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Intestical permeability and contractility in murine colitis

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We developed an *in vitro* organ bath method to measure permeability and contractility simultaneously in murine intestinal segments. To investigate whether permeability and contractility are correlated and influenced by mucosal damage owing to inflammation, BALB/c mice were exposed to a 10% dextran sulphate sodium (DSS) solution for 8 days to induce colitis. The effect of pharmacologically induced smooth muscle relaxation and contraction on permeability was tested *in vitro*. Regional permeability differences were observed in both control and 10% DSS-treated mice. Distal colon segments were less permeable to ^{3}H-mannitol and ^{14}C-PEG 400 molecules compared with proximal colon and ileum. Intestinal permeability in control vs. 10% DSS mice was not altered, although histologic inflammation score and IFN-γ pro-inflammatory cytokine levels were significantly increased in proximal and distal colon. IL-1β levels were enhanced in these proximal and distal segments, but not significantly different from controls. Any effect of pharmacologically induced contractility on intestinal permeability could not be observed. In conclusion, intestinal permeability and contractility are not correlated in this model of experimentally induced colitis in mice. Although simultaneous measurement in a physiological set-up is possible, this method has to be further validated.

Key words: permeability, contractile activity, dextran induced colitis, mannitol, polyethylene glycol 400, mice

Introduction

The intestinal epithelium demonstrates different properties, providing both barrier and transport functions to luminal molecules. A defective barrier function may contribute to changes in intestinal permeability. As a result of this impaired barrier function, antigen access can initiate and perpetuate inflammation.[1] Moreover, studies in animal models indicate a causal relationship between the presence of mucosal inflammation and altered sensory-motor function.[2] In human inflammatory bowel disease (IBD) alterations in intestinal permeability[3] and motility[2,4] are both reported. However, it remains unclear whether these factors play an initiating role in the pathogenesis of human IBD or are secondary results of released inflammatory mediators.

In clinical practice and research, several intestinal permeability tests are used. Monitoring urinary excretion of orally administered test markers provides information on the intestinal barrier function. Frequently used markers are the cylindrical polymer polyethylene glycol 400 (PEG 400)[5] with a cross-sectional diameter of 5.3 Å, the globular shaped sugar alcohol mannitol[6] with a cross-sectional diameter of 6.7 Å and chromium-51-labelled EDTA[3,5–7] with a cross-sectional diameter of approximately 11 Å.[8,9]

Current *in vitro* animal studies of intestinal permeability use flat tissue sheets, mounted in Ussing chambers,[10] while contractility is studied in smooth muscle strips immersed in organ baths.[11] Intestinal permeability in *in vivo* animal models is mostly studied in perfused intestinal segments,[12] giving a more physiological approach. However, operational systems to study intestinal permeability and contractility simultaneously in whole colonic segments, including the mucosa, longitudinal and circular layers and neuronal plexus are not available. Therefore our aim was to develop an *in vitro* organ bath method to measure permeability and contractility simultaneously in normal and inflamed intestinal segments of mice. The degree of inflammation was determined by macroscopic scoring, histology and measuring cytokine levels in different intestinal segments.

Methods

Animals

Female BALB/c mice (20–22 g, IFFA Credo, France) were used in this study. They were kept individually

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on chopped wood bedding in polystyrene cages under a 12-h day/night cycle at 20–22°C, and with a relative humidity of 50% Mice were permitted free access to a standard mouse chow (Hope Farms, Woerden, The Netherlands) and DSS-supplemented or normal tap water.

The experiments were carried out after approval of the Ethics Committee for the use of experimental animals of the Rotterdam University Hospital (protocol 118–97–01).

Experimental design

Colitis was induced by adding 10% (wt/vol) DSS (Mw>500 000, Pharmacia Biotech AB, Uppsala, Sweden) to the drinking water for 8 days. The control group received normal tap water throughout the study. At day 7 all animals were deprived from food overnight and killed by cervical dislocation on day 8. Prior to study initiation and before deprivation, body weight was measured.

