The rectal mucosa-associated microflora in patients with ulcerative colitis

M. GILLIAN HARTLEY, M. J. HUDSON,* E. T. SWARBRICK,† M. J. HILL, A. E. GENT,‡
M. D. HELLIER§ and R. H. GRACE||

Pathology Division, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, SP4 0JG, †New Cross Hospital, Wolverhampton, WV10 0QP, ‡The General Infirmary, Salisbury, SP2 7SX, §Princess Margaret Hospital, Swindon, SN1 4JU and ||The Royal Hospital, Wolverhampton, WV2 1BT

Summary. The rectal mucosa-associated flora (MAF) of patients with ulcerative colitis has been studied in 25 patients with newly diagnosed disease, 20 with relapse of existing disease, and 44 who were in remission. Patients with active disease were re-examined twice during treatment. The MAF was simpler and less dense than the microflora of faeces. Obligate anaerobes usually predominated in the MAF although the ratio of obligate anaerobes to facultative species was lower than that found in faeces. Viable counts of the total flora and of its constituent genera varied considerably between patients. Counts of the total flora, of obligate anaerobes (including bifidobacteria, eubacteria and clostridia), and facultative organisms and micro-aerobes (enterobacteria and lactobacilli) were reduced in patients with active disease compared with those with inactive disease; corresponding carriage rates were also lower. Counts and carriage rates increased during treatment and approached those found in quiescent disease. The alterations in the MAF were especially marked in patients experiencing their first attack of ulcerative colitis. The relationship between these alterations and the aetiology and pathogenesis of this disease remains unclear.

Introduction

The aetiology of ulcerative colitis (UC) is unknown,¹ but the characteristic and unexplained chronic inflammatory response strongly suggests that the pathological basis of this disease is chronically altered intestinal immune response.² This alteration includes chronic immunocyte infiltration and activation, local immune complex deposition, complement activation and auto-immune phenomena.^{3,4} The nature and sources of the provoking antigen or antigens involved in these processes are not known and may differ between individual patients. In the acute stages, UC shares many pathological features with infectious colitis but thereafter follows a characteristic chronic and relapsing course with chronic inflammatory lesions and epithelial destruction in the absence of infection by enteric pathogens.⁵ Chronically damaged intestinal mucosa would be expected to be more permeable to provoking antigens or metabolites, thus perpetuating the lesion.

The intestinal lesions of UC are confined to the mucosa of the colon and rectum and occur only in an intact and functional large bowel.⁵ Therefore, it is likely that the intestinal microflora plays an important

rôle in the pathogenesis of UC. The mechanisms for such an involvement are not known but could include mucosal invasion, by acting directly as provoking microbial antigens, by the production of antigens from the metabolism of endogenous or dietary substrates, or by the elaboration of toxins and metabolites toxic to the cells of the colorectal epithelium. The majority of investigations into the rôle played by intestinal microbes in UC have studied the faecal flora. Despite numerous investigations over the last half century, no aetiological agent has been identified nor have any consistent alterations in the balance or composition of this flora been found.^{5,6}

The mucosa of the colon and rectum of man harbours a distinct microflora that is closely associated with the mucosal surfaces and crypts and lies beneath the mucus blanket. This mucosa-associated flora (MAF) has been characterised in individuals with no significant bowel disease^{7,8} and in patients with idiopathic inflammatory bowel disease^{9–11} and neoplasia.⁹ The MAF occupies a unique anatomical position from whence to exert direct and local damage to the mucosa or, alternatively, to protect these tissues against invasion or damage by luminal microbes or their metabolites. This flora is likely to play an important rôle in the modulation of colonisation resistance in the large intestine. Analysis of the MAF should therefore yield important clues to the rôle of microbes in the aetiology and pathogenesis of UC.

This paper reports the characterisation of the rectal MAF of patients at first presentation with active UC. Previous studies have shown that there is considerable variation in the rectal MAF between individual patients.¹¹ To minimise this problem, we monitored the MAF of each patient twice during the treatment of their colitis; the results were compared with those from patients with relapse of UC and also patients with colitis in remission.

