Pharmaceutical Research, Vol. 25, No. 5, May 2008 (© 2007)
DOI: 10.1007/s11095-007-9501-3

Research Paper

A Tertiary Amino-Containing Polymethacrylate Polymer Protects Mucus-Covered Intestinal Epithelial Monolayers Against Pathogenic Challenge

Simon Keely,[1] Lee-Anne B. Rawlinson,[1] David M. Haddleton,[2] and David J. Brayden[1,3]

Received October 1, 2007; accepted November 9, 2007; published online November 29, 2007

Purpose. We examined the cytoprotective influences of the mucoadhesive polymer, poly(DMAEMA), on human mucus-producing intestinal epithelial monolayers against two bacterial exotoxins and *S. typhimurium*. Direct anti-bacterial effects were also assessed against *S. typhimurium*.
Methods. In the presence and absence of mucus, untreated or poly(DMAEMA)-exposed monolayers were challenged with *S. typhimurium* or supernatants containing either cholera (CTx) or *C. difficile* toxins. Assays included LDH, cytokine secretion, cyclic AMP (cAMP) and microscopy to visualise bacterial adherence by monolayers. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of poly(DMAEMA) against *S. typhimurium* were established, along with a time–kill study.
Results. CTx and *C. difficile* toxin induced LDH release from E12 monolayers. CTx also elevated intracellular epithelial cAMP, while *S. typhimurium* induced basolateral IL-8 secretion. Pre-treatment of E12 monolayers with poly(DMAEMA) reduced these effects, but only in the presence of mucus. The polymer co-localised with *S. typhimurium* in mucus and reduced bacteria–epithelia association. Poly (DMAEMA) was directly bactericidal against *S. typhimurium* at 1 mg/ml within 30 min.
Conclusions. Poly(DMAEMA) may have potential as a non-absorbed polymer therapeutic against infection. These effects were mediated by a combination of physical interaction with mucus and by direct bacterial killing.

KEY WORDS: anti-bacterial polymers; bacterial resistance; HT29 monolayers; living radical polymerisation; poly(2-(dimethylamino-ethyl) methacrylate.

INTRODUCTION

The growing resistance of bacterial species to antibiotic therapies is an issue of worldwide concern. Poor therapeutic management and contamination of the food chain, coupled with increasingly adaptive bacterial strains has led to the emergence of "super bugs" such as methicillin-resistant *Staphylococcus aureus* (MRSA) (1). This has led to a need to shift away from antibiotics and a focus on investigating new types of antimicrobial drugs with novel ways to deliver them in high concentrations to the site of infection (2). The intestinal epithelium is important for initiation and regulation of immune responses against pathogens and their metabolites (3). It achieves this through the innate immune system, an inherent mechanism of resistance to disease, which provides physical and chemical barriers to the passage of pathogens from the lumen to the bloodstream (4). The first barrier encountered by a pathogenic agent is the mucus-gel layer, a dynamic, interactive mucosal defensive system active at the mucosal surfaces (5). Intestinal barrier defences secrete peptides with anti-microbial, anti-fungal and/or anti-viral activity, which reside in the epithelial mucus-gel layer (6). Host-originating molecules that inactivate bacteria include cationic anti-microbial peptides such as defensins and cathelicidins and bacteriolytic enzymes such as lysozyme. The cationic charge facilitates binding to anionic bacterial cell envelopes. Recently, a small synthetic peptide modelled on a host-defence defensin was shown to protect mice against a lethal bacterial challenge through modulation of the innate immune response (7). Overlying the intestinal epithelium, the mucus-gel itself confers a degree of protection to the underlying epithelial cells. Alteration of mucus-gel viscosity has been shown to impede the migration of pathogenic bacteria towards the mucosal surface (8). Thus far, there has been little focus on therapeutics that mimic or enhance either of these defensive properties. While anti-microbial peptides are one possible group to further investigate, they tend to be labile and unstable and are expensive to produce in pharmaceutical grade quantities (9).

In contrast, synthetic polymers are relatively easy to produce and relatively inexpensive to make (10). Synthesised

[1] School of Agriculture, Food Science and Veterinary Medicine and UCD Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland.
[2] Department of Chemistry, University of Warwick, Coventry, CV4 7AL, UK.
[3] To whom correspondence should be addressed. (e-mail: david.brayden@ucd.ie)

ABBREVIATIONS: LDH, lactate dehydrogenase; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; poly(DMAEMA), poly(2-(dimethylamino-ethyl) methacrylate.

poly(propyleneimine) dendrimers (11), amphiphilic polymethacrylates (12) and biomimetic amphiphilic agents (13) are polymers that mimic defence peptides and have been shown to exert anti-microbial properties. Similarly, the naturally occurring mucoadhesive polymer, chitosan, has broad antimicrobial properties in gel and particulate format (14). Identification and application of non-absorbed antimicrobial polymers may alleviate the growing risk from antibiotic-resistant bacteria. Encouraging studies with synthetic non-absorbed polymers such as polyethylene glycol (PEG) have shown that prophylactic administration of PEG by lavage conferred protection to mice against lethal sepsis upon challenge with *Pseudomonas aeruginosa* (15). Cationic polymers including poly(hexamethylenebiguanide) are also bactericidal against *P. aeruginosa* (16).

Poly(2-(dimethylamino-ethyl) methacrylate, Poly(DMAEMA), is a mucoadhesive cationic polymer that adheres to the intestinal epithelial-derived mucus (17) and increases mucus-gel barrier function (18). Confocal microscopy studies showed that the polymer co-localised with the mucus layer. We hypothesised that increased mucus gel density was the reason for the impeded passage of drugs across mucus-covered human E12 monolayers (18). There is also *in vitro* evidence to suggest that poly(DMAEMA) may have potential as an antimicrobial agent (19). When applied as a surface coating to inert surfaces, poly(DMAEMA) inhibits growth and colonisation by *B. subtilis* and *E. coli* (20) and *P. aeruginosa* (21). These properties indicate that the polymer may have potential as a non-absorbed topically-administered mucoadhesive antimicrobial agent, which might eventually have potential for treatment of dental caries, wounds to the skin, oral and gastrointestinal mucositis or inflammatory bowel disease. The polymer has the advantage of being non-cytotoxic to human epithelial cells (18) and ultimately of being formulated into particulate gels and pastes. To date, there has been few attempts to investigate anti-microbial effects of poly(DMAEMA) in biological systems.

The aims of this study were to examine the effects of the polymer in prevention of bacterial-induced epithelial damage in E12 monolayers. Monolayers were challenged with two bacterial exotoxins and an invasive bacterium. The cytoprotective potential of poly(DMAEMA) against these different types of challenges was examined by pre-treating monolayers with poly(DMAEMA) followed by examination of epithelial function through a selection of biochemical and metabolic endpoints, as well as by measurement of the effect of the polymer on bacterial adherence and uptake. Finally, we investigated direct antimicrobial affects of poly(DMAEMA) against *S. typhimurium* for which minimum inhibitory concentration (MIC), minimum bacteriocidal concentrations (MBC) and time–kill curves were determined.

