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The faecal flora of patients with Crohn's disease

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SUMMARY

The faecal flora of patients with Crohn's disease was compared with that of healthy subjects. In patients with terminal ileitis, numbers of anaerobic gram-negative and coccoid rods (species of *Eubacterium* and *Peptostreptococcus*) were higher than in the controls whereas anaerobic gram-positive rods and cocci and aerobes occurred in normal numbers. The composition of the flora was neither influenced by duration of the disease nor by ileocaecal resection. In healthy subjects and patients, a chemically defined diet induced only slight changes in the flora. Thus, the flora in terminal ileitis although stable was permanently abnormal.

In patients with Crohn's colitis, abnormally low numbers of anaerobes were found in patients with severe, bloody diarrhoea while aerobic counts were normal. The flora in patients with mild colitis was similar to that in terminal ileitis. It is suggested that the abnormal flora composition might be an expression of the genetic predisposition to Crohn's disease.

INTRODUCTION

Most of the bacteriological studies of Crohn's disease concern the relationship between colonization of the small intestine and malabsorption. In some of these investigations (Vince *et al.* 1972; Mallory *et al.* 1973), the faecal flora was also studied and no significant differences between patients and healthy controls were found. Numbers of coliform bacteria were increased in the faeces of patients studied by Gorbach *et al.* (1968) but West *et al.* (1974) reported normal numbers. These findings suggest that the faecal flora of Crohn's patients is similar to that of healthy people, but the knowledge of this flora is only based on fragmentary data collected from some twenty-five patients.

In the present investigation, the anaerobic flora of patients with Crohn's disease was compared with that of healthy subjects using an exacting method of anaerobic culture. The anaerobes were assigned to one of four broad groups, namely gram-negative rods, gram-positive rods, cocci and coccoid rods. Thus, no more than a general classification was attempted and it is clear that only gross differences, if present, would be detected.

In a disease like Crohn's, the intestinal flora might be influenced by the localization and course of the disease. To see whether localization had an effect

on the flora, patients were selected in which either terminal ileitis or colitis predominated. The series included patients in which either ileocaecal resection or colectomy had been performed before stool collection and others in which these operations were not required. Different localizations and stages of the disease were therefore represented. In addition, the effect on the faecal flora of a chemically defined, liquid diet, known to be useful in the treatment of inflammatory bowel disease, was studied. Preliminary results have been reported by Wensinck (1975, 1976).

MATERIALS AND METHODS

Subjects and diet

Twenty apparently healthy laboratory workers were studied, eight females and 12 males, aged 20 to 59 with a median age of 25 years. The patient group consisted of 19 females and 10 males, aged between 18 and 50 with a median age of 29 years. All patients were undergoing treatment either in the Department of Internal Medicine II or Surgery of the University Hospital Dijkzigt, Rotterdam, The Netherlands.

In 19 patients, the terminal ileum was the principal site of inflammation. At the time of stool collection seven patients had been treated by ileocaecal resection, eight not; the faecal flora of four patients was cultured before and after ileocaecal resection.

In 10 patients the disease was confined to the colon. Two of them had previously been treated by colectomy; in five the operation was performed a few weeks after stool collection and in three it was not required. Data on the length of time between stool collection and onset of disease or operation are presented in Tables 2, 3 and 4.

In all but one of the operated cases the diagnosis was confirmed by the histopathological findings; granulomas were present in 12 of the 18 surgical specimens. One case was classified as atypical colitis.

The patients did not receive sulphasalazine nor corticosteroids at the time of stool collection and special dietary measures were not taken.

The effect of a chemically defined, liquid diet on the faecal flora was studied in five healthy subjects and five patients with terminal ileitis. The diet (Vivonex) was supplied in 80 g bags yielding 300 kcal per bag. The controls built up the diet over three to four days and then consumed it for one week, using seven to 10 bags per day. No other food was taken during the diet period. In both controls and patients, stool collection started when the diet had been taken for at least 4 days. The materials for the control subjects were kindly provided by Norwich Benelux, Utrecht, The Netherlands.

Stool dilution

Stools were diluted and plated within 2 h of passage. Samples of 1·5 g were suspended in a Universal container (28 ml) with 25 ml of diluting fluid and 10 g of glass beads (3 mm in diameter) and agitated for 15 min on a Whirlimixer (Fisons Scientific Apparatus). Three serial 100-fold dilutions were prepared from the suspensions. The diluent contained (per l distilled water): tryptone (Oxoid), 5 g; glucose, 5 g; $K_2HPO_4.3H_2O$, 3 g; KH_2PO_4, 0·5 g; NaCl, 5 g; cysteine hydro-

chloride, 0·5 g; resazurin 0·002 g. The pH was adjusted to 7·2 and 100-ml volumes were sterilized for 10 min at 121 °C.

