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In vivo transfer of an R factor within the lower gastro-intestinal tract of sheep

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SUMMARY

The transfer of an R factor from donor *E. coli* introduced into the rumen of adult sheep to strains of the coliform microflora resident post rumen in the lower gastro-intestinal tract was found to be greatly increased when the animals were subjected to a short period of starvation (*ca.* 24–48 h). This also resulted in coliform organisms containing the resistance determinants of the R factor being excreted for much longer periods, sometimes for months afterwards. As no antibiotic treatment was given to the animals during these experiments, possession of the R factor should have conferred no selective advantages on the host cells and other plasmids could possibly be transferred similarly *in vivo* in sheep or other ruminants and perhaps also within the gut of monogastric animals.

INTRODUCTION

Previous experiments at this laboratory showed that transfer of an R factor from donor to recipient bacteria introduced into the rumen of sheep could take place readily if the organisms were able to multiply to large numbers. This occurred when a short period of starvation (24–48 h) was imposed on the animals. The ruminal environment appeared to be altered to the extent that it no longer exerted an inhibitory effect on the growth of the organisms introduced and *in vivo* transfer was demonstrated between *E. coli* organisms (Smith, 1975) and from *E. coli* donors to salmonella recipients (Smith, 1977). During these experiments it was noticed that exconjugant organisms (recipient cells containing the R factor) were sometimes present in the faeces of an animal before such organisms were detected in the rumen. This indicated that *in vivo* transfer might also be occurring post rumen in the lower gastro-intestinal tract. The present paper describes experiments to demonstrate more clearly the *in vivo* transfer of R factors between coliform organisms within the lower gastro-intestinal tract of sheep.

MATERIALS AND METHODS

Bacterial cultures

The donor and recipient *E. coli* organisms were the same as those used in experiments reported previously (Smith, 1975). The donor *E. coli* contained an R factor which conferred resistance to streptomycin (25 μg/ml) and sulphadimidine

(500 μg/ml) and the prospective recipient was a chromosomal mutant resistant to nalidixic acid (50 μg/ml).

Plating media

The total number of coliform organisms was estimated by surface plating samples on MacConkey agar (Oxoid). Streptomycin (25 μg/ml) and sulphadimidine (500 μg/ml) were added to the medium to determine the number of coliforms containing the R factor, including non-lactose fermenting variants. The prospective recipient coliforms were estimated using the same medium with the addition of nalidixic acid (50 μg/ml). All three inhibitory agents were included in the medium to determine the number of exconjugant organisms present, i.e. recipient cells which had received the R factor.

Inoculation of animals and sampling techniques

The method of introducing *E. coli* organisms directly into the rumen of an adult Merino wether (3–4 years old) with a sampling device, the technique by which samples of rumen fluid were withdrawn at the required intervals of time, and the collection of faeces from the experimental animal, were the same as described previously (Smith, 1975). Once more, care was taken to minimize the possibility of transfer occurring from donor to recipient organisms after the rumen and faecal samples were taken from the experimental animal. Faeces (10 g) were blended in 0·1 % peptone water (90 ml) on a Sunbeam blender for 30 s at 20000 rev./min. Dilutions of the faecal suspension or of rumen fluid were made in 0·1 % peptone water.

Implantation of prospective recipient E. coli *in carrier animals*

Previous experiments showed that *E. coli* introduced into the rumen of sheep subjected to a short starvation period could grow for a short time after the animal was re-fed and sometimes appeared to colonize the lower gastro-intestinal tract. Although the organisms introduced into the rumen of fasted sheep disappeared quickly from this site when the animals were again fed daily, they were sometimes excreted continuously in the faeces for long periods afterwards. This occurred both with a chromosomal mutant of *E. coli* resistant to nalidixic acid (Smith, 1975) and with *Salmonella lomita* organisms (Smith, 1977). Therefore, using this technique, attempts were made to produce carrier animals excreting prospective recipient *E. coli* which were resistant to nalidixic acid by chromosomal mutation. However, this proved more difficult than expected.

Nalidixic acid resistant *E. coli* (*ca.* 10^8–10^{10} cells) were administered directly into the rumen of sheep which were then subjected to a 3-day starvation period and re-fed. Over a period of several months a total of 34 animals were treated in this way. Of these, only seven became long-term carriers. An animal was judged to be a carrier if it continuously excreted large numbers of resistant *E. coli* in the faeces for more than 3 weeks after the same organisms were no longer detected in the rumen. The administration of nalidixic acid before or at the same time as the nalidixic acid resistant organisms were introduced into the rumen of some animals

had no discernible effect on whether the animal became a carrier, and this practice was discontinued. No explanation can be advanced why relatively few of the treated animals became carriers of the introduced organisms. The different carrier animals continued to excrete the implanted *E. coli* for various periods, the longest being approximately 105 days. The implanted *E. coli* formed only part of the total coliform flora present in the faeces of the experimental animals and usually varied from about 0·01 % to approximately 100 % of the lactose-fermenting colonies able to grow on MacConkey agar.