MATERIALS AND METHODS

Materials

All tissue culture reagents were from Gibco (Biosciences, Ireland). Cholera toxin (CTx) was from Sigma-Aldrich, Ireland. Tissue culture filters and plates were from Corning Costar (Fannin Healthcare, Ireland). The cyclic-AMP (cAMP) ELISA (Cat#DE0450) and IL-8 ELISA (Cat#MAB208) kits, as well as the detection antibody, biotinylated IL-8 (Cat. #BAF208), were from R&D Systems (UK). The lactate dehydrogenase (LDH) kit, cholera toxin (CTx) and N-acetyl cysteine (NAC) were obtained from Sigma Aldrich, UK.

Polymer Synthesis

The poly(methacrylate) used was a quaternised derivative of poly(2-(dimethylamino)-ethyl methacrylate (DMAEMA) monomer (10), untagged or tagged with the fluorescent probe hostasol. Hostasol is a fluorescent marker in the green spectrum range (465 nm excitation/570 nm emission). Polymer synthesis was carried out using living radical polymerisation exactly in accordance with our previously described methods (17,18). The molecular weight of poly(DMAEMA) was 12,300 Da and the poly-dispersity index was 1.08 (18). This batch was used in most studies. A second batch was used in MIC, MBC and time–kill studies and it had a molecular weight of 6,600 Da and a poly-dispersity index of 1.16.

Cell Culture

HT29-MTX-E12 (E12) is a mucus-producing sub-clone of the human adenocarcinoma intestinal cell line, HT29, which has been used in drug transport studies (22). E12 cells between passages 50 and 57 were seeded at a density of 2×10^4 cells/filter on 1 cm^2 Transwell® polycarbonate membrane inserts (pore size 3 μm, Corning Costar cat.# 3402) as previously described (18). The cells were fed apically and basolaterally every 2 days and differentiated to form monolayers over 21 days. Monolayer integrity was examined by measuring transepithelial electrical resistance (TEER) across confluent cell monolayers 21 days post-seeding, and directly before and after adhesion experiments. TEER was measured using an EndOhm® electrode system.

Toxin Studies

E12 monolayers were left untreated or treated with 0.1 mg/ml poly(DMAEMA) for 30 min under physiological conditions. The apical medium was then replaced with either DMEM–HEPES medium, 1 ng/500 μl CTx in DMEM–HEPES or 10 ng/500 μl CTx in DMEM–HEPES and incubated for 2 h at physiological conditions. Binding of CTx to GM1 membrane receptors increases intracellular cAMP levels (23). The physiological buffer was supplemented with 1 mM 3-isobutyl-1-methylxanthine (IBMX) to prevent metabolism by phosphodiesterase (24). The apical medium was sampled for LDH assay. Cells were lysed with 0.1 M HCl and the lysate was centrifuged for 10 min at $600\times g$. Cell lysates were dried in an evaporation centrifuge (Concentrator 5301, Eppendorf) and assayed for cAMP. Cells were also stimulated with the adenylate cyclase activator, forskolin (10 μM), as a positive control. *Clostridium (C.) difficile* supernatants were taken from a 24 to 72-h anaerobic culture and passed through a 0.4 μm filter to remove cells. Each sample was assayed for the EC_{50} required to produce E12 cell death by LDH assay to provide a dilution factor. Supernatants were diluted in DMEM–HEPES. E12 monolayers were pre-treated with 0, 0.01, 0.1 or 1 mg/ml poly(DMAEMA) in the apical compartment for 30 min in DMEM–HEPES medium. The apical medium was then

replaced with either fresh DMEM–HEPES medium or supernatants and incubated for 12 h. The apical medium was periodically sampled for LDH assay.

S. Typhimurium Culture and 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 for 3 h at 37°C resulting in a bacterial population of 1×10^8 CFU/ml. *S. typhimurium*-infected LB broth (1×10^8 CFU/ml) was centrifuged at $3{,}000\times g$ for 10 min. The resulting pellet was re-suspended in DMEM–HEPES buffer to give a concentration of 1.25×10^7 CFU/ml. The apical side of the monolayers were exposed to poly (DMAEMA) for 30 min and the apical medium was then replaced and washed, leaving only mucus-bound polymer. The apical medium was then replaced with *S. typhimurium*-infected DMEM–HEPES. Plates were then either incubated at 37°C with bacteria for 5 min (fluorescent imaging), 10 min (immunohistochemistry) or for 24 h (IL-8 studies). The apical medium was replaced with fresh uninfected DMEM–HEPES buffer in controls. Basolateral samples were taken at regular intervals over 24-h and stored for ELISA. Monolayer integrity was examined by measuring the TEER of monolayers after 24-h (data not shown). All microscopy was carried out using a Nikon Eclipse E400 Light/Fluorescent Microscope with Nikon Digital Camera (Model:DXM 1200) and captured using Nikon ACT-1 imaging software (version 2.0).

Preservation of E12 Mucus-Gel for Histology

Monolayers were washed in DMEM–HEPES medium. Wafer-thin strips (approximately 200 μm thick) were cut from frozen blocks of chicken liver with a razor and the monolayer-membranes were gently and compactly sandwiched between the liver-wafers. The resulting tissue-monolayer "sandwiches" were snap-frozen in liquid nitrogen. This tissue sandwiching process prevented loss of the mucus-gel layer. The tissues were embedded in optimal cutting temperature compound, cut into 20 μm cross sections with a cryo-microtome and transferred to glass slides. To visualise the mucus-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.

Fluorescent Staining of Bacteria

In order to fluorescently visualize *Salmonella*, the bacteria were prelabeled with 5- (and 6-) carboxytetramethylrhodamine, succinimidyl ester [5-(6)-TAMRA, SE] (catalogue no. C-1171; Molecular Probes). TAMRA stain (1 μl of 10 mg/ml) was added to a 1 ml bacterial suspension to give a final concentration of 10 μg/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 (25). Bacterial preparations and challenge were performed as described above and Transwell® membranes of infected E12 cells were cut from their frames and fixed and processed for bacterial quantification. Samples were stained for *Salmonella* (Mouse anti-*S. typhimurium*-*Abcam* #ab13633) and counterstained with haematoxylin.

Quantification of Bacterial Adherence and Uptake

Bacteria were counted manually on transverse sections under a light microscope. For each monolayer, bacteria were counted in 20 random fields, each 500 μm in length, and the average number of bacteria calculated. Each section width was 10 μm and bacterial adhesion per 1 cm^2 Transwell membrane was calculated. Results were expressed as bacteria/cell and were calculated based on an average count of 5×10^5 E12 cells/cm^2.

IL-8 ELISA

A 96-well Elisa plate was coated with anti-human IL-8 (40 μg/μl in PBS) capture monoclonal antibody per well and incubated overnight at 4°C. Thereafter all incubations were at room temperature. The plate was washed and blocked for 1 h. Standards and samples were added and the plate was incubated for 2 h. The plate was washed, and detection antibody (biotinylated anti-human IL-8, 20 ng/ml in assay diluent) was added and incubated for 2 h. The wash steps were repeated and the wells were incubated with Streptavidin-HRP solution (Sigma cat #S5512) for 20 min in the dark. The plate was washed and developed with 1% tetramethylbenzidine (TMB) for 20 min. The reaction was stopped with 1 M H_2SO_4 and the optical density (OD) was read on an ELISA plate reader at 450 nm with the reference filter at 570 nm.