Anaerobic culture

From the final dilution of the stool sample, 0·1 ml was plated in anaerobic culture flasks (Wensinck & Ruseler-van Embden, 1971). The following non-selective medium was used: tryptone (Oxoid), 15 g; glucose, 5 g; soluble starch, 1 g; yeast extract, 3 g; meat extract (Oxoid), 10 g; $K_2HPO_4 . 3H_2O$, 3 g; KH_2PO_4, 0·5 g; NaCl, 5 g; $MgSO_4 . 7H_2O$, 0·5 g; cysteine hydrochloride, 0·5 g; resazurin, 0·002 g; agar (Difco), 17·5 g. The materials were dissolved in one litre of an extract of erythrocytes prepared as follows: sheep erythrocytes were mixed with a tenfold volume of distilled water, the pH was adjusted to 6·8 and the mixture was heated at 100 °C for 15 min, cooled and filtered. The components were dissolved in the clear, brownish-coloured filtrate, the pH was adjusted to 7·2 and the medium sterilized for 10 min at 121 °C. When sufficiently cool, the flasks were connected to the gas supply and a mixture of 10 % CO_2 in nitrogen was allowed to stream through the flasks. When the flasks had cooled to about 45 °C, a vitamin solution was added (1 %; v/v) and the medium allowed to solidify. The vitamin solution contained (per l distilled water): *p*-aminobenzoic acid, 100 mg; biotin, 0·03 mg; calcium pantothenate, 1·2 g; folic acid, 100 mg; niacin, 100 mg; pyridoxal phosphate, 100 mg; riboflavin, 100 mg; thiamine HCl, 100 mg. The solution was adjusted to pH 6·8 and sterilized by filtration.

Enumeration of anaerobes

The surface of the medium was sufficiently large to permit the development of 100 to 150 well-separated colonies. When the diluted faecal sample was expected to yield higher numbers of viable organisms (as was the case with patients' samples) the three dilutions used were 1/100, 1/100 and 1/200. Gram stains of all colonies were made and the counts were multiplied by the dilution factors to give numbers per g faeces (wet weight).

Aerobic counts

Appropriate dilutions were plated on sheep blood agar plates and the colonies counted after 24 h incubation at 37 °C.

Identification of anaerobes

The inoculated flasks were incubated for 48 h at 37 °C and the organisms grown assigned to one of four groups according to colonial aspect, gram reaction and morphology. Gram-negative rods: round or irregular colonies, 3–8 mm in diameter, transparent; morphological features of *Bacteroides* or *Fusobacterium*. Gram-positive rods: round colonies, 5–10 mm in diameter, opaque with brownish colour; morphological features of *Bifidobacterium*, *Eubacterium aerofaciens* or other rods. Cocci: round colonies, 1 mm in diameter or smaller, transparent; small gram-positive cocci in masses or chains. Coccoid rods: colonies like those of gram-positive rods but smaller; gram-positive ovoid, pointed or lozenge-shaped cells in pairs or chains in which elongated cells may predominate. A number of gram-positive isolates were identified by fermentation tests and analysis of fermentation end-products

according to Holdeman, Cato & Moore (1977). These tests showed that the assignment of isolates to *Bifidobacterium* or *Eubacterium aerofaciens* on colonial aspect and morphology was correct and these organisms were, therefore, listed separately. The gram-positive coccoid rods were found to belong to at least 4 species, namely *Streptococcus intermedius*, *Peptostreptococcus productus*, *Eubacterium rectale* and *Eubacterium contortum*.

Presentation and comparison of results

From all healthy subjects and patients with terminal ileitis at least two stool samples were analysed and from the resulting counts, median values per individual were calculated for each group of organisms. From these data, medians and ranges were calculated per group of subjects.

The Mann–Whitney U-test and the tables in Siegel (1956) were used in the statistical comparison of results; *P*-values were derived from two-tailed tests. The Spearman rank correlation test was used to calculate correlation coefficients.