Drugs

Sodium nitroprusside (0.1 M) and carbachol (0.1 M) both from Sigma (St Louis, MO, USA) were dissolved in distilled water and stored at 4°C until use.

Tissue preparation

Upon sacrifice, the intestines were removed from the abdominal cavity. A segment of ileum most proximal to the caecum and a proximal and distal colon segment were taken and immediately immersed in standard Krebs buffer (pH 7.4) containing in mmol/l: 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$. $2H_2O$, 1.2 $MgSO_4$.7 H_2O, 1.2 KH_2PO_4, 25 $NaHCO_3$ and 8.3 glucose. Each segment was directly cannulated with stainless-steel cannulas, mounted horizontally in a 5 ml double-walled perspex organ bath (Fig. 1). The organ baths, warmed to 37°C, were filled with standard Krebs buffer and continuously gassed with carbogen (95% O_2 and 5% CO_2). The intestinal lumen was filled with Krebs buffer containing marker molecules; 0.25 µCi/ml ^{3}H-mannitol (NEN Life Science Products, Hoofddorp, The Netherlands), with a specific activity of 22.4 Ci/mmol and 0.25 µCi/ml ^{14}C-PEG 400 (Amersham Life Science, 's-Hertogenbosch, The Netherlands), with a specific activity of 0.135 µCi/mol. The distal cannula was connected to a low pressure sensor (Dépex, De Bilt, The Netherlands), which measures pressure differences between a passive port, set at 15 mmHg (registration range: ±10 mmHg), and the intraluminal pressure of the intestinal segment. The signal was recorded by using Multiple Channel Registration (MCR) computer-software.

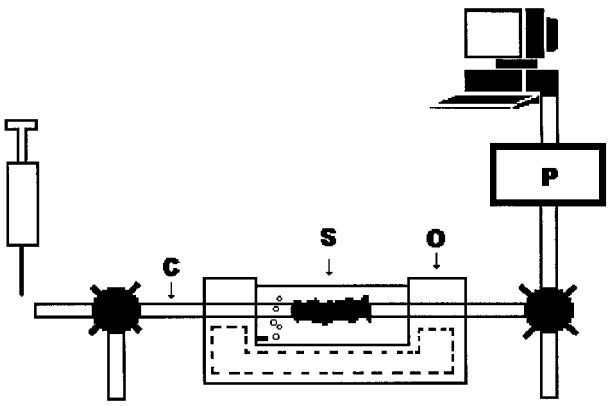

FIG. 1. Schematic presentation of the *in vitro* model. The intestinal segment (S) is cannulated with stainless-steel cannulas (C) and mounted in a double-walled organ bath (O) that contains carbogenated standard Krebs buffer at 37°C. The intestinal lumen is filled with standard Krebs buffer containing marker molecules via a syringe. The distal cannula is connected to a low pressure sensor (P). Contractility is recorded as pressure difference compared to a reference port and registered by Multiple Channel Registration computer-software.

Permeability measurement *In vitro*

Permeability measurement started at t=0 by replacing the organ bath fluid with 5 ml of fresh, carbogenated Krebs buffer. Every subsequent 5 min, 1 ml samples were taken from the serosal reservoir for marker analysis, directly followed by exchange of the entire organ bath fluid. This procedure was continued for 55 min. The samples were collected in 20 ml Econo glass vials (Packard Instrument BV, Groningen, The Netherlands) and 7 ml of scintillation fluid (Pico-Fluor 15; Packard Instrument BV, Groningen, The Netherlands) was added. Each vial was counted for radioactivity by β liquid scintillation counting (1500 TRI-CARB Liquid Scintillation Analyzer; Packard Instrument BV, The Netherlands).

Contractility measurement *In vitro*

Running parallel with the permeability measurements, contractile activity was monitored by measuring intraluminal pressure (mmHg) in each segment. At t=25, 30 and 35 min, smooth muscle relaxation was induced by adding a single dose of 50 µl nitroprusside (0.1 M; final concentration 10^{-3} M) to the organ bath directly after buffer exchange. At t=40, 45 and 50 min smooth muscle contraction was induced following the same procedure with a single dose of 50 µl carbachol (0.1 M; final concentration 10^{-3} M).

