Effect of clindamycin on the ability of a continuous culture of colonic bacteria to ferment carbohydrate

C A EDWARDS, B I DUERDEN AND N W READ

From the Departments of Medical Microbiology and Physiology, University of Sheffield, Sheffield

SUMMARY A continuous culture model of the proximal colon was used to study the effect of clindamycin on the ability of colonic bacteria to ferment carbohydrate. Six steady state anaerobic cultures of human faeces, in a medium simulating ileostomy effluent, were treated with 26 µg/ml clindamycin. They were paired with six untreated cultures, run under identical conditions. Clindamycin treatment eliminated the anaerobic bacteria, significantly decreased osmolality and the output of volatile fatty acids, particularly propionic acid and increased the residual carbohydrate concentration. Doubling the amount of carbohydrate in the medium increased osmolality and the production of volatile fatty acid, though the response of clindamycin treated cultures was less than that of untreated cultures. Attempts to introduce *Clostridium difficile* into three pairs of cultures were successful in only two cultures after administration with clindamycin and when a heavy inoculum (10^6 – 10^9 organisms) had been used.

Diarrhoea is an important side effect of the administration of broad spectrum antibiotics. In recent years, ideas concerning the mechanism of antibiotic associated diarrhoea have centred on the opportunist colonisation of the colon with *Clostridium difficile*.¹ This organism, however, is responsible for only one third of the cases of antibiotic associated diarrhoea^{2,3} and has been recovered in large numbers from healthy infants and some healthy adults. An alternative theory is that the reduction in anaerobic colonic bacteria by antibiotics may impair the absorption of carbohydrates by reducing their fermentation of volatile fatty acids⁴ which are rapidly absorbed.^{5,6} The unfermented sugars would not be absorbed and would retain fluid in the colonic lumen by virtue of their osmotic activity, resulting in osmotic diarrhoea.

We have developed a simple bacteriological model of the proximal colon, in which faecal material from normal subjects was cultured in a medium based on the composition of ileostomy effluent for long periods of time under anaerobic conditions and constant pH in a continuous flow system.⁷ The aim of this study was to investigate the effect of clindamycin on the fermentation of carbo-

hydrates in our model; six cultures were treated with clindamycin and the bacterial survival and yield of volatile fatty acids was monitored. *C. difficile* was also introduced into the cultures to ascertain whether pretreatment with clindamycin enabled the organism to become established in culture.

Methods

PROXIMAL COLON MODEL

The apparatus comprised a Gallenkamp modular fermenter (model FBL, London) with some adaptations (Fig. 1). An inoculum of 20–50 g of fresh human faeces was homogenised in 250 ml of pre-reduced sterile medium and placed in a one litre fermentation vessel (A) within one hour of collection. The medium was based on established knowledge of ileostomy effluent⁸ and consisted of 60 mmol NaCl, 40 mmol NaHCO₃, 10 mmol KCl, 10 g/l tryptone (Oxoid), 5 g/l porcine bile extract (Sigma), 5×10^{-5} g/l hemin, 1.2 g/l starch, 5 g/l glucose, 6 g/l maltose. The sugars and hemin were sterilised by filtration and then mixed aseptically with the rest of the medium which had been autoclaved at 1.03×10^5 Pa at 121°C for 20 minutes. Thirteen millilitres of fresh medium was introduced over a period of two minutes every hour and excess culture was simultaneously removed by the same peristaltic pump (B), maintaining the culture

Address for correspondence: N W Read, Clinical Research Unit, Royal Hallamshire Hospital, Sheffield S10 2JF.

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Fig. 1 Apparatus for the continuous culture of human faecal bacteria. A is the one litre fermentation vessel; B is the peristaltic pump and timing device for the infusion of medium and removal of excess culture; C is the pH control unit which infused NaHCO_3 when necessary; D is the sample tube; E is the pH meter and F is the electrometer used to measure Eh.

volume constant at 300 ml. Anaerobic conditions were attained by gassing the medium and fermentation vessels with 95% N_2 /5% CO_2 and by using tubing which was impermeable to gases (butyl XX tubing Esco (Rubber) Ltd, Middlesex). Hydrogen ion concentration was controlled by a pH stat unit (C) which infused 1.5 mol- NaHCO_3 when necessary. The cultures were stirred magnetically at 100 rev/min and the whole apparatus was housed in a constant temperature room at 37°C. Daily 20 ml samples were taken with a 60 ml catheter syringe through the glass sample tube (D).