Materials and methods

Patients and samples

Samples were obtained from patients presenting for diagnosis or treatment at four District General Hospitals participating in a collaborative research programme into various aspects of diagnosis and management of idiopathic inflammatory bowel disease (The Inflammatory Bowel Disease Study Group). The protocols for the present study were approved by local Ethics Committees and informed consent was obtained from each patient.

Ulcerative colitis was diagnosed by conventional clinical criteria and investigations included routine sigmoidoscopy and rectal biopsy. Diagnosis was confirmed by typical histology of involved tissues and supported by findings from radiological and endoscopic examinations when these were indicated.

Rectal biopsy tissue samples were taken at routine sigmoidoscopy. No bowel preparation was used. Lubricant gel (K-Y Lubricating Jelly, Johnson and Johnson Ltd) was applied sparingly to the sigmoidoscope when necessary. Biopsy samples were taken from the rectosigmoid junction of 25 patients at first presentation with untreated, active UC. Samples of rectal faeces and venous blood were also taken for related studies. Further biopsy samples were obtained from each patient after treatment for 3 and 12 weeks with sulphasalazine and steroids as appropriate. A comparable set of samples was taken from 20 patients with relapse of previously diagnosed and treated ulcerative colitis. Where possible, a complete set of three biopsy samples, or at least two, was obtained from each of these patients. For comparison, single biopsy samples were taken at routine follow-up examination of 44 patients with quiescent UC; ethical considerations precluded taking a comparable series of samples from these patients. Disease severity at the time each sample was taken was determined from patient records of relevant clinical symptoms. Patients were classified as quiescent colitics if they had been free of overt clinical disease for at least 6 months.

Sample storage

Rectal biopsy samples were placed, unwashed, in peptone broth containing glycerol as a cryoprotectant and immediately snap frozen in liquid nitrogen or on

dry ice for subsequent storage at below -35°C .^{11,12} Serum and faeces were stored frozen without treatment.

Microbiological techniques

Biopsy samples were thawed rapidly within the oxygen-free atmosphere of a flexible-film anaerobic chamber and processed by established procedures.^{11,13,14} Media and solutions were steamed, or heated in a microwave oven to free them from dissolved oxygen immediately before transfer into the anaerobic chamber for immediate use or storage overnight. Heat-sensitive sterile solutions were prepared within the chamber by membrane filtration. All plastic ware (petri dishes, 7-ml sample bottles, micropipette tips, inoculating loops, stomacher bags, etc.) was stored in the chamber for at least 18 h (overnight) to remove adsorbed oxygen.

Each biopsy sample was washed thoroughly by vortex mixing in three changes of c. 5 ml of anaerobic diluent to remove loosely adherent faecal material and mucus. The diluent was made isotonic to minimise absorption of water by the tissue and contained peptone to maximise recovery of gut bacteria.^{15,16} The composition of the diluent was proteose-peptone (Oxoid) 1% in Ringer solution with cysteine HCl 0.5 g/L, dithiothreitol 0.3 g/L and resazurin 2 mg/L. The weighed biopsy sample was macerated in a known volume of diluent in a Colworth Stomacher. Triplicate 25- μl samples of a decimal dilution series prepared in brain-heart infusion broth were used to inoculate lightly dried agar media by a modification of the technique of Miles, Misra and Irwin¹⁷ as described previously.¹¹ A range of non-selective, selective and differential media were inoculated (table I).^{18,19,20} Portions of the dilution series were also treated with Tyndallised absolute ethanol (50% v/v final concentration) for 1 h at room temperature to select spore-forming clostridia and bacilli^{11,21} and triplicate 50- μl samples were used to inoculate egg-yolk agar.

Extremely oxygen-sensitive obligate anaerobes were sought and subcultured on M10-AC, a habitat-simulating medium prepared and incubated only within the anaerobic chamber. M10-AC was the pre-reduced and anaerobically sterilised (PRAS) medium 10 of Caldwell and Bryant²² including modifications suggested by Mitsuoka²³ and others.^{15,24} It was further modified by including proteose peptone, pectin and partially-purified hog-gastric mucin as complex nutrients and 3-phenylpropionic acid (hydrocinnamic acid)²⁵ in the volatile fatty acid supplement. The complete medium was re-formulated as two double-strength stock solutions for ease of preparation, storage and use within an anaerobic chamber. The double-strength broth base contained the following (/L): glucose, cellobiose, soluble potato starch, pectin (Sigma), and purified hog gastric mucin (type III, Sigma), 1 g of each; tryptone (Oxoid L42) and proteose-peptone (Oxoid L85), 2 g of each; neutralised