RESULTS

Healthy subjects

The results obtained in healthy subjects are presented in Table 1. Anaerobic gram-negative and gram-positive rods occurred in equal numbers and formed more than 70% of the flora. *Eubacterium aerofaciens* and bifidobacteria were present in about 75% of the samples, and in 15 subjects these organisms were found in all samples. In one subject (two samples) *E. aerofaciens*, and in another one (five samples) bifidobacteria were not found. Together, the organisms formed about 30% of the group of gram-positive rods, the majority of which belonged to species not identified. Cocci were present in all samples from 19 subjects but coccoid rods were found much less frequently; in 11 subjects none of the 35 samples yielded coccoid rods and in only four were the median numbers higher than 9 ($\log_{10}$ of numbers per g faeces). Table 1 further shows that total numbers may be four times higher in one subject than in another. The proportional composition of the flora, however, was similar. The ratio between anaerobic and aerobic counts varied from 45 to 2500 with a median value of 330. This wide range is mainly due to variation in aerobic counts.

Patients

Results were different in patients with terminal ileitis and colitis and are, therefore, reported separately.

Terminal ileitis

There were eight patients in which ileocaecal resection had not been performed and was not necessary for a period of at least 1·5 years after stool collection. Table 2 shows that the faecal flora differed in three respects from the normal flora. Numbers of anaerobic gram-negative and coccoid rods were higher and, other organisms being present in normal numbers, total counts were also higher.

In seven patients, ileocaecal resection had been performed before stool culture and their faecal flora (Table 3) differed from the normal flora by higher numbers of anaerobic gram-negative and coccoid rods and, as a consequence, by higher total numbers.

Table 1. *Faecal flora of healthy subjects*

	Log_{10} of number per g faeces	
	Median	Range
Anaerobes		
Total	10·65	10·34–10·95
Gram-negative rods	10·20	9·92–10·60
Gram-positive rods	10·21	9·77–10·66
Eubacterium aerofaciens	9·42	< 8·50–10·26
Bifidobacterium	9·37	< 8·50–10·34
Other	9·97	9·30–10·51
Cocci	9·54	< 8·50–10·31
Coccoid rods	< 8·50	< 8·50–9·60
Aerobes		
Total	7·91	7·30–9·57

Number of subjects 20, number of samples 71, median number of samples per subject 3 (range 2–8).

Table 2. *Faecal flora of patients with terminal ileitis (no ileocaecal resection)*

	Log_{10} of number per g faeces		
	Median	Range	Significance*
Anaerobes			
Total	10·80	10·67–10·96	0·02
Gram-negative rods	10·61	10·40–10·78	< 0·002
Gram-positive rods	10·21	10·08–10·46	—
Cocci	9·60	8·65–9·90	—
Coccoid rods	9·56	8·82–9·71	< 0·002
Aerobes			
Total	8·07	6·54–9·20	—

* *P*-values of difference from normal numbers; values not recorded when $P > 0{\cdot}1$.
Number of patients 8, number of samples 23. Median interval between onset of disease and culture 6 years (range 1–19).

Table 3. *Faecal flora of patients with terminal ileitis (after ileocaecal resection)*

	Log_{10} of number per g faeces		
	Median	Range	Significance*
Anaerobes			
Total	10·91	10·84–11·09	< 0·002
Gram-negative rods	10·78	10·61–10·89	< 0·002
Gram-positive rods	10·15	9·48–10·86	—
Cocci	9·52	8·82–10·22	—
Coccoid rods	9·72	9·82–9·84	< 0·002
Aerobes			
Total	7·95	6·91–9·00	—

* *P*-values of difference from normal numbers: values not recorded when $P > 0{\cdot}1$.
Number of patients 7, number of samples 24. Median interval between onset of disease and culture 7·5 years (range 1–15); median interval between operation and culture 16 months (range 7–44).

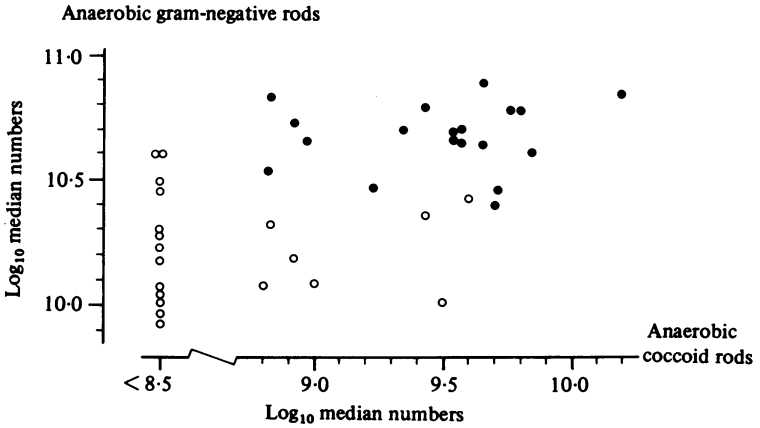

Fig. 1. Median numbers of anaerobic gram-negative and coccoid rods in faeces from healthy subjects (○) and patients with terminal ileitis (●).