CHEMICAL ASSAYS

The pH and redox potential (Eh) of the culture were monitored daily with a pH glass combination electrode (Gallenkamp, London) and an Eh platinum combination electrode (Russell CM RPT, Fife, Scotland), connected to a pH meter (FBL 20, Gallenkamp) (E) and high impedance electrometer (model 602, Keithley, Cleveland, OH) (F) respectively.

Volatile fatty acid concentrations were measured using a gas chromatograph (no 3700 Varian, Palo

Alto, CA) fitted with a 2 metre column of 4 mm internal diameter, packed with 10% AT 1200 and 1% H_3PO_4 on chromosorb WAW (80–100 mesh). Column temperature was 121°C and gas flow rates were air 300 ml/min, H_2 30 ml/min and N_2 30 ml/min. The samples (3 μl) included an internal standard of valeric acid. External standards of acetic, propionic, n-butyric and valeric acids were used to calibrate the responses. The output of the flame ionisation detector was processed by a Hewlett Packard integrator (model 3390 MA), which integrated the areas under the peaks.

Osmolality was measured by depression of the freezing point in an osmometer (model no 3WII, Advanced Instruments, MA).

The carbohydrate concentration of the cultures was assayed by hydrolysis with 0.5 mol sulphuric acid, followed by incubation with glucose oxidase for one hour at 37°C. Solutions were read at 420 nm on a spectrophotometer (Pye Unicam no SP6-550).

BACTERIAL IDENTIFICATION

Daily samples from the cultures were used to inoculate a series of selective and non-selective

media for the identification of facultative and anaerobic organisms. Blood agar, Sabouraud agar (Oxoid) and MacConkey agar (Oxoid) plates were incubated aerobically at 37°C for 48 hours. Blood agar (Oxoid), Rogosa agar (Oxoid), reinforced Clostridial medium (Oxoid) with cotton blue,⁹ BM-Kanamycin¹⁰ and BM-Kanamycin/Vancomycin, and cycloserine cefoxitin fructose agar (CCFA)¹¹ were incubated anaerobically at 37°C for 48 hours in a Whitley anaerobic jar (Don Whitley Scientific, Shipley, W. Yorkshire, England) with an atmosphere of 90% H₂/10% CO₂ using an evacuation replacement method.¹² After 48 hours, representative colonies of each type were subcultured. These were then identified according to colony morphology, cell morphology and a variety of conventional tests.¹³⁻¹⁵

The numbers of different bacteria were estimated in a semi-quantitative manner using a standard loop plating technique and a scoring system of 0-5. A score of 1 was gained if the organism grew in the pool equivalent to approximately 10⁴ colony forming units/ml, a score of 2 if it appeared in the first set of streaks and so on to 5 (> 10⁸ colony forming units/ml)¹⁶

EFFECT OF CLINDAMYCIN ON CARBOHYDRATE FERMENTATION

Six cultures were allowed to reach steady state for at least seven days before clindamycin was administered in three 8 mg bolus doses every day at 0900, 1400, and 1800 for periods of 5-21 days. We used this dose of clindamycin on the assumptions that 10% of an oral dose of clindamycin reaches the colon¹⁷ and that the chyme flow rate to the colon is 1500 ml/day.¹⁸ As our cultures had a flow rate of

approximately 300 ml/day we used 1/50 of a normal oral dose of 1200 mg/day divided into three bolus injections. Two cultures were maintained at pH 7 and four at pH 6. Each culture was paired with an untreated control culture, which was seeded with the same inoculum and run under the same conditions.

In three pairs of cultures the starch, maltose, and glucose components of the medium were doubled after steady state had been reached at least five days after the clindamycin treatment was started.

INOCULATION WITH *C. DIFFICILE*

In another three sets of paired cultures, an inoculum of 4 × 10³ to 1.4 × 10⁹ organisms (determined by the spread plate method¹⁹ from an overnight broth culture of *C. difficile* was introduced after steady state had been maintained for at least five days. Samples from the cultures were monitored for the survival of the *C. difficile*, and if no survival occurred the conditions were altered to favour survival; the size of the clostridial inoculum was increased, the pH was changed from 6 to 7, the treatment with clindamycin was terminated, and ampicillin was used in an attempt to suppress *Streptococcus faecalis* which has been reported to inhibit the growth of *C. difficile*.²⁰ Cultures were monitored for the establishment of *C. difficile* for at least three days after each inoculation. Each pair of cultures was used for more than one inoculation of *C. difficile*.