Table I. Bacteriological media*Media incubated anaerobically*

M10-AC; non-selective, pre-reduced, habitat-simulating (see *Materials and methods*).
 Modified Brain-Heart Infusion (BHI) blood agar; non-selective; contains volatile fatty acids to improve habitat-simulating properties.¹⁸
 BHI-kanamycin-vancomycin; selective for *Bacteroides* spp.¹⁹.
 BHI-rifampicin; selective for *Eubacterium* spp. and *C. ramosum*.
 Bifidobacterium Agar;¹⁴ with maltose, fructose and lactose.
 BHI-cefoxitin-cycloserine (Oxoid); selective for *C. difficile*.
 Willis and Hobbs egg-yolk medium for clostridia.
 Fastidious Anaerobe Agar (Lab M Ltd) with spectinomycin and polymyxin; selective for intestinal anaerobic spirochaetes.²⁰

Media incubated micro-aerobically

Columbia blood bi-layer agar (Oxoid); non-selective.
 Mitis-Salivarius Agar (Difco) for oral streptococci.
 Rogosa's SL medium (Oxoid) for lactobacilli.
 Skirrow's Campylobacter blood agar (Oxoid).

Media incubated aerobically

Columbia blood bi-layer agar (Oxoid); non-selective.
 MacConkey's agar (Oxoid) for enterobacteria and enterococci.
 Gentamicin-thallos acetate-carbonate agar for Group D streptococci.
 CIN agar (Oxoid); selective for *Yersinia* spp.

Media from reference¹¹ except where indicated.

liver digest (Oxoid L27) and yeast extract (Oxoid L21), 1 g of each; ascorbic acid 1 g; antifoam (Toray Silicone SH5535) 1 ml; haemin solution (0.5 g/L in 0.1 M NaOH) 20 ml; vitamin K₁ (1 g/L in ethanol) 2 ml; cresyl violet acetate (2 g/L) 1 ml; M10 Mineral Solutions I and II, 75 ml of each; trace minerals and trace vitamin solutions,²⁶ 20 ml of each; M10 volatile fatty acid supplement 6.2 ml and 3-phenylpropionic acid 2 mg. The double-strength agar was purified agar (Oxoid L28) 3% prepared in ACES-KOH (5 mM final concentration, pH 7.0) with resazurin 2 mg/L. The autoclaved, double-strength stock solutions were stored within the anaerobic chamber. For use, equal volumes of the broth and melted agar (50°C) were combined, cysteine HCl 0.5 g/L, dithiothreitol 0.3 g/L and sodium bicarbonate 2 g/L were added as filter-sterilised (0.2 µm) solutions, and the complete medium was poured as plates. The concentration of bicarbonate in the complete medium was selected to give a final pH of 7.2 ± 0.2 after equilibration in the anaerobic chamber atmosphere of CO₂ c. 10%.

Inoculated media, except M10-AC and *Clostridium difficile*-selective agar, were removed from the anaerobic chamber for incubation. Anaerobic plates were removed inside anaerobic jars containing active palladium catalyst. Inoculated media were incubated under the appropriate atmospheres at 37°C. *Yersinia* spp. were sought on selective medium incubated at 30°C. Plates incubated in air or in a micro-aerobic incubator (CO₂ 10%, O₂ 5% in N₂, >90% RH) were examined after 2 days, plates incubated anaerobically (CO₂ 10% in H₂, >90% RH) after 5–7 days. M10-AC plates were incubated within the anaerobic chamber atmosphere (CO₂ 10%, H₂ 10% in N₂, >90% RH) for

5–8 days. Intestinal spirochaetes were sought microscopically in the macerate and by anaerobic culture on plates of selective medium which were examined weekly for 3 weeks.

