FACTORS THAT MAY PREVENT TRANSFER OF ANTI-BIOTIC RESISTANCE BETWEEN GRAM-NEGATIVE BACTERIA IN THE GUT

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JAPANESE workers first noted that transfer of multiple drug resistance between members of Enterobacteriaceae did not take place as readily in the intestinal tract as in the laboratory (Akiba *et al.*, 1961). Subsequent literature suggests that R-factor transfer rarely occurs in the human gut except during chemotherapy. Smith (1969) obtained evidence in a few instances of transfer of resistance from ingested R-factor bearing organisms to a resident recipient in a single individual in the absence of antibiotics. Wiedemann, Knothe and Döll (1970), on the other hand, failed to detect transfer in three healthy persons from ingested resistant organisms to the resident bowel flora. In the study of Anderson, Gillespie and Richmond (1973), R-factor transfer did not occur in the absence of chemotherapy in four subjects who had ingested very large numbers of potential donor and recipient organisms even though the plasmids concerned could be freely transferred in a broth medium.

In the present investigation, possible reasons for the almost complete inhibition of R-factor transfer in the gut in the absence of chemotherapy have been studied in the laboratory with the plasmids that had been used in the human experiments (Anderson *et al.*, 1973).

MATERIALS AND METHODS

Media

MacConkey bile-lactose agar was prepared with and without antibiotics as already described (Anderson *et al.*, 1973). Media containing ampicillin, nalidixic acid, ampicillin with nalidixic acid, tetracycline, or tetracycline with nalidixic acid, were used for these studies. Brain-heart Infusion Broth was prepared from a dehydrated base (Difco, Bacto). Cysteine-serum broth was prepared as described by Hofstad and Kristoffersen, 1971. Cultures of *Bacteroides fragilis* were maintained by weekly subculture on Oxoid DST Agar supplemented with saponin-lysed oxalated horse blood (5%).

Transfer experiments were usually performed in nutrient broth prepared from Difco Nutrient Broth base (1.6%) and sodium chloride (0.5%). Nutrient broth was also prepared containing sodium taurocholate (0.8%, w/v) or sodium glycocholate (0.8%, w/v). A slurry of cellulose powder (Whatman, chromatographic grade, 4.1%, w/v) in sterile nutrient broth was heated to 100° for 30 min. and then cooled immediately before use to destroy any bacteria present. Slurries of formalin-killed *Escherichia coli*, *B. fragilis*, and suspensions of various living organisms in nutrient broth were prepared as described below.

Received 26 Mar. 1974; accepted 31 May 1974.

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Organisms and R factors

Two F-like f_i^+ repressed R factors were used in this work (see Anderson *et al.*, 1973). One (R-1), conferred resistance to ampicillin, chloramphenicol, kanamycin, streptomycin and sulphonamides; the other (R-222-1), determined resistance to chloramphenicol, streptomycin, sulphonamides, and tetracyclines.

A nalidixic-acid-resistant laboratory strain of *E. coli* (NAR) was used as a recipient for R factors.

The experimental R factors were introduced from a donor organism of different colonial appearance into each of two strains of *E. coli* that had been isolated from human faeces (Anderson *et al.*, 1973). The new resistant strains derived from faecal organisms were then isolated and purified on media containing appropriate antibiotics.

Two strains of *B. fragilis* from human faeces were identified by biochemical reactions and antibiotic-disk-sensitivity tests, and grown by means of anaerobic-culture techniques that have already been described (Anderson and Sykes, 1973).

An isolate of *Streptococcus faecalis* was obtained from infected urine, and isolates of *Staphylococcus albus* and *Neisseria catarrhalis* from sputum: all these organisms were identified (Cowan and Steele, 1965) after purification and grown in bulk in cysteine-serum broth or brain-heart infusion broth.

Factors affecting R-factor transfer

Presence of B. fragilis. Stationary-phase cultures of one or other of the two strains of *B. fragilis* in cysteine-serum broth were centrifuged, and the precipitated bacteria resuspended in nutrient broth to provide suspensions containing suitable densities of viable cells. Overnight cultures of donor strains of *E. coli* containing plasmids R-1, or R-222-1, and of the recipient *E. coli* NAR, were each diluted 1 in 10 with fresh, warmed (37°C) broth immediately before use.