Macroscopy and histology

Upon sacrifice, the removed intestines were macroscopically examined. Signs of inflammation were scored. The macroscopic score ranged from 0–8, which represents the sum of scores from 0 to 2 for weight gain, appearance, diarrhoea and occult blood.

Table 1. Histologic grading of colitis

Feature graded	Grade	Description
Inflammation	0	None
	1	Mild
	2	Moderate
	3	Severe
Damage/necrosis	0	None
	1	Mild (superficial)
	2	Moderate (involving muscularis mucosa)
	3	Severe (transmural, involving muscularis propria)
Regeneration	3	None
	2	Focal migration and mitotic features
	1	Broad, multifocal re-epithelialization
	0	Complete re-epithelialization

Specimens of proximal, middle and distal colon were taken for histology. The sections were fixed in 3.6% buffered formalin, pH 7, and embedded in paraffin wax. Sections cut at 5-μm thickness, and stained with haematoxylin and azafloxin were examined under a light microscope using a 40× magnification. A colitis activity score was used to score each section in a blind fashion based on the method of Dieleman *et al.*[13] and as detailed in Table 1. The total histological activity score ranged from 0 to 9, which represents the sum of scores from 0 to 3 for inflammation, damage/necrosis and regeneration.

Cytokine bioassays

Small tissue sections of proximal and distal colon were taken for determination of IFN-γ, TNF-α and IL-1β cytokine synthesis. After adding 1 ml of standard Krebs buffer, the tissue was homogenized for ± 10 s using an Ultra-Turrax (Polytron, Switzerland). The suspension was centrifuged (400 × ***g***, 10 min, 4°C) and the supernatant was stored at −20°C until analysis. The cytokine concentration in the supernatant was determined by means of ELISA and expressed in pg/mg tissue.

For IFN-γ, a specific mouse IFN-γ ELISA kit was obtained from HyCult biotechnology BV (Uden, The Netherlands). Microplates pre-coated with monoclonal antibody specific for mouse IFN-γ were incubated with 150 μl sample or standard for 1 h at 37°C. After three washing steps, 100 μl of biotinylated antibody reagent mixed with streptavidin conjugate was added. Microplates were incubated for 1 h at 37°C. After three times washing, 100 μl para-nitrophenyl-phosphate (PNPP) substrate solution was added to the wells. Microplates were developed at 37°C for 30 min in the dark. To stop the enzymatic reaction 30 μl NaOH (3 M) was added. The optical density was measured spectrophotometrically at 450 nm using a Microplate Reader (model 3550, Bio-Rad Laboratories, Richmond, USA). The detection range was between 175 and 7000 pg/ml. For the assay of TNF-α, a specific mouse TNF-α ELISA kit was obtained from Biotrak (Amersham Life Science, UK). Microplates pre-coated with a solid phase monoclonal antibody specific for mouse TNF-α were incubated with 50 μl sample or standard along with 50 μl biotinylated antibody reagent for 2 h at room temperature. After washing five times to remove unbound sample proteins and biotinylated antibody, 100 μl of streptavidin conjugated to horseradish peroxidase (HRP) was added to the wells. Microplates were incubated for 30 min at room temperature and washed five times. Hereafter, 100 μl of TMB substrate solution was added. The microplates were developed at room temperature for 30 min in the dark. To stop the enzymatic reaction 100 μl sulphuric acid (0.18 M) was added to the wells. The optical density of each well was determined spectrophotometrically by using the Microplate Reader at 450 nm. The detection range was between 50 and 2450 pg/ml.

IL-1β was determined by using a high sensitivity IL-1β assay from Biotrak (Amersham Life Science, UK). Microplates pre-coated with a solid phase monoclonal antibody specific for mouse IL-1β were incubated with 50 μl sample or standard along with 50 μl biotinylated antibody reagent for 2 h at room temperature. After three washing steps, 100 μl of streptavidin-HRP conjugate was added to the wells. The microplates were incubated for 30 min at room temperature. After three washing steps, 100 μl of TMB substrate solution was added. Microplate development and measurement was similar to the TNF-α assay. The detection range was between 15.6 and 1000 pg/ml.