MIC and MBC of Poly(DMAEMA)

MICs were calculated as the lowest concentration of polymer to completely inhibit growth of the bacterial cultures examined. The method of calculation was adapted from the microdilution broth dilution procedure as outlined in the Clinical and Laboratory Standards Institute (formerly NCCLS) protocol (26). Two strains of *S. typhimurium* (Strain A and Strain B, bovine origin) were seeded on microtitre plates at a density of 5×10^5 CFU/ml in tryptic soy broth (TSB) at 37°C. Cells were incubated with varying concentrations of poly(DMAEMA) in fresh media. Plates were incubated at 37°C for 18 h and growth was determined at 600 nm. For MBC calculations (27), bacteria were then spread on bacterial agar plates and incubated at 37°C for 24 h. MBC's were calculated as the lowest concentrations of poly(DMAEMA) that produced 99.9% reduction in resulting CFU values compared to initial inoculum. Poly(DMAEMA) was considered bactericidal if the MBC was ≤4× the MIC concentration (28).

Cytotoxicity Assays

Cytotoxicity was assessed by release of LDH from cell monolayers as previously described (18). Apical samples were centrifuged to remove debris and assayed for LDH release. LDH concentrations were expressed as percentage LDH release relative to treatment with Triton-X 100 in 30 min. The haemolytic assessment of poly(DMAEMA) was adapted from the protocol outlined by Shin *et al.* (29). Briefly, blood samples were drawn from male Wistar rats. Erythrocytes

were collected by centrifugation (2,000×*g*, 5 min, 4°C) and washed three times in PBS. The final pellet was re-suspended in PBS (4% *w*/*v*) and 100 µl aliquots were plated in 96-well microtitre plates. Cells were exposed to poly(DMAEMA) for 1 h at 37°C and were then centrifuged at 1,000×*g* for 5 min. Aliquots (100 µl) of the supernatant were transferred to a fresh 96 well microtitre plate where haemoglobin release was measured spectrophotometrically at 414 nm. Percent haemolysis was calculated relative to that detected with 1% Triton-X 100.

Time–Kill Studies

Time–kill studies were adapted from the time–kill method for determining bactericidal activity as outlined in the NCCLS protocol (27). *S. typhimurium* (strain A) were inoculated into flasks at 5×10^5 CFU/ml and incubated for 90 min in TSB at 37°C, 170 rpm. An initial sample was taken for colony counting. Poly(DMAEMA) at ×0.1, 0.25, 0.5, and ×1 the MIC, or medium alone was added to the flasks. Incubation was continued at 37°C, 170 rpm and samples taken at 0.5, 1, 1.5, 2, 4, 6 and 24 h for colony counts. Bacteria were grown on tryptic soy agar for all time points except 24 h which were grown on the selective media, xylose lysine deoxycholate agar, to ensure resulting colonies were *Salmonella*. Viable counts were calculated to give CFU/ml and time–kill curves plotted of $\log_{10}$ CFU/ml against time. A bactericidal effect was defined as a $\geq$3 $\log_{10}$ decrease in CFU/ml after 24 h.

Statistics

Values are given as mean ± S.E. of the mean. Comparisons were made using Student's *t*-tests, paired or unpaired as appropriate.

RESULTS

Poly(DMAEMA) Reduces Cell Killing by *C. Difficile* Toxins

We examined the influence of E12 exposure to poly (DMAEMA) on cytotoxicity induced by *C. difficile* toxins. Cells were challenged with a range of supernatant dilutions and the dilution of toxins that returned 50% LDH release into the apical compartment buffer from monolayers was selected as the challenge. *C. difficile* toxins bind to epithelial cells causing disruption of actin filaments, rearrangement of the cell cytoskeleton and breakdown of intracellular junctions (30). Pre-treatment of E12 monolayers with 0.1 and 1 mg/ml poly(DMAEMA) significantly reduced the LDH release induced by *C. difficile* toxins in a concentration-dependent manner after 12 h by more than 50% (Fig. 1). In the absence of monolayer exposure to *C. difficile* toxin and poly (DMAEMA), the control LDH release value was 98±1% in 30 min.

Poly(DMAEMA) Prevents the cAMP Elevation and Cell Death Induced by Cholera Toxin

Poly(DMAEMA)-exposed E12 monolayers were challenged with cholera toxin (CTx) and LDH release and the resulting intracellular cAMP levels were measured. Both 1 and 10 ng/ml CTx induced significant cAMP levels in E12 cells, of the same order as those seen with the adenylate cyclase activator, 10 µM forskolin (225±20 pmol/ml). The increase in cAMP induced by CTx was statistically reduced by 30–50% by pre-exposing monolayers to both 0.1 and 1 mg/ml poly(DMAEMA) for 30 min followed by CTx challenge for 120 min (Fig. 2A, data shown for 1 mg/ml polymer). CTx induced levels of cytotoxicity of 9 and 14% at polymer concentrations of 1 and 10 ng, respectively. This relatively low level of cytotoxicity is consistent with the action of CTx, which induces electrogenic chloride and fluid secretory transport across intestinal cells but does not actively kill cells (23). Exposure of E12 monolayers to poly(DMAEMA) at concentrations of 0.1 and 1.0 mg/ml decreased the cytotoxic effects of both the 1 ng and 10 ng CTx challenges to 2–3%, similar to those of polymer-treated cells (Fig. 2B, data shown for 1 mg/ml polymer).

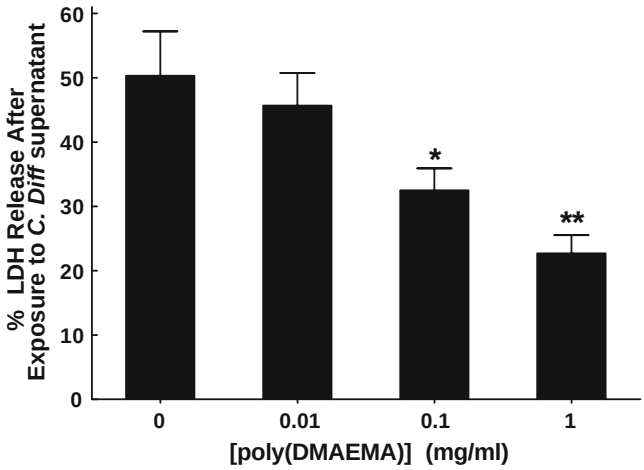

Fig. 1. Concentration-dependent effects of poly(DMAEMA) against LDH release induced by *C. difficile* supernatant on E12 monolayers at 12 h following incubation with increasing concentrations of polymer for 30 min. N=7 per group; *single asterisk* P<0.05, *double asterisk* P<0.01 *vs C. difficile* alone.

