

Paracellular permeability is increased by basal lipopolysaccharide in a primary culture of colonic epithelial cells; an effect prevented by an activator of Toll-like receptor-2

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Abstract:	Lipopolysaccharide (LPS), which generally activates Toll-like receptor 4 (TLR-4), is expressed on commensal colonic bacteria. In a number of tissues, LPS can act directly on epithelial cells to increase paracellular permeability. Such an effect in the colon would have an important impact on the understanding of normal homeostasis and of pathology. Our aim was to use a novel primary culture of colonic epithelial cells grown on Transwells to investigate whether LPS, or Pam3CSK4, an activator of Toll-like receptor-2 (TLR-2), affected paracellular permeability. Consequently, [14C] mannitol transfer and transepithelial electrical resistance (TEER) were measured. The preparation consisted primarily of cytokeratin-18 positive epithelial cells that produced superoxide, stained for mucus with periodic acid-Schiff reagent, exhibited alkaline phosphatase activity and expressed TLR-2 and TLR-4. Tight junctions and desmosomes were visible by transmission electron microscopy. Basally, but not apically, applied LPS from <i>Escherichia</i>

	<p>coli increased the permeability to mannitol and to a 10-kDa dextran, and reduced TEER. The LPS from <i>Helicobacter pylori</i> increased paracellular permeability of gastric cells when applied either apically or basally, in contrast to colon cells, where this LPS was active only from the basal aspect. A pan-caspase inhibitor prevented the increase in caspase activity caused by basal <i>E. coli</i> LPS, and reduced the effects of LPS on paracellular permeability. Synthetic Pam3CSK4 in the basal compartment prevented all effects of basal <i>E. coli</i> LPS. In conclusion, LPS applied to the base of the colonic epithelial cells increased paracellular permeability by a mechanism involving caspase activation, suggesting a process by which perturbation of the gut barrier could be exacerbated. Moreover, activation of TLR-2 ameliorated such effects.</p>



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ABSTRACT

Lipopolysaccharide (LPS), which generally activates Toll-like receptor 4 (TLR-4), is expressed on commensal colonic bacteria. In a number of tissues, LPS can act directly on epithelial cells to increase paracellular permeability. Such an effect in the colon would have an important impact on the understanding of normal homeostasis and of pathology. Our aim was to use a novel primary culture of colonic epithelial cells grown on Transwells to investigate whether LPS, or Pam₃CSK₄, an activator of Toll-like receptor-2 (TLR-2), affected paracellular permeability. Consequently, [¹⁴C] mannitol transfer and transepithelial electrical resistance (TEER) were measured. The preparation consisted primarily of cytokeratin-18 positive epithelial cells that produced superoxide, stained for mucus with periodic acid-Schiff reagent, exhibited alkaline phosphatase activity and expressed TLR-2 and TLR-4. Tight junctions and desmosomes were visible by transmission electron microscopy. Basally, but not apically, applied LPS from *Escherichia coli* increased the permeability to mannitol and to a 10-kDa dextran, and reduced TEER. The LPS from *Helicobacter pylori* increased paracellular permeability of gastric cells when applied either apically or basally, in contrast to colon cells, where this LPS was active only from the basal aspect. A pan-caspase inhibitor prevented the increase in caspase activity caused by basal *E. coli* LPS, and reduced the effects of LPS on paracellular permeability. Synthetic Pam₃CSK₄ in the basal compartment prevented all effects of basal *E. coli* LPS. In conclusion, LPS applied to the base of the colonic epithelial cells increased paracellular permeability by a mechanism involving caspase activation, suggesting a process by which perturbation of the gut barrier could be exacerbated. Moreover, activation of TLR-2 ameliorated such effects.

Keywords: Colon, Lipopolysaccharide, Permeability, Toll-like receptor, Primary culture

INTRODUCTION

A single layer of colonic epithelial cells, linked together by tight and adherens junctions, forms the barrier that prevents access of the enteric bacterial flora into the internal host tissues. Importantly, perturbation of the gut barrier is increasingly being linked with disease. Thus, in both Crohn's disease¹⁻³ and in ulcerative colitis,⁴ there is increased permeability through tight junctions and in Crohn's disease changes in permeability may predict relapse.⁵ A current view is that the cytokines released into the mucosa in these inflammatory bowel diseases activate signalling pathways that ultimately effect changes in the tight junctions.⁶⁻⁸

Cytokine release may be caused by pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) which, for example, induces release of tumour necrosis factor-alpha (TNF- α) from macrophages. Generally, the cellular receptor for LPS is Toll-like receptor-4 (TLR-4). In the gut, messenger RNA (mRNA) for TLR-4 is present not only in macrophages but has been found in highly purified colonic epithelial cells from humans^{9,10} and mice,¹¹ as has TLR-4 protein.^{11,12} In lung,¹³ corneal¹⁴ and cholangiocyte¹⁵ epithelial cells LPS directly increases paracellular permeability. We therefore hypothesized that LPS might act directly on colonic epithelial cells to increase paracellular permeability. A further issue is that since epithelial cells border the gut lumen, the presence of TLR-4 must be reconciled with normal homeostasis in the presence of commensal bacteria, many of which are Gram-negative, and thus will express LPS. Recent suggestions are an intracellular location of TLR-4,¹⁶ sequestration of TLR-4 into crypts¹⁷ and the influence of regulation of TLR-4 signalling.¹⁸ Alternatively, expression of TLR-4 protein may normally be low, but be increased in pathological conditions such as Crohn's disease

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3 and ulcerative colitis.¹² A further possibility, which is investigated here, is that
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5 responses of colonic epithelial cells might be polarized, whereby any effect of LPS
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7 applied to the apical lumen-facing aspect differs from that of LPS applied to the basal
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9 aspect.

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12 Toll-like receptor 2 (TLR-2) mRNA has been detected in human and mouse
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14 colonic epithelial cells^{10,11}, as has the protein in mouse colonic epithelial cells.¹¹
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16 Effects of TLR-2 can be investigated by using the synthetic TLR-2 ligand, *N*-
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18 palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteinyl-(*S*)-seryl-(*S*)-lysyl(*S*)-
19
20 lysyl-(*S*)-lysyl-(*S*)-lysine (Pam₃CSK₄). In Caco-2 cells this ligand produced a
21
22 transient increase in transepithelial electrical resistance (TEER), which was
23
24 associated with a redistribution of the tight junction-associated protein ZO-1.¹⁹
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26 Consequently, we have investigated the effects of Pam₃CSK₄ on colonic cells in the
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28 presence and absence of LPS.
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35 Investigations of the effects of LPS using adenocarcinoma cell lines is
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37 problematic possibly because of the low expression of TLR-4²⁰ or of accessory
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39 proteins needed for receptor activation. For example, LPS does not affect TEER in
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41 Caco-2 cells,¹⁹ and Caco-2 cells produced very little interleukin-8 (IL-8) in response
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43 to LPS.²⁰ Thus, we decided to build on our experience of primary culture of guinea
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45 pig gastric cells on Transwells^{21,22} to develop a preparation using colonic epithelial
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47 cells in which the effects of LPS on paracellular permeability could be explored by the
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49 apical to basal transfer of mannitol, and from measurement of TEER. As described
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51 herein, we find that basally, but not apically applied, LPS increases permeability and
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53 that this effect is inhibited by a pan-caspase inhibitor and by the synthetic TLR-2
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55 ligand Pam₃CSK₄.
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MATERIALS AND METHODS

Animals and materials

Dunkin-Hartley guinea pigs of 200-300g were obtained from Harlan UK, Bicester, Oxon. All experimental work complied with the UK Animals (Scientific Procedures) Act 1986 and had been approved by the Aston University Bioethics committee. The LPS of *Escherichia coli* 0111:B4 was obtained from Sigma (Poole, UK). *Helicobacter pylori* LPS was prepared from the type strain (NCTC 11637) as described previously.²³ Synthetic Pam₃CSK₄ was obtained commercially (InvivoGen, San Diego, USA), and dissolved in sterile, endotoxin-free water before use. *N*-benzyloxycarbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone (Z-VAD-FMK) was obtained from R&D systems (Abingdon, UK). Unless otherwise stated, all other reagents including antibodies were obtained from Sigma.

