Measurement of in vivo rectal mucosal cytokine and eicosanoid production in ulcerative colitis using filter paper

E Carty, M De Brabander, R M Feakins, D S Rampton

Abstract

Background—Excessive mucosal generation of cytokines and eicosanoids has been reported in vitro in ulcerative colitis (UC) using traumatising biopsy techniques, and in vivo using time consuming rectal dialysis.

Aims—To validate a simple filter paper technique to profile rectal mucosal production of cytokines and eicosanoids in vivo in patients with UC compared with controls.

Patients—Forty one patients with UC (21 with active disease) and 16 controls were studied.

Methods—In vitro, recovery of known concentrations of cytokine or mediator applied to filter papers was measured by ELISA following incubation in buffer. In vivo, patients and controls had filter papers apposed to the rectal mucosa briefly through a rigid sigmoidoscope. Filter papers were then incubated prior to assay by ELISA.

Results—In vitro validation studies showed that the filter paper technique could be used to measure mucosal release of interleukin-1β (IL-1β), tumour necrosis factor α (TNF-α), thromboxane B_2 (TXB$_2$), and prostaglandin E_2 (PGE$_2$), but not interferon γ (IFN-γ). Mucosal release of IL-1β, TNF-α, TXB$_2$ and PGE$_2$ were significantly increased in active UC (p=0.001) and correlated directly with disease activity (p=0.02).

Conclusions—The filter paper technique confirmed increased rectal mucosal release of cytokines and eicosanoids in UC, in proportion to disease activity. The simplicity, safety and speed of the technique make it a practicable option for use in the outpatient clinic to study the pathogenesis of inflammatory bowel disease, and potentially its response to treatment.

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Keywords: cytokines; eicosanoids; ulcerative colitis; rectal dialysis

Digestive Diseases Research Centre, St Bartholomew's and the Royal London School of Medicine and Dentistry, London, UK
E Carty
D S Rampton

Janssen Research Foundation, Jan Palfijn Hospital, Merksem, Belgium
M De Brabander

Department of Histopathology, St Bartholomew's and the Royal London School of Medicine and Dentistry, London, UK
R M Feakins

Correspondence to:
Dr E Carty, Digestive Diseases Research Centre, St Bartholomew's and the Royal London School of Medicine, 2 Newark St, London E1 2AD, UK.
Email: E.Carty@mds.qmw.ac.uk

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Although the aetiology of inflammatory bowel disease (IBD) remains obscure, its pathogenesis is gradually being unravelled. Numerous studies have indicated the importance of excessive mucosal generation of cytokines (for reviews see Sartor[1] and Nielsen and Rask-Madsen[2]) and inflammatory mediators such as eicosanoids (for reviews see Nielsen and Rask-Madsen,[2] Rampton and Collins,[3] and Targan and colleagues[4]). Conventional therapy of IBD with corticosteroids and aminosalicylates may act by modulating the profile of cytokines and eicosanoids produced[1–4] and novel agents are being targeted specifically at excessive production of proinflammatory cytokines (e.g. tumour necrosis factor α (TNF-α[5])) and eicosanoids (e.g. thromboxane[6] and leukotriene B_4[7]).

Existing methods to quantify mucosal generation of cytokines and lipid mediators have several disadvantages. In vitro biopsy methods using tissue obtained by endoscopic biopsy have demonstrated increased levels of prostaglandins,[8] thromboxane,[9] leukotrienes,[10] platelet activating factor,[11] and proinflammatory cytokines[12 13] in IBD. However, these methods are traumatising and may be associated with a small risk of bleeding and perforation, particularly in patients with active ulcerative colitis (UC). Furthermore, the trauma of biopsy collection may itself alter the profile of mediators generated by the mucosa.

The most widely used alternative method, in vivo rectal dialysis, has been used to quantify mucosal production of eicosanoids,[14 15] electrolytes,[16] histamine[17] and interleukin-8.[18] This method is safe for patients, but is a lengthy procedure, taking up to four hours to complete. Additionally, rectal dialysis can be used for detection of only those mediators and cytokines which are small enough to penetrate the dialysis membrane. In some studies the necessity to discard samples showing faecal contamination has been another major disadvantage of this method.

The third major option, rectal perfusion,[19] is tedious, technically difficult and may itself alter mucosal function.