Poly(DMAEMA) Inhibits *S. Typhimurium* Adherence and Uptake by E12 Monolayers

In order to assess whether poly(DMAEMA)'s cytoprotective nature was limited to exotoxins, control- and poly (DMAEMA)-exposed E12-monolayers were treated with fluorescently labelled *S. typhimurium* and adhesion and invasion of the bacteria to E12 monolayers assessed. Adherence (Fig. 3A) and invasion (Fig. 3B) were examined microscopically. Adherence to and uptake by poly(DMAEMA)-pretreated monolayers were significantly reduced compared to polymer-untreated controls (Fig. 3C, D; P=0.005; P=0.04 respectively). Normal histological processes such as formalin fixation or snap-freezing readily disrupt the mucus-gel layer and these fixation techniques usually result in the capture of only cytoplasmic and residual surface mucins (31). In order to preserve the E12 mucus-gel, membranes were cut from the Transwell® and sandwiched in chicken liver wafers before snap-freezing. Wafer-thin slices of liver tissue were found to provide the best results for embedding due to ease of

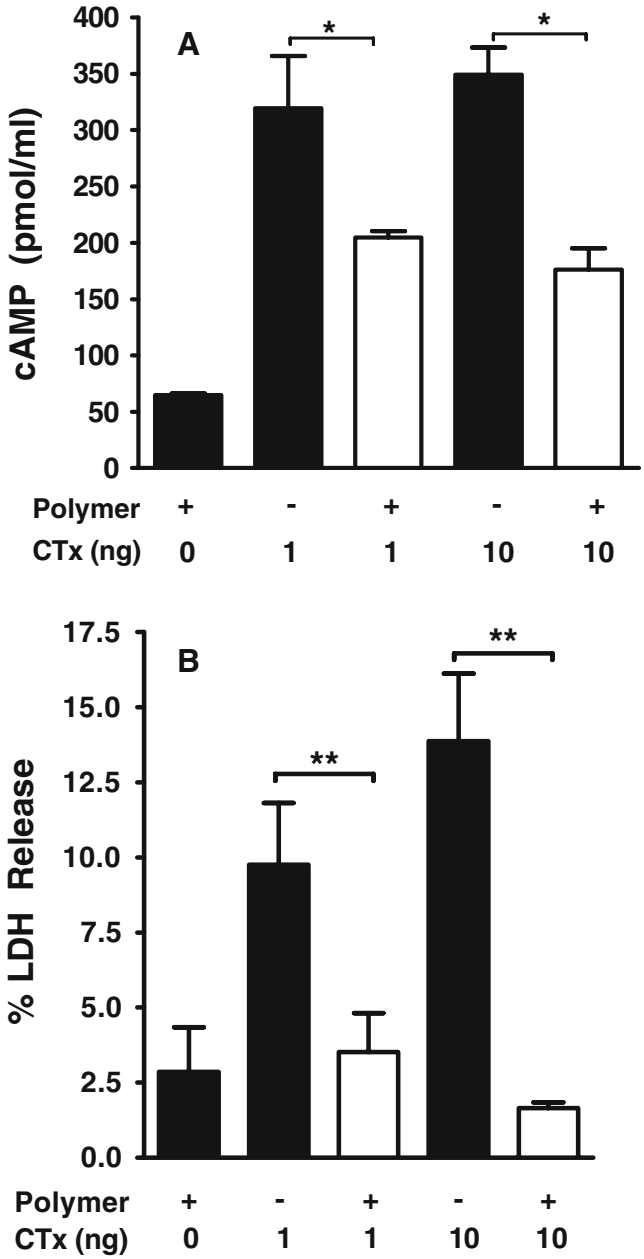

Fig. 2. **A** CTx-induced cAMP increase in E12 monolayers. **B** CTx-induced LDH release. Assays were with and without pre-treatment poly(DMAEMA), 1 mg/ml. All incubations were for 120 min. $N=6$ per group; *single asterisk* $P<0.05$, *double asterisk* $P<0.0001$ *vs* CTx alone.

manipulation and density of the liver tissue. Following a 5 min challenge, bacteria were observed co-localised with the gel (Fig. 4A). Similarly, in poly(DMAEMA)-treated (1.0 mg/ml) E12 monolayers infected with bacteria, there was co-localisation of poly(DMAEMA) throughout the E12 mucus-gel, but *Salmonella* were excluded (Fig. 4B). The mucus-gel layer was previously shown to be required for poly(DMAEMA) to bind the E12 monolayer (18) and the combination of gel with polymer appears also to be important in allowing the polymer to reduce bacterial access to the epithelium.

S. Typhimurium-Induced IL-8 secretion from E12 is Prevented with Poly(DMAEMA)

E12 monolayers were challenged with *S. typhimurium* and examined after 24 h for additional indices of *S. typhimurium* pathogenicity *in vitro*. *S. typhimurium* challenge induced significantly higher IL-8 secretion from E12 monolayers than controls not exposed to bacteria. Pre-incubation of E12 monolayers with 0.1 mg/ml poly(DMAEMA) prior to 24 h of *S. typhimurium* exposure decreased the ability of the bacteria to induce IL-8 secretion from the basolateral side of monolayers to 122±11 pg/ml compared to a value of 159±8 pg/ml for monolayers exposed to bacteria alone ($N=18$; $P<0.001$).

Direct Inhibitory Effects of Poly(DMAEMA) on *S. Typhimurium* Growth

Actions of poly(DMAEMA) on *S. typhimurium* growth were examined. Poly(DMAEMA) reduced *S. typhimurium* growth at a threshold concentration of 0.25 mg/ml within 30 min and was bactericidal at 1 mg/ml (Fig. 5). This may indirectly contribute to poly(DMAEMA)'s ability to reduce *S. typhimurium*-associated IL-8 secretion in bacterial-exposed E12 monolayers. While there was evidence of resistant bacteria growing back at 24 h for low concentrations of poly (DMAEMA), this did not occur at 1 mg/ml polymer. MIC and MBCs were also carried out on two different *S. typhimurium* isolates, strains A and B. The MICs obtained for both isolates were 1 mg/ml and the MBCs were 1 and 4 mg/ml for the strain A and B, respectively. The polymer was also found to induce negligible haemolysis on rat red blood cells at the highest MIC concentration of poly(DMAEMA) used, in agreement with its non-cytotoxic actions in E12 epithelia (18) (Table I).

DISCUSSION

Based on previous work indicating that poly(DMAEMA) augments the cytoprotective function of the mucus-gel layer (18), we investigated whether poly(DMAEMA) could prevent intestinal monolayer damage induced by two bacterial toxins and by bacteria, which must migrate through the mucus-gel to the epithelium. The criterion for protection against the two toxins was a reduction in LDH release following pre-incubation of monolayers with the polymer, in addition, for CTx there was attenuation of the increase in intracellular cAMP. For *Salmonella*, poly(DMAEMA) prevented attachment and uptake by epithelia, reduced IL-8 secretion and inhibited bacterial growth. We previously demonstrated that treatment of E12 monolayers with poly(DMAEMA) reduces the passage of a paracellular marker FD-4 across the monolayer and caused minimal cytotoxicity to the epithelium (18). Confocal microscopy suggests that muco-integration of poly(DMAEMA) into the mucus-gel layer may decrease the molecular weight cut off of the mucus layer, normally 600–700 Da (32). Similarly, calcium-mediated mucin cross-linking also reduces diffusion of 500 nm microspheres through salivary mucus (33), and other studies have shown that mucus viscosity limits bacterial migration to intestinal epithelium (8). Interaction of potentially harmful compounds and toxins with the intestinal epithelium may therefore be reduced by poly(DMAEMA) via actions on the mucus-gel layer.

