The effect of carbon dioxide on the sensitivity of *Bacteroides fragilis* to certain antibiotics *in vitro*

H. R. INGHAM, J. B. SELKON, A. A. CODD, AND J. H. HALE

From the Regional Public Health Laboratory, Newcastle upon Tyne

SYNOPSIS The effect of 10% carbon dioxide on the sensitivity to four antibiotics of 10 strains of *Bacteroides fragilis* was studied. The minimum inhibitory concentrations of erythromycin and lincomycin hydrochloride for these strains were four to 32 times higher, when grown in hydrogen plus 10% carbon dioxide, than the values obtained when the strains were grown in pure hydrogen. A similar effect was obtained by growing the strains in hydrogen on acid medium. Except for *Haemophilus influenzae* and *Clostridium tertium* the sensitivity to erythromycin and lincomycin hydrochloride of other species of bacteria examined was not affected by the atmosphere in which the tests were carried out. 7-Chlorolincomycin and rifamycin B diethylamide, to which the strains of *B. fragilis* were uniformly sensitive, were not significantly affected by additional carbon dioxide.

The possible mechanisms underlying this phenomenon and its clinical implications are discussed, and a case report describing the successful use of erythromycin in the treatment of a cerebral abscess due to *B. fragilis* is presented.

In a recent study in this laboratory of the sensitivity to antibiotics of *B. fragilis* the majority of strains were found to be inhibited by 0.15 µg/ml of erythromycin and by 0.55 µg/ml of lincomycin hydrochloride (Ingham, Selkon, Codd, and Hale, 1968). After this work had been completed hydrogen plus 10% carbon dioxide was substituted for pure hydrogen in the anaerobic technique. Strains of *B. fragilis* isolated on routine culture now appeared to be relatively resistant to erythromycin and lincomycin hydrochloride when their sensitivity was examined by the disc diffusion method. A more detailed investigation of this phenomenon was carried out, the results of which are reported here. The opportunity was also taken to examine the susceptibility of *B. fragilis* to two new antibiotics, namely, 7-chlorolincomycin and rifamycin B diethylamide.

Materials and Methods

STRAINS

The following strains were obtained from the National Collection of Type Cultures, Colindale, London: *Bacteroides fragilis* 9343, *Clostridium welchii* 8246, *Cl. sporogenes* 276, *Cl. septicum* 547, *Cl. histolyticum* 503, *Cl. tertium* 541, *Cl. tetani* 279, and the Oxford staphylococcus strain 6571. *B. fragilis* strains B558 and B571 were kindly supplied by Dr S. M. Finegold, Department of Medicine, Wadsworth Veterans Hospital, California, USA. *B. fragilis* strains 15, 17, 21, 26, 29, 38, and 41 were described in the previous study (Ingham *et al*, 1968). One strain each of *Haemophilus influenzae*, Lancefield group A streptococcus, and *Streptococcus faecalis* were isolated in this laboratory and identified by standard procedures.

ANTIBIOTICS SENSITIVITY TESTS

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Doubling dilutions of each of the four antibiotics were prepared in nutrient broth at pH 7, except for rifamycin B diethylamide where the diluent was phosphate buffer at pH 6. The four series of doubling dilutions were incorporated

Strain	Minimum Inhibitory Concentration ($\mu\text{g/ml}$)							
	Erythromycin		Lincomycin		7-Chlorolincomycin		Rifamycin B Diethylamide	
	Hydrogen	Hydrogen and 10% Carbon Dioxide	Hydrogen	Hydrogen and 10% Carbon Dioxide	Hydrogen	Hydrogen and 10% Carbon Dioxide	Hydrogen	Hydrogen and 10% Carbon Dioxide
Oxford staphylococcus	0.15	0.3	0.5	0.5	0.07	0.15	0.008	0.008
B558	0.3	2.5	1.1	2.2	0.3	0.3	0.15	0.3
B571	0.3	1.25	0.26	4.4	0.6	0.6	0.15	0.3
NCTC 9343	0.3	2.5	0.5	4.4	0.15	0.3	0.15	0.3
15	0.15	2.5	0.26	4.4	0.35	0.3	0.15	0.15
17	0.3	2.5	0.5	4.4	0.15	0.15	0.15	0.6
21	0.3	2.5	0.5	2.2	0.15	0.3	0.035	0.15
26	0.15	5.0	0.26	4.4	0.15	0.3	0.07	0.3
29	0.15	1.25	0.26	1.1	0.035	0.15	0.15	0.3
38	0.3	5.0	0.5	2.2	0.07	0.15	0.15	0.3
41	0.3	1.25	0.26	4.4	0.3	0.6	0.07	0.3