Statistical analysis

Data are presented as means (SEM). The statistical significance of differences was determined by using the Mann–Whitney ranking test. $P < 0.05$ was considered statistically significant.

Results

All mice developed a mild colitis after drinking 10% DSS for 8 days. The macroscopic score for appearance, diarrhoea, occult blood and weight gain was significant increased ($P < 0.01$) in 10% DSS-treated animals compared with controls (Table 2). Microscopic examination of tissue from different intestinal regions showed a significantly increased score ($P < 0.05$) in proximal and distal colon segments of 10% DSS-treated animals versus controls.

The production of IFN-γ in inflamed proximal and distal colon tissue was significant increased ($P < 0.05$) compared with controls. IL-1β levels in inflamed

Table 2. Experimentally induced colitis by 10% DSS administration to BALB/c mice. The degree of inflammation determined by macroscopic scoring and histology for proximal, middle and distal colon segments of control ($n = 5$) and 10% DSS treated mice ($n = 7$)

Group	Macroscopic score	Microscopic score		
		Proximal	Middle	Distal
Control	0.4 (0.3)	4.0 (0.6)	3.0 (0.9)	3.4 (1.2)
10% DSS	5.6 (0.6)*	6.4 (0.6)**	5.7 (0.7)	6.4 (0.5)**

*Significantly different ($P < 0.05$) from control. **Significantly different ($P < 0.01$) from control. Results are shown as mean score (SEM).

Table 3. Experimentally induced colitis by 10% DSS administration to BALB/c mice. IFN-γ and IL-1β levels in proximal, middle and distal colon segments of control ($n = 5$) and 10% DSS treated mice ($n = 7$)

Segment	[IFN-γ] (pg/mg tissue)		[IL-1β] (pg/mg tissue)	
	Control	10% DSS	Control	10% DSS
Proximal	2.7 (1.8)	27.2* (7.0)	0.2 (0.2)	1.5 (0.4)
Middle	1.7 (1.0)	12.5 (4.4)	3.7 (3.3)	2.1 (0.8)
Distal	0	37.2* (27.7)	2.0 (1.0)	2.4 (0.8)

*Significantly different ($P < 0.05$) from control. Results are shown as mean (SEM).

proximal and distal colon were raised, but not significantly different from controls (Table 3). TNF-α levels were under detection limit in intestinal tissue of both 10% DSS-treated and control animals (data not shown).

Passage of the marker molecules, from the luminal to the serosal side, was reproducible measured with the *in vitro* organ bath method. Figure 2 shows representative permeation profiles of a 10% DSS treated (B) and control (A) animal. Regional differences in permeability were observed. Distal colon segments were less permeable to ^{3}H-mannitol and ^{14}C-PEG 400 molecules compared with proximal colon and ileum in both groups. Exposure to 10% DSS-induced enhanced permeability in the ileum segment, but did not alter permeability in the inflamed proximal and distal regions. The intestinal segments showed a higher passage of ^{3}H-mannitol than of ^{14}C-PEG 400 molecules in both groups. Simultaneously with permeability measurement, contractile activity was monitored in time (Fig. 3). Smooth muscle contraction is observed as an increase of intraluminal pressure, while relaxation of the smooth muscles was reflected by a decline in pressure. Periodic buffer exchange caused a revival of smooth muscle activity in proximal and distal colon segments, but this phenomenon was not observed in the ileum. Ileum intraluminal pressure diminished during the experiments possibly due to the large relaxing properties of the smooth muscles in these