The flora of four patients was cultured before and after ileocaecal resection. Table 4 records the time relationships between onset of disease, stool collection and operation and gives the pre- and post-operative results from faecal cultures. Numbers of anaerobic gram-negative and coccoid rods were higher than normal values in both periods; total numbers, however, were higher than normal before operation only.

The data presented in Tables 2–4 show that, irrespective of the course of terminal ileitis, the faecal flora contained higher numbers of anaerobic gram-negative and coccoid rods than are normally found, and that these differences were statistically highly significant. To characterize the flora, the combined data of all 19 patients are presented in Table 5. Numbers of *E. aerofaciens* and bifidobacteria, which are not given in Tables 2–4, are here seen to be normal.

When the ranges of numbers of gram-negative and coccoid rods in healthy subjects (Table 1) and patients (Table 5) are compared it is seen that they overlap. The question then arises whether a faecal flora should be considered normal or abnormal. Fig. 1 illustrates this point and shows that normal and patients' floras, characterized by the two parameters of difference, formed two clusters. The flora of one healthy subject should be classified as abnormal and two floras (one healthy subject and one patient) were on the borderline. The difference between the two floras, therefore, is not an absolute one.

Fig. 1 is based on median numbers of anaerobic gram-negative and coccoid rods resulting from several stool cultures from each individual. The variation between samples was determined by comparing the numbers of anaerobic gram-negative and coccoid rods in two stools from 10 healthy subjects and 10 patients, collected with an interval of 7 to 14 days. Table 6 shows that the differences between numbers in first and second samples were not statistically significant. Numbers of gram-negative rods in paired samples from healthy subjects were correlated but those of gram-negative and coccoid rods in patients' samples were not. In the individual patient, therefore, numbers vary more than in healthy subjects.

Table 4. *Faecal flora of patients with terminal ileitis (before and after ileocaecal resection)*

	Before operation (log_{10} of numbers per g faeces)			After operation (log_{10} of numbers per g faeces)		
	Median	Range	Significance*	Median	Range	Significance*
Anaerobes						
Total	10·88	10·62–11·11	$0·1 > P > 0·05$	10·68	10·57–11·01	—
Gram-negative rods	10·67	10·47–10·85	$< 0·02$	10·53	10·39–10·75	$< 0·02$
Gram-positive rods	10·22	9·94–10·56	—	10·04	9·70–10·45	—
Cocci	9·26	< 8·50–10·20	—	9·51	< 8·50–10·20	—
Coccoid rods	9·61	9·23–10·18	$< 0·02$	9·48	9·00–9·70	$< 0·02$
Aerobes						
Total	9·04	8·47–9·23	0·02	7·03	6·96–9·20	—

* *P*-values of difference from normal numbers; values not recorded when $P > 0·1$.

Number of patients 4, number of samples 18 before and 20 after operation. Median interval between onset of disease and culture 4 years (range 2–12). Median interval between culture and operation 4 months (range 1–10) before and 18 months (range 3–45) after operation.

Table 5. *Faecal flora of patients with terminal ileitis*

	Log_{10} of number per g faeces		
	Median	Range	Significance*
Anaerobes			
Total	10·85	10·60–11·09	< 0·002
Gram-negative rods	10·66	10·40–10·89	< 0·002
Gram-positive rods	10·19	9·48–10·86	—
Eubacterium aerofaciens	9·42	< 8·50–10·22	—
Bifidobacterium	9·00	< 8·50–10·00	—
Other	9·99	9·36–10·66	—
Cocci	9·58	< 8·50–10·22	—
Coccoid rods	9·56	8·52–9·95	< 0·002
Aerobes			
Total	8·27	6·54–9·23	—

* *P*-values of difference from normal numbers; values not recorded when $P > 0·1$.
Number of patients 19, number of samples 85, median number of samples per subject 4 (range 2–10).

