THE MICROBIAL FLORA OF THE RECTAL MUCOSA AND FAECES OF PATIENTS WITH CROHN'S DISEASE BEFORE AND DURING ANTIMICROBIAL CHEMOTHERAPY

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SUMMARY. The faecal flora and mucosa-associated flora (MAF) of rectal biopsy material from 12 patients with active Crohn's disease were studied before and during treatment with a combination of metronidazole and cotrimoxazole given orally for at least 2 weeks. The total faecal flora was greater than the MAF although the proportions of bacterial groups were similar. The changes observed during treatment were: obligate anaerobes such as *Bacteroides* spp. decreased in faeces ($p < 0.05$) and in MAF ($p < 0.02$); the total count of facultative bacteria increased in the faeces ($p < 0.002$) but not in the MAF. *Streptococci*, predominantly enterococci, increased significantly in faeces ($p < 0.001$) and in MAF ($p < 0.02$) such that they became predominant components of these floras. Facultative gram-negative bacilli were unaltered in faeces but significantly reduced in the MAF ($p < 0.05$). Sporing clostridia were infrequently isolated from the MAF but were significantly reduced in the faeces ($p < 0.01$).

During the treatment period, eight of the 12 patients showed clinical improvement, but this could not be related to the site or extent of disease or to specific changes in faecal flora or MAF. This combination of antibacterial agents causes profound alterations to the bacterial flora of mucosa and faeces and these changes may help to define the role of bacteria in the pathogenesis of Crohn's disease.

INTRODUCTION

Recent studies have confirmed the presence of a relatively simple, cultivable microflora attached to or intimately associated with animal or diseased human colorectal mucosa (Peach *et al.*, 1978; Hartley, Neumann and Richmond, 1979; Marks *et al.*, 1979; Edmiston, Avant and Wilson, 1982; Croucher *et al.*, 1983). Such an association suggests that this mucosa-associated flora (MAF) might have a greater potential for involvement in intestinal disease than the flora of the lumen, and may be particularly important in the study of the idiopathic inflammatory bowel diseases. Electronmicroscopy studies have shown intramural bacteria to be present in

unaffected colorectal submucosa of some patients with Crohn's disease (Aluwihare, 1971), and others have shown that patients with this disease have increased serum antibody levels to various intestinal bacteria (Brown and Lee, 1974; Tabaqchali, O'Donoghue and Bettelheim, 1978; Helphingstine *et al.*, 1979; Persson and Danielsson, 1979; Wensinck and van de Merwe, 1981; Gump *et al.*, 1981).

Recent reports of the usefulness of antimicrobial agents in the treatment of some patients with Crohn's disease (Moss, Carbone and Kressel, 1978) and the efficacy of metronidazole in particular (Ursing and Kamme, 1975; Blichfeldt *et al.*, 1978; Kasper, Sommer and Kuhn, 1979; Bernstein *et al.*, 1980; Brandt *et al.*, 1982; Ursing *et al.*, 1982) further support a role for the involvement of the intestinal bacterial flora in the aetiology, exacerbation or maintenance of Crohn's disease.

The purpose of the present study was to examine the MAF and faecal flora of a small group of patients with active Crohn's disease before and during treatment with a short combined course of metronidazole and cotrimoxazole.

MATERIAL AND METHODS

Patients and sample collection. Twelve patients gave informed consent and were entered into the study. All were considered to have active Crohn's disease as judged by the diagnostic criteria of Lennard-Jones, Ritchie and Zohrab (1976). The relevant clinical details are given in table I. Patients 6, 7, 11 and 12 ceased taking sulphasalazine at the start of the study but no patient had taken other antimicrobial agents for 2 weeks before entering the study. Only two patients continued to take prednisolone (15 mg/day).

Patients were treated for 10–14 days with a combination of metronidazole (Flagyl) 200 mg thrice daily and cotrimoxazole (Septrin; sulphamethoxazole 800 mg and trimethoprim 160 mg) twice daily. A rectal biopsy and a sample of rectal faeces were taken at routine sigmoidoscopy at the start of the study and at its termination. Faecal samples were not obtained from one patient. The samples were immediately placed in weighed 7-ml screw-capped bottles containing 4.5 ml of a cryoprotective glycerol broth, frozen and stored at below -25°C . This procedure permits the storage of faeces and mucosa for at last 6 months with minimal loss of bacteria (Crowther, 1971; Marks *et al.*, 1979). All samples were analysed within 4 months of collection.