In 1996, Hendel and colleagues[20] described a method of assessing mucosal production of interleukin-1β (IL-1β) and IL-1 receptor antagonist by direct application, via a sigmoidoscope, of a small strip of filter paper to the inflamed rectal mucosa for a period of up to one minute. Specimen collection on filter paper has been used for a variety of body fluids, including blood (e.g. dried blood for phenylketonuria[21] and cytomegalovirus DNA[22]

in neonates) and dental crevicular fluid for eicosanoids.[23] The potential advantages of the use of filter paper include its simplicity, safety, speed, non-traumatic nature, and low cost.

The aim of this study was to assess the validity of the in vivo filter paper method for quantification of rectal mucosal release of not only IL-1β[20] but also of interferon γ (IFN-γ), TNF-α, and the eicosanoids thromboxane B_2 (TXB$_2$) and prostaglandin E_2 (PGE$_2$). If adequately validated, the filter paper technique could prove useful for further elucidation in vivo of the pathogenesis of IBD, and for the assessment of both the mode of action of, and response to, conventional and novel therapies.

Methods

PATIENTS

Twenty one patients with active UC (defined as a mucosal score of 2 or 3 using a standardised sigmoidoscopic score[24]) and 20 patients with inactive UC (score 0 or 1[24]) were studied. Disease activity was assessed clinically using the Powell-Tuck score[25] and histologically using a semiquantitative score described by Savery-muttu and colleagues.[26] Sixteen patients undergoing routine sigmoidoscopic assessment because of a change in bowel habit, abdominal pain or rectal bleeding, who were found to have normal rectal mucosa endoscopically and histologically, acted as controls. Details of age, sex, disease extent, and current drug treatments are shown in table 1.

Sample volume restricted the measurement of all mediators in every patient and the lack of clinical requirement for rectal biopsy limited the availability of histology. The number of patients in each group for each result is stated throughout.

All patients gave informed consent and the study was approved by the East London and City Health Authority Research Ethics Committee.

FILTER PAPER PROCEDURE

Patients were studied during the course of a routine outpatient clinic; no bowel cleansing preparation was used. Through a standard rigid sigmoidoscope, a piece of filter paper (Whatmann No 42, 7 mm×30 mm) was apposed to the rectal mucosa until visibly soaked with luminal secretion (up to 60 seconds). The filter paper was then removed and placed in a vial containing 1 ml of Tris

buffer 0.1 M pH 7.4 in normal saline with human serum albumin (0.3%), sodium azide (0.01%), and Tween (0.002%). The vial was placed on a rocker and agitated gently for 24 hours at 4°C. Contaminating particles were then removed by centrifugation and the solution stored at −70°C until analysis.

VALIDATION STUDIES

In vitro recovery of a known concentration of cytokine or eicosanoid was assessed by adding known volumes of standard onto the filter paper, placing the paper in buffer, and incubating for 24 hours, as above. The supernatant was then assayed to measure the concentration recovered.

To assess if rectal luminal bacteria might degrade the cytokine or eicosanoid, in vitro recovery of a known quantity of cytokine or eicosanoid from a filter paper was measured after incubation for 24 hours in buffer containing either 10^9 bacteria (*E coli* and *Streptococcus faecalis*) per 1 ml or control buffer containing the same volume of sterile culture broth (Brain heart infusion broth; Oxoid CM25, Basingstoke, UK).

To assess intraindividual variation, two filter papers were apposed to different parts of the rectal mucosa during a single sigmoidoscopy in the same patient for six patients with active UC, six patients with inactive UC and four control patients.

To investigate if a greater volume of fluid, and consequently artefactually more cytokine or lipid mediator, was collected on the filter paper in patients with active UC, the weight of fluid collected for 10 patients with active disease, 12 with inactive disease and eight controls was measured.

RECTAL DIALYSIS

In vivo rectal dialysis was used as a comparator for the filter paper technique on eight occasions in two patients. A standard method was used.[14] A 12 cm long dialysis bag (volume 4 ml; Visking seamless cellulose tubing 8/32) filled with an isotonic saline based buffer containing 10% dextran was gently inserted into the patient's rectum. The bag was withdrawn after two hours and the contents emptied into a test tube and immediately frozen at −70°C until analysis. TXB$_2$ concentration in the fluid was measured by ELISA. The molecular weights of IL-1β (17.4 kD) and TNF-α (17 kD) were too high to allow penetration through the dialysis membrane (molecular weight cut off of the dialysis membrane used was 12–15 kD): comparison of rectal dialysis and the filter paper technique for the cytokines was therefore not performed.