Table 1 A comparison of the sensitivity to erythromycin, lincomycin hydrochloride, 7-chlorolincomycin, and rifamycin B diethylamide, of 10 strains of *Bacteroides fragilis* cultured in hydrogen and hydrogen plus 10% carbon dioxide

nutrient agar plus 5% defibrinated horse blood at 50°C. Plates were dried for two hours at 37°C and inoculated with suspensions of *B. fragilis* in nutrient broth, using a phage typing apparatus. These suspensions were prepared by emulsifying sufficient growth from a 48-hour culture on blood agar in 2 ml of broth to produce a just visible turbidity; this was then diluted 1 part in 5 parts. Such suspensions were shown to contain approximately 10^6 organisms per ml. An overnight broth culture of the Oxford staphylococcus diluted 1 in 500 in broth was used as the control. The tests were carried out in duplicate, one series being incubated for 48 hours at 37°C in an atmosphere of pure hydrogen and the other in hydrogen plus 10% carbon dioxide in a Mackintosh and Fildes anaerobic jar fitted with a cold catalyst. The minimum inhibitory concentration was defined as the lowest concentration producing a marked decrease in colony size or permitting the growth to normal size of not more than 19 colonies.

Minimum bactericidal concentrations of 7-chlorolincomycin and rifamycin B diethylamide were determined by inoculating the suspensions of *B. fragilis* with a wire loop over an area 1 cm in diameter on standard grade Oxoid membrane filters lying on the surface of blood agar plates containing doubling dilutions of these antibiotics. The plates were incubated in hydrogen plus 10% carbon dioxide for 48 hours at 37°C when the MICs were determined. Those filters on which growth was inhibited or absent were transferred to antibiotic-free blood agar plates which were then incubated in the same atmosphere for a further 48 hours at 37°C. Bactericidal endpoints were read using 20 or more colonies as the definition of significant growth. The quantity of antibiotics carried over by the filters to the antibiotic-free plates was

determined by inoculating the filters after transfer with one loopful of 1 in 500 dilution of an overnight broth culture of the Oxford staphylococcus. Inhibition of growth of the Oxford staphylococcus after incubation of the filters for 48 hours at 37°C on the antibiotic-free plates was related to the concentration of antibiotic in the plates from which the filter has been transferred, and as the MIC of the organism was known the percentage of antibiotic carried over could be calculated.

Disc sensitivity tests were performed in duplicate on 5% blood agar plates using paper discs containing erythromycin 5 μg and lincomycin hydrochloride 2 μg . Strains were inoculated on to the medium with a wire loop so as to produce a semi-confluent growth after incubation in hydrogen or hydrogen plus 10% carbon dioxide at 37°C for 48 hours when the sizes of the zones of inhibition were measured.

Results

Preliminary examination of the sensitivity to erythromycin and lincomycin hydrochloride of 10 strains of *B. fragilis* by the disc technique revealed that all strains showed considerable reduction in the size of the zones of inhibition produced by these antibiotics when cultured in hydrogen plus 10% CO_2 instead of pure hydrogen. In many instances colonies were present within the zone of inhibition of growth often adjacent to the edge of the disc. The sensitivity to erythromycin and lincomycin hydrochloride of the other species of bacteria examined did not appear to be significantly affected by the atmosphere in which the tests were carried out, except *Cl. tertium* and *H. influenzae* which were less sensitive to lincomycin hydrochloride and erythromycin

Strain	7-Chlorolincomycin		Rifamycin B Diethylamide	
	MIC	MBC	MIC	MBC
Oxford				
staphylococcus	0.15	5.0	0.008	0.017
B558	0.3	0.6	0.3	>2.5
B571	0.6	1.25	0.3	2.5
NCTC 9343	0.3	0.3	0.3	1.25
15	0.3	1.25	0.15	>2.5
17	0.15	0.3	0.6	>2.5
21	0.3	0.3	0.15	0.6
26	0.3	0.3	0.3	1.25
29	0.15	0.3	0.3	0.6
38	0.15	0.3	0.3	>2.5
41	0.6	1.25	0.3	>2.5

Table II The minimum inhibitory and minimum bactericidal concentrations of 7-chlorolincomycin and rifamycin B diethylamide for strains of *B. fragilis* grown in hydrogen plus 10% carbon dioxide

respectively when cultured in hydrogen plus 10% carbon dioxide.

The results of the plate sensitivity tests showed that when the plates were incubated in hydrogen plus 10% carbon dioxide the MICs of erythromycin and lincomycin hydrochloride for the 10 strains of *B. fragilis* examined were four to 32 times greater than those obtained when the tests were carried out in pure hydrogen (Table I). The minimum inhibitory concentrations of these antibiotics for the Oxford *staphylococcus* were not significantly different under these cultural conditions. The minimum inhibitory concentrations of 7-chlorolincomycin and rifamycin B diethylamide for these strains of *B. fragilis* when grown in hydrogen plus 10% CO₂ were the same as, or two to four times greater than, those obtained in pure hydrogen.