Table 6. *Numbers of anaerobic gram-negative and coccoid rods in repeat stool samples*

	Medium log_{10} of numbers per g faeces (range)	
	Gram-negative rods	Coccoid rods
Healthy subjects*		
First sample	10·24 (9·82–10·45)	< 8·50 (< 8·50–9·45)
Second sample	10·35 (10·00–10·76)	< 8·50 (< 8·50–9·51)
U	32; $P > 0·1$	—
r	0·82; $P = 0·014$	—
Patients*		
First sample	10·72 (10·46–10·98)	9·28 (8·82–9·70)
Second sample	10·75 (10·28–11·02)	9·36 (8·82–10·18)
U	48; $P > 0·1$	43; $P > 0·1$
r	0·12; $P = 0·726$	0·49; $P = 0·140$

* Numbers of healthy subjects and patients 10; interval between first and second samples 7 to 14 days.

Colitis

There were two patients in which colectomy had not been performed before stool collection and was not necessary for at least 10 months afterwards. Two patients had undergone the operation one and four years respectively before stool culture and six patients had been operated upon a few weeks afterwards. The results were grouped according to total numbers cultured (Table 7). The samples from eight patients yielded total numbers of 10 (log_{10} of numbers per g faeces) or less, whereas aerobic counts were normal. The flora of two patients differed in the same respect from the normal flora, as did the flora of patients with terminal ileitis, namely by higher numbers of anaerobic gram-negative and coccoid rods resulting in higher total numbers.

Table 7. *Faecal flora of patients with Crohn's colitis*

	Total number < 10		
	Log_{10} of number per g faeces		
	Median	Range	
Anaerobes			
Total	9·86	< 9·00–10·08	
Aerobes			
Total	8·73	7·91–9·16	
	Total numbers > 10		
	Log_{10} of number per g faeces		
	Median	Range	Significance*
Anaerobes			
Total	10·79	10·62–11·00	< 0·02
Gram-negative rods	10·64	10·23–10·70	0·02
Gram-positive rods	10·23	9·99–10·52	—
Cocci	9·62	8·92–9·97	—
Coccoid rods	9·45	9·34–9·90	< 0·02
Aerobes			
Total	8·86	8·18–9·43	—

* *P*-values of difference from normal numbers; values not recorded when $P > 0·1$.
Number of patients with low total numbers (< 10) 8; number of samples 12. Number of patients with high total numbers (> 10) 2; number of samples per subject 2.

Table 8. *Faecal flora of healthy subjects and patients with terminal ileitis on conventional and chemically defined diets*

	Log_{10} of median numbers per g faeces(range)	
	Conventional diet	Chemically defined diet
Healthy subjects		
Anaerobes		
Total	10·72 (10·59–10·84)	10·80 (10·22–10·93)
Gram-negative rods	10·45 (10·08–10·49)	10·57 (9·89–10·70)
Gram-positive rods	10·45 (10·15–10·49)	10·18 (9·77–10·71)
Cocci	9·60 (9·33–9·80)	9·07 (< 8·50–9·48)
Coccoid rods	8·63 (< 8·50–8·91)	< 8·50 (< 8·50–8·50)
Aerobes		
Total	8·01 (7·30–8·41)	7·89 (7·00–8·14)
Patients		
Anaerobes		
Total	10·94 (10·67–11·11)	10·54 (10·42–11·23)
Gram-negative rods	10·76 (10·40–10·85)	10·36 (10·11–11·10)
Gram-positive rods	10·31 (10·18–10·46)	10·08 (9·62–10·43)
Cocci	9·78 (9·48–10·20)	8·74 (< 8·50–9·40)
Coccoid rods	9·53 (8·92–10·18)	9·28 (9·05–10·11)
Aerobes		
Total	7·42 (6·54–9·23)	8·57 (7·20–8·97)

Number of healthy subjects 5; number of samples 28 when on conventional and 19 when on chemically defined diet. Number of patients 5; number of samples 13 when on conventional and 11 when on chemically defined diet.

Effect of a chemically defined diet on the faecal flora

The faecal flora of five healthy subjects and five patients with terminal ileitis in whom ileocaecal resection had not been performed were studied when on a chemically defined, liquid diet. The results are presented in Table 8 together with those obtained during a conventional diet period. There were no significant changes in the composition of the flora of healthy subjects. The only change in the patients' flora was a tenfold decrease of numbers of anaerobic cocci ($P = 0{\cdot}05$). The numbers of subjects studied was small and consequently differences between numbers of gram-negative rods did not reach statistical significance. The numbers of coccoid rods, however, were significantly higher in the patient than in the control floras during the conventional and the chemically defined diet period ($P = 0{\cdot}02$). From these data it is concluded that only minor changes in the faecal flora occurred when a chemically defined diet was fed.