Primary culture of colonic cells

Guinea pigs were anaesthetized with intramuscularly delivered Hypnorm (Janssen, Oxford, UK) at a dose of 1 ml/kg, and with intraperitoneal diazepam (3.75 mg/kg). The colon was removed from the rectum to a point 5 cm from the caecum. Subsequently, colonic mucosa was scraped from the muscle, minced with fine scissors, and incubated for 20 min in Dulbecco's Modified Eagle Medium:F12 Nutrient mixture, 1:1 (DMEM:F12, Invitrogen, Paisley, UK), containing bovine serum albumin (2 g/l), gentamicin (50 µg/ml), amphotericin B (2.5 µg/ml), and pronase E (0.5 mg/ml, Merck, Lutterworth, UK) at 37°C with shaking (120 cycles/min) in a mixed atmosphere of O₂:CO₂ (95%:5%). After centrifugation for 2 min (200 x g, 15°C), the pellet was incubated with the same medium for 20 min, except that collagenase type

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3 I (0.4 mg/ml) replaced pronase. The digest was filtered through nylon mesh (150
4 μm), again centrifuged for 2 min (100 x g and 15°C), and resuspended in culture
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6 medium composed of DMEM:F12 containing foetal calf serum (10% v/v), penicillin
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8 (100 u/ml), streptomycin (100 $\mu\text{g/ml}$), amphotericin B (2.5 $\mu\text{g/ml}$), epidermal growth
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10 factor from mouse sub-maxillary glands (20 ng/ml), recombinant human insulin (10
11
12 $\mu\text{g/ml}$) and hydrocortisone 21-hemisuccinate (150 nmol/l). After a further
13
14 centrifugation (75 x g, 15°C, 2 min), the pellet, which comprised crypt fragments (\geq
15
16 20 cells) and single cells, was resuspended in culture medium (at 24,000 crypt
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18 fragments/ml). The suspension (0.5 ml) was added onto Transwell membranes (12
19
20 mm diameter, polycarbonate membrane, 0.4 μm pore size) supplied by Corning
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22 (Appleton Woods, Birmingham, UK) which had been coated with collagen IV from
23
24 human placenta.²¹ Culture medium (1.5 ml) was present in the basal compartment.
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26 Media were changed after 1, 3 and 6 d of culture using culture medium with serum
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28 reduced from 10% to 4% (v/v). Experiments were performed eight days after culture
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30 was initiated.
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39 Viability of cells at the end of a 5 h experiment was assessed by replacing the basal
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41 medium 30 min before termination with one which was identical except for the
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43 presence of the cell permeable nuclear stain bisbenzimidazole (H33258, 5 $\mu\text{g/ml}$) and the
44
45 normally cell membrane impermeant propidium iodide (PI, 2 $\mu\text{g/ml}$). After 30 min
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47 Transwell membranes were removed, mounted in phosphate-buffered saline (PBS),
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49 and examined under a fluorescence microscope with successively uv and green
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51 illumination. Damaged cells were identified as those with nuclei exhibiting both blue
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53 (H33258) and red (PI) fluorescence.
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Paracellular permeability and TEER of colonic cells

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3 D-[1-¹⁴C]Mannitol (GE Healthcare, Little Chalfont, UK) was introduced (0.9 μ Ci/ml
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5 15.5 μ mol/l) into the apical compartment, and medium from the lower compartment
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7 was removed after 1 h for estimation of mannitol transfer by scintillation counting.
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10 This basal measurement was performed on all Transwells and was used for
11
12 normalization. Subsequently, reagents were added to apical and basal
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14 compartments as required and transfer of Transwells to new basal medium was
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16 made at times determined by the experimental design. Apparent permeability (P_{app})
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18 to mannitol was calculated as described by Tavelin et al.²⁴ Fluorescein
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20 isothiocyanate- (FITC-) labelled dextran 10 kDa (FD10) was present in the apical
21
22 compartment (1 mg/ml), and its presence in the basal compartment was measured
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24 by using a fluorescence plate reader (Molecular Devices, Sunnyvale, CA) with
25
26 excitation at 485 nm, emission at 530 nm and cut-off at 515 nm. At the beginning and
27
28 the end of experiments TEER was measured by using an EVOM voltohmmeter
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30 (World Precision Instruments, Sarasota, FL, USA) and chopstick electrodes as
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32 described previously.²¹
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41 *Gastric epithelial cells*

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43 Culture of gastric cells and measurement of TEER and paracellular permeability was
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45 as described in detail previously.²¹ Briefly, cells were isolated from minced guinea
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47 pig gastric mucosa by sequential digestion with pronase and collagenase, and after
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49 centrifugation, suspended in RPMI 1640 medium containing foetal calf serum (10%
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51 v/v), penicillin (100 u/ml), streptomycin (100 μ g/ml), amphotericin B (2.5 μ g/ml) and
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53 epidermal growth factor from mouse sub-maxillary glands (20 ng/ml) at 10^6 cells/ml.
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55 The suspension (0.5 ml) was added to Transwell membranes (12 mm diameter,
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57 polycarbonate membrane, 0.4 μ m pore size), which had been coated with collagen
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3 IV, and cultured for 3 d, after which time the cells were fully confluent and were used
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5 for experiments.
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10 *Culture of Caco-2 cells*

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12 Caco-2 cells, obtained from the American Type Culture Collection (ATCC, Rockville,
13 MD), were plated on polycarbonate Transwells at 2×10^5 cells/cm² and were grown
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15 in DMEM containing heat-inactivated foetal calf serum (10% v/v), penicillin (100 u/ml)
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17 and streptomycin (100 µg/ml) for 21 days at 37°C in 5% CO₂ in air.
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23 *Histochemistry*

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27 To obtain vertical sections through Transwell preparations they were fixed for 10 min
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29 at room temperature in 4% (w/v) paraformaldehyde in PBS, washed in PBS at 4°C,
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31 and then infiltrated with a 10% (w/v) solution of gelatin (bloom number 300) for 15
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33 min at 37°C. Membranes were stacked, cooled to 4°C, embedded in OCT (optimal
34
35 cutting temperature) compound and frozen in liquid nitrogen. Sections (10 µm) were
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37 cut using a cryostat (Bright, Huntingdon, UK) and transferred to slides coated
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39 previously with gelatin (0.1% w/v) and chromium potassium sulphate (0.01% w/v).
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44 The use of gelatin enabled the retention of sections on the Transwell membrane and
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46 on the microscope slide. Alternatively, cells were detached from Transwells by using
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48 trypsin/ethylenediamine tetraacetic acid (EDTA) fixed in 4% (w/v) paraformaldehyde
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50 in PBS and transferred to microscope slides by using a Cytocentrifuge (Shandon,
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52 UK). Sections were stained with conventional haematoxylin and eosin stain, periodic
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54 acid-Schiff reagent²¹, or alkaline phosphatase by soaking in PBS containing Tween-
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56 20 (0.2% v/v) for 10 min, rinsing in PBS and incubating with Tris buffer (0.1 M, pH
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58 8.8) containing Fast Red TR (1 mg/ml) and Naphthol AS-MX phosphate (0.5 mg/ml)
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3 for 30 min at room temperature. Nuclei were stained with 4', 6'-diamidino-2-phenyl
4 indole dihydrochloride (DAPI, 2 µg/ml).
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10 *Fluorescence immunocytochemistry*