The minimum bactericidal concentrations of 7-chlorolincomycin for *B. fragilis* were two to four times the MICs; rifamycin B diethylamide did not exert any consistent bactericidal effect against *B. fragilis* although it was bactericidal for the Oxford *staphylococcus* at a concentration twice the minimum inhibitory concentration. The carry over of both 7-chlorolincomycin and rifamycin B diethylamide by the membrane filter was less than 3% (Table II).

The effect of 10% CO₂ on erythromycin was studied by preparing in triplicate 5% blood agar plates containing doubling dilutions of the antibiotics. One set of plates was immediately inoculated with one drop (0.03 ml) of a 1 in 500 dilution of an overnight broth culture of the Oxford *staphylococcus* and incubated aerobically at 37°C for 18 hours when the MIC was found to be 0.15 µg/ml. The other two series of plates were pre-incubated in hydrogen or hydrogen plus 10% CO₂ for 18 hours at 37°C before inoculation with the Oxford *staphylococcus*. These plates were incubated aerobically for a further 18 hours when the MIC of erythromycin was found to be 0.15 µg/ml in both series. It was thus not possible to demonstrate any loss in activity of erythromycin after a previous incubation in the presence of 10% carbon dioxide.

In order to determine whether the lower pH resulting from the incubation of media in hydrogen plus 10% CO₂ could be responsible for the increase in resistance of *B. fragilis* to erythromycin and lincomycin hydrochloride 5% horse blood agar plates were prepared at pH 7.0, 6.5 and 6.0 respectively. The sensitivity to these antibiotics of three strains of *B. fragilis* and several other species of bacteria was examined on these media using discs containing erythromycin 5 µg and lincomycin 2 µg, the plates being incubated in pure hydrogen for three days at 37°C. At pH 6.5 and 6.0 the three strains of *B. fragilis* were much more resistant to both antibiotics than at pH 7.0. A similar but less marked effect was observed with *H. influenzae* and the Oxford *staphylococcus*. The sensitivities of *Strep. faecalis* and *Strep. pyogenes* did not vary greatly over the pH range tested (Table III).

Discussion

The sensitivity *in vitro* of *B. fragilis* to erythromycin as reported by different authors has varied considerably. Thus, the majority of the

Strain	Diameter of Zone of Inhibition (mm)					
	pH 6.0		pH 6.5		pH 7.0	
	Erythromycin	Lincomycin	Erythromycin	Lincomycin	Erythromycin	Lincomycin
Oxford staphylococcus	5	3.5	7	4.5	11	6
<i>Bacteroides fragilis</i>						
B.558	— ¹	—	2	—	11	5.5
NCTC 9343	—	—	—	—	12.5	5
21	—	—	2	—	9.5	5.5
<i>H. influenzae</i>	—	—	10	10	13.5	12
Group A streptococcus	5.5	7	7	5.5	8	7
<i>Strep. faecalis</i>	6.5	—	6	—	8.5	—

Table III The effect of pH on the sensitivity to erythromycin and lincomycin hydrochloride of several species of bacteria.¹

¹All plates were incubated for three days in pure hydrogen at 37°C.

²No zone of inhibition of growth.

Author(s)	Number of Strains	Minimum Inhibitory Concentration of Erythromycin (µg/ml)	
		Range	Median Value
Garrod (1955)	23	2->4	>4
Gillespie and Guy (1956)	10	0.06-4	0.25-0.5
Hoogendijk (1965)	13	0.25-2	2
Finegold <i>et al</i> (1967)	47	0.4-400	6.2
Ingham <i>et al</i> (1968)	26	0.03-0.3	0.15

Table IV The sensitivity of *Bacteroides fragilis* to erythromycin

strains examined by Garrod (1955), Hoogendijk (1965), and Finegold, Harada, and Miller (1967) had MICs of greater than 1 µg/ml, and most of the strains examined by Gillespie and Guy (1956) had minimum inhibitory concentrations of 0.5 µg/ml or more, whereas the majority of the strains examined by Ingham *et al* (1968) had MICs of 0.15 µg/ml (Table IV). In all these studies except that of Ingham *et al* (1968), the sensitivity tests were carried out anaerobically in the presence of 5% or 10% carbon dioxide. The present report has shown that it was the addition of CO₂ which was responsible for the difference between the results of the sensitivity to erythromycin obtained by Ingham *et al* (1968) and these other authors. Similarly the difference between the MIC of lincomycin for *B. fragilis* found by Ingham *et al* (1968), namely, 0.55 µg/ml and of 6.2 µg/ml found by Finegold *et al* (1967), has also been shown by the present report to be due to the addition by the latter authors of 10% CO₂ to the anaerobic environment used to culture this organism. About the same degree of reduction in sensitivity to erythromycin and lincomycin as was produced by the addition of 10% carbon dioxide was produced by altering the pH of the culture medium from 7.0 to 6.0. Furthermore, this effect of pH, like that of carbon dioxide, did not apply equally to all organisms. It was most marked with *B. fragilis*, nearly as marked with *H. influenzae*, but much less apparent with the Oxford staphylococcus, a group A *streptococcus*, and *Streptococcus faecalis*. This suggests that this effect of the carbon dioxide results from its increasing the acidity of the culture medium. The alternative hypothesis that the carbon dioxide reduces the sensitivity of the *B. fragilis* and *H. influenzae* by stimulating an alternative biochemical pathway is unlikely since the addition of carbon dioxide did not significantly reduce the activity of 7-chlorolincomycin, a derivative of lincomycin. Furthermore there is previously published evidence that erythromycin has reduced activity at an acid pH (Haight and Finland, 1952; Garrod and Waterworth, 1956) and it has been suggested that this results from alterations in physicochemical factors concerned in the penetration of antibiotics into the bacterial cell. Thus, Sabbath, Gerstein, Loder, and Finland (1968)