Bacteriology. Quantitative bacterial analysis was performed within a flexible film anaerobic chamber maintained at room temperature and filled with an atmosphere of 10% hydrogen in

TABLE I

Clinical features of patients with Crohn's disease given cotrimoxazole and metronidazole

Subject no.	Age	Sex	Duration of disease (years)	Localisation of disease	Other treatment	Clinical outcome
1	24	M	2	Duodenum, jejunum, anus	Nil	Improved
2	25	M	11	Terminal ileum, rectum , anus	Codeine	Improved
3	36	M	4	Descending colon, rectum	Nil	Improved
4	30	F	2	Colon, rectum , anus	Prednisolone	Improved
5	23	M	2	Terminal ileum, colon, rectum	Prednisolone	Improved
6	52	F	5	Terminal ileum	Nil	No change
7	24	M	1	Terminal ileum, colon, rectum	Nil	Improved
8	23	F	5	Terminal ileum, rectum , anus	Codeine	Improved
9	31	F	9	Small bowel, asc. colon, rectum , anus	Nil	No change
10	50	F	18	Terminal ileum	Nil	No change
11	23	F	2	Terminal ileum	Nil	Improved
12	20	F	1	Terminal ileum, rectum	Nil	No change

nitrogen which was kept oxygen-free by circulation over palladium-coated alumina catalyst (Drasar, 1974). Cryoprotective sample broths were transferred into the chamber and rapidly thawed at 37°C on an electrically heated aluminium block before the samples were processed.

Biopsy material was carefully removed and, in a vortex mixer, washed free from adherent mucus and faeces in three changes of reduced diluent broth—Brain Heart Infusion Broth (Oxoid) containing Yeast Extract (Oxoid) 5 g/L and the reducing agents cysteine HCl 0.5 g/L and sodium formaldehyde sulfoxylate 0.3 g/L diluted to 10% in full-strength Ringer's lactate solution (Oxoid), containing the reducing agents and resazurin 1 mg/L, pH 7.0. Reduced broth and diluent were steamed free from dissolved oxygen and cooled within the chamber before use. The use of isotonic diluent prevented swelling of the biopsy tissue and consequent changes in sample weight during washing. Each washed sample was lightly dried on sterile filter paper (Whatman no. 1), weighed on an electronic balance (Sartorius 1212MP) and macerated for 5 min in reduced diluent broth in a Colworth Stomacher 80 (Seward Laboratories Ltd, Blackfriar's Road, London SE1 9UG). A decimal-dilution series in reduced broth was prepared from the macerate. Rapidly thawed faecal samples in cryoprotective broth were vortexed and reweighed and decimal-dilution series of these were prepared in reduced broth.

Triplicate 25- μ l portions of each dilution were used to inoculate a range of selective and non-selective media by a modification of the method of Miles, Misra and Irwin (1938) and a repetitive micro-dispenser (Oxford Micro-Doser, BCL). The selective and non-selective media were similar to those described by Borriello, Hudson and Hill (1978) with the following exceptions: veillonellae were sought on the medium of Rogosa (1956) but with vancomycin (7.5 mg/L) replacing the streptomycin. Rifampicin agar (Sutter, Citron and Finegold, 1980) was prepared by the addition of rifampicin 50 mg/L (as a solution in 50% dimethyl sulfoxide) to supplemented Brain Heart Infusion-blood agar. Enterococci were sought on the medium of Donnelly and Hartman (1978) and oral streptococci on Mitis-Salivarius Agar (Difco). Freshly prepared agar plates were stored for at least 18 h in anaerobic conditions before use. All media intended for the isolation of obligate anaerobes were supplemented with haemin (5 mg/L), menadione or vitamin K₁ (1 mg/L) and reducing agents. Clostridial spores were sought by treatment of selected dilutions with sterile ethanol (at a final concentration of 50% v/v) for 1 h at room temperature (Koransky, Allen and Dowell, 1978) before triplicate 50- μ l portions were dispensed on to lactose egg-yolk agar medium (Willis and Hobbs, 1959).

Aerobically incubated media were examined after at least 18 h at 36°C in air. Media for microaerophilic growth were incubated in 10% CO₂ in air and examined after 48 h, except Rogosa's lactobacillus agar which was incubated in 10% air in CO₂.

Plates for anaerobic incubation were removed from the chamber in sealed milking-machine pails (Drasar, 1974) or pressure cookers (Gargan and Phillips, 1978) converted for use as anaerobic incubation vessels and containing room-temperature deoxygenating catalyst. The gas in each vessel was replaced with 20% CO₂ in hydrogen. Anaerobic plates were examined after incubation for at least 72 h.