Bacterial colonies growing on each medium were counted and, where possible, differentiated at least to genus level. Some less easily differentiated isolates, including the anaerobic gram-positive cocci and the non-bifidobacterial, non-eubacterial, anaerobic gram-positive rods, were grouped phenotypically only. If similar isolates were recovered on both selective and non-selective media, the count from the latter was used in calculations. Aerobes, facultative organisms and micro-aerophiles were identified by conventional methods. Obligate anaerobes were identified by absence of, or very poor, aerobic or micro-aerobic growth, morphology in gram-stained smears, fatty acid end-product analysis by gas-liquid chromatography¹⁴ and various selected biochemical tests.²⁴ Representative isolates from each sample were stored frozen at –35°C in nutrient broth containing dimethylsulphoxide (DMSO) 10% as cryoprotectant for subsequent studies.

Statistical analyses

Bacterial counts are expressed as log₁₀ cfu/g biopsy tissue (wet weight)²⁷ and the data were analysed by appropriate statistical methods—analysis of variance (ANOVA), Student's *t* test, χ^2 -test, and linear regression. Differences of *p* < 0.05 were deemed to be significant. Two-sided tests were used unless otherwise indicated. Multiple linear regression was used to determine the effect on bacterial counts of clinical variables such as extent of disease, severity and treatment regimens. For each patient, the response variable (whether bacterial count or clinical variable) was assumed to be independent at each of the three time points.

To be able to include those samples that apparently lacked a particular bacterial group (i.e., none detected), we substituted into our calculations an arbitrary value of one half of the lower limit of detection, calculated for that sample.

Results

All but two rectal mucosal biopsy samples yielded a cultivable microflora; the range of logarithmic counts was from 8.6/g of tissue (4.0 × 10⁸/g) to below the limit of detection (c. 3.0/g, 1 × 10³/g) and the median count was 6.1 g (1.3 × 10⁶/g). Regression analysis on the total flora and major genera present indicated that there was no significant loss of bacterial viability as a result of frozen storage for up to 36 months (*p* = 0.3). Similarly, there was no evidence for any selective loss of individual genera during storage.

Bacterial genera present in the MAF were similar to those found in the normal faecal flora but differed

in several respects, notably in a lower ratio of obligate anaerobes to facultative organisms, reduced numbers of bacteria, reduced complexity and an altered balance of genera (data not shown). Obligate aerobes (bacilli and micrococci), yeasts and fungi were isolated only rarely.

Although specific enrichment procedures were not used, enteric bacterial pathogens were not isolated, with the exception of *C. difficile*; this was isolated from three patients (two with "new" and one with relapsed UC) during active treatment with sulphasalazine. Intestinal spirochaetes and campylobacters were neither observed nor cultured from any sample.

Table II summarises the rectal MAF of 25 patients with newly diagnosed, active UC before specific treatment was given. The flora was composed predominantly of obligate anaerobes, particularly anaerobic gram-positive cocci and bacteroides. The transformed data shows good correspondence of means and median values for the counts. The wide variation in counts of the flora between individual patients is evident from the estimates for the spread of the data. This variability was seen in each of the patient groups and limited the sensitivity of statistical comparisons between groups.

Table III shows the microbiological data for the same 25 "new" UC patients before and twice during treatment. The median values and ranges are omitted from this table for simplicity. Treatment with sulphasalazine or topical steroids, or both, was very effective and all patients improved clinically. The complexity of the flora of each patient appeared to increase after

treatment, as judged by an increase in the variety of colony types present. The total flora, counts and carriage rates of the majority of the genera increased during this treatment period, although only achieving statistical significance for the unidentified facultative and anaerobic gram-positive rods. The increase in total count, and counts of total obligate anaerobes, bacteroides, eubacteria and streptococci just failed to reach statistical significance ($0.1 > p > 0.05$). Multivariate analysis showed that these alterations were unrelated to the treatment used ($p > 0.5$). Counts and carriage rates of the MAF after 3 weeks were generally intermediate between the 0 and 12-week values.

Table IV summarises the rectal MAF from 44 patients with quiescent disease. The flora was composed predominantly of obligate anaerobes, particularly bifidobacteria, bacteroides and anaerobic gram-positive cocci. There were neither marked nor significant differences between the flora of these patients and that of the "new" patients studied at 12 weeks. However, counts or carriage rates, or both, of bifidobacteria, eubacteria, clostridia, propionibacteria, lactobacilli and enterobacteria were significantly higher than the values for untreated, active disease (table II) and, as such, follow the trend observed in these patients during treatment. In contrast, anaerobic gram-positive cocci, bacteroides, streptococci and staphylococci were isolated less frequently.