observed that the total concentration of erythromycin required to inhibit the growth of *Escherichia coli* increased as the pH fell whereas the antibacterial activity of the un-ionised fraction remained constant over the pH range 5.5 to 8.5. These authors and others (Abraham and Duthie, 1946; Crane, 1921; Albert, 1968) suggest that it is the un-ionised molecules which enter the bacterial cell. However, Albert (1968), studying the antibacterial action of acridines, has shown that ionised molecules are not inactive but that their activity is reduced by the presence of hydrogen ions which, he suggests, compete for important anionic groups on the surface of the bacterial cell. The greater susceptibility of *B. fragilis* and *H. influenzae* to the effect of carbon dioxide on the activity of erythromycin and lincomycin, compared with other organisms studied, may be due to differences at the site on the bacterial wall where absorption of these antibiotics occurs. A somewhat similar explanation has been advanced by Eagle, Levy, and Fleischman (1952) for the difference in the effect of pH on the action of tetracycline against *Staphylococcus aureus* and *Escherichia coli*.

The marked influence of 5 to 10% CO₂ and of pH on the sensitivity of *B. fragilis* to erythromycin and lincomycin poses the question whether a sensitivity test to these agents in the presence of added CO₂ is more appropriate in relation to conditions pertaining *in vivo* than a test carried out in the presence of pure hydrogen as described in our previous report (Ingham *et al*, 1968). The partial pressure of CO₂ in mm of mercury varies from 40 mm in arterial blood to 46 mm in venous blood. Although this is equivalent to approximately 5% CO₂ only a small proportion of this is in solution, the remainder being in the form of bicarbonate or combined with protein as carbamino compounds (Campbell and Dickinson, 1960). Pus may on occasion have a pH below 6.0 (Rous, 1925). On the other hand the pH of body fluids is 7.4 so that in healthy tissue, and this includes the extending edge of an inflammatory site, the action of these two antibiotics would be unimpaired. The answer to this question has clearly to be sought from a controlled trial of lincomycin and 7-chlorolincomycin. In the absence of this evidence it may perhaps be safer to use the latter agent in preference to lincomycin since its action on *B. fragilis in vitro* is not significantly affected by the concentrations of carbon dioxide and variations in pH likely to be encountered *in vivo*.

Although concentrations of CO₂ attained in the body under certain conditions might be sufficient to cause significant impairment of the antibacterial activity of erythromycin, clinical experience in the use of this antibiotic suggests that it has been effective. The following case history is presented in support of this statement.

A male patient, aged 53 years, was admitted with a right temporal lobe abscess secondary

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to right chronic otitis media. The abscess was drained and a mastoidectomy performed. Culture of pus aspirated from the abscess yielded a scanty growth of *Proteus* species and coliforms and a heavy growth of *Bacteroides fragilis*; the latter organism was sensitive to tetracycline and chloramphenicol. Treatment with chloramphenicol 250 mg six-hourly for 10 days resulted in a marked clinical improvement, and aspirates obtained from the abscess cavity were sterile on culture. Chloramphenicol was withdrawn and sulphamethazine substituted. Relapse subsequently occurred with accumulation of pus which yielded *B. fragilis* on culture. Treatment with chloramphenicol, 500 mg six-hourly, and frequent surgical drainage over a period of three weeks failed to eradicate the infection. Further drainage of the abscess was carried out and erythromycin, 100 mg intramuscularly eight-hourly, given in addition to chloramphenicol. Within 10 days of commencing therapy with erythromycin the patient became afebrile and there was no further accumulation of pus. The administration of chloramphenicol was stopped at this stage and erythromycin was given orally in a dose of 250 mg six-hourly for a further four weeks.

The patient was discharged three months after admission and there has been no recurrence of infection during the past 18 months.

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