Results

REDOX POTENTIAL

The redox potential of all control cultures was maintained at a value more negative than -270 mV,

Table 1 Metabolic indices of six continuous cultures of faecal organisms treated with clindamycin and six control cultures tested in parallel

Parameter	Results of test cultures			Results of control cultures during same period		
	Preliminary control period	During clindamycin treatment	P	Preliminary control period	Control in parallel with treatment period	P
Redox potential (mV)	-388 ± 24	-378 ± 24	NS	-373 ± 13	-364 ± 15	NS
Osmolality (mmol/kg)	500 ± 34	457 ± 44	<0.05	472 ± 34	456 ± 29	NS
Acetic acid output (mmol/day)	17.9 ± 3.0	14.3 ± 1.1	<0.01	17.2 ± 2.2	18.5 ± 3.5	NS
Propionic acid output (mmol/day)	11.6 ± 2.3	0.86 ± 0.3	<0.001	13.8 ± 1.8	15.1 ± 1.7	NS
n-Butyric acid output (mmol/day)	2.0 ± 0.8	0.4 ± 0.2	NS	1.0 ± 0.4	0.9 ± 0.5	NS
Total volatile fatty acid output (mmol/day)	32.1 ± 2.7	15.3 ± 1.0	<0.001	32.5 ± 1.0	34.1 ± 2.4	NS
Residual carbohydrate concentration (g/l)	0.00 ± 0.00	0.70 ± 0.3	<0.05*	0.03 ± 0.03	0.06 ± 0.06	NS

Results are expressed as Mean ± SEM. Statistical significance by Student's *t* test. Four samples were analysed at each stage for each culture.

*Wilcoxon's rank sum test.

a level suitable for the growth of anaerobic bacteria. Neither the addition of clindamycin nor doubling the carbohydrate input (in treated or untreated cultures) produced any consistent change in the redox potential (Table 1).

OSMOLALITY

The osmolality of normal sterile medium was 285 mmol/kg. After steady state had been attained, cultures at pH 7 had an average osmolality of between 532–588 mmol/kg and those at pH 6 had an average osmolality of 404–441 mmol/kg. The osmolality of four out of six cultures (three at pH 6 and one at pH 7) decreased after clindamycin was administered ($p < 0.05$).

Clindamycin also affected the response of the osmolality to an increase in carbohydrate (Fig. 2).

Fig. 2 Osmolality and total volatile fatty acid (vfa) profiles of a typical control culture (—●—●—) and a paired clindamycin treated culture (---○---○---) before and after the carbohydrate supply was doubled. The box indicates the period when the carbohydrate supply was doubled. The clindamycin was added to the treated culture on day seven.

The osmolality of all three cultures increased when the carbohydrate input was doubled, by 56–250 mmol/kg (14–60%) in control cultures and by 47–141 mmol/kg (14–36%) in clindamycin treated cultures. In each pair, the increase was higher in the untreated cultures. In all except one culture, the rise in osmolality was greater than that caused by the presence of the extra carbohydrate (50 mmol/kg).

VOLATILE FATTY ACID PRODUCTION

The mean total volatile fatty acid production of control cultures (32.5 ± 1.0 mmol/day, mean $\pm$ SEM), was similar to that in the test cultures before addition of clindamycin (32.1 ± 2.7 mmol/day). After clindamycin treatment total volatile fatty acid production fell to 15.3 ± 1.0 mmol/day which was significantly lower than the level maintained in the control cultures (Table 1) or in the same cultures before addition of clindamycin ($p < 0.001$). This reduction was largely caused by a decrease in the output of propionic acid from 11.6 ± 2.3 mmol/day to 0.9 ± 0.3 mmol/day ($p < 0.001$). The effect of clindamycin on acetic acid production was variable; in four cultures, (three at pH 6 and one at pH 7) the output was decreased, whereas it increased in the two cultures in which the osmolality had not declined after clindamycin treatment. Clindamycin also abolished the production of n-butyric acid in the three cultures in which it was produced. In control cultures, production of n-butyric acid declined but did not disappear as the culture was maintained.

When the carbohydrate input was doubled, volatile fatty acid output increased in all three control cultures (by 30–125%) and in two of the clindamycin treated cultures (Fig. 2). This was mainly caused by an increase in acetic acid output in both cases. The average increase in total volatile fatty acid output in the clindamycin treated cultures (5.3 ± 3 mmol/day) was less than that in the control cultures (22.0 ± 6 mmol/day) (Table 2) but the result was not statistically significant.

Table 2 Effect of an increase in carbohydrate input on three control and three clindamycin treated continuous cultures of human faeces.