ASSAYS

All eicosanoids and cytokines were measured in duplicate on unextracted fluid using commercial enzyme linked immunosorbent assay (ELISA) kits. Serial dilution of selected samples revealed parallelism with the assay standards and standards made up in incubation buffer. TXB$_2$ standards made up in the same dilution of 10% dextran (1 in 5) as the

Table 1 Age, sex, anatomical disease extent, and treatment of patients using the filter paper technique. Disease activity was assessed using sigmoidoscopic score (scores 2 and 3 are active UC, scores 0 and 1 are inactive UC[24])

	Ulcerative colitis		
	Active	*Inactive*	*Controls*
n	21	20	16
Age (years) (median (IQR))	43 (33–53)	51 (40–66)	49 (36–55)
Sex (M/F)	11/10	11/9	9/7
Disease extent			
Distal colitis	14	8	—
Left sided colitis	4	9	—
Extensive colitis	3	3	—
Treatment			
Oral or topical 5ASA	20	17	0
Oral or topical steroids	8	4	0
Azathioprine	1	1	0

Table 2   Assay characteristics

Mediator	ELISA kit supplier	Sensitivity	Cross reactivity	Intra-assay CV	Interassay CV
IL-1β	Medgenix	2 pg/ml*	None*	13.5%	4.6%*
IFN-γ	Medgenix	0.03 iu/ml*	None*	3.5%	7.9%*
TNF-α	Medgenix	3 pg/ml*	None*	8.1%	9.9%*
TXB$_2$	R&D Systems	8 pg/ml	7.1% for 2,3,dinor TXB$_2$* <0.01% for PGE$_2$*	6.8%	18%
PGE$_2$	R&D Systems	36 pg/ml	70% for PGE$_1$* <0.1% for TXB$_2$*	4.6%	24%

*Manufacturer's data. CV, coefficent of variation.

Table 3   In vitro recovery of known amounts of eicosanoids and cytokines from filter papers incubated in buffer alone, buffer containing colonic bacteria, and buffer containing sterile culture broth. Median (IQR) percentage recovery is shown for in vitro recovery from buffer alone; for recovery from buffer containing bacteria or sterile culture broth, mean percentage recovery is shown

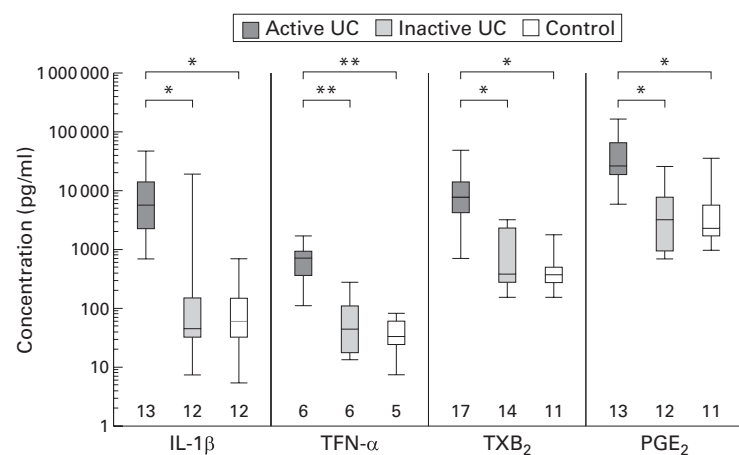| Mediator	Recovery from buffer (%)	n	Recovery in the presence of colonic bacteria (%)	n	Recovery in the presence of culture broth (%)	n
IL-1β	84 (76–91)	8	82	2	78	2
IFN-γ	15 (12–18)	6	Not assessed	2	Not assessed	2
TNF-α	88 (75–106)	6	92	2	125	2
TXB$_2$	99 (95–107)	6	99	2	87	2
PGE$_2$	96 (92–99)	4	110	2	101	2

*Figure 1   Rectal mucosal release of interleukin 1β (IL-β), tumour necrosis factor α (TNF-α), thromboxane B$_2$ (TXB$_2$), and prostaglandin E$_2$ (PGE$_2$), measured by the filter paper technique in active ulcerative colitis (UC), inactive UC (defined by sigmoidoscopic score[24]) and in controls. Box and whisker plots are shown where the box is the interquartile range, the horizontal line the median, and the whiskers show the highest and lowest values. Numbers of patients studied are shown above the x axis. *p<0.0001, **p<0.001.*