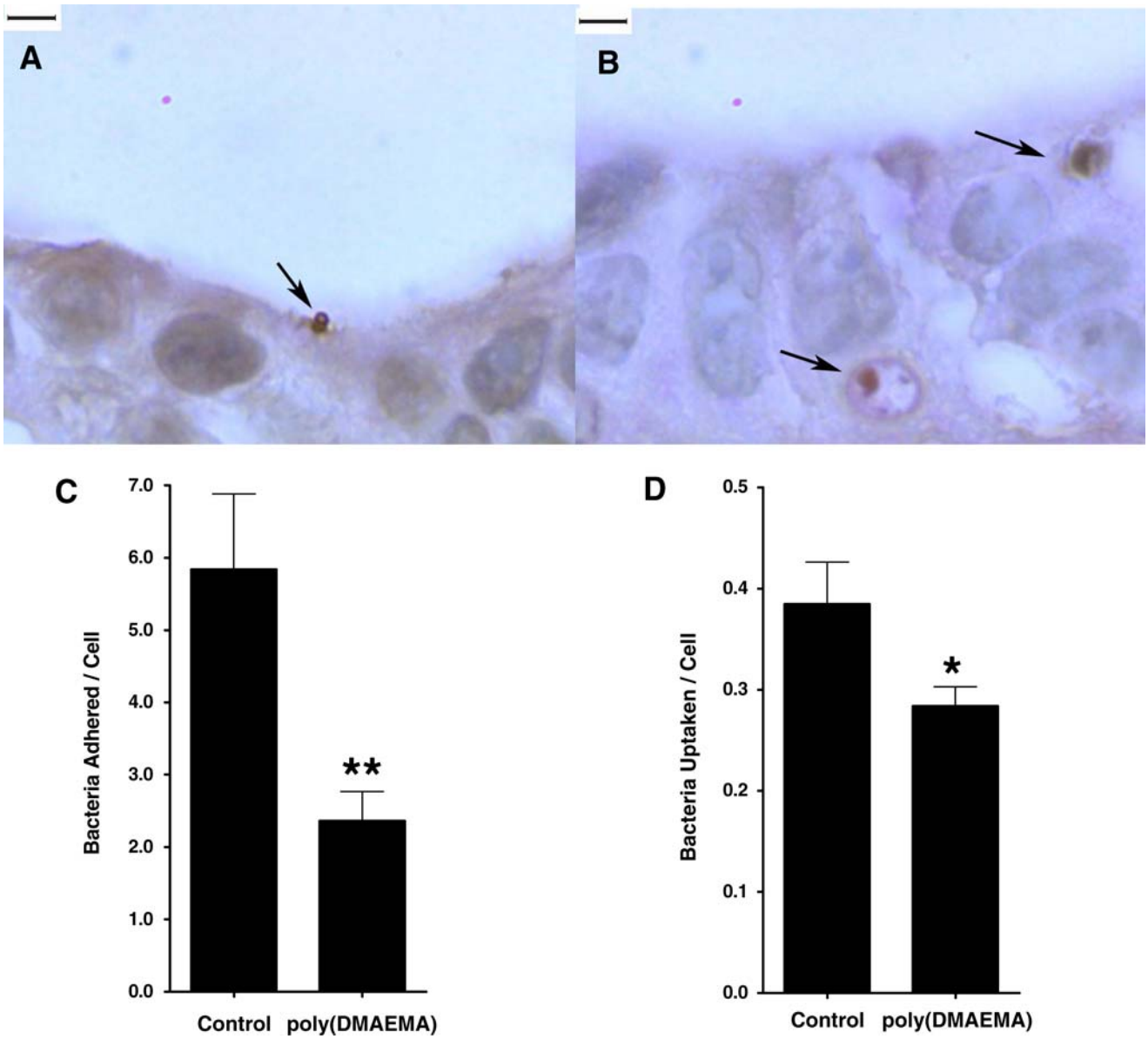

Fig. 3. Interaction of *S. typhimurium* (indicated by *arrows*) with E12 monolayers in the presence and absence of poly(DMAEMA; 1 mg/ml). **A** Adherence. **B** Uptake. **C** Quantitative analysis of adherence. **D** Quantitative analysis of *S. typhimurium* uptake. *Single asterisk* $P<0.05$, *double asterisk* $P<0.005$ *vs* untreated. $N=11$ per group. *Horizontal bars* $=10$ μm.

A non-absorbed epithelial-protective compound may avoid some disadvantages of antibiotic therapies concerning bacterial resistance from systemic delivery. For example, tolevamer sodium is a polymer with inherent drug-like properties and is in Phase III trials as an oral therapy against *C. difficile* toxins A and B (34). Tolevamer acts by chelating the toxins and preventing epithelial cell–toxin interactions (35). By a different mechanism dependent on integration into mucus, poly(DMAEMA) prevented the cytotoxic effects of *C. difficile* supernatants and also prevented CTx interaction with GM1 receptors on the E12 cell surface. *C. difficile* toxins A and B are high molecular weight molecules over 300 kDa in size (36) while CTx is approximately 84 kDa in size (37), so poly(DMAEMA) may lower molecular weight cut-off point for toxin permeation through the gel. In addition to limiting access to the epithelium, poly(DMAEMA) has direct actions as an antimicrobial agent. Poly(DMAEMA) acts as an antimicrobial surface coating, prohibiting growth of *B. subtilis* and *E. coli* and impeding the binding of *P. aeruginosa* to glass, paper and polymer discs (20,21). Other studies have used PEG as a non-absorbed intestinal prophylactic agent against bacterial challenge *in vivo* (15), while non-absorbed oral antibiotics including rifamycin are being investigated for localised treatment of intestinal infection (38). Polymeric conjugation to antibiotics is a logical next step.

Interactions between the enteric pathogen *S. typhimurium* and intestinal epithelia provoke an acute inflammatory response, mediated in part by epithelial cell secretion of IL-8 and other pro-inflammatory molecules from the basolateral membrane (39). This response is partly due to the activation