DISCUSSION

Improvement of anaerobic techniques, in particular the use of pre-reduced media, has increased total numbers of bacteria cultured from faeces from less than 10 ($\log_{10}$ of numbers per g faeces, wet weight) to 10·7–11 (Finegold, Attebery & Suter, 1974; Gossling & Slack, 1974; Moore & Holdeman, 1974; Mitsuoka & Ohno, 1977). Total numbers as estimated by microscopic counts are between 11 and 11·5 and, assuming that most bacteria in faeces are viable, one can reasonably conclude that total viable counts of 11 are close to the maximum possible. In the present investigation, the median number of anaerobes cultured per g faeces from healthy subjects was 10·65. Though not optimal, this yield equals that reported in other recent studies (Speck, Calloway & Hadley, 1970; Crowther *et al.* 1973; Bornside & Cohn, 1975; Drasar, Jenkins & Cummings, 1976) and can be considered sufficient when a detailed inventory is not intended. Our findings that anaerobic gram-negative and gram-positive rods occurred in equal numbers and that *E. aerofaciens* and bifidobacteria formed about 30% of the gram-positive rods agree reasonably well with data in the cited literature. The numbers of anaerobic cocci reported vary widely. Some investigators only mention *Veillonella* which occurs in small numbers (Aries *et al.* 1971; Hill *et al.* 1971; Crowther *et al.* 1973) while others report $\log_{10}$ of numbers as high as 10 for *Peptostreptococcus* and *Ruminococcus* (Gossling & Slack, 1974; Moore & Holdeman, 1974; Mitsuoka & Ohno, 1977). We separately listed cocci and coccoid rods on the basis of cultural and morphological characteristics, and the combined numbers represent cocci as reported by others.

In patients with terminal ileitis, numbers of anaerobic gram-negative and coccoid rods were higher while the other organisms (gram-positive rods, cocci and aerobes) occurred in normal numbers. As a result, total numbers were higher. The differences with normal values were statistically highly significant. When median numbers are compared, the increase of gram-negative anaerobes was threefold and that of coccoid rods at least 10-fold (Tables 1 and 5). The composition of the flora was independent of the clinical condition as judged by the necessity of ileocaecal resection (Tables 2, 3 and 4) and was not significantly altered by the chemically defined diet (Table 8). The latter finding agrees with results in healthy subjects (Attebery, Suter & Finegold, 1972; Crowther *et al.* 1973; Bounous & Devroede,

1974; Bornside & Cohn, 1975; Axelsson & Justesen, 1977). The sum of evidence, therefore, indicates that the flora of patients with terminal ileitis is stable and permanently abnormal.

In Crohn's colitis, the flora is markedly influenced by the severity of diarrhoea. The flora of two patients was closely similar to that in terminal ileitis (Table 7) and, although these patients suffered from diarrhoea, their loosely formed stools contained only small amounts of blood and mucus. Log_{10} of total numbers in the faeces of the other eight patients was 10 or less; their stools were liquid and bloody. These findings suggest that essentially the same flora is present in both terminal ileitis and colitis but in the latter, severe and bloody diarrhoea may prevent the establishment of the characteristic anaerobic flora of Crohn's disease. A similar breakdown of the anaerobic part of the flora has also been observed in other cases of diarrhoea, whether aspecific or caused by *Vibrio cholerae* or enteropathogenic *Escherichia coli* (Moore, Cato & Holdeman, 1969; Gorbach *et al.* 1970, 1971). The suggestion that the flora in terminal ileitis and colitis is essentially the same is strengthened by the fact that Crohn's patients react to the presence of coccoid rods in the intestinal tract by the production of agglutinating antibodies (Wensinck & Van de Merwe, 1981). The percentage of patients in which antibodies are found is even higher in the colitis group.

Speculations as to the mechanisms leading to a selective increase of numbers of anaerobic gram-negative and coccoid rods in Crohn's disease should take into account that the observed changes are not necessarily specific for this disease. In view of the remarkable stability of the individual flora, Drasar, Jenkins & Cummings (1976) suggested that genetic constitution should be considered as a controlling factor. Thus, a peculiar intestinal flora might be an expression of the genetic predisposition to Crohn's disease.

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