Two of the animals which became carriers also continuously had large numbers of coliforms (not resistant to nalidixic acid) in the rumen. Possibly these organisms were shed constantly from the buccal or nasal cavities or from the tonsils. Neither of these animals was used for further experimental work.

No antibiotics were administered to any of the animals during the course of an actual experiment. Although sometimes nalidixic acid was given by mouth to assist in implanting the prospective recipient *E. coli* in the lower gastro-intestinal tract, none of the carrier animals were used for at least several weeks afterwards when there should have been no residual influence from this drug affecting transfer of the R factor *in vivo*. Neither streptomycin nor sulphadimidine was administered to the animals at any time and rumen and faeces from each animal were tested before every experiment to ensure that no organisms resistant to these antibiotics and capable of growth on MacConkey agar were present.

Preparation of non-lactose fermenting strains of donor E. coli

Several cultures of *E. coli* were isolated from different samples of sheep faeces. Using the method described by Lin, Lerner & Jorgensen (1962) a non-lactose fermenting strain was prepared from each culture by treatment with ethyl methanesulphonate (Sigma Chemical Co.). An R factor was transferred *in vitro* into each non-lactose fermenting strain from a normal, lactose-positive *E. coli* donor. The non-lactose fermenting, antibiotic resistant strains were then tested with a competent recipient organism resistant to nalidixic acid to ensure that each was capable of acting as a donor organism able to transfer the R factor to other organisms.

In vitro *transfer to* E. coli *organisms isolated from experimental sheep*

Five *E. coli* cultures isolated from the faeces of each of eight adult Merino wethers, none of which contained antibiotic resistant bacteria, were tested with at least one strain of the prepared series of non-lactose fermenting donor *E. coli* organisms. At least two of the *E. coli* cultures obtained from each of five animals were found to accept the R factor *in vitro* from these donor *E. coli* and the animals from which they were isolated were retained for *in vivo* transfer experiments. The other three animals were released.

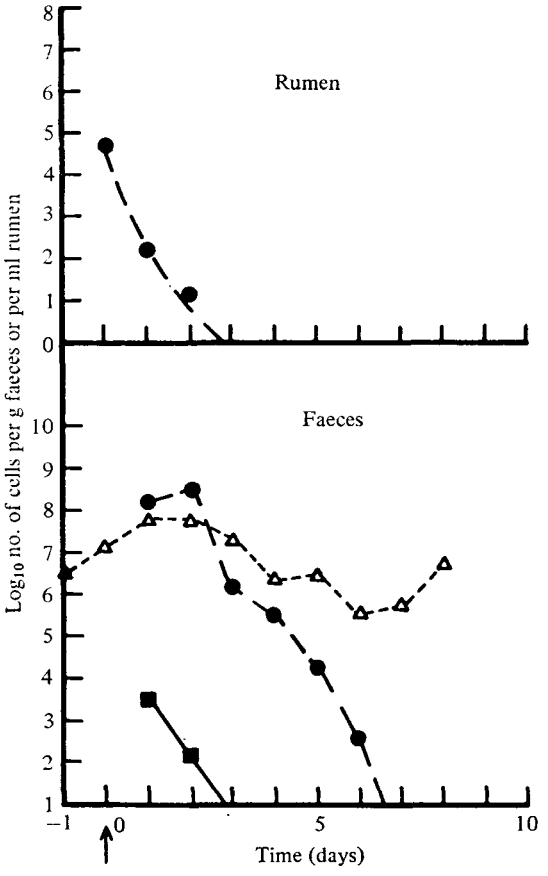

Fig. 1. Number of exconjugant cells (■) detected in the faeces of a sheep fed daily after *ca.* $2{\cdot}6 \times 10^8$ cells of donor *E. coli* (●) were introduced into the rumen (↑). The recipient organism (△) was a nalidixic acid resistant *E. coli* implanted in the lower gastro-intestinal tract.

RESULTS

Transfer of an R factor in vivo *to implanted* E. coli *recipients in animals fed daily*

Two animals, each excreting large numbers of nalidixic acid resistant *E. coli* (10^5–10^8 cells/g faeces) were used. The implanted recipient *E. coli* was not detected in the rumen of either animal at any stage during the experiments. Both animals behaved similarly and the results obtained with one are shown in Fig. 1. Donor organisms ($2{\cdot}6 \times 10^8$ cells) introduced into the rumen decreased rapidly and were no longer detectable 3 days later. Large numbers of coliforms resistant to streptomycin and sulphadimidine were present in the faeces 24 h after the *E. coli* organisms containing the R factor were introduced into the rumen. Exconjugant organisms, resistant to all three antibiotics, were also detected in the faeces at this time. Only small numbers of these exconjugant organisms were present and were detected only for 2 days. The numbers of coliforms resistant to streptomycin and sulphadimidine also decreased rapidly in the faeces after 2 days and were no longer detectable after 6 days. Both of the experimental animals continued to excrete large numbers (10^5–10^8 cells/g faeces) of the nalidixic acid resistant *E. coli*.