Colonies on various media were noted, counted and representatives subcultured and gram-stained by the modification of Preston and Morrell (1962). Aerobic and microaerophilic isolates were identified by conventional criteria (Cowan, 1974). Anaerobic isolates were identified at least to genus level by morphology, biochemical properties, fermentation end-products and growth on selective or differential media (Holdeman, Cato and Moore, 1977; Dowell *et al.*, 1977; Sutter *et al.*, 1980). Volatile fatty acid end products were analysed by gas-liquid chromatography (GLC) and the 'head-space' technique described by Drasar (1974); non-volatile fatty acids were methylated (Holdeman *et al.*, 1977) and examined by conventional GLC procedures (Drasar, 1974).

Statistical methods. Logarithmic counts of bacteria (log₁₀ cfu/g wet weight) were compared (Best, 1970) by the paired Student's *t*-test and by linear regression analysis.

RESULTS

Tables II and III summarise the main groups of bacteria isolated from mucosa and faecal samples respectively before and during antimicrobial chemotherapy with

TABLE II
Faecal flora in patients with Crohn's disease given cotrimoxazole and metronidazole

Bacterial group	Bacterial count (mean log ₁₀ cfu/g wet weight $\pm$ SD)		Significance (p)
	before therapy	during therapy	
Total flora	8.61 $\pm$ 1.59	9.03 $\pm$ 0.69	NS
Total obligate anaerobes	8.56 $\pm$ 1.69	7.33 $\pm$ 2.18	NS
<i>B. fragilis</i> group	7.87 $\pm$ 2.70	5.72 $\pm$ 2.20	<0.05
Veillonellae	4.33 $\pm$ 1.89	<2.5	<0.01
Sporing clostridia	4.21 $\pm$ 0.98	3.14 $\pm$ 1.00	<0.01
Total facultative anaerobes	6.97 $\pm$ 1.46	8.86 $\pm$ 0.78	<0.002
Enterobacteria	6.35 $\pm$ 2.04	6.16 $\pm$ 2.43	NS
Enterococci	6.04 $\pm$ 1.04	8.81 $\pm$ 0.87	<0.001
Lactobacilli	4.91 $\pm$ 1.77	6.31 $\pm$ 1.61	NS

NS = not significant

TABLE III
Rectal mucosa-associated flora (MAF) in patients with Crohn's disease given cotrimoxazole and metronidazole

Bacterial group	Bacterial count (mean log ₁₀ cfu/g wet weight $\pm$ SD)		Significance (p)
	before therapy	during therapy	
Total flora	6.39 $\pm$ 1.18	5.88 $\pm$ 0.86	NS
Total obligate anaerobes	6.36 $\pm$ 1.28	5.17 $\pm$ 1.50	<0.05
<i>B. fragilis</i> group	5.64 $\pm$ 1.19	4.41 $\pm$ 1.62	<0.02
Veillonellae	3.34 $\pm$ 0.61	<3.0	<0.05
Sporing clostridia	3.09 $\pm$ 0.19	3.12 $\pm$ 0.28	NS
Total facultative anaerobes	5.27 $\pm$ 0.88	5.54 $\pm$ 0.63	NS
Enterobacteria	5.00 $\pm$ 1.12	4.10 $\pm$ 1.16	<0.05
Enterococci	4.43 $\pm$ 1.00	5.40 $\pm$ 0.55	<0.02
Lactobacilli	3.20 $\pm$ 0.48	3.25 $\pm$ 0.63	NS

NS = not significant

cotrimoxazole and metronidazole. Recognised intestinal pathogens were not isolated from any specimen although specific enrichment culture for *Yersinia* spp., salmonellae and shigellae were not used. Faecal samples were not assayed for *Clostridium difficile* cytotoxin but *C. difficile* was not among the clostridia isolated. Obligate aerobes were not isolated from any patient and therefore the non-selective anaerobic count represents the total viable bacterial count. Yeasts were isolated from one patient in both pre-treatment samples.