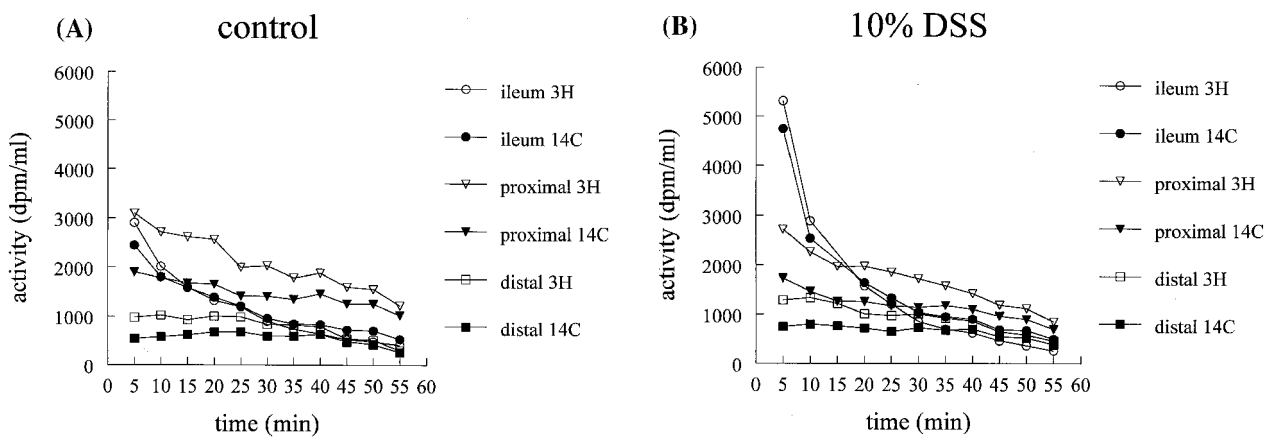

FIG. 2. Permeability monitored with ^{3}H-mannitol (3H) and ^{14}C-PEG 400 (14C) from luminal towards serosal side in different intestinal segments of a control (A) and 10% DSS-treated mouse (B).

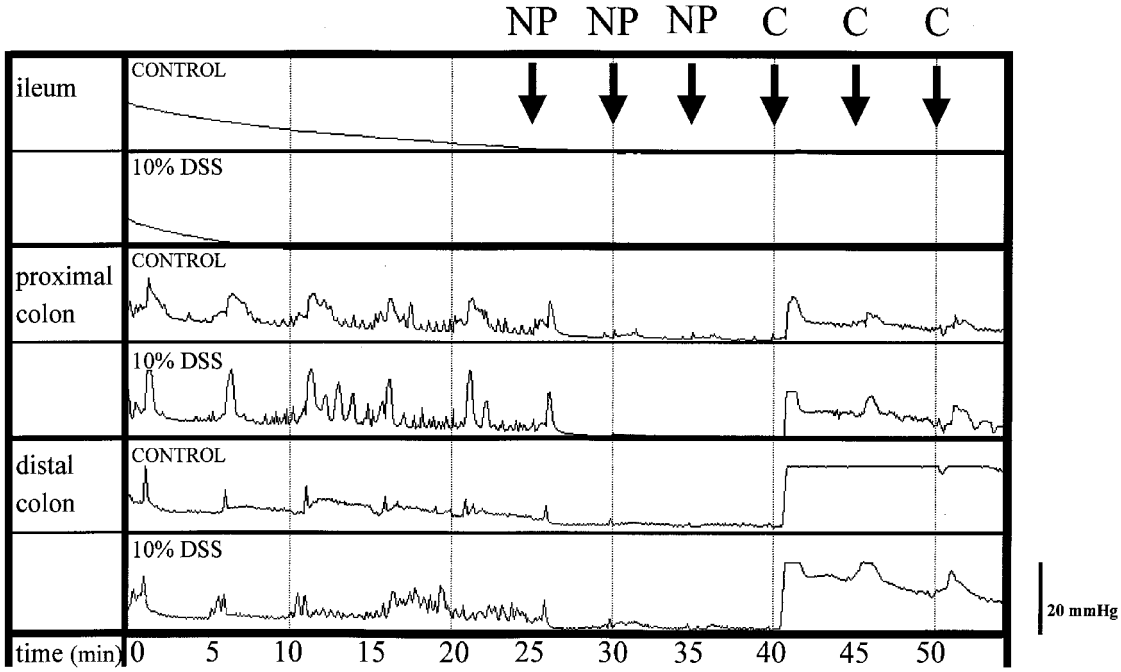

FIG. 3. Contractility, simultaneously measured with permeability (see Fig. 2) and serosal influenced by nitroprusside (NP) and carbachol (C) at various time points.

segments. Serosal additions of sodium nitroprusside induced a rapid decrease in smooth muscle activity, while repeated addition of carbachol resulted in a sustained smooth muscle contraction. Any effect of these pharmacologically induced contractile alterations on changes in intestinal permeability could not be observed.