There was no indication that the composition of the flora was influenced by either the severity of the most recent attack, the anatomical extent of previous

Table II. Rectal MAF of 25 patients with untreated active UC

MAF	Carriage rate (%)	Bacterial count ($\log_{10}$ cfu/g wet weight)			
		Mean	SD	Median	Range*
Total flora	100	5.41	0.89	5.32	3.69-7.48
Obligate anaerobes	96	5.19	1.03	5.23	ND-7.45
<i>Gram-positive</i>					
Actinomyces	4	—	—	—	ND-4.38
Anaerobic cocci	88	4.76	1.08	4.59	ND-7.20
Bifidobacteria	60	4.03	1.06	3.89	ND-6.90
Clostridia	44	3.37	0.51	3.29	ND-4.79
Eubacteria	43	3.81	1.04	3.39	ND-6.51
Propionibacteria	4	—	—	—	ND-4.46
Other rods	52	3.65	0.78	3.39	ND-5.12
<i>Gram-negative</i>					
Bacteroides	92	4.51	0.90	4.53	ND-6.20
Fusobacteria	4	—	—	—	ND-5.60
Veillonella	4	—	—	—	ND-3.48
Facultative aerobic organisms	100	4.49	0.94	4.51	3.20-6.00
<i>Gram-positive</i>					
Bacilli	8	—	—	—	ND-3.56
Lactobacilli	24	3.45	0.84	3.18	ND-5.36
Other rods	20	3.29	0.63	3.18	ND-5.56
Streptococci	88	3.91	0.68	3.66	ND-5.38
Staphylococci	60	3.57	0.76	3.30	ND-4.95
<i>Gram-negative</i>					
Enterobacteria	36	3.50	0.84	3.22	ND-5.63
Anaerobe: aerobe ratio		mean 16:1 (median 6:1)			

ND, not detected; —, not calculated.

* Where carriage rate was <20%, only the range is given; ratios are rounded to the nearest integer.

Table III. The rectal MAF of patients with newly diagnosed UC before and during treatment

MAF	Mean bacterial count (log ₁₀ cfu/g) (SD) and percentage carriage rate*					
	untreated		3 weeks treatment		12 weeks treatment	
Total flora	5.41	(0.89) 100%	5.64	(0.99) 100%	5.96	(1.05) 100%
Obligate anaerobes	5.19	(1.03) 96%	5.48	(0.98) 100%	5.79	(1.20) 96%
<i>Gram-positive</i>						
Actinomyces	ND-4.38	4%	ND-5.38	8%	ND	
Anaerobic cocci	4.76	(1.08) 88%	4.68	(1.03) 84%	5.03	(1.10) 88%
Bifidobacteria	4.03	(1.06) 60%	4.15	(1.02) 64%	4.36	(1.27) 71%
Clostridia	3.37	(0.51) 44%	3.65	(0.88) 44%	3.70	(0.78) 38%
Eubacteria	3.81	(1.04) 43%	3.39	(0.83) 48%	4.24	(1.21) 67%
Propionibacteria	ND-4.46	4%	ND-4.42	12%	ND-6.92	17%
Other rods	3.65	(0.78) 52%	3.94	(1.03) 44%	4.77	(1.45)† 79%‡
<i>Gram-negative</i>						
Bacteroides	4.51	(0.90) 92%	4.75	(1.09) 84%	5.08	(1.10) 96%
Fusobacteria	ND-5.60	4%	ND-5.20	8%	ND-6.15	4%
Veillonella	ND-3.48	4%	ND-5.34	16%	ND-4.99	9%
Facultatives/Aerobes	4.49	(0.94) 100%	4.79	(0.93) 100%	4.96	(0.94) 96%
<i>Gram-positive</i>						
Bacilli	ND-3.56	8%	ND-3.88	12%	ND-3.32	9%
Lactobacilli	3.45	(0.84) 24%	3.74	(0.91) 48%	3.74	(0.95) 38%
Other rods	3.29	(0.63) 20%	3.66	(1.03) 32%	3.85	(0.96) 46%
Streptococci	3.91	(0.68) 88%	4.18	(0.94) 88%	4.41	(1.14) 75%
Staphylococci	3.57	(0.76) 60%	3.48	(0.59) 40%	3.55	(0.57) 50%
<i>Gram-negative</i>						
Enterobacteria	3.50	(0.84) 36%	3.65	(0.79) 40%	3.75	(0.86) 46%
Anaerobe: aerobe ratio§	16:1	(6:1)	20:1	(8:1)	244:1	(7:1)

ND, not detected.