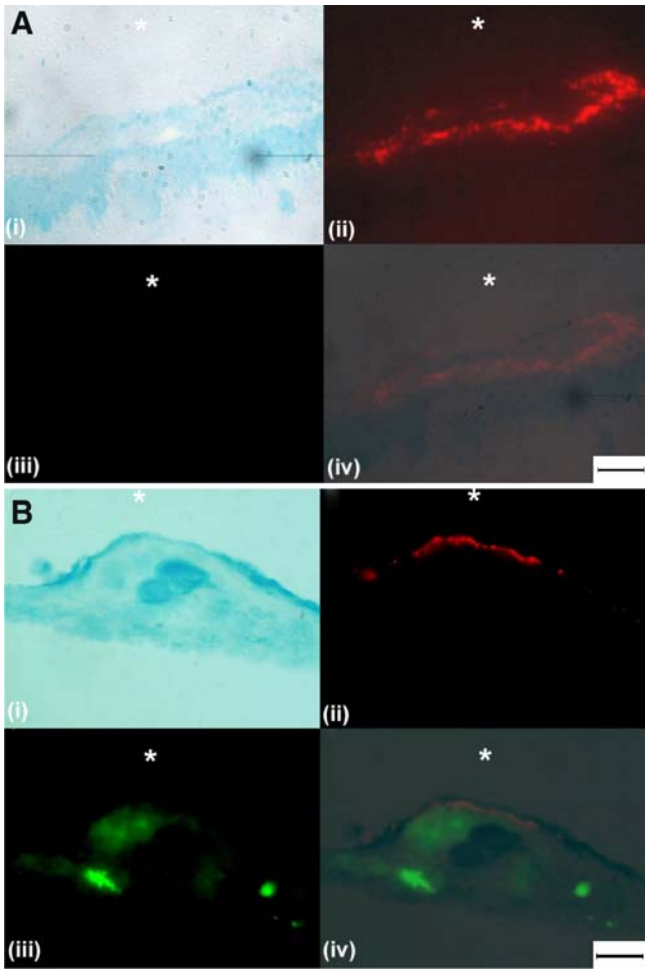

Fig. 4. A Light and fluorescent microscopy images of *Salmonella*-infected E12 monolayers. *(i)* Alcian blue stained E12 mucus-gel under light microscope. *(ii)* Fluorescent red TAMRA/SE stained *Salmonella*. *(iii)* green fluorescent field, showing an absence of poly(DMAEMA). *(iv)* Composite overlay of *(i)*, *(ii)* and *(iii)*. **B** Light and fluorescent microscopy images of *Salmonella* infected E12 monolayers pre-treated with poly(DMAEMA). *(i)* Alcian blue stained E12 mucus-gel under light microscope. *(ii)* Fluorescent red TAMRA/SE-stained *Salmonella*. *(iii)* Green fluorescent hostosol-labelled poly(DMAEMA). *(iv)* Composite overlay of images *(i)*, *(ii)* and *(iii)*. *Asterisk* denotes apical side of epithelial monolayer. *Horizontal bar*=10 μm.

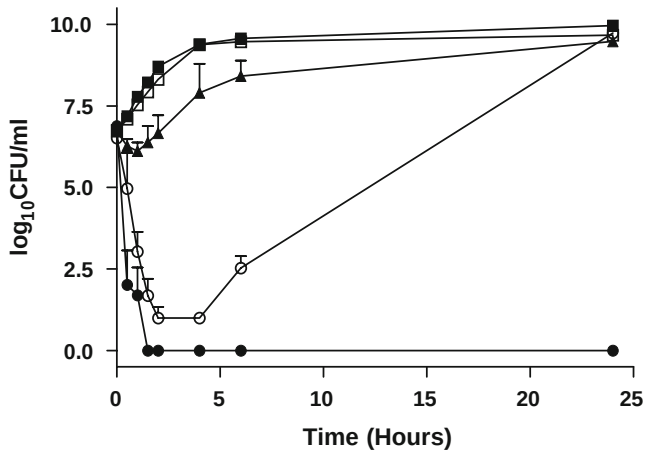

Fig. 5. Time–kill curve of effect of poly(DMAEMA) on *S. typhimurium* growth. *Closed square*=0 poly(DMAEMA), *open square*=0.1 mg/ml poly(DMAEMA), *closed triangle*=0.25 mg/ml poly(DMAEMA), *open circle*=0.5 mg/ml poly(DMAEMA), *closed circle*=1 mg/ml poly (DMAEMA). *N*=3 in each group. Note that from 1.5 h the values for the 1 mg/ml group were zero.

of Toll-like receptor-5 (TLR5) by interaction with bacterial flagellin resulting in the subsequent activation of the transcription factor NF-kappaB in a MyD-88 dependent manner (40). As TLR5 is expressed exclusively on the basolateral side of epithelial cells (41), flagellin-promoted inflammation could arise from apical-to-basolateral translocation of small bacterial epitopes and peptides. E12 monolayers challenged with *S. typhimurium* have increased TLR5 expression (unpublished data), correlating with increased levels of IL-8 secretion to the basolateral side. In order to reach the epithelium, *S. typhimurium* must penetrate the intestinal mucus-gel layer, but there is evidence that the mucus gel is an inhibitor of *S. typhimurium* movement (42). When *Salmonella* reach the epithelium, they adhere to cell membranes causing actin rearrangement and forming ruffles in the cell membrane through which they may invade (43). Previous tissue cell culture studies examining the interaction of epithelial cells and *S. typhimurium* have mainly focused on Caco-2 and HT29 cell lines as intestinal epithelial models. As these lines do not secrete mucus or form a mucus-gel layer, the role of mucus in *Salmonella* translocation cannot be fully investigated. E12 mucus gel layers however both impede the transport of lipophilic drugs (44) and limit the cytotoxicity of cationic polymers similar to data seen in excised tissue models (18). Thus, E12 monolayers may be a physiologically relevant in vitro cell culture model for examining host–pathogen epithelial interactions.

Prior exposure of E12 monolayers to poly(DMAEMA) reduced adhesion, uptake and the subsequent provocation of IL-8 secretion due to *S. typhimurium* challenge. Decrease in *Salmonella*-associated IL-8 secretion from E12 monolayers arising from pre-treatment with poly(DMAEMA) occurred only in the presence of a mucus-gel layer. In combination with the polymer, mucus is likely to limit accessibility of *S. typhimurium* and flagellin components to the E12 monolayer and prevent IL-8 secretion being stimulated. Fluorescent imaging of *Salmonella* and poly(DMAEMA) within the gel layer also suggested that poly(DMAEMA) limits *Salmonella* migration through the mucus-gel similar to the mechanism of the toxin interaction with the polymer-enhanced mucus gel. Regarding effects on bacterial growth, populations of *Salmonella* were reduced by over 99.9% after 30 min co-incubation with the polymer and an MIC value of 1 mg/ml was obtained against two strains.

Table I. MICs and MBCs for Poly(DMAEMA) Against *S. Typhimurium*

Organism (Gram)	MIC[a]	MBC[a]	(%) Haemolysis
S. typhimurium (Strain A)	1	1	1.1 (±0.4)
S. typhimurium (Strain B)	1	4	1.1 (±0.4)

[a] MIC and MBC are given in mg/ml. Percent haemolysis refers to capacity to lyse rat erythrocytes at the MIC concentration. *N*=2–3 on triplicate measurements.

The exact nature of the interaction between poly (DMAEMA) and bacteria is not currently known, but many defensin-like peptides that inactivate bacteria are also cationic (6). Poly(DMAEMA) halts growth and impedes bacterial adherence and invasion of epithelial cells. These actions are reminiscent of the bactericidal effects of the cationic polymer, chitosan, which causes alterations to the outer membrane of Gram-negative bacteria leading to increased sensitivity to surfactants (45,46). Importantly, *Salmonella* mutants with a cationic outer membrane are less susceptible to the antimicrobial action of chitosan (45). This is consistent with the growth inhibitory effects the cationic poly(DMAEMA) polymer had for the Gram negative *S. typhimurium*, a bacterium which has more anionic membranes due to the presence of LPS.

The ability of a mucoadhesive polymer such as poly (DMAEMA) to act not only as an antimicrobial agent but also as an epithelial barrier enhancer, could lead to its eventual use if formulated as a prophylactic. Immune-enhancing anti-bacterial approaches including probiotic drinks are widely accepted by patients and some strains have proved successful in management of acute infectious diarrhoea (47). Since the polymer can be conjugated to drug cargoes (48), poly(DMAEMA) could potentially act to protect inflamed or compromised epithelium while also delivering antibiotic/probiotic agents. Finally, poly(DMAEMA) may be synthesised by an economic and relatively simple process (10) and may not have the toxicity of many natural host defence peptides (49). Furthermore, as its major action is to combine with the mucus gel to defend mucosal surfaces against toxin and bacterial attachment to epithelia, induction of bacterial resistance appears unlikely.