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12 Transwell membranes were fixed in 4% (w/v) paraformaldehyde in PBS and they, or
13 cytocentrifuge preparations, were permeabilized by incubation in Triton X-100 (0.1%
14 v/v) in PBS for 10 min. After blocking with serum from the appropriate species (10%
15 v/v) in PBS for 1 h, samples were incubated with either mouse anti-cytokeratin-18,
16 (clone CY-90) monoclonal antibody (Mab), 1:400; mouse anti-human macrophages
17 (clone MAC 387) Mab, 1:100 (Serotec Oxford, UK); mouse anti- α smooth muscle
18 actin (clone 1A4) Mab, 1:400; or goat anti-vimentin antibody, 1:20 for 1 h at room
19 temperature. Incubation with the appropriate conjugated secondary antibodies (FITC-
20 labelled goat anti-mouse IgG, 1:64; FITC-conjugated rabbit anti-goat IgG, 1:400; and
21 tetramethyl rhodamine-labelled goat anti-mouse IgG, 1:128) was for 1 h at room
22 temperature; after which preparations were washed and mounted in Vectashield hard
23 set (Vector laboratories, Peterborough, UK). Negative controls were obtained by
24 omitting the primary antibody. Counting of apoptotic nuclei on Transwell membranes
25 was performed using a Zeiss AxioCam HRc digital camera (Jena, Germany) with a
26 x40 objective, by capturing six successive fields moving in from the edge of the filter,
27 coding the output, and then counting total and apoptotic nuclei (which were
28 characterized by condensation and fragmentation of chromatin). Confocal images
29 were obtained with a Zeiss LSM 510 confocal microscope.
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58 *Electron microscopy*

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3 Transwell membranes were fixed for 1 h at room temperature in glutaraldehyde
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5 (2.5% v/v) in sodium cacodylate buffer (0.1M, pH 7.4). Samples were post-fixed in
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7 osmium tetroxide (1% w/v) in the same buffer, dehydrated and embedded in LR
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9 white resin (Agar Scientific, Essex, UK). Sections, approximately 70 nm thick, were
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11 stained with ethanolic uranyl acetate and Reynold's lead citrate, before observation
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13 in a Jeol 1200 EXII transmission electron microscope.
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18 19 *Immunoblotting*

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21 Transwell membranes were rapidly extracted with 100 µl of electrophoresis sample
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23 buffer at 95°C in a microfuge tube and then maintained at this temperature for 3 min.
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25 Samples were sonicated at an amplitude of 10 µm for 10 s. After sodium dodecyl
26
27 sulphate polyacrylamide gel electrophoresis and transfer of separated proteins to
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29 polyvinylidene difluoride (PVDF) membranes, immunoblotting was initiated by
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31 incubation with blocking solution (defatted milk powder 5% w/v) in Tris-buffered
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33 saline [Tris (20 mM), NaCl (137mM), pH 7.6] containing Tween-20 (0.1% v/v) (TBS-
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35 Tween) for 1 h at room temperature. Primary antibodies used were rabbit anti-mouse
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37 TLR-4 (sc-30002; 1:200) and rabbit anti-human TLR-2 (sc-10739; 1:100) (both
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39 obtained from Santa Cruz, Heidelberg, Germany) diluted in blocking solution and
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41 incubated for 1 h at room temperature. After washing in TBS-Tween, incubation with
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43 secondary goat anti-rabbit IgG peroxidase conjugate (sc-2004, Santa Cruz) diluted
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45 1:1000 in blocking solution was also for 1 h at room temperature. Immunoreactant
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47 bands were detected by enhanced chemiluminescence (Pierce, Northumberland,
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49 UK). Blots were then stripped and reprobed with rabbit anti-actin (Sigma A2066,
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51 1:2000) to check for equality of loading between lanes.
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Caspase activity and superoxide production

Superoxide production by intact cells was measured as superoxide dismutase inhibitable reduction of cytochrome C.²¹ Caspase 3-like activity on Transwell membranes, that had been rinsed in PBS, frozen in liquid nitrogen, and stored at -70 °C, was determined as described previously.²⁵ Briefly, the release of fluorescent product from Ac-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin was measured using a plate-reading spectrofluorimeter (Molecular Devices Ltd; Wokingham, UK). Alkaline phosphatase activity was extracted from frozen Transwell membranes, by thawing into extraction buffer (pH 8.0) containing Tris (10 mM), NaCl (150 mM) and Triton X-100 (0.5% v/v). The extract (50 µl) was transferred to 150 µl of assay buffer (pH 9.2) comprising glycine (42 mM), MgCl₂ (5 mM) and *p*-nitrophenylphosphate (4.5 mM), and the mixture incubated for 1 h at 37 °C. The reaction was terminated by addition of 50 µl of NaOH (1 M) and the absorbance read at 405 nm.

RESULTS

Colonic epithelial cells form a tight monolayer on Transwells

The TEER increased markedly between three and six days of culture (Fig. 1A) but the subsequent increase was smaller (Fig. 1A). Although TEER varied somewhat between cultures, intra-culture variation was generally much less (Fig. 1B). There was a steep relationship between TEER and apparent permeability (P_{app}). The mean P_{app} was similar to that found with a variety of colon cell lines derived from adenocarcinomas, which are known to form tight monolayers (Fig. 1C)^{24, 26-30}

Examination of vertical sections through Transwell preparations by light microscopy revealed a thin layer of cells (Fig. 2 A) with basally located nuclei (Fig. 2

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3 B, C). The dimensions of the nuclei were similar to that of surface epithelial cells in
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5 sections of colonic mucosa but their orientation was rotated through 90° (compare
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7 Fig. 2 C and D). Sections stained for mucus with periodic acid-Schiff reagent (Fig. 2
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9 E) as did both colonic epithelial cells and goblet cells in sections of colonic mucosa
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11 (Fig. 2 F). The surface of the Transwell preparation stained for alkaline phosphatase
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13 (Fig. 2 G) as did surface regions of colonic mucosa (Fig. 2 H). The enzyme activity of
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15 alkaline phosphatase was 91 ± 5 nmol/h/mg protein. Superoxide production (83 ± 21
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17 nmol/h/mg protein, n=4), was much higher than in Caco-2 cells³¹ and similar to that of
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19 guinea pig³¹ and human colonocytes³² in short-term culture. A low power fluorescent
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21 microscope image of cells fixed *in situ* on Transwells showed fairly evenly spaced
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23 nuclei and a 'lawn' of cytokeratin positive cells (Fig. 3 A, B), with more detail being
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25 shown by confocal microscopy (Fig. 3 C-E). To quantify the proportion of the various
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27 cell types, they were detached from the Transwell membrane and transferred to
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29 microscope slides by using a cytocentrifuge. A large proportion of cells were positive
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31 for the epithelial marker cytokeratin-18 ($94 \pm 2\%$, n=3; Fig. 3 F, G) and for mucin as
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33 determined by periodic acid-Schiff staining ($96 \pm 1\%$, n=3, Fig. 3 H, I). A small
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35 number of cells were positive for vimentin ($3.3 \pm 0.44\%$ n=4 Fig. 3 J, K). These cells
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37 were also positive for α -smooth muscle actin and were therefore probably
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39 myofibroblasts.³³ Macrophages were undetectable.
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48 Transmission electron microscopy showed flattened interleaved cell plates
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50 with an intact and continuous apical surface but with extensive basolateral spaces.
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52 Nuclei were mainly basal (Fig. 4 A, B). Desmosomes and tight junctions were visible
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54 (Fig. 4 C). The flattened nature of the primary culture may result from the lack of any
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56 constraint to lateral spreading and to rapid inhibition of cell division on contact.
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58 Caco-2 cells appear more columnar in culture and this may result from the much
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3 greater nuclear density that can be obtained with these cells than with the primary
4 culture (Fig. 4 D and E).
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7 The Transwell preparation expressed both TLR-4 and TLR-2 after 8 d of
8 culture and incubation for 5 h in the absence (Fig. 5 left-hand two lanes) or presence
9 (Fig. 5 right-hand two lanes) of basal LPS (10^{-7} g/ml). The amount of these proteins
10 appeared unaffected by the presence of LPS.
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17 *Basal but not apical LPS increased tight junction permeability and apoptosis*