In each experiment a mixture of diluted suspension of donor organism (2 ml), diluted suspension of recipient organism (2 ml) and prewarmed (37°C) dense suspension (6 ml) of one of the strains of *B. fragilis* was incubated at 37°C in screw-capped bottles (30 ml). Viable counts of donor and recipient organisms, and of organisms resulting from plasmid transfer, were quantitatively determined (Miles, Misra and Irwin, 1938) on a suitable range of antibiotic-containing media. Counts were made at the start of the experiment, and again at 4 h and in some cases at 24 h. Transfer frequency was expressed as the number of conjugants per donor organism after incubation for 4 h.

Supernate from cultures of B. fragilis. Supernatant fluid was obtained by centrifugation of 40-h stationary-phase cultures of *B. fragilis* in cysteine-serum broth. Organisms remaining in this fluid were removed by filtration through a Sartorius 0.45 μ m membrane filter (Sartorius, 34 Göttingen, West Germany). In each experiment, the diluted suspension of donor organism in nutrient broth (2 ml), prepared as already described, was mixed with the diluted suspension of the recipient organism (2 ml) and the filtered culture supernatant (6 ml). Fresh cysteine-serum broth (6 ml) was used as a control. Experiments were otherwise similar to those used to study the effect of *B. fragilis* upon transfer rates.

Supernates from more concentrated suspensions of *B. fragilis* were prepared by incubating saline-washed suspensions ($c. 2 \times 10^{10}$ viable organisms per ml) in nutrient broth at 37°C for 2 h. The supernate was obtained from this suspension as described above.

Killed B. fragilis or E. coli. Stationary-phase (40 h) cultures of *B. fragilis* in cysteine-serum broth (or 18-h cultures of *E. coli* in nutrient broth) were concentrated by centrifugation and resuspended in about 10% of their original volume of formol-saline (2% v/v) for 20 min. The killed organisms were concentrated again by centrifugation, washed twice with fresh saline to remove residual formaldehyde, and finally resuspended in a suitable volume of nutrient broth. Rates of R-factor transfer were studied by methods already described for analogous experiments with suspensions of viable *B. fragilis*.

Bile salts and cellulose-powder slurry. The method was similar to that already described for experiments with viable cultures of *B. fragilis*. Diluted suspensions of donor (2 ml) and

recipient (2 ml) organisms in nutrient broth were added to 6-ml volumes of nutrient broth containing either cellulose powder, sodium taurocholate, or sodium glycocholate to give the final concentrations of these substances shown in the table.

Living Strep. faecalis, Staph. albus, and N. catarrhalis. Overnight broth cultures of each of these organisms were concentrated by centrifugation and resuspended by stirring well during slow addition of nutrient broth. The effect of these organisms upon rates of conjugation was determined in experiments similar to those with *B. fragilis*.

Anaerobic conditions. Hydrogen was bubbled through cysteine-serum broth (500 ml) contained in screw-capped bottles (540 ml) to remove air. Overnight broth cultures (9 ml) of donor and recipient organisms were added to each bottle; the bottles were filled to the top with fresh oxygen-free medium and the cultures mixed by rotating the bottles. Viable counts were determined at intervals as in similar experiments with *B. fragilis*. After each sample had been removed, the bottles were filled to the top with fresh medium to displace air. Portions (20 ml) of the same donor-recipient-broth mixture in cotton-plugged conical flasks (250 ml) served as "aerobic" controls.

RESULTS AND DISCUSSION

The aerobic Gram-negative bacteria that are known to be involved in R-factor transfer form only a small minority of the intestinal flora, which is predominantly anaerobic (see Drasar, Shiner and McLeod, 1969; Gall, 1970). Since *Bacteroides* spp. form the main component of the gut flora, the effect of these organisms upon R-factor transfer was studied. Populations of donor and recipient organisms were chosen that were similar to those that might be found in faeces. The addition of suspension of *B. fragilis* of somewhat lower density than those normally found in faeces completely stopped R-factor transfer (no detectable transfer even after 24 h), whilst the addition even of quite small numbers of this organism had some effect (the table). Filtrates of *B. fragilis* cultures, or dense suspensions of formalin-killed organisms had relatively little effect on transfer (the table). The effect of *B. fragilis* may be due to the production of an unstable metabolite, or to a component that is destroyed by formalin, which either specifically inhibits conjugation or suppresses metabolism.