In summary, poly(DMAEMA) has potential as a non-absorbed antimicrobial therapy. Poly(DMAEMA) prevented the cytotoxic effect of two high molecular weight toxins, CTx and *C. difficile* toxins on cultured human intestinal epithelial monolayers bearing a mucus covering. It also reduced *Salmonella* adherence, invasion and IL-8 secretion in the presence of the mucus-gel layer. An additional direct antimicrobial action of poly(DMAEMA) was also demonstrated against *S. typhimurium in vitro*. *In vivo* studies will be designed to show the potential of poly(DMAEMA) as a mucoadhesive antimicrobial prophylactic against intestinal bacterial pathogens.

ACKNOWLEDGMENTS

The authors are grateful to Dr. D. Drudy of University College Dublin (UCD) for providing the *S. typhimurium* bovine isolate and for culturing *C. difficile* for collection of the supernatants. Thanks also to Dr. Rebecca O'Mahony, Centre for Food Safety, UCD, for the *S. typhimurium* food isolate, and to Dr. G. Mantovani for synthesising the poly (DMAEMA) at the University of Warwick, UK.

REFERENCES

1. A. Vindel, P. Trincado, E. Gomez, R. Cabrera, T. Boquete, C. Sola, S. Valdezate, and J. A. Saez-Nieto. Prevalence and evolution of methicillin-resistant Staphylococcus aureus in Spanish hospitals between 1996 and 2002. *J. Clin. Microbiol.* **44**:266–270 (2006).
2. C. Weidenmaier, S. A. Kristian, and A. Peschel. Bacterial resistance to antimicrobial host defenses—an emerging target for novel anti-infective strategies? *Curr. Drug Targets* **4**:643–649 (2003).
3. P. J. Sansonetti. War and peace at mucosal surfaces. *Nat. Rev. Immunol.* **4**:953–964 (2004).
4. G. Hecht. Innate mechanisms of epithelial host defense: spotlight on intestine. *Am. J. Physiol.* **277**:C351–C358 (1999).
5. A. P. Corfield, D. Carroll, N. Myerscough, and C. S. Probert. Mucins in the gastrointestinal tract in health and disease. *Front. Biosci.* **6**:D1321–D1357 (2001).
6. J. M. Otte, K. Kiehne, and K. H. Herzig. Antimicrobial peptides in innate immunity of the human intestine. *J. Gastroenterol.* **38**:717–726 (2003).
7. M. G. Scott, E. Dullaghan, N. Mookherjee, N. Glavas, M. Waldbrook, A. Thompson, A. Wang, K. Lee, S. Doria, P. Hamill, J. J. Yu, Y. Li, O. Donini, M. M. Guarna, B. B. Finlay, J. R. North, and R. E. Hancock. An anti-infective peptide that selectively modulates the innate immune response. *Nat. Biotechnol.* **25**:465–472 (2007).
8. A. Swidsinski, B. C. Sydora, Y. Doerffel, V. Loening-Baucke, M. Vaneechoutte, M. Lupicki, J. Scholze, H. Lochs, and L. A. Dieleman. Viscosity gradient within the mucus layer determines the mucosal barrier function and the spatial organization of the intestinal microbiota. *Inflamm. Bowel Dis.* **13**:963–970 (2007).
9. L. Zhang, and T. J. Falla. Antimicrobial peptides: therapeutic potential. *Expert Opin. Pharmacother.* **7**:653–663 (2006).
10. D. M. Haddleton, M. C. Crossman, B. H. Ana, D. J. Duncalf, A. M. Heming, D. Kukulj, and A. J. Shooter. Atom transfer polymerization of methyl methacrylate mediated by alkylpyridylmethanimine type ligands, copper(I) bromide, and alkyl halides in hydrocarbon solution. *Macromolecules* **32**:2110–2119 (1999).
11. C. Z. Chen, and S. L. Cooper. Interactions between dendrimer biocides and bacterial membranes. *Biomaterial* **23**:3359–3368 (2002).
12. K. Kuroda, and W. F. DeGrado. Amphiphilic polymethacrylate derivatives as antimicrobial agents. *J. Am. Chem. Soc.* **127**:4128–4129 (2005).
13. G. N. Tew, D. Liu, B. Chen, R. J. Doerksen, J. Kaplan, P. J. Carroll, M. L. Klein, and W. F. DeGrado. De novo design of biomimetic antimicrobial polymers. *Proc. Natl. Acad. Sci. U. S. A.* **99**:5110–5114 (2002).
14. O. Felt, A. Carrel, P. Baehni, P. Buri, and R. Gurny. Chitosan as tear substitute: a wetting agent endowed with antimicrobial efficacy. *J. Ocul. Pharmacol. Ther.* **16**:261–270 (2000).
15. L. Wu, O. Zaborina, A. Zaborin, E. B. Chang, M. Musch, C. Holbrook, J. Shapiro, J. R. Turner, G. Wu, K. Y. Lee, and J. C. Alverdy. High-molecular-weight polyethylene glycol prevents lethal sepsis due to intestinal *Pseudomonas aeruginosa*. *Gastroenterology* **126**:488–498 (2004).
16. M. Werthen, M. Davoudi, A. Sonesson, D. P. Nitsche, M. Morgelin, K. Blom, and A. Schmidtchen. Pseudomonas aeruginosa-induced infection and degradation of human wound fluid and skin proteins *ex vivo* are eradicated by a synthetic cationic polymer. *J. Antimicrob. Chemother.* **54**:772–779 (2004).
17. A. J. Limer, A. K. Rullay, V. S. Miguel, C. Peinado, S. Keely, E. Fitzpatrick, S. D. Carrington, D. Brayden, and D.M. Haddleton. Fluorescently tagged star polymers by living radical polymerisation for mucoadhesion and bioadhesion. *React. Funct. Polym.* **66**:51–64 (2006).
18. S. Keely, A. Rullay, C. Wilson, A. Carmichael, S. Carrington, A. Corfield, D. M. Haddleton, and D. J. Brayden. *In vitro* and *ex vivo* intestinal tissue models to measure mucoadhesion of poly (methacrylate) and *N*-trimethylated chitosan polymers. *Pharm. Res.* **22**:38–49 (2005).
19. M. W. Chapman, and W. K. Hadley. The effect of polymethylmethacrylate and antibiotic combinations on bacterial viability. An *in vitro* and preliminary *in vivo* study. *J. Bone Joint Surg. Am.* **58**:76–81 (1976).
20. S. B. Lee, R. R. Koepsel, S. W. Morley, K. Matyjaszewski, Y. J. Sun, and A. J. Russell. Permanent, nonleaching antibacterial surfaces. 1. Synthesis by atom transfer radical polymerization. *Biomacromolecules* **5**:877–882 (2004).
21. A. B. Lowe, M. Vamvakaki, M. A. Wassall, L. Wong, N. C. Billingham, S. P. Armes, and A. W. Lloyd. Well-defined