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20 During 4-5 h after exposure to basally applied *E. coli* LPS, transfer of mannitol from
21 the apical to the basal compartment was increased (Fig. 6A). The dose response
22 curve for this effect was “bell-shaped” with LPS showing a maximal action at 10^{-7}
23 g/ml. Basal LPS produced a decrease in TEER, the magnitude of which correlated
24 with the increase in permeability to mannitol (Fig. 6B). The effect of 10^{-7} g/ml LPS on
25 transfer of mannitol increased with time (Fig. 6C). The presence of LPS in the apical
26 compartment affected neither permeability to mannitol nor TEER (Fig. 6 A-C). Basal
27 application of LPS (10^{-7} g/ml) increased apoptotic activity as evidenced by an
28 increase in caspase 3-like activity, in contrast to apical application (Fig. 6 D), and in
29 the proportion of cells showing nuclear condensation and fragmentation (Fig. 6 E-F).
30 Overall viability at the end of a 5h experiment was assessed by the proportion of
31 bisbenzimidazole stained nuclei that did not stain with propidium iodide and was > 99% in
32 both the absence and presence of basal LPS (10^{-7} g/ml).
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54 *The effect of LPS on permeability to mannitol and FD 10 was proportionate*

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56 Treatment of T84 cells with cytokines caused a much bigger increase in permeability
57 to FD10, than to mannitol.³⁴ Basally applied *E. coli* LPS (10^{-7} g/ml) increased
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3 permeability to mannitol, which has a hydrodynamic radius of 4.1 Å,³⁵ and to FD10
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5 with a hydrodynamic radius of 23 Å (Fig. 7).³⁶ However, the magnitude of the change
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7 in permeability to these probes induced by LPS was very similar (Fig. 7 insert).
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12 *H. pylori* LPS increases paracellular permeability when applied to either the apical or
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14 basal sides of gastric cells but only affects colon cells from the basal aspect
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18 In order to establish whether the lack of response to apically applied LPS was a
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20 particular feature of colon cells, we undertook a comparison with gastric cells. We
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22 used a previously established preparation of gastric surface epithelial cells²¹, and
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24 LPS from the gastric pathogen *H. pylori*. The bioactivities of LPS from *H. pylori* are
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26 generally less potent than that from *E. coli*,^{23, 37, 38} but at concentrations of 10⁻⁶ g/ml
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28 and above, it increased permeability to mannitol and decreased TEER when applied
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30 either to the apical or the basal aspect of gastric epithelial cells (Fig. 8). In contrast,
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32 *H. pylori* LPS only affected transfer of mannitol and TEER when applied to the basal
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34 aspect of colon cells (Fig. 8).
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41 *Inhibition of caspase activation reduces the effect of LPS on paracellular permeability*
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43 *and TEER*
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47 As shown in Fig. 9, when the pan-caspase inhibitor Z-VAD-FMK was present in the
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49 basal compartment of the Transwell, 5 h incubation with *E. coli* LPS did not increase
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51 caspase activity, in contrast to when Z-VAD-FMK was absent. Furthermore, the
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53 effects of LPS on permeability to mannitol and TEER were significantly reduced
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55 (P<0.05 and P<0.01, respectively).
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3 *The synthetic TLR-2 agonist, Pam₃CSK₄ does not mimic the effects of LPS but rather*
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5 *inhibits them*
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9 When applied to either the apical or basal aspect of the preparation Pam₃CSK₄ did
10 not significantly affect permeability to mannitol, TEER or caspase activity (Fig. 10,
11 open bars). Nevertheless, when Pam₃CSK₄ was present in the basal compartment,
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13 10⁻⁷ g/ml basal *E. coli* LPS did not significantly increase permeability to mannitol or
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15 caspase activity, or significantly decrease TEER. With Pam₃CSK₄ in the apical
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17 compartment there was still a significant effect of basally applied LPS on TEER and
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19 caspase activity (Fig. 10 B-C) but not on mannitol transfer. However, in the presence
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21 of apical Pam₃CSK₄ the effect of LPS on TEER was significantly reduced from a
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23 decrease of 55 ± 8% to 39 ± 6% (n=4, % calculated with respect to appropriate
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25 control, P<0.05 by paired t-test). In summary, basally applied Pam₃CSK₄ completely
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27 abolished all effects of LPS on paracellular permeability, and apically applied
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29 Pam₃CSK₄ effected a partial inhibition of LPS action.
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39 DISCUSSION

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43 The primary culture procedure used in this study gave rise to a confluent monolayer
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45 the tight junctions of which exhibit permeability to mannitol similar to that of
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47 adenocarcinoma cell lines (Fig. 1C). The initial TEER is somewhat higher than the
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49 3300 Ω.cm² of T84 cells,³⁹ and considerably higher than that of Caco-2 cells.^{24,29}
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52 The other only apparent report of a primary colon culture, with separated apical and
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54 basal compartments, used weanling rats and had a much lower TEER, but was not
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56 characterized as to cell type.⁴⁰ The key features of this present procedure are: (i) the
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58 use of guinea pig colonic mucosa which disaggregates rapidly compared to mouse
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3 mucosa, (ii) usage of pronase to remove mucus during the disaggregation of tissue,⁴¹
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5 and (iii) utilization of Transwells which promote long-term cell survival much better
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7 than a plastic substratum. Surface, but not crypt, guinea pig colonic epithelial cells
8
9 express Nox 1, which enables them to produce superoxide.³¹ Alkaline phosphatase
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11 activity and apical membrane staining are features of colonic epithelial cells.⁴²
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13 Colonic epithelial cells contain MUC4,⁴³ and are periodic acid-Schiff positive.³¹ The
14
15 production of superoxide, the presence of alkaline phosphatase and the high
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17 proportion of cells staining positively with periodic acid-Schiff reagent and for
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19 cytokeratin-18, indicate that the present preparations contain primarily colonic
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21 epithelial cells, resembling surface rather than crypt cells. Cells were rather
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23 flattened, about half the height, of guinea-pig colonocytes in intact mucosa.⁴⁴ Such
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25 flattening is found in primary cultures of jejunal⁴⁵ and tracheal cells,⁴⁶ and probably
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27 reflects the tendency of epithelial cells to spread as in wound healing until they reach
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29 contact when there is immediate inhibition of cell division. Attempts to increase the
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31 cell density by plating increased numbers of crypt fragments did not enhance
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33 monolayer formation possibly because fragments attached to each other rather than
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35 to the Transwell membrane. In a three dimensional system *in vivo* there is
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37 considerable lateral pressure which may induce a more columnar appearance by the
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39 cells.
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48 The bell-shaped dose response curves of *E. coli* LPS are characteristic of
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50 many actions of LPS mediated via TLR-4, e.g. in resected human ileal mucosa⁴⁷ and
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52 in the murine intestinal epithelial cell line m-IC_{cl2}.¹⁶ Furthermore, the low
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54 concentration at which LPS is effective implies a specific effect via TLR-4, and
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56 contrasts with the effect of high concentrations of apically applied LPS on Caco-2
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58 cells which appear to be mediated by CD14 and not TLR-4.⁴⁸ The comparison
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3 between the effects of highly purified LPS from *H. pylori* on stomach and colon cells,
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5 both grown on Transwells, shows that the effect of LPS on colon cells is specific to
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7 tissue, but not to the bacterial source of LPS. Furthermore, the results fit with the
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9 expectations derived from current knowledge of the relationship between the tissues
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11 and microorganism expressing the LPS. Thus, *H. pylori* is a gastric pathogen and an
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13 increase in permeability of tight junctions to allow delivery of antibodies to the
14
15 bacterium would seem an appropriate physiological response. In contrast, the
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17 colonic epithelium has to tolerate a high luminal LPS load from the commensal
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19 enteric flora that are present and a lack of response to apical LPS would therefore
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21 seem appropriate. The significance of the effect of basal LPS on gut permeability
22
23 needs further exploration but could clearly be of importance in exacerbating a
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25 defective permeability barrier.
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31 The action of LPS on paracellular permeability is probably not mediated
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33 indirectly by cytokines such as TNF- α and interferon-gamma (IFN- γ). This is
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35 because, firstly, the effect of LPS observed here is evident after 2 h of exposure,
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37 whereas 24 h of exposure to such cytokines is required for an effect on paracellular
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39 permeability in intestinal (Caco-2) cells to be evident.⁸ Secondly, in contrast to the
40
41 effect of LPS found in the present study, cytokines act preferentially on a subset of
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43 pores to increase the permeability of higher molecular mass probes (FD 10) to a
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45 greater extent than mannitol.³⁴ Thirdly, the effect of cytokines can occur in the
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47 absence of the activation of caspases,⁴⁹ in contrast to the caspase-dependent
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49 phenomenon observed here. The dependence of the LPS-mediated increase in
50
51 paracellular permeability on caspase activation may reflect increased leakage
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53 through apoptotic cells,^{39,50} or caspase-mediated cleavage and activation of Rho-
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55 associated protein kinase 1 (ROCK-1)⁵¹ which can enhance paracellular
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3 permeability.⁵² Such issues are the focus of our future studies. Overall, the findings
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5 suggest that the current paradigm that the effects of PAMPs on gut permeability are
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7 primarily mediated by the action of cytokines released from leukocytes⁶⁻⁸ may have to
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9 be modified to include direct effects on epithelial cells of one such pattern molecule,
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11 i.e. LPS.
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15 Synthetic Pam₃CSK₄ is a specific ligand for TLR-2.⁵³ The Pam₃CSK₄ ligand
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17 has been reported to increase TEER in Caco-2 cells¹⁶, but no significant effect of this
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19 agent was found in the present preparation of colon cells. This disparity may reflect
20
21 the transient effect of Pam₃CSK₄ in Caco-2 cells or differences between the
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23 preparations. Nevertheless, beneficial effects of Pam₃CSK₄-induced responses on
24
25 the permeability barrier were observed in this study, namely the prevention of the
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27 deleterious effect of *E. coli* LPS. The prevention of LPS-induced apoptosis by
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29 Pam₃CSK₄ is echoed by its inhibition of spontaneous apoptosis in sheets of mouse
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31 small intestine in primary culture⁵⁴. Finally, our finding that Pam₃CSK₄ delivered from
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33 the apical side was active in countering the effect of basal LPS is consistent with the
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35 use of orally delivered Pam₃CSK₄ to ameliorate the effect of dextran sodium
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37 sulphate-induced colitis in mice.⁵⁴ There are, thus, parallels between the present
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39 work and that of others^{16, 54} which suggest a beneficial effect of Pam₃CSK₄ on the
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41 permeability barrier. Furthermore, the work raises issues related to the cross-talk
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43 between TLR-2 and TLR-4 in the colon in response to PAMPs.
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51 In conclusion, LPS increased paracellular permeability of colonic epithelial
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53 cells only when added from the basal side by a mechanism which involved caspase
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55 activation. Perturbation of the gut barrier, which allows access of LPS to the base of
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57 colonic epithelial cells, could therefore result in a further exacerbation in paracellular
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59 permeability. The lack of effect of apically applied LPS on paracellular permeability
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3 may contribute to the ability of the colon to exist in harmony with the enteric flora. Of
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5 note, Pam₃CSK₄, an activator of TLR-2-mediated responses, prevented all effects
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7 induced by basally applied LPS, which suggests a possible protective effect of this
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9 agent in the colon.
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For Peer Review