* Where carriage rate was <20%, only the range is given.

† Significantly different ($p < 0.001$) from patients with "new" untreated UC.‡ Significantly different ($p < 0.05$) from patients with "new" untreated UC.

§ Mean (median) ratio rounded to integer.

Table IV. Rectal MAF of 44 patients with equivalent UC

MAF	Carriage rate (%)	Bacterial count (log ₁₀ cfu/g wet weight)			
		Mean	SD	Median	Range
Total flora	98	6.19*	0.83	6.22	ND-8.29
Obligate anaerobes	98	6.08*	0.85	6.07	ND-8.29
<i>Gram-positives</i>					
Actinomyces	9	—	—	—	ND-5.45
Anaerobic cocci	77	4.85	1.13	5.04	ND-6.95
Bifidobacteria	91†	5.19*	1.10	5.28	ND-7.32
Clostridia	61	4.02†	0.95	3.94	ND-6.15
Eubacteria	84*	4.59*	1.02	4.58	ND-6.83
Propionibacteria	27†	3.63	0.90	3.26	ND-6.53
Other rods	45	3.92	0.92	3.58	ND-6.04
<i>Gram-negatives</i>					
Bacteroides	84	4.95	1.19	4.87	ND-8.23
Fusobacteria	7	—	—	—	ND-4.11
Veillonella	7	—	—	—	ND-5.53
Facultatives/Aerobes	91	5.07†	0.85	5.16	ND-7.33
<i>Gram-positives</i>					
Bacilli	7	—	—	—	ND-4.32
Lactobacilli	57†	4.01†	0.90	3.88	ND-5.83
Other rods	27	3.49	0.68	3.23	ND-5.45
Streptococci	80	4.32	0.95	4.10	ND-7.18
Staphylococci	39	3.48	0.69	3.25	ND-5.77
<i>Gram-negatives</i>					
Enterobacteria	59	4.01†	0.99	3.83	ND-6.77
Anaerobe: aerobe ratio		mean 137:1 (median 9:1)			

ND, not detected.

* Significantly greater ($p < 0.001$) than patients with "new" untreated UC.† Significantly greater ($p < 0.05$) than patients with "new" untreated UC.

Table V. The rectal MAF of patients with relapsed UC, studied before and during treatment

MAF	Mean bacterial count (log ₁₀ cfu/g) (SD) and percentage carriage rate*								
	untreated			3 weeks treatment			12 weeks treatment		
Total flora	5.95	(1.04)	100%	5.99	(1.02)	94%	5.84	(1.17)	100%
Obligate anaerobes	5.85	(1.10)	100%	5.87	(1.22)	94%	5.76	(1.19)	88%
Gram-positive									
Actinomyces	ND-5.90		15%	ND			ND-5.20		5%
Anaerobic cocci	4.88	(1.29)	85%	5.25	(1.39)	82%	5.10	(1.13)	94%
Bifidobacteria	4.78	(1.23)	80%	4.56	(1.22)	77%	4.38	(0.80)	88%
Clostridia	3.67	(0.72)	60%	3.71	(0.78)	59%	3.72	(0.87)	31%
Eubacteria	4.14	(0.98)	75%	4.30	(1.31)	65%	4.47	(0.86)	81%
Propionibacteria	3.71	(1.17)	25%	3.58	(0.79)	24%	3.46	(0.55)	25%
Other rods	4.13	(1.33)	50%	4.16	(1.23)	47%	4.34	(1.13)	56%
Gram-negative									
Bacteroides	4.92	(1.33)	80%	5.04	(1.39)	82%	5.14	(1.19)	75%
Fusobacteria	ND-5.15		5%	ND			ND		
Veillonella	ND-5.18		5%	ND-5.63		6%	ND-5.94		13%
Facultative/Aerobes	4.87	(0.90)	95%	5.02	(0.95)	88%	4.80	(0.99)	88%
Gram-positive									
Bacilli	3.01	(0.84)	25%	ND-3.11		6%	ND		
Lactobacilli	4.01	(1.18)	50%	3.82	(0.66)	59%	4.30	(1.00)	63%
Other rods	3.53	(0.81)	30%	3.68	(0.78)	29%	3.74	(0.79)	31%
Streptococci	4.24	(0.88)	85%	4.51	(0.99)	82%	4.28	(0.78)	81%
Staphylococci	3.49	(0.49)	65%	3.56	(0.73)	29%	3.73	(0.64)	44%
Gram-negative									
Enterobacteria	3.60	(0.96)	40%	3.98	(1.05)	53%	3.58	(0.65)	44%
Anaerobe:aerobe ratio§	51:1	(6:1)		43:1	(8:1)		23:1	(14:1)	