Discussion

No previous studies have been able to measure permeability and contractility simultaneously in intestinal segments. We developed a new *in vitro* organ bath method in which reliable data were obtained in a physiological set-up. Intestinal permeability was measured with ^{3}H-mannitol and ^{14}C-PEG 400 molecules, while smooth muscle contractility was online monitored by registration of intraluminal pressure.

Okayasu *et al.*[14] developed a new experimental model for ulcerative colitis in mice. In our hands the applied animal model in which 10% DSS was administered via the drinking water to BALB/c mice for 8 days, induced a reproducible mild colitis. Macroscopic signs of inflammation were reflected by diarrhoea, occult blood and loss of body weight. Contrary to the results of Okayasu *et al.*[14] not only the distal colon was affected, but also proximal colon was significantly more inflamed in 10% DSS treated mice. This may be caused by the difference in molecular weight of the administered DSS. In the present study dextran with a molecular weight of >500 000 was used, while in the experiments of Okayasu *et al.*[14] a dextran fraction with a molecular weight of 54 000 was used to induce colitis.

After 8 days of 10% DSS administration IFN-γ pro-inflammatory cytokine levels were significantly increased in proximal and distal colon segments. IL-1β levels were also raised in these sections, but not significantly different from controls. Besides, TNF-α levels were undetectable in colonic tissue on day 8. These results confirmed observations of van Dijk *et al.*[15] who found that IL-1β and TNF-α cytokines were detectable at peak levels on day 2 in 10% DSS-treated BALB/c mice and were subsequently downregulated thereafter.

Despite the significant increased degree of inflammation in proximal and distal colon segments, no difference in permeability to ^{3}H-mannitol or ^{14}C-PEG 400 molecules was observed. According to Hollander[8] intestinal permeability of water-soluble compounds is dependent on the specific size of the marker molecules based on the hypothesis of tight junctional differences between intestinal villi and crypts. It was proposed that smaller and more resistant tight junctions at the tips of villi can be penetrated by smaller compounds. Larger compounds (around 10 Å) can only penetrate the crypt tight junctions where access from the lumen is more limited. Mannitol and PEG 400 are permeability markers of nearly equal size (6.7 and 5.3 Å respectively). The permeation route of mannitol is proposed to be paracellular. The pathway of PEG 400 is not so clearly defined and is possibly paracellular and/or transcellular.[9] Our results showed nearly identical permeation profiles for both marker molecules. Mannitol crossed the intestinal barrier in slightly larger amounts compared with PEG 400,

resulting in mannitol/PEG 400 permeation ratios ≈1.3 (data not shown). To discriminate between the two proposed tight junctional permeability routes, mannitol or PEG 400 and a larger marker molecule should be used in forthcoming experiments.

In addition, no effect of the pharmacological altered intraluminal pressure was seen on the permeation profiles. Sodium nitroprusside, a nitric oxide-donor inducing smooth muscle relaxation via cellular increased cGMP levels and carbachol known as a muscarinic agonist inducing smooth muscle contraction were possibly not able to alter intestinal permeability. Other pathways, like 5-HT_3 receptor agonists, triggering the fast opening of cation selective channels to induce contraction and for instance salbutamol as a selective β_2-receptor agonist inducing relaxation should be investigated.

In summary, we developed a new operational *in vitro* organ bath method in which simultaneous measurement of intestinal permeability and contractility in a physiological set-up is possible. However, despite the significantly enhanced degree of intestinal inflammation, altered permeability and contractility was not demonstrated, nor correlated in this model of experimentally induced colitis in mice. Further validation of this method is recommended.

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