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Soluble plantain fibre blocks adhesion and M-cell translocation of intestinal pathogens☆

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Abstract

Dietary fibres may have prebiotic effects mediated by promotion of beneficial bacteria. This study explores the possibility that soluble plant fibre may also improve health by inhibiting epithelial adhesion and translocation by pathogenic bacteria. We have focussed on soluble non-starch polysaccharide (NSP) from plantain bananas (*Musa* spp.) which previous studies showed to be particularly effective at blocking *Escherichia coli* epithelial adherence. In vitro and ex vivo studies assessed the ability of plantain NSP to inhibit epithelial cell adhesion and invasion of various bacterial pathogens, and to inhibit their translocation through microfold (M)-cells and human Peyer's patches mounted in Ussing chambers. Plantain NSP showed dose-related inhibition of epithelial adhesion and M-cell translocation by a range of pathogens. At 5 mg/ml, a concentration readily achievable in the gut lumen, plantain NSP inhibited adhesion to Caco2 cells by *Salmonella* Typhimurium (85.0±8.2%, $P<.01$), *Shigella sonnei* (46.6±29.3%, $P<.01$), enterotoxigenic *E.coli* (56.1±23.7%, $P<.05$) and *Clostridium difficile* (67.6±12.3%, $P<.001$), but did not inhibit adhesion by enteropathogenic *E.coli*. Plantain NSP also inhibited invasion of Caco2 cells by *S.* Typhimurium (80.2 ± 9.7%) and *Sh. sonnei* (46.7±13.4%); $P<.01$. Plantain NSP, 5 mg/ml, also inhibited translocation of *S.* Typhimurium and *Sh. sonnei* across M-cells by 73.3±5.2% and 46.4±7.7% respectively ($P<.05$). Similarly, *S.* Typhimurium translocation across Peyer's patches was reduced 65.9±8.1% by plantain NSP ($P<.01$). Soluble plantain fibre can block epithelial adhesion and M-cell translocation of intestinal pathogens. This represents an important novel mechanism by which soluble dietary fibres can promote intestinal health and prevent infective diarrhoea.

Keywords: Dietary fibre; Diarrhoea; Enteric infections; Peyer's patches; M (microfold) cell; Mucosal immunology

1. Introduction

It has long been thought that a high intake of dietary fibre promotes intestinal health. Burkitt noted the low rates of bowel cancer and diverticular disease in Africans and thought this might be due to a rapid colonic transit time related to the bulking effects of fibre [1]. Subsequent studies showing that not all fibre sources provided equivalent defence against colon cancer implied more complex protective mechanisms [2] notably fermentation of fibre to generate short chain fatty acids such as butyrate that act as a carbon and energy source for the colonic epithelium [3]. Another mechanism that has attracted attention has been the "prebiotic" effect — an effect that is mediated by promotion of beneficial bacteria [4]. Here we explore an alternative hypothesis - that dietary fibre, particularly soluble fibre, may inhibit adhesion, invasion and translocation of pathogenic bacteria.

Pathogens may induce diarrhoea either as a consequence of invasion and inflammation or by release of toxins. Even for those that release toxins, close proximity to or adhesion to the mucosa is likely greatly to enhance their effect. Anything that prevents their

Abbreviations: ANOVA, analysis of variance; CFU, colony forming units; DMEM, Dulbecco's modified Eagle's medium; FAE, follicle-associated epithelium; FBS, fetal bovine serum; LB, Luria-Bertani; M-cell, membranous/microfold cell; MOI, multiplicity of infection; NSP, non-starch polysaccharides; PBS, Phosphate-buffered saline; PP, Peyer's patches; TEER, trans-epithelial electrical resistance; TEM, transmission electron microscopy.

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close apposition to the mucosa may therefore have a beneficial or preventative effect. Natural defences will include the mucus layer but, although this is continuous in the healthy colon, it is discontinuous in the small intestine, particularly overlying the Peyer's patches where mucus-secreting goblet cells are relatively sparse [5].