Table 4   Correlation coefficients and p values of significance for the relationship between rectal mucosal release of cytokines and eicosanoids measured with the filter paper technique, and sigmoidoscopic,[24] clinical activity,[25] and histological[26] scores in patients with ulcerative colitis

Score	Mediator	n	Correlation coefficient (r)	p Value
Sigmoidoscopic score				
	IL-1β	25	+0.71	0.0001
	TNF-α	12	+0.76	0.005
	TXB$_2$	31	+0.80	<0.0001
	PGE$_2$	25	+0.75	<0.0001
Clinical activity score				
	IL-1β	25	+0.43	0.0007
	TNF-α	12	+0.89	0.02
	TXB$_2$	31	+0.69	<0.0001
	PGE$_2$	25	+0.51	0.009
Histological score				
	IL-1β	17	+0.76	0.04
	TNF-α	10	+0.71	<0.0001
	TXB$_2$	16	+0.87	<0.0001
	PGE$_2$	16	+0.64	0.01

rectal dialysis samples also revealed parallelism with standards made up in assay buffer. Details of the commercial companies from which the ELISA kits were obtained, sensitivity, cross

reactivity and precision (intra- and interassay coefficients of variation) are shown in table 2. The intra-assay coefficient of variation was calculated on duplicate measurements of 16 samples (eight highest and eight lowest) using a method described by Bland and Altman.[27] Similarly, the interassay coefficients of variation for TXB$_2$ and PGE$_2$ were calculated on repeated duplicate measurements from two samples in five assays using the same statistical method.[27]

STATISTICAL METHODS

Statistical comparisons between groups were made using the Mann-Whitney U test (two-tailed). Correlations between two sets of data were assessed by Spearman's rank correlation (two-tailed). The coefficient of variation of paired results was calculated as described by Bland and Altman.[27] Comparison of the two methods of measurement (filter paper method and rectal dialysis) was performed using a simple correlation rather than a method to test agreement described by Bland and Altman,[28] as the aim was to investigate if the values obtained using the filter paper technique reflected those of rectal dialysis rather than resembled them in magnitude.

In the in vivo validation studies to assess the influence of the volume of fluid collected by the filter paper, results are expressed as pg/mg of mucosal fluid collected; all other results are expressed as concentration (pg/ml of incubation fluid).

Results

IN VITRO VALIDATION

Data for eicosanoid and cytokine recovery after addition to the filter paper in vitro and incubation for 24 hours in buffer at 4°C are shown in table 3. Adequate recoveries were achieved for IL-1β, TNF-α, and the eicosanoids TXB$_2$ and PGE$_2$. However, there was low recovery of IFN-γ. For IFN-γ, repeated experiments using a four hour incubation also gave low median (interquartile range (IQR)) recovery of 18% (16–38%) (n=6). The low recovery of IFN-γ invalidated further attempts at in vivo study of this cytokine using the filter paper technique.

Recovery of cytokines and lipid mediators after addition to the filter paper in vitro and its incubation for 24 hours in buffer containing bacteria or sterile culture broth is shown in table 3. Colonic bacteria in the buffer did not appear to degrade the cytokines or eicosanoids.

CYTOKINES IN ULCERATIVE COLITIS

The median concentration of IL-1β in filter paper incubation fluid in active UC was 155-fold that of controls (p<0.0001) and 277-fold that of patients with inactive UC (p<0.0001) (fig 1). The concentration of IL-1β correlated directly with sigmoidoscopic, clinical activity, and histological activity scores (table 4).

The median concentration of TNF-α in filter paper incubation fluid in active UC was 23 times that of controls (p=0.003) and 20 times that of patients with inactive UC (p=0.001) (fig

Table 5 Weight of fluid collected on the filter paper and the corrected IL-1β, TNFα, and eicosanoid levels (for weight) for patients with active and inactive ulcerative colitis(defined by sigmoidoscopic score) and in controls. Median (IQR) are shown

