across epithelial mucosa. Such a coordinated process, which may contribute to the gel-phase transition necessary for gel formation was also shown to influence the kinetics of experimental challenge with bacteria. These data provide a novel insight into the importance of the mucous-gel layer as an innate defence to pathogenic challenge of the intestinal mucosa. E12 monolayers may therefore serve as a useful tool for future studies in assessing the role of mucus shedding in host-pathogen interactions.

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