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Legends to Figures

Fig. 1. Transepithelial electrical resistance (TEER) and paracellular permeability of colonic cells. (A) Increase in TEER with culture time. Data are means \pm SEM from 10 experiments. (B) Initial TEER and its relationship to basal permeability to mannitol for eight-day cultures. Data points are means \pm SEM for 14 plates from 7 experiments with 12 wells on each plate. (C) Comparison between apparent permeability to mannitol from different preparations of colon cells. Superscripts after the cell type indicate the source of the information (reference list).

Fig. 2. Light microscopy pictures of cryostat sections (10 μ m) through Transwell membranes (A-C, E, G) and colonic mucosa (D, F, H, apical surfaces to the left). (A) Phase contrast view of unstained tissue. (B) The same section as in (A) under UV illumination showing nuclei stained with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) (the position of the membrane is shown by the horizontal white lines). Sections stained with haematoxylin and eosin (C), with haematoxylin (D), with periodic acid-Schiff reagent (E) and (F) (goblet cells indicated by arrows in F), and for alkaline phosphatase (arrows) (G) and (H). (A-E) and (G) are all at the same magnification and which can be judged from the thickness of the Transwell membrane (10 μ m). (F) and (H) are shown at 25% relative to the other pictures to enable the whole depth of the mucosa to be visible.

Fig. 3. Examination of cells grown for 8 days on Transwells and fixed *in situ* (A-E) or detached, and transferred to microscope slides by use of a cytocentrifuge (F-K). (A) Low power image showing nuclei stained with DAPI. (B) The same cells as in (A)