See footnote to table III.

disease or the time in remission (up to 13 years). Sulphasalazine was the long term treatment used by 21 (48%) of the 44 patients with quiescent disease and this appeared to have no selective effect on the MAF.

Table V shows the bacterial counts for the comparative group of 16 patients with relapse of existing colitis examined before and twice during treatment. The trend in alterations to the MAF in these relapse patients, although less pronounced and not achieving statistical significance, was similar to that observed in the equivalent samples from the patients with newly diagnosed UC. Counts of both obligate anaerobes and facultative organisms were reduced, particularly the bifidobacteria, clostridia, eubacteria and enterobacteria. Anaerobic gram-positive cocci, streptococci and staphylococci were isolated more frequently.

Considered as a group, the relapse patients appeared to have more severe disease than was seen in the newly diagnosed patients. Treatment with sulphasalazine or steroids, or both, was again successful in achieving symptomatic improvement and remission of colitis. Although eight (50%) of the 16 patients presenting with relapsed UC were taking sulphasalazine as a maintenance treatment at the time of their relapse, their colitis was no less severe and this treatment did not appear to affect the nature of the MAF.

The composition of the MAF for different patient groups was also determined with respect to the predominant genus or phenotypic group found in each sample, and thus was independent of the density of the flora. Anaerobic gram-positive cocci and bacteroides were the predominant organisms in active disease, whether newly diagnosed or relapsed UC, whereas bifidobacteria predominated in quiescent

disease (table VI). Genera other than those indicated, including eubacteria, clostridia, lactobacilli and unidentified anaerobic gram-positive rods ("other genera") were predominant in relatively few samples; there was no obvious relationship between any component of this group and disease activity.

We found no obvious relationship between altered bowel function (stool consistency, bowel frequency and presence of frank blood in stool) and either qualitative or quantitative changes in the MAF or the predominant flora in our patients with UC, regardless of disease activity or treatment.

Discussion

Most investigations into the rôle of the intestinal flora in the aetiology and pathogenesis of UC have concentrated on the faecal flora.^{5,6,28-34} The study of the pathogenic or protective effects of the MAF of the colorectum might be of greater relevance due to its unique anatomical position. However, the composition and control of this flora are poorly understood.

We chose to study the MAF of rectal biopsy tissue taken at routine sigmoidoscopy because no bowel preparation whatsoever is required for this procedure. Alternative sources of tissue would be colonoscopic biopsy samples or resected tissue taken during abdominal surgery. The use of purgatives, laxatives, antimicrobial agents and enemas before such procedures would be expected to affect the flora, although one study³⁵ found the MAF to be less altered by such procedures than the luminal flora.

The method adopted for washing the tissue sample free of loosely adherent mucus and faecal material is

Table VI. The predominant flora of the MAF: the percentage of samples in each patient group in which a given major bacterial group predominated

Predominant§ bacterial group	Percentage of samples from						
	new UC at (weeks)			quiescent UC (n = 44)	relapsed UC at (weeks)		
	0 (n = 25)	3 (n = 25)	12 (n = 24)		0 (n = 20)	3 (n = 17)	12 (n = 16)
Anaerobic cocci	40%	29%	17%	23%	35%	24%	47%
Bacteroides	36%	17%	38%	20%	40%	41%	38%
Bifidobacteria	0%	8%	17%*	39%†	20%†	12%	13%
Streptococci	16%	21%	0%*	5%	10%	12%	0%
Other genera	12%	33%	42%*	18%	20%	24%	13%

* Significantly different ($p < 0.05$) from patients with "new" untreated UC.† Significantly different ($p < 0.001$) from patients with "new" untreated UC.