"Membranous" or "microfold" cells (M-cells) are specialized epithelial cells that account for about 5–10% of the dome epithelium that overlies Peyer′spatches in the distal ileum, and lymphoid follicles, their smaller equivalent in the colon [5]. They are the major site of both antigen and microorganism sampling in the gut and also serve as the principal portal of entry for pathogens, such as mycobacteria, *Listeria* spp., *Vibrio cholerae, Salmonella* spp. and *Shigella* spp. These are translocated across M-cells and delivered to the underlying macrophages [6,7]. Previously, we have validated an in vitro derived M-cell model and have shown that translocation of Crohn′s disease mucosal *Escherichia coli* isolates across M-cells is inhibited by soluble plant fibres, particularly plantain (banana) fibre [8]. Furthermore, the effects were verified in ex vivo studies of human follicle-associated epithelium (FAE) taken from resected intestinal tissue of patients undergoing surgery [8], indicating potential for a therapeutic benefit from dietary supplementation with soluble plantain fibre in Crohn′s disease [9,10]. We have now used these models to investigate the potential protective effects of soluble plantain fibre against M-cell translocation by pathogens.

Bacteria that cause toxin-mediated diarrhoea include enterotoxigenic *E. coli* (ETEC), the commonest cause of traveller′s diarrhoea, and *Clostridium difficile*, the major cause of antibiotic-associated diarrhoea. *C. difficile* mediates damage by local release of enterotoxin (*toxin A*) and cytotoxin (*toxin B*) [11]. Close proximity of *C. difficile* to the host epithelium is almost certainly necessary to produce toxic effects [12] and preventing these interactions should therefore be of therapeutic benefit.

Here we show that soluble plantain fibre at concentrations achievable in vivo is able to prevent the adhesion in vitro to intestinal epithelial cells of *Salmonella enterica* serovar Typhimurium, *Shigella sonnei*, ETEC and *C. difficile*. We also show that soluble plantain fibre can inhibit epithelial cell invasion and translocation across M-cells by *S*. Typhimurium and *Sh. sonnei.*

2. Materials and methods

2.1. Sampling of human Peyer's patches

Tissue specimens from macro- and microscopically normal terminal ileum were obtained from four patients [two women and two men, median age 79.5 (range 52–89) years] who were undergoing right hemicolectomy for colon cancer. All patients had no signs of generalised disease and none had received preoperative chemo- or radiotherapy. The study was approved by the Regional Human Ethics Committee; Linköping, Sweden. All patients had given their informed written consent.

2.2. Bacterial strains and growth conditions

S. Typhimurium LT2, *Sh. sonnei* and the enteropathogenic *E. coli* (EPEC) strains D55 and E2348/6 were all obtained from stocks held within the Department of Clinical Infection, Microbiology and Immunology, University of Liverpool. ETEC C410 (serotype O160, ST^+ and LT^+) was kindly supplied by Dr. Godfrey Smith (Medical Microbiology, Royal Liverpool & Broadgreen University Hospitals NHS Trust, UK). All were cultured on Luria Bertani (LB) agar plates with overnight incubation in air, at 37 °C. *C. difficile* Type 027 (strain 080042), also supplied by Dr. Godfrey Smith, was grown on Fastidious Anaerobe Agar (Lab M, Bury, UK) under anaerobic conditions. *S.* Typhimurium LT2, transformed with a plasmid carrying the enhanced green fluorescent protein gene e*gfp* (pEGFP; BD Biosciences-Clontech, Mountain View, CA, USA), was used in experiments examining bacterial translocation across ex vivo human FAE.

Prior to infection of cultured epithelial cells, bacteria were washed three times, resuspended in sterile phosphate-buffered saline (PBS) and adjusted to an OD_{550nm} equating to 1×10^9 CFU/ml.

2.3. Soluble plantain fibre

Soluble non-starch polysaccharide (NSP) from plantain, the banana family (*Musa* spp.) member that is usually cooked as a vegetable, was provided by Provexis Plc (Windsor, UK). Soluble NSP was obtained from Green plantain (ripeness stage 1) flour produced in Ecuador from locally grown cultivars *Musa* AAB (Horn) var. Dominico, with a ratio of acidic:neutral polysaccharides of ~9:1. The molecular weight distribution of the polysaccharides is between 900 and ~5000 kDa [8]. Plantain was selected as it had previously been found to inhibit adhesion of colonic mucosa-associated *E. coli* to intestinal epithelial cells and translocation across M-cells in vitro [8,13]. Concentrations tested were within the range of intraluminal concentrations that would be readily achievable with dietary supplementation, [8].