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3 showing extensive staining for cytokeratin-18. (C) Nuclei and (D) and (E) x, y
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5 confocal images, respectively, in and above the plane of the nuclei showing the
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7 presence of cytokeratin-18. (F) Nuclei and (G) cytokeratin-18-positive cells on
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9 cytocentrifuge preparations. (H) Nuclei and (I) corresponding periodic acid-Schiff
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11 stained cells. (J) Nuclei and (K) corresponding vimentin-positive cell (arrow).
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17 **Fig. 4.** Transmission electron microscopy of vertical sections through the Transwell
18 preparation. (A) Image showing a cross section of the monolayer resting on the filter.
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20 (B) Higher magnification image showing a basally located nucleus (arrow) and a
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22 cluster of granules (*). (C) Microvilli, desmosomes (twin white arrows) and apical
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24 junction complexes (single black arrow) were visible. (E) and (F) Nuclear density
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26 achieved, respectively, with the present primary culture and with Caco-2 cells.
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33 **Fig. 5.** Immunoblots for TLR-4 and TLR-2 with corresponding actin loading controls
34 for cells incubated for 5h in the absence (control) or presence of LPS (10^{-7} g/ml).
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41 **Fig. 6.** (A) Effect of the concentration of apical LPS (open bars) and basal LPS (filled
42 bars) from *E. coli* on permeability to mannitol, and (B) on transepithelial electrical
43 resistance (TEER). Permeability was measured over a 1 h period starting 4 h after
44 exposure to LPS was initiated. The TEER was measured 5 h after exposure to LPS
45 was started. (C) The change in the rate of mannitol transfer from apical to basal with
46 time of exposure to LPS (10^{-7} g/ml). (D) Effect of the concentration of LPS on
47 caspase 3-like activity measured 5 h after the start of exposure to LPS. In (A-D) data
48 are means \pm SEM from four experiments; * $P < 0.05$, ** $P < 0.01$ after analysis of
49 variance followed by Dunnett's test for comparison with results in the absence of LPS
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3 (A, B, D) or with the data for 0-1 h (C). (E) Nuclei on Transwells stained with 4', 6-
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5 diamidino-2-phenylindole dihydrochloride (DAPI) after 5 h incubation without or with
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7 LPS (10^{-7} g/ml) in the basal compartment. Arrows point to apoptotic nuclei exhibiting
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9 features of condensation and fragmentation. (F) The proportion of apoptotic cells on
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11 Transwells after incubation for 5 h in the absence or presence of basal LPS (10^{-7}
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13 g/ml). Data are means \pm SEM of six Transwells from three experiments **P<0.01 by
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15 t-test.
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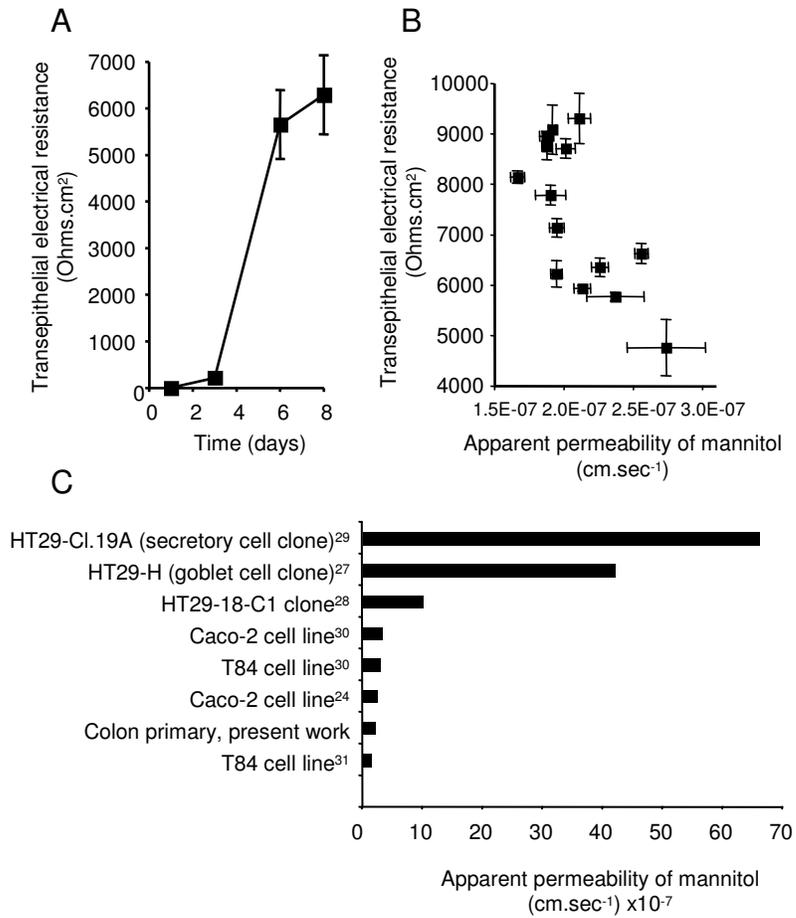
22 **Fig. 7.** Effect of basally applied *E. coli* LPS (10^{-7} g/ml) (filled bars) compared to
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24 controls (open bars) on the apical to basal transfer of fluorescein isothiocyanate-
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26 labelled dextran 10 kDa (FD10) and mannitol. Results, for the period 4-5 h after
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28 exposure to LPS was initiated, are means \pm SEM from four experiments; *P<0.05
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30 and **P<0.01 for comparison with control by paired t-test. The insert shows a lack of
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32 difference between FD10 and mannitol in the magnitude of the effect of LPS on their
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34 transfer.
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41 **Fig. 8.** Comparison between gastric and colonic epithelial cells of the effect of
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43 incubation with apically applied (open bars) and basally applied (filled bars) LPS from
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45 *H. pylori*. (A) Apical to basal transfer of mannitol and (B) the transepithelial electrical
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47 resistance (TEER). Data were collected between 2-4 h after exposure of gastric cells
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49 to LPS, and from 4-5 h after exposure of colonic cells to LPS. The data are means \pm
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51 SEM from three experiments for gastric cells and four experiments with colon cells.
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53 *P<0.05 and **P<0.01 for comparison with no LPS by analysis of variance and
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55 Dunnett's test.
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3 **Fig. 9.** Action of the pan-caspase inhibitor Z-VAD-FMK (50 μ M) in the basal
4 compartment (shaded bars) on the actions of basal *E. coli* LPS (10^{-7} g/ml). Mannitol
5 transfer was measured between 4-5 h after exposure to LPS was initiated and
6 transepithelial electrical resistance (TEER) and caspase 3-like activity after 5 h.
7 Results are means \pm SEM from four experiments and were obtained by calculating
8 the effect of LPS in the presence and absence of Z-VAD-FMK. This inhibitor had no
9 effect on mannitol transfer, TEER or caspase 3-like activity in the absence of LPS.
10 *P<0.05, **P<0.01, ***P<0.001 for comparisons between the effect of LPS in the
11 presence and absence of Z-VAD-FMK by a paired t-test.
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27 **Fig. 10.** Effect of the TLR-2 agonist Pam₃CSK₄ (20 μ g/ml). (A) Permeability to
28 mannitol, (B) transepithelial electrical resistance (TEER) and (C) caspase 3-like
29 activity in the absence (open bars) and presence (filled bars) of basal *E. coli* LPS (10^{-7}
30 g/ml). Results are means \pm SEM of four experiments. *P<0.05, **P<0.01 for effect
31 of LPS in the absence of Pam₃CSK₄, or its presence in basal, or in apical
32 compartments, by analysis of variance and a Newman-Keuls test. The same test
33 showed no significant effect of Pam₃CSK₄ on mannitol transfer, TEER or caspase 3-
34 like activity with respect to controls (no addition).
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Fig.1