§ In some samples there was no single predominant bacterial group, hence the values for each patient group do not total 100%.

clearly an important consideration in determining the nature of the flora. We found logarithmic counts of < 3.0 – $8.3/g$ wet weight in samples from the rectosigmoid. Although gentler washing techniques have been used in other studies^{7,8} this did not result in large differences in counts. Logarithmic counts of 5.5 – $9.9/g$ were reported from unwashed colonoscopic biopsy specimens,⁹ depending on the biopsy site, with 6.7 – $8.4/g$ in samples from the sigmoid colon. Lightly-washed tissue samples removed surgically from four sudden-death patients⁸ yielded 6.2 – $8.4/g$ depending on site, with 6.5 – $8.4/g$ in samples from the sigmoid colon. In agreement with these and other studies,^{11,35} we found considerable variation in the colorectal MAF between individuals; similar variation is found in the faecal microflora.^{36,37} Direct comparisons between studies are problematic because of differences in patients, treatments, types of samples, anaerobic technique and media used.

Our results indicate that the bacterial flora of the colorectal mucosal surface is not appreciably altered by bowel dysfunction such as bleeding and diarrhoea in contrast to the faecal flora of patients with inflammatory bowel disease.^{11,33,34,38} This adds support to the hypothesis that alterations to the MAF in patients with UC might be of greater significance to the pathogenesis of this disease than changes in the faecal flora.

It is interesting to note that we saw neither overgrowth by, nor loss of, any individual component of the flora in active disease or during treatment, although in active disease the numbers and complexity of the flora were reduced and the predominant genera were altered. With the exception of *C. difficile* found in three patients during treatment, enteric pathogens were not isolated. Patients with inflammatory bowel disease (IBD) given antimicrobial treatment, including sulphasalazine, suffer from loss of colonisation resistance as evidenced by detection of *C. difficile* or its toxin.^{39–41} However, the consensus view appears to be that this is no different from comparable non-IBD patients exposed to similar treatments; in patients with IBD the infection is normally self-limiting and of

doubtful pathological significance. Our three patients followed an unremarkable clinical course and appeared unaffected by mucosal colonisation with *C. difficile*. Spirochaetes or campylobacters were neither observed nor cultured from our patients, in contrast to the characteristic spirochaetal overgrowth of the rectal MAF in the proctitis associated with gay-bowel syndrome.²⁰ We did not observe the unusual large spiral or helical bacteria described by Croucher *et al.*;⁸ however, we did not examine the biopsy specimens microscopically before the maceration step which might have physically disrupted these bacteria.

Although it was evident that individual patients tended to carry phenotypically similar isolates through sequential samples, we found specific and significant changes in the MAF of patients suffering their first attack of colitis compared to the flora in quiescent UC. The effect of treatment was particularly interesting: the proportion of samples in which anaerobic gram-positive cocci and facultative streptococci were the predominant flora decreased during treatment, whereas the proportion with bifidobacteria predominant increased and approached the frequency found in the quiescent disease group. It is possible that speciation may reveal more subtle changes within this flora with respect to disease activity. The MAF of relapsed UC patients was also altered but not to the same degree as that seen in patients at first presentation; this is not inconsistent with chronic inflammatory bowel disease causing a persistent alteration in this flora.

Enterobacteria, predominantly *Escherichia coli*, have been linked to colitis in various studies.^{28,42–45} They were isolated infrequently from the MAF in our patients and always in low numbers. There were significantly fewer *E. coli* in untreated patients with active disease. This study shows that other bacterial groups, such as the bacteroides and aerobic and anaerobic gram-positive cocci were isolated far more commonly from patients with active colitis, whether at first onset or upon relapse; these may be more relevant candidates for future studies into the rôle of micro-organisms in UC.

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