2.4. Epithelial cell culture

The human colorectal adenocarcinoma cell-line Caco2 (#86010202) and the human Burkitt's lymphoma cell-line Raji B (#85011429) were purchased from the European Collection of Animal Cell Culture (Public Health Laboratory Service, Wiltshire, UK). Caco2 Clone 1 cells (Caco2-cl1), kindly provided by Dr. Elisabet Gullberg (University Hospital Linköping, Sweden), were originally obtained from Dr. Maria Rescigno (European Institute of Oncology, Milan, Italy) [14]. Both Caco2 and Caco2-cl1 were grown and maintained in Dulbecco′s modified Eagle′s medium (DMEM) supplemented with 10% v/v fetal bovine serum (FBS), 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The Raji B cell-line was maintained in RPMI-1640 medium supplemented with 10% FBS, 8 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Raji B cells were seeded at $3x10^5$/ml and every third day cell suspensions were allowed to settle: Two thirds of the media was replaced with fresh culture media. Every ninth day, cells were split 1:3.

All cells were maintained at 37 °C with 5% CO_2 in a humidified atmosphere. Culture medium and supplements were supplied by Sigma-Aldrich excepting FBS (Invitrogen; Paisley, Scotland).

2.5. Adherence to, and invasion of Caco2 monolayers

Bacterial strains were tested for their ability to adhere to, and/or invade, Caco2 cells in the presence of soluble plantain NSP. Cells were maintained in complete Dulbecco′s modified Eagle medium (Sigma) at 37 °C, 5% CO_2. Cells were initially seeded into 24-well tissue culture plates (Corning/Costar, High Wycombe, UK) at 5×10^4 cells per well and grown overnight in Dulbecco′s modified Eagle medium (without antibiotics). The monolayers were then washed twice with sterile PBS. Bacteria were grown overnight on agar, washed twice in sterile PBS, before being added to the well. Bacteria were added to each monolayer to a multiplicity of infection (MOI) of 10. For EPEC studies, bacteria were initially added at MOI of 10 but due to low adherence of EPEC to Caco2 cells, studies were repeated at higher MOI of 100 and 500.

After 4 h of infection at 37 °C, cell monolayers were washed 3 times with sterile PBS. To determine bacterial invasion, cells were treated with fresh culture medium containing 100 μg/ml gentamicin to kill extracellular bacteria. After 1 h at 37 °C, the monolayers were again washed 3 times in sterile PBS. Parallel cell monolayers without gentamicin treatment were used to calculate bacterial adhesion. All monolayers were lysed by adding 1% v/v Triton X-100 for 5 min to release internalized bacteria. Tenfold dilutions of the cell lysate were performed, and 50 μl from each was plated onto LB agar plates. Plates were incubated at 37 °C, and colony-forming units were counted after 24 h.

Giemsa microscopy was performed on cells grown on 13 mm glass coverslips in 24 well culture plates. Monolayers were washed with sterile PBS and medium replaced with DMEM without antibiotics, supplemented with 10% FBS and 1% D-mannose. Cells were pre-treated with plantain NSP (10 mg/ml) or saline vehicle for 30 min, and then inoculated with bacteria (grown overnight in static suspension in LB broth containing 1% v/v D-mannose to inhibit type 1 fimbrial adhesins) at MOI 10 for 90 min to 4 h. Cells were washed three times with sterile PBS to remove non-adherent bacteria, fixed with 70% ethanol and stained with 10% Giemsa for 20 min.

2.6. Bacterial translocation across M-cells

M-cells were generated on Millicell-PCF 3 μm pore size Transwell filters (Millipore Ltd; Watford, UK) by co-culture of Caco2-cl1 cells (grown on the apical aspect) and Raji B lymphocytes (on the basolateral aspect). Parallel Caco2-cl1 monocultures (without Raji B cells in the basal compartment) were also generated on Transwell inserts and maintained as for M-cells. TEER was measured throughout, using an EVOM epithelial voltohmmeter (World Precision Instruments, Stevenage, UK), to monitor monolayer integrity. Translocation of *S.* Typhimurium and *Sh. sonnei,* coupled with transmission electron microscopy (TEM), was used to confirm successful generation of M-cells in vitro as previously described [8].

For all M-cell and Caco2-cl1 monoculture translocation experiments, DMEM medium was prepared with 10% FBS and 4 mM L-glutamine only (i.e., without antimicrobial agents). For studies examining the effect of soluble dietary fibres, fresh DMEM (0.5 ml) containing plantain NSP (0-50 mg/ml), was applied to the apical aspect of the cells for 30 min at 37 °C. Confluent monolayers were then infected for 4 h, with