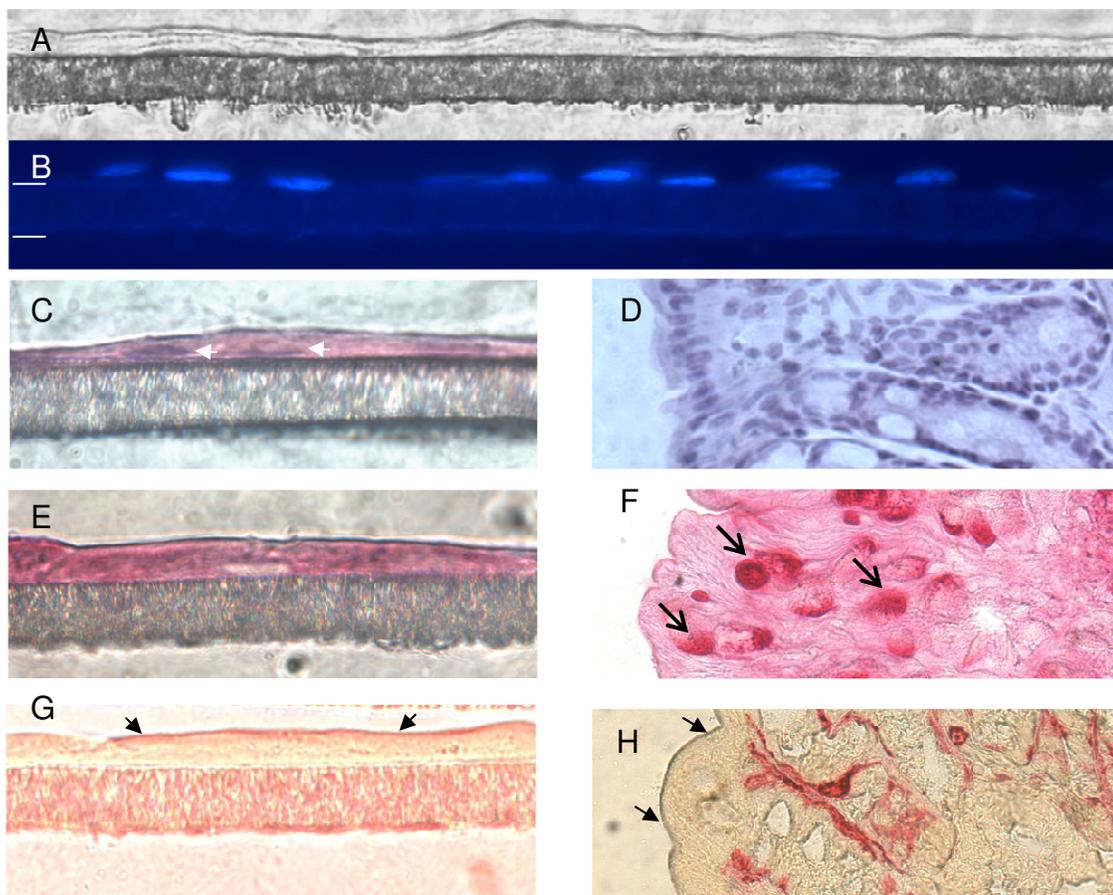


Fig. 2

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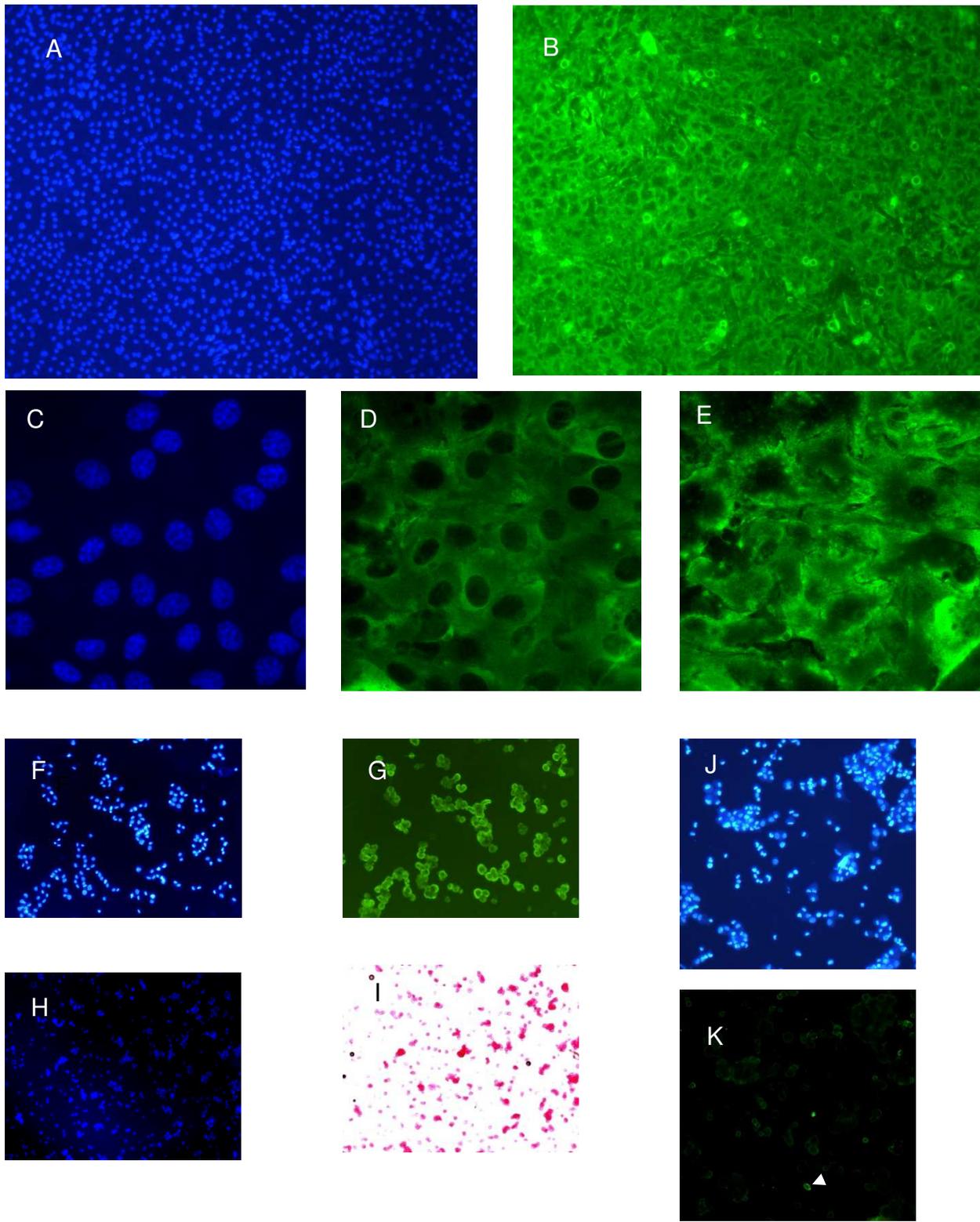