The effect of dense suspensions of *E. coli*, or a slurry of cellulose powder upon transfer frequencies (the table) suggests that inhibition of conjugation in the gut is unlikely to be due entirely to the purely mechanical effects of inert material.

Conjugation is known to be inhibited in broth cultures by bile salts such as sodium taurocholate (Koyama and Akiba, 1961; Wiedemann, 1972). These may act by lowering surface tension, because sodium dodecyl sulphate, an anionic detergent, has a similar effect (Yokota and Akiba, 1961), although other authors (Chaloupecky and Zahradnik, 1972) found that compounds lowering surface tension did not influence transmission *in vitro*. The effect of bile salts and bile pigments upon R-factor transfer in the gut is difficult to assess in the laboratory, because bile salts undergo a variety of chemical changes during passage down the gut. A realistic laboratory model for bile salt or bile pigment action cannot therefore be provided and laboratory findings should be viewed with some reservation. Nevertheless bile salts do have some inhibitory effect upon conjugation *in vitro* (the table), and may do so *in vivo*.

Wiedemann (1972) found that *Strep. faecalis* inhibited R-factor transfer. Although this effect was confirmed here, it was not marked even with much higher concentrations of this organism than are normally found in faeces.

TABLE
Laboratory studies of factors that may explain the low frequency of
R-factor transfer in the human gut*

Factor tested	Mean transfer frequency† of	
	donor plasmid R-1	donor plasmid R-222-1
Nutrient broth control	6.0×10^{-3} (32; $2.4 \rightarrow 12 \times 10^{-3}$)	7.6×10^{-3} (32; $2.0 \rightarrow 12.2 \times 10^{-3}$)
Nutrient broth containing c. 2×10^{10} <i>Bacteroides fragilis</i> per ml	$< 10^{-8}$ (8; no detectable transfer)	$< 10^{-8}$ (8; no detectable transfer)
Nutrient broth containing $3-5 \times 10^9$ <i>B. fragilis</i> per ml	3.2×10^{-3} (6; $0.2 \rightarrow 3.8 \times 10^{-3}$)	3.0×10^{-3} (6; $0.6 \rightarrow 4.0 \times 10^{-3}$)
Nutrient broth containing c. 2×10^{10} formalin-killed <i>B. fragilis</i>	4.4×10^{-3} (4; $2.2 \rightarrow 8.0 \times 10^{-3}$)	4.6×10^{-3} (4; $2.0 \rightarrow 7.0 \times 10^{-3}$)
Supernate from nutrient broth that had been incubated for 2 h with c. 2×10^{10} <i>B. fragilis</i>	4.0×10^{-3} (1)	4.8×10^{-3} (1)
Supernate from 40-h stationary-phase culture of <i>B. fragilis</i> in cysteine-serum broth	2.6×10^{-3} (1)	4.2×10^{-3} (1)
Nutrient broth containing c. 2×10^{11} formolised <i>Escherichia coli</i> per ml	5.4×10^{-3} (4; $4.0 \rightarrow 6.4 \times 10^{-3}$)	5.6×10^{-3} (4; $3.0 \rightarrow 7.2 \times 10^{-3}$)
Nutrient broth containing a suspension of cellulose powder 2.5% (w/v)	6.8×10^{-3} (1)	7.9×10^{-3} (1)
Nutrient broth containing sodium taurocholate 0.5% (w/v)	3.6×10^{-3} (4; $2.0 \rightarrow 5.8 \times 10^{-3}$)	1.7×10^{-3} (4; $1.1 \rightarrow 2.6 \times 10^{-3}$)
Nutrient broth containing sodium glycocholate 0.5% (w/v)	5.5×10^{-3} (1)	3.2×10^{-3} (1)
Nutrient broth containing c. 2×10^9 <i>Streptococcus faecalis</i> per ml	2.9×10^{-3} (3; $1.9 \rightarrow 4.2 \times 10^{-3}$)	3.1×10^{-3} (3; $2.0 \rightarrow 5.6 \times 10^{-3}$)
Nutrient broth containing c. 1×10^{10} <i>Staphylococcus albus</i> per ml	1.9×10^{-3} (1)	3.2×10^{-3} (1)
Nutrient broth containing c. 1×10^{10} <i>Neisseria catarrhalis</i> per ml	7.3×10^{-3} (1)	7.8×10^{-3} (1)
Cysteine-serum broth incubated aerobically	1.2×10^{-2} (3; $0.8 \rightarrow 1.7 \times 10^{-2}$)	1.5×10^{-2} (3; $1.0 \rightarrow 2.2 \times 10^{-2}$)
Cysteine-serum broth incubated anaerobically	1.2×10^{-2} (3; $0.9 \rightarrow 1.7 \times 10^{-2}$)	1.6×10^{-2} (3; $0.8 \rightarrow 2.6 \times 10^{-2}$)