The total viable counts in faeces were significantly higher than those from the rectal mucosa ($p < 0.001$) before and during treatment. No significant difference was found for the ratio of obligate to facultative anaerobes in the MAF and faeces (average of 12 fold and 39 fold respectively). When compared with the pretreatment results, the antimicrobial chemotherapy caused no significant change to the total cultivable flora or total obligate anaerobes of the faeces (table II) although total facultative bacteria

FIG. 1.—Faecal flora before and during antimicrobial drug treatment: (a) total cultivable flora, (b) total obligate anaerobes and (c) total facultative anaerobes.

increased significantly ($p < 0.002$, fig. 1). Individual groups of obligate anaerobes were significantly reduced in number (table II, fig. 2), *Bacteroides* spp. by 2 logarithms ($p < 0.05$) and veillonellae becoming undetectable ($p < 0.01$). Lactobacilli and bifidobacteria were variably but not significantly altered by the treatment regimen. Enterobacteriaceae, mainly *Escherichia coli*, were unaltered by the treatment regimen

FIG. 2.—Faecal flora—selected bacterial groups before and during antimicrobial drug treatment: (a) *Bacteroides* spp., (b) enterobacteria and (c) total streptococci.

(table II, fig. 2). Faecal streptococci increased significantly ($p < 0.001$) and became the dominant component of the faecal flora.

The treatment had no effect on the total cultivable flora of the rectal mucosa (table III, fig. 3) nor on the total count of facultative bacteria. Obligate anaerobes were reduced ($p < 0.05$) and *Bacteroides* spp. significantly so (fig. 4, $p < 0.02$). Veillonellae and clostridia were not found in some patients, although in the patients who harboured them veillonellae fell below the limit of detection during antibiotic treatment ($p < 0.05$). Streptococci increased ($p < 0.02$) to become the predominant component of the MAF and Enterobacteriaceae were significantly reduced in number ($p < 0.05$). In faeces and in MAF, the treatment regimen significantly reduced the ratio of obligate to facultative bacteria to less than unity ($p < 0.001$ and $p < 0.02$ respectively).

Eight of the 12 patients showed clinical improvement during the treatment period and this appeared unrelated to the site, extent or duration of the disease (table I), and could not be ascribed to particular changes in the faecal or mucosa-associated florae.

DISCUSSION

There has long been interest in the possible role of the intestinal microflora in Crohn's disease, either as an aetiological agent or as a factor exacerbating the effects of some other agent. Evidence strongly suggests that an immunological mechanism is involved in pathogenesis because immunoglobulin-bearing immunocytes are increased in diseased ileal and colonic tissue (Brandtzaeg and Baklien, 1976; Meijer, Bosman and Lindeman, 1979). Elevated levels of antibody have been demonstrated in the serum of patients with Crohn's disease directed against a wide range of normal intestinal bacteria including *E. coli* (Tabaqchali *et al* 1978; Persson and Danielsson, 1979), *Bacteroides* spp. (Brown and Lee, 1974; Helphingstine *et al.*, 1979; Persson and

FIG. 3.—Rectal mucosa-associated flora (MAF) before and during antimicrobial drug treatment: (a) total cultivable flora, (b) total obligate anaerobes and (c) total facultative anaerobes.

FIG. 4.—Rectal mucosa-associated flora (MAF)—selected bacterial groups before and during antimicrobial drug treatment: (a) *Bacteroides* spp., (b) enterobacteria and (c) total streptococci.

Danielsson, 1979; Gump *et al.*, 1981) and *Eubacterium* spp. and *Peptostreptococcus* spp. (Matthews *et al.*, 1980; Wensinck and van de Merwe, 1981). The presence of these circulating antibodies is consistent with infection or antigenic stimulation secondary to the breakdown of the mucosal barrier, with exacerbation of local and distant inflammation by the consequences of antigen-antibody interaction such as complement activation, lysozyme release and production of other chemical mediators.

There have been few previous studies on the faecal or mucosa-associated flora in Crohn's disease. Gorbach *et al.* (1968) noted a significant increase in coliforms, and concomitant increase in total aerobic organisms, in the faeces of patients with Crohn's disease compared with normal persons, and a significantly reduced number of anaerobic lactobacilli. Vince *et al.* (1972) reported a reduced number of aerobic coliform bacteria in the faeces of patients with Crohn's disease compared with normal persons, but did not comment on other bacteria. West *et al.* (1974) were unable to detect any differences between the faecal flora of patients with Crohn's colitis and a control group. Significant increases in the number of coliforms and aerobic lactobacilli were noted in the colonic contents of patients with Crohn's disease compared with a control surgical group of patients with non-inflammatory bowel disease studied by Keighley *et al.* (1978). They also noted that there was no difference in flora attributable to disease distribution in the patients with Crohn's disease. In contrast to their study, ours did not show coliform bacteria to be such a high proportion of the total faecal flora. Wensinck *et al.* (1981), elaborating on an earlier report (Wensinck, 1975), studied the predominant flora of patients with Crohn's disease and found that in those with ileal disease or mild Crohn's colitis the total cultivable flora was increased due to an increase in the populations of gram-negative anaerobic rods and gram-positive anaerobic coccoid-rods. In patients with severe Crohn's colitis, however, a marked diminution in total cultivable flora was observed. These workers did not comment on

other bacterial groups such as coliforms, streptococci or lactobacilli. Ruseler-van Embden and Both-Patoir (1983) have recently confirmed the observation that the obligately anaerobic gram-negative rods are increased as a proportion of the total cultivable faecal flora of patients with Crohn's disease and that this was largely attributable to an increase in numbers of *B. vulgatus* and others of the *B. fragilis* group.