Parameter	Results of untreated cultures		Results of clindamycin treated cultures	
	Basal conditions	Double carbohydrate	Basal conditions	Double carbohydrate
Redox potential (mV)	-347 ± 8	-377 ± 30	-365 ± 13	-379 ± 25
Osmolality (mmol/kg H ₂ O)	427 ± 9	600 ± 40	368 ± 17	471 ± 46
Total volatile fatty acid output (mmol/day)	30.3 ± 4.5	42.4 ± 5.2	15.5 ± 2.6	18.8 ± 3.0
Acetic acid output (mmol/day)	14.1 ± 1.5	25.0 ± 4.0	15.2 ± 2.6	18.4 ± 3.2
Propionic acid output (mmol/day)	15.9 ± 3.1	15.9 ± 2.5	0.3 ± 0.2	0.3 ± 0.1
Residual carbohydrate concentration (g/l)	0.22 ± 0.22	1.26 ± 1.07	1.51 ± 0.78	3.67 ± 1.31

Results are expressed as Mean $\pm$ SEM.

CARBOHYDRATE CONCENTRATION

The carbohydrate concentration of the cultures under basal conditions was very low (undetectable in most cases) (Table 1). After administration of clindamycin the level rose in every case ($p < 0.05$). In those cultures where the carbohydrate supply was doubled, the carbohydrate concentration of the clindamycin cultures rose further and reached higher levels than were present in the untreated cultures under the same conditions (Table 2). The difference was, however, not statistically significant.

BACTERIA

Before administration of clindamycin the cultures supported a range of bacterial species (Table 3), which resembled that found in the original inocula. Most species, *Escherichia coli*, *Klebsiella* spp., *Str faecalis* and *Bacteroides* spp either retained their original populations or increased in number while grown in culture. Some species, *Clostridium* spp and diphtheroids maintained their population or decreased in number. The lactobacilli and bifidobacteria were not detected in any culture after three days. Each culture contained three or four species of bacteroides, usually from the *B fragilis* group, and the *Clostridium* species present were *C perfringens* and *C septicum*. *C difficile* was not found in any of the original inocula nor did it spontaneously appear in any culture.

Treatment with clindamycin eliminated or considerably reduced the anaerobes, though some *Bacteroides* spp (*B vulgatus* and *B variabilis*) gradually re-established themselves seven to 13 days after the start of the clindamycin treatment. When tested, these were found to be resistant to clindamycin up to a concentration of 0.8 mg/ml. There was no consistent effect of clindamycin on the other bacteria (Table 3), though the numbers of at least one facultative species increased after clindamycin

treatment. *Klebsiella* spp increased in four cultures and *Str faecalis* increased in three. *C difficile* did not appear in any culture after administration of clindamycin.

When the carbohydrate supply of the cultures was doubled, there was no consistent change in most bacterial species. The exception was *E coli* which gained higher scores in all three cultures. This increase in *E coli* population did not occur in any of the clindamycin treated cultures.

INOCULATION WITH *C DIFFICILE*

Seven attempts were made to establish *C difficile* in control and in clindamycin treated cultures. Three sets of paired cultures were used. Inoculation with 4×10^3 organisms and 3×10^4 organisms did not result in growth of the organisms, whether the pH was maintained at pH 6 or pH 7. Because the concentration of clindamycin was above the minimal inhibitory concentration for *C difficile*,²¹ clindamycin treatment was discontinued in the next set of cultures before inoculation with 4.5×10^6 organisms. Recovery of *C difficile* was achieved at a score of one in the culture that had been treated with clindamycin.

Finally, in the third experiment the cultures were pretreated with ampicillin at two doses (10 µg/ml and 30 µg/ml) to inhibit the *Str faecalis* which is reported to suppress the growth of *C difficile* *in vitro*,²⁰ the clindamycin was discontinued and the culture was inoculated with 1.4×10^9 organisms. The ampicillin had no effect on either the clindamycin treated culture or the control culture. *C difficile* was recovered for three days after inoculation and on the final day isocaproic acid, which is produced by *C difficile* and was not previously present, was detected in the culture fluid. *C difficile* was never recovered from cultures that had not been treated with clindamycin.

Table 3 Effect of 26 µg/ml clindamycin on the mean scores gained by bacterial species in six continuous cultures of faecal bacteria

Bacterial species	Mean score gained by bacterial in individual cultures during Control period						Clindamycin period					
	1	2	3	4	5*	6*	1	2	3	4	5*	6*
<i>E coli</i>	2	3	3	4	3	4	3	4	3	4	3	3
<i>Klebsiella</i> spp	2	1	0	4	0	3	3	3	1	4	2	3
<i>Proteus</i> spp	1	0	0	0	0	0	1	0	0	0	0	0
<i>Str faecalis</i>	1	1	2	1	3	1	1	2	2	4	3	3
Diphtheroids	0	0	0	0	1	1	0	0	0	0	1	1
<i>Clostridium</i> spp†	1	0	1	0	2	0	0	0	0	0	0	0
<i>Bacteroides</i> spp†	3	4	3	4	3	4	1	0	0	0	0	0

Cultures marked * were maintained at pH 7, the others at pH 6. † indicate anaerobic species.