* See text for details of R factors, organisms, and experimental technique.

† Defined in *Methods* section; figures in brackets show the number of individual determinations and the observed range of values.

Similarly *Staph. albus* and *N. catarrhalis*, which are not normally found in large numbers in the gut, had little effect upon conjugation.

The partial pressure of oxygen in human intestinal gas is low (Askeveld, 1956) and the predominant flora is strictly anaerobic. Nevertheless, transfer rates of both R factors used in this study were found to be the same under both aerobic and anaerobic conditions. Although Stallions and Curtiss (1972) obtained similar findings, other workers (Fisher, 1957*a*; Egawa *et al.*, 1961; Moodie and Woods, 1973) have found that anaerobiosis may inhibit conjugation under certain conditions. It is not certain, therefore, that anaerobic conditions do not inhibit R-factor transfer in the gut.

Growth of *E. coli* in mixed culture was almost completely prevented by dense suspensions of *B. fragilis* and, as has been suggested, suppression of metabolism in *E. coli* under such conditions provides a possible explanation for the inhibition of conjugation. This view is confirmed by experimental studies, which have indicated that energy provided by oxidative phosphorylation is probably essential for R-factor transfer (Fisher, 1957*a* and *b*; Egawa *et al.*, 1961). The other factors studied, which had a less marked effect upon conjugation, depressed growth rates to as little as 10% of that of the controls.

The presence of dense cultures of *B. fragilis* thus provides a satisfactory explanation for the inhibition of conjugation in the human gastro-intestinal tract in the absence of antibiotics. Other factors that inhibit conjugation to a lesser degree may reinforce the effects of *B. fragilis*. Other gut anaerobes such as *Veillonella* spp. and Gram-positive cocci, which are also very numerous, may well have a similar effect to *B. fragilis*, and there is obviously a need for further studies in this field.

Therapeutic courses of ampicillin or tetracycline increased populations of resistant organisms, arising in the gut by conjugation, from undetectable to very large numbers (Anderson *et al.*, 1973). It is possible that as well as selecting for resistant organisms, antibiotics may also act indirectly by interfering with the Gram-positive or Gram-negative anaerobic flora which may normally suppress R-factor transfer.

SUMMARY

Antibiotic-resistance transfer between populations of donor and recipient strains of *Escherichia coli* was completely inhibited in broth by dense suspensions of *Bacteroides fragilis*. Comparable amounts of inert bacterial matter (formolised suspensions of *E. coli* or *B. fragilis*), or smaller numbers of viable *B. fragilis*, *Streptococcus faecalis*, *Staphylococcus albus*, *Neisseria catarrhalis*, or solutions of sodium taurocholate or glycocholate were only moderately inhibitory. Anaerobiosis had no effect upon plasmid transfer. Population densities of enteric organisms in these studies were similar to those found in faeces. The presence of dense cultures of *B. fragilis* provide a satisfactory explanation for the almost total inhibition of conjugation in the human gut. Other factors inhibiting conjugation to a lesser degree may reinforce the effect of *B. fragilis* *in vivo*.

As well as selecting for resistant organisms, antibiotics may also indirectly

increase populations of R-factor-bearing organisms in the gut by interfering with the anaerobic flora and so permit an increase in the frequency of conjugation.

I am very grateful to Professor W. A. Gillespie for his interest and to Miss M. A. C. Jones for technical help. M. A. C. J. was supported by a Programme Grant to Professor M. H. Richmond from the Medical Research Council.

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