	Ulcerative colitis						
	Active	n	Inactive	n	Controls	n
Weight of mucosal fluid (mg)	42.7 (31.2–57.3)	10	18.5 (14.4–24.1)*	12	19.3 (16.5–23.8)*	8
Corrected cytokine/eicosanoid level						
IL-1β (pg/ml mucosal fluid)	57.9 (28.4–121)	6	1.5 (0.8–2.7)**	6	1.3 (0.9–4.7)*	5
TNF-α (pg/ml mucosal fluid)	8.4 (3.2–11.9)	6	0.68 (0.34–3.5)**	6	0.36 (0.28–0.81)**	5
TXB₂ (pg/ml mucosal fluid)	192 (123–249)	6	22.9 (8.6–37.8)*	6	10.6 (8.8–14.8)*	4
PGE₂ (pg/ml mucosal fluid)	1574 (869–2184)	5	88.8 (53.4–263)**	4	102 (49–310)**	4

*$p<0.0001$, **$p=0.02$ compared with active UC

1). TNF-α release showed a positive correlation with sigmoidoscopic, histological, and clinical activity scores (table 4).

EICOSANOIDS IN ULCERATIVE COLITIS
Median concentrations of TXB_2 and PGE_2 in rectal mucosal fluid in active UC were 39- and 12-fold those of controls ($p<0.0001$ for both) and 35-fold and 10-fold those of patients with inactive UC ($p<0.0001$ for both) (fig 1). Release of TXB_2 and PGE_2 correlated directly with sigmoidoscopic, histological, and clinical activity scores (table 4).

IN VIVO VALIDATION
The coefficients of variation[27] for IL-1β, TXB_2, and PGE_2 concentrations from the paired filter paper samples were 61% (n=5), 60% (n=6), and 43% (n=5), respectively, in the active disease group, 16% (n=5), 30% (n=6), and 20% (n=4) in the inactive group, and 30% (n=4), 72% (n=4), and 35% (n=4) in the control group. For TNF-α, the coefficient of variation was 5% (n=6, three active patients, one inactive and two controls).

The median weight of fluid collected on the filter papers from patients with active disease was significantly greater than that collected from inactive patients and controls ($p<0.0001$) (table 5). There was a significant positive correlation between disease activity measured by sigmoidoscopic score and the weight of mucosal fluid collected ($r=+0.58$, $p<0.0001$). However, when the concentrations of IL-1β, TNF-α, TXB_2, and PGE_2 measured were corrected for the weight of fluid collected, differences in the amounts of cytokine and eicosanoid between the clinical groups remained highly significant with higher amounts of IL-1β, TNF-α, TXB_2, and PGE_2 per mg of mucosal fluid collected in the active group compared with the inactive ($p=0.003$, $p=0.02$, $p<0.0001$, and $p=0.0002$, respectively) and control ($p<0.0001$, $p=0.0021$, $p<0.0001$, and $p=0.0009$, respectively) groups (table 5). The percentages of the differences between the samples from patients with active UC and controls that could be explained by increased mucosal fluid weight for IL-1β, TNF-α, TXB_2, and PGE_2 were 0.8%, 3%, 3.8%, and 8.3%.

COMPARISON WITH RECTAL DIALYSIS
In each of two patients with active UC, the filter paper technique was compared with in vivo rectal dialysis[14] on four occasions over eight days. The filter paper was collected just before the start of a two hour rectal dialysis. For four
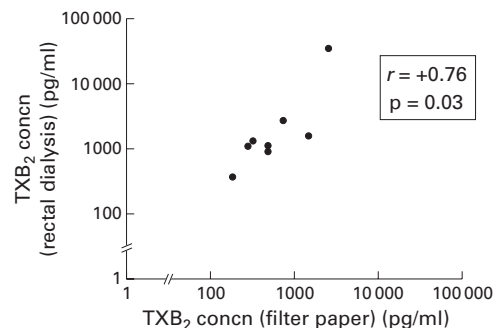

Figure 2 Correlation of measurements of rectal mucosal thromboxane (TXB_2) release using the filter paper method and rectal dialysis. Two patients with active ulcerative colitis underwent the filter paper technique and two hour rectal dialysis on eight occasions. The x axis shows the values obtained by the filter paper method and the y axis the values obtained by rectal dialysis.

serial measurements of mucosal TXB_2 release over eight days with the filter paper technique and rectal dialysis in each patient, there was a positive correlation between the results obtained by the two methods ($r=+0.76$, $p=0.03$) (fig 2).

Discussion
In this study, we have confirmed the validity of the filter paper method for studying release of IL-1β into rectal mucosal fluid and have extended its application to the measurement of TNF-α and the eicosanoids TXB_2 and PGE_2.