The MAF in Crohn's disease has been studied by Peach *et al.* (1978) and compared with the MAF in a control group. They were unable to find a statistical difference between diseased and normal tissue in the total aerobic or anaerobic flora, in selected bacterial groups or in the ratio of anaerobes to aerobes, but did note that enterobacteria were more commonly associated with Crohn's tissue. Comparison with luminal contents was not made.

The study reported here compares the faecal flora with the flora intimately associated with the colorectal mucosa. Although the MAF is numerically less than the faecal flora by a factor of at least 100, it contains the same major genera. The total cultivable faecal flora of these patients was lower than that found for healthy persons studied by similar methods by a factor of at least 10. This may be attributable to the effects of severe diarrhoea in this series of patients, 60% of whom had colorectal involvement and all of whom had acute Crohn's disease as judged by clinical criteria. Although analysis based on the presence of severe diarrhoea was not made, it seems likely that the reduced faecal flora was similar to that observed by Wensinck *et al.* (1981) in the patients with colorectal disease in their series.

There has recently been considerable interest in the use of antibacterial agents in the management of Crohn's disease. Broad-spectrum agents have not been subjected to controlled investigation, but Moss *et al.* (1978) found that various such drugs, given over extended periods, resulted in marked clinical and radiological improvement in 20 of 25 patients. Metronidazole, a drug with high activity against most obligate anaerobes was introduced into the treatment of Crohn's disease by Ursing and Kamme (1975). The first controlled trial (Blichfeldt *et al.*, 1978) in a small group of 22 patients showed benefit only in patients with colorectal involvement. A more striking response in patients with chronic perineal disease has been claimed by Bernstein *et al.* (1980), who performed an uncontrolled prospective study of 21 patients, continuing treatment with metronidazole for up to 21 months when tolerated. Ten of the patients showed complete and five showed advanced perineal healing. In a follow-up report to this last study (Brandt *et al.*, 1982) and in another uncontrolled trial with metronidazole (Kasper *et al.*, 1979), relapse of disease has been found to be the normal result of withdrawal of treatment but remission may be reinstituted with reintroduction of metronidazole therapy. The co-operative Crohn's disease study in Sweden (Rosen *et al.*, 1982; Ursing *et al.*, 1982) showed that in double-blind crossover conditions metronidazole is at least as effective as sulphasalazine in the treatment of Crohn's disease, whatever the site of disease.

Krook *et al.* (1981a) have shown that metronidazole markedly decreased the number of *Bacteroides* spp. in faeces of patients with Crohn's disease. Such an effect was not found in healthy controls (Krook, 1981), but correlated with clinical response in patients with Crohn's disease (Krook, Jarnerot and Danielsson, 1981b). The streptococcal component of the faecal flora rose significantly in patients and controls but did not result in changes in the total aerobic or anaerobic flora.

Using a combination of metronidazole and cotrimoxazole we have corroborated

the analyses of Krook and colleagues and have shown furthermore that the MAF is similarly altered with the additional observations that coliforms and anaerobic bacteria other than bacteroides were suppressed. These additional changes may be in part attributable to the inclusion of cotrimoxazole in our treatment regime. It is pertinent to note that serological studies have shown that an elevated immune response is demonstrable against *E. coli* and *Bacteroides* spp. in patients with Crohn's disease (Brown and Lee, 1974; Tabaqchali *et al.*, 1978; Helphingstine *et al.*, 1979; Persson and Danielsson, 1979).

We conclude that it is important to obtain more evidence of the clinical usefulness of antibacterial drugs in Crohn's disease, in particular to establish whether the already proven benefit of metronidazole is enhanced by combining it with wider-spectrum agents and to establish whether there is a correlation between demonstrable bacterial changes and therapeutic response. Such studies would greatly increase our understanding of the role played by bacteria in the pathogenesis of Crohn's disease. A multicentre trial with these aims is now in progress.

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