Fig. 3

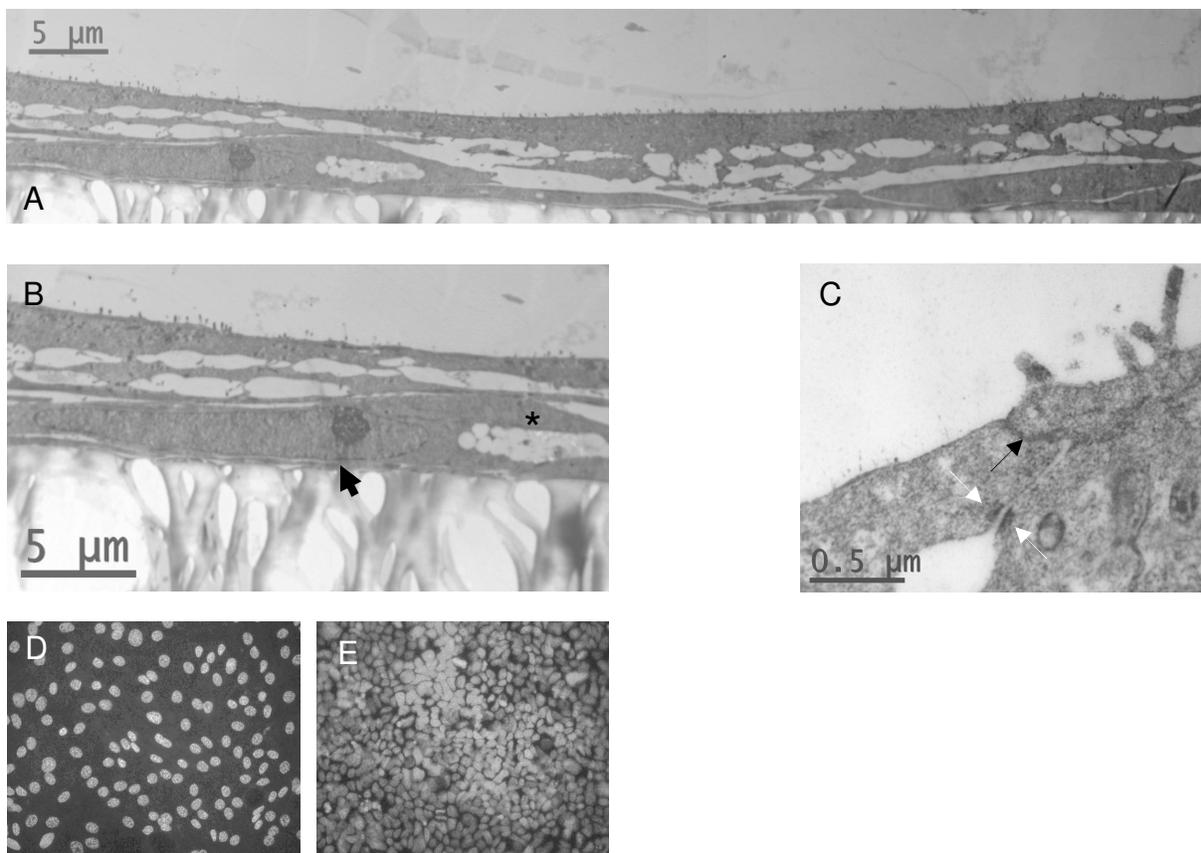


Fig. 4

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Fig. 5

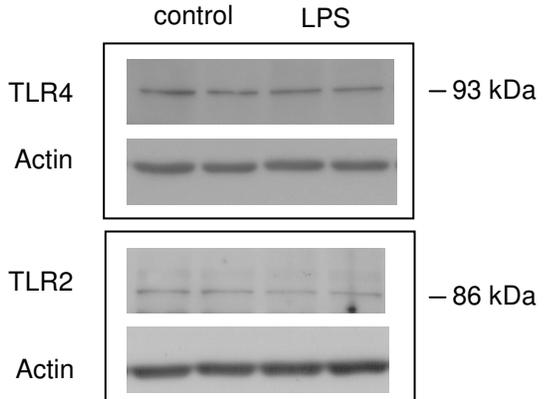
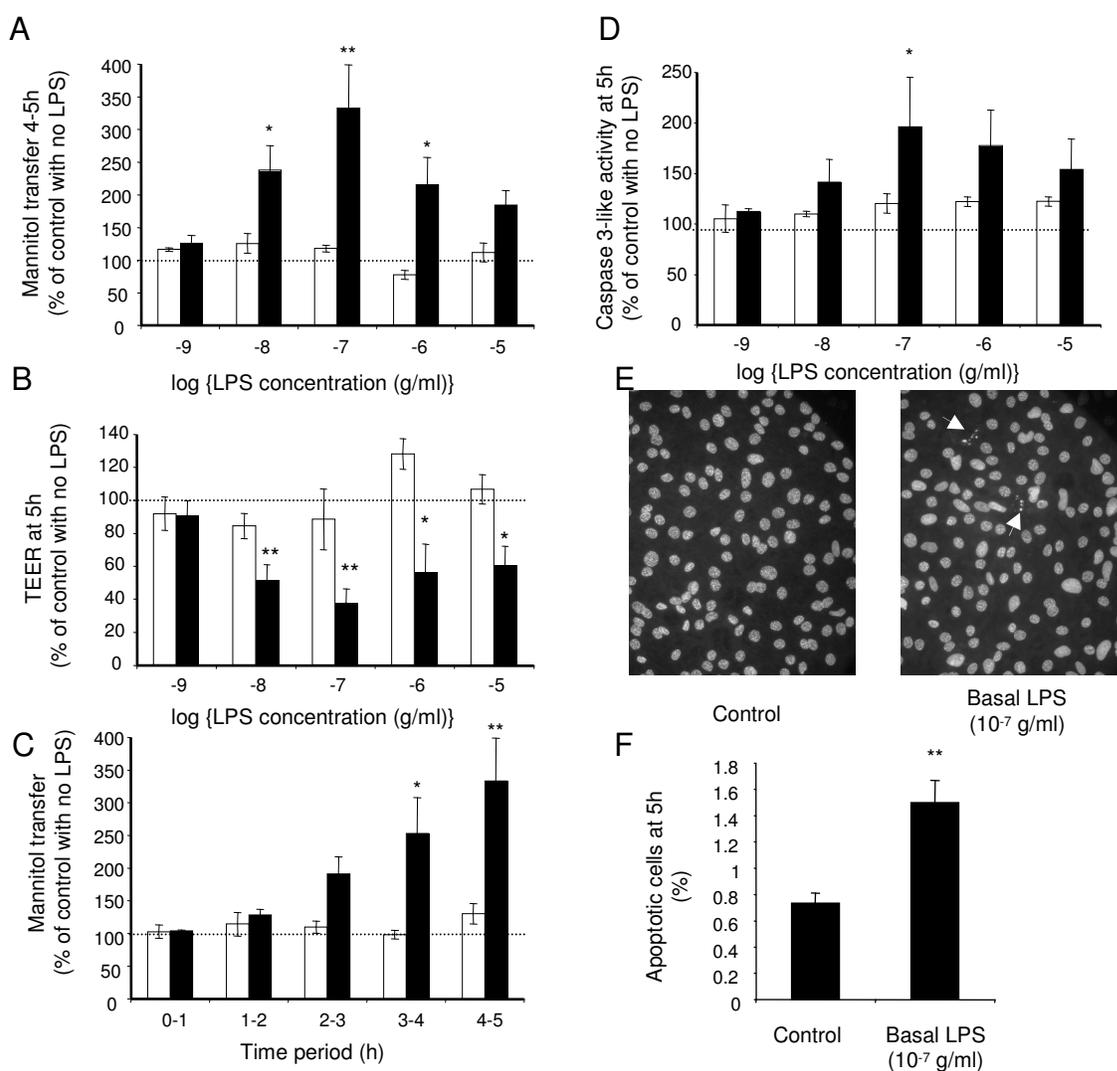


Fig. 6



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Fig. 7

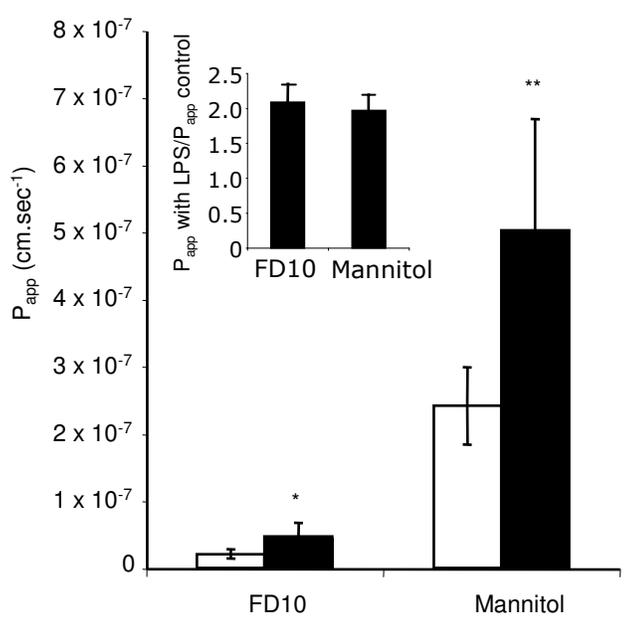
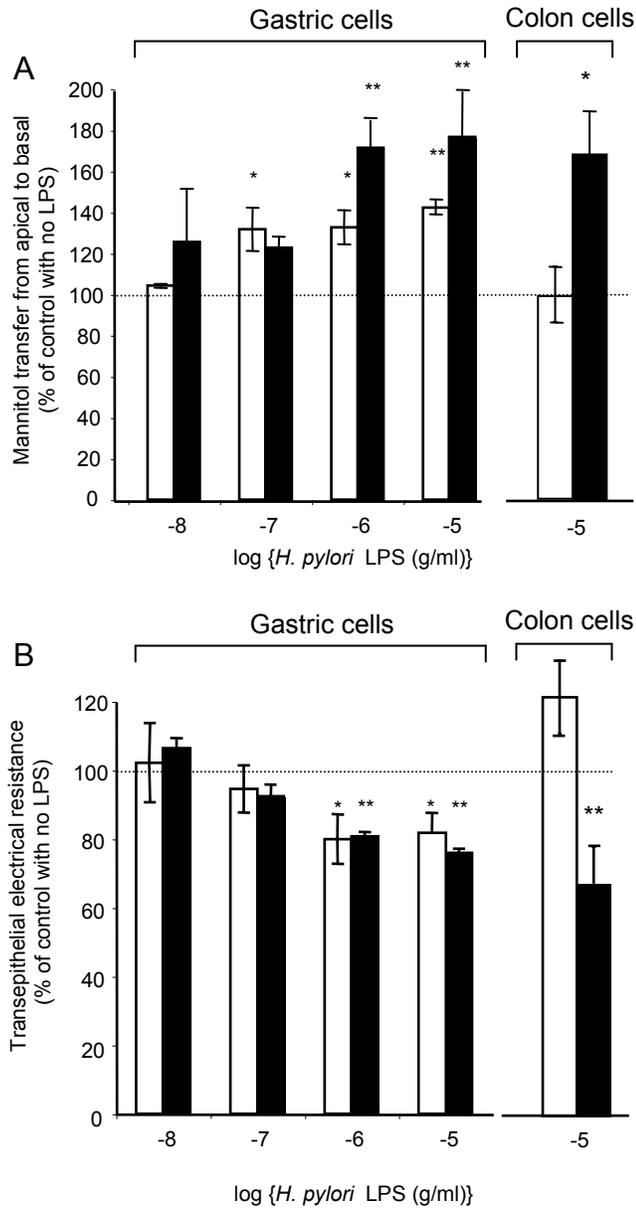


Fig. 8



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Fig. 9

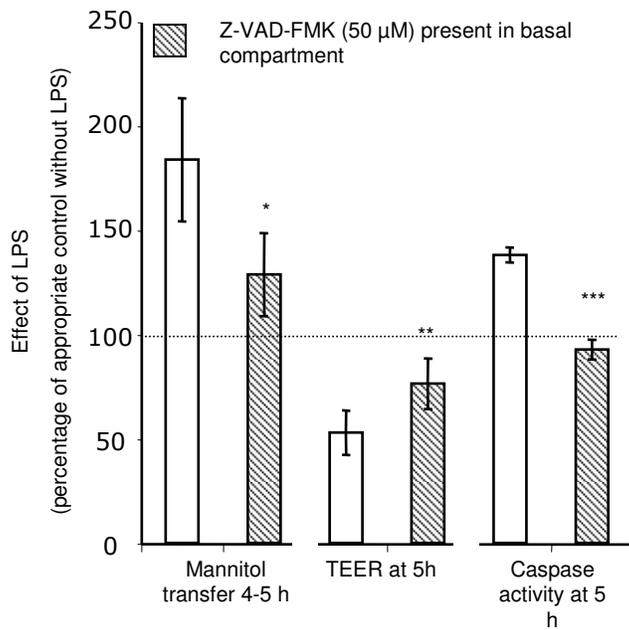


Fig. 10