sulfobetaine-based statistical copolymers as potential antibioadherent coatings. *J. Biomed. Mater. Res* **52**:88–94 (2000).
22. I. Behrens, P. Stenberg, P. Artursson, and T. Kissel. Transport of lipophilic drug molecules in a new mucus-secreting cell culture model based on HT29-MTX cells. *Pharm. Res.* **18**:1138–1145 (2001).
23. T. Ma, J. R. Thiagarajah, H. Yang, N. D. Sonawane, C. Folli, L. J. Galietta, and A. S. Verkman. Thiazolidinone CFTR inhibitor identified by high-throughput screening blocks cholera toxin-induced intestinal fluid secretion. *J. Clin. Invest.* **110**:1651–1658 (2002).
24. J. X. Zhu, G. H. Zhang, N. Yang, D. K. Rowlands, H. Y. Wong, L. L. Tsang, Y. W. Chung, and H. C. Chan. Activation of apical CFTR and basolateral Ca(2+)-activated K+ channels by tetramethylpyrazine in Caco-2 cell line. *Eur. J. Pharmacol.* **510**:187–195 (2005).
25. K. T. Giannasca, P. J. Giannasca, and M. R. Neutra. Adherence of Salmonella typhimurium to Caco-2 cells: identification of a glycoconjugate receptor. *Infect. Immun.* **64**:135–145 (1996).
26. Clinical and Laboratory Standards Institute. Methods for dilutionantimicrobial susceptibility tests for bacteria that grow aerobically;Approved Standard—Seventh Edition. CLSI document M7-A7. CLSI, Wayne, PA, USA. (2006).
27. National Committee for Clinical Laboratory Standards. Methods for determining bactericidal activity of antimicrobial agents; Approved Guideline. NCCLS document M26-A. NCCLS, Wayne, PA, USA. (1999).
28. G. L. French. Bactericidal agents in the treatment of MRSA infections—the potential role of daptomycin. *J. Antimicrob. Chemother.* **58**:1107–1117 (2006).
29. S. Y. Shin, S.H. Lee, S. T. Yang, E. J. Park, D. G. Lee, M. K. Lee, S. H. Eom, W. K. Song, Y. Kim, K. S. Hahm, and J. I. Kim. Antibacterial, antitumor and hemolytic activities of alpha-helical antibiotic peptide, P18 and its analogs. *J. Pept. Res.* **58**:504–514 (2001).
30. C. Pothoulakis, and J. T. Lamont. Microbes and microbial toxins: paradigms for microbial–mucosal interactions II. The integrated response of the intestine to Clostridium difficile toxins. *Am. J. Physiol. Gastrointest. Liver Physiol.* **280**:G178–G183 (2001).
31. N. Jordan, J. Newton, J. Pearson, and A. Allen. A novel method for the visualization of the *in situ* mucus layer in rat and man. *Clin. Sci. (Lond)* **95**:97–106 (1998).
32. I. Matthes, F. Nimmerfall, J. Vonderscher, and H. Sucker. Mucus models for investigation of intestinal absorption mechanisms. 4. Comparison of mucus models with absorption models *in vivo* and *in situ* for prediction of intestinal drug absorption. *Pharmazie* **47**:787–791 (1992).
33. B. D. Raynal, T. E. Hardingham, J. K. Sheehan, and D. J. Thornton. Calcium-dependent protein interactions in MUC5B provide reversible cross-links in salivary mucus. *J. Biol. Chem.* **278**:28703–28710 (2003).
34. W. Braunlin, Q. Xu, P. Hook, R. Fitzpatrick, J. D. Klinger, R. Burrier, and C. B. Kurtz. Toxin binding of tolevamer, a polyanionic drug that protects against antibiotic-associated diarrhea. *Biophys. J.* **87**:534–539 (2004).
35. R. H. Barker Jr., R. Dagher, D. M. Davidson, and J. K. Marquis. Review article: tolevamer, a novel toxin-binding polymer: overview of preclinical pharmacology and physicochemical properties. *Aliment. Pharmacol. Ther.* **24**:1525–1534 (2006).
36. N. M. Sullivan, S. Pellett, and T. D. Wilkins. Purification and characterization of toxins A and B of Clostridium difficile. *Infect. Immun.* **35**:1032–1040 (1982).
37. M. W. Bitensky, M. A. Wheeler, H. Mehta, and N. Miki. Cholera toxin activation of adenylate cyclase in cancer cell membrane fragments. *Proc. Natl. Acad. Sci. U. S. A.* **72**:2572–2576 (1975).
38. J. A. Adachi, and H. L. DuPont. Rifaximin: a novel nonabsorbed rifamycin for gastrointestinal disorders. *Clin. Infect. Dis.* **42**:541–547 (2006).
39. A. T. Gewirtz, T. A. Navas, S. Lyons, P. J. Godowski, and J. L. Madara. Cutting edge: Bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J. Immunol.* **167**:1882–1885 (2001).
40. Y. Yu, H. Zeng, S. Lyons, A. Carlson, D. Merlin, A. S. Neish, and A. T. Gewirtz. TLR5-mediated activation of p38 MAPK regulates epithelial IL-8 expression via post-transcriptional mechanism. *Am. J. Physiol. Gastrointest. Liver Physiol.* **285**: G282–G290 (2003).
41. A. Haque, F. Bowe, R. J. Fitzhenry, G. Frankel, M. Thomson, R. Heuschkel, S. Murch, M. P. Stevens, T. S. Wallis, A. D. Phillips, and G. Dougan. Early interactions of Salmonella enterica serovar typhimurium with human small intestinal epithelial explants. *Gut* **53**:1424–1430 (2004).
42. K. Sakamoto, Y. Mori, H. Takagi, H. Iwata, T. Yamada, N. Futamura, T. Sago, T. Ezaki, Y. Kawamura, and H. Hirose. Translocation of Salmonella typhimurium in rats on total parenteral nutrition correlates with changes in intestinal morphology and mucus gel. *Nutrition* **20**:372–376 (2004).
43. M.A. Jepson, B. Kenny, and A. D. Leard. Role of sipA in the early stages of Salmonella typhimurium entry into epithelial cells. *Cell. Microbiol.* **3**:417–426 (2001).
44. I. Behrens, P. Stenberg, P. Artursson, and T. Kissel. Transport of lipophilic drug molecules in a new mucus-secreting cell culture model based on HT29-MTX cells. *Pharm. Res.* **18**:1138–1145 (2001).
45. I. M. Helander, E. L. Nurmiaho-Lassila, R. Ahvenainen, J. Rhoades, and S. Roller. Chitosan disrupts the barrier properties of the outer membrane of gram-negative bacteria. *Int. J. Food Microbiol.* **71**:235–244 (2001).
46. H. Liu, Y. Du, X. Wang, and L. Sun. Chitosan kills bacteria through cell membrane damage. *Int. J. Food Microbiol.* **95**:147–155 (2004).
47. D. A. Lemberg, C. Y. Ooi, and A. S. Day. Probiotics in paediatric gastrointestinal diseases. *J. Paediatr. Child Health* **43**:331–336 (2007).
48. S. M. Ryan, L. Tao, D. M. Haddleton, and D. J. Brayden. Oral polymeric conjugate system for salmon calcitonin: cytotoxicity studies. *Proc. Intern. Symp. Control. Rel. Bioact. Mater.* **33**:A870 (2006).
49. G. Batoni, G. Maisetta, S. Esin, and M. Campa. Human beta-defensin-3: a promising antimicrobial peptide. *Mini Rev. Med. Chem.* **6**:1063–1073 (2006).