Discussion

The ability of the colon to ferment unabsorbed carbohydrate is difficult to investigate because of the inaccessibility of the proximal colon, where most fermentation occurs. We have developed an *in vitro* model of the proximal colon, in which faecal bacteria are cultured in conditions simulating those found in the colon. This model has been used to investigate the action of clindamycin on bacterial content and metabolism.

We have attempted to mimic the environment of the proximal colon as closely as practically possible by using a medium based on ileostomy effluent, by using a faecal inoculum which contains the same types of bacteria as are present in the proximal colon²² and by maintaining an anaerobic atmosphere. An infusion of sodium bicarbonate simulated colonic secretion of bicarbonate in the colon. The dilution rate of approximately one per day was chosen as this was successful in growing mixtures of rumen bacteria and rat colonic bacteria in continuous cultures.^{23 24}

It is very difficult to mimic all aspects of the colonic environment, and our model differs in several important aspects. The cultures were essentially liquid unlike caecal contents which are more solid. There was little provision of bacterial attachment sites. The clearance of volatile fatty acid from the colon by absorption could not be reproduced; products of bacterial metabolism could only escape from the system via the outflow cannula. The carbohydrate supply was of simple standard sugars whereas it is known that glycoproteins, mucin and complex polysaccharides are all digested by colonic bacteria.²⁵⁻²⁷ Our cultures were unable to support populations of lactobacilli and bifidobacteria which are important components of the colonic flora. Also the scores gained by the bacteroides and enterobacteria were similar in some cultures. This is different from the situation *in vivo* where the anaerobes outnumber the aerobes. This result may indicate another difference between our cultures and the colon but may also be because of the insensitivity of our culture techniques. The scores gained by these groups were similar to those gained by the same bacteria in the original inoculum. Despite these considerations, our model maintained a range of bacteria from the faecal inoculum in steady populations for up to 28 days, and the cultures actively metabolised sugars to volatile fatty acids which accumulated in proportions very similar to those found *in vivo*.⁴

Treatment with clindamycin suppressed the anaerobic flora in our cultures, reduced the output of volatile fatty acid, particularly propionic acid, and

reduced the ability of the cultures to increase production of volatile fatty acid in response to an increase in supply of carbohydrate. These findings were associated with an increase in the residual amount of unfermented carbohydrate in our cultures. Previous reports have shown that clindamycin suppresses the colonic anaerobic flora *in vivo*.²⁸ Our results indicate that clindamycin inhibits the fermentation of carbohydrate. As appreciable quantities of carbohydrate normally enter the colon each day^{8 29} suppression of fermentation would cause carbohydrate to remain in the lumen where it could cause diarrhoea by an osmotic mechanism.

Treatment with clindamycin also changed the pattern of the volatile fatty acid profile, suppressing volatile fatty acid production by the anaerobic bacteria. Propionic acid production was almost abolished and, in the cultures in which it was produced, n-butyric acid production was also eliminated. This could be important for the metabolism of the colonic enterocytes which may use n-butyric in preference to glucose as their main energy source.³⁰

The possibility that clindamycin treatment facilitates the growth of endogenous or exogenous *C. difficile* was not confirmed in our studies. Clindamycin treatment did not result in the appearance of *C. difficile* in any culture. Moreover, seven attempts to 'infect' clindamycin-treated cultures with *C. difficile* resulted in success only when clindamycin was discontinued and the inoculum contained a very large number of organisms. *C. difficile* was never established in untreated cultures. This suggests that factors other than the suppression of the normal flora are important for the establishment of *C. difficile*. It is possible that lack of mucosa or attachment sites may have prevented growth of *C. difficile* as attachment is often a prerequisite for pathogens.^{31 32} The high dilution rate, and presence of *Str. faecalis* may have also inhibited the establishment of *C. difficile* in the culture, though these factors might also be present in the colon of a patient with diarrhoea.

In summary, these studies have shown that clindamycin suppresses human colonic anaerobic bacteria in mixed continuous culture, reducing volatile fatty acid production and changing the pattern of volatile fatty acid produced. Similar effects occurring *in vivo* would compromise the ability of the colon to ferment and absorb carbohydrate and would result in diarrhoea if the patient is eating normally. This mechanism may explain why *C. difficile* cannot be implicated in many cases of antibiotic associated diarrhoea.

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