The results of the in vitro recovery experiments indicated that the use of the filter paper technique in vivo was feasible for IL-1β, TNF-α, TXB_2, and PGE_2. However, recovery of IFN-γ from the filter paper in vitro, regardless of its incubation time, was very low. The inability to elute this cytokine from the filter paper satisfactorily precluded reliable evaluation of rectal mucosal release in vivo using the filter paper method.

The paired in vivo validation experiments showed that there was considerable intraindividual variation for the filter paper method, particularly for those patients with active UC and the control group. The coefficient of variation (CV) of paired filter papers from the same individuals (intraindividual variation) was higher than the reported CV for rectal dialysis (9–37%[14 29]) and biopsy incubation techniques (6–20%[10 30 31]). However, the very large difference in the median values for IL-1β, TNF-α, TXB_2, and PGE_2 release between the active UC and the inactive UC or control groups makes lack of reproducibility of the filter paper method unimportant for most applications.

The greater weight of mucosal fluid collected on the filter paper of those patients with active UC accounts for a small proportion (<10%) of the differences in IL-1β, TNF-α, TXB$_2$, and PGE$_2$ levels between the patient groups. In practice, however, the advantage of the minor increase in accuracy provided by correcting for the volume of fluid collected on the filter paper is outweighed by the need to weigh it before and after mucosal apposition. Accordingly, apart from table 5, our results are presented uncorrected for the weight of fluid collected on the filter paper.

As the final validation procedure, we showed that the filter paper method gave results for TXB$_2$ which, although lower in magnitude, closely paralleled those obtained by in vivo rectal dialysis, the technique often considered to be the best method for measuring mediator levels in vivo.[14 15]

Excess mucosal production of IL-1β and eicosanoids in active UC, which has previously been demonstrated using other methods,[1 15] has been confirmed using this filter paper technique. Mucosal release of IL-1β and eicosanoids correlated well with disease activity, however measured.

Existing data on mucosal production of TNF-α in IBD are contentious. In UC, as in Crohn's disease (CD), there are conflicting reports of TNF-α mRNA levels in mucosal biopsies. Some studies show increased expression in both active[32] and inactive[33] disease, while others report no increase in either active[34] or inactive[35 36] UC: the apparent discrepancies between these studies may be explained by methodological differences. Mucosal biopsies from patients with active UC, cultured in vitro, released increased amounts of TNF-α compared with controls.[37] Luminal TNF-α concentrations, investigated using colonic perfusion studies in adults[38 39] and by assay of faeces in children,[40] were increased in active UC. A recent report suggested that TNF-α production by cultured lamina propria mononuclear cells in vitro can predict clinical relapse in CD.[41] Our study is the first to demonstrate increased luminal TNF-α concentrations using a simple, repeatable in vivo method.

For measurement of mucosal release of a mediator, cytokine or other substance by the filter paper technique, it must be shown in in vitro experiments to be adsorbed onto the filter paper, adequately recoverable from the filter paper, stable during incubation with mucosal fluid contaminated incubate, and present at a detectable concentration. These conditions were achieved for IL-1β, TNF-α, TXB$_2$, and PGE$_2$. The advantages of the filter paper over other methods are primarily its safety, ease, and speed; furthermore, it is free from the molecular weight restriction of rectal dialysis. With the filter paper method, the rectal mucosal fluid is directly sampled and thus, unlike rectal dialysis, prolonged insertion of the filter paper for equilibration is not required.

The filter paper method could be applied to any mediator for which the above criteria are met. It could also be used to study mediator release from involved mucosa in other diseases, including infectious colitis and collagenous colitis in which increased eicosanoid release has been shown.[15 42] In CD, the filter paper method is likely to be most useful when applied to overtly abnormal mucosa. However, in patients with CD and macroscopically normal rectal mucosa, abnormalities have been reported including, for example, in histology,[43 44] mucosal thromboxane production,[9] and vasoactive intestinal peptide content.[45] This suggests that using the filter paper method in patients with CD without rectal involvement may provide interesting results for some cytokines or mediators.

In summary, this simple, safe, speedy, and inexpensive filter paper method can be used in the outpatient clinic for assessment of rectal mucosal release of a range of cytokines and other mediators. The simplicity and low cost of the method also make it applicable in areas of the world where medical facilities are limited. The technique may prove helpful to investigate further the pathogenesis of UC. It also has the potential to shed new insights into the mode of action and to monitor the effects of current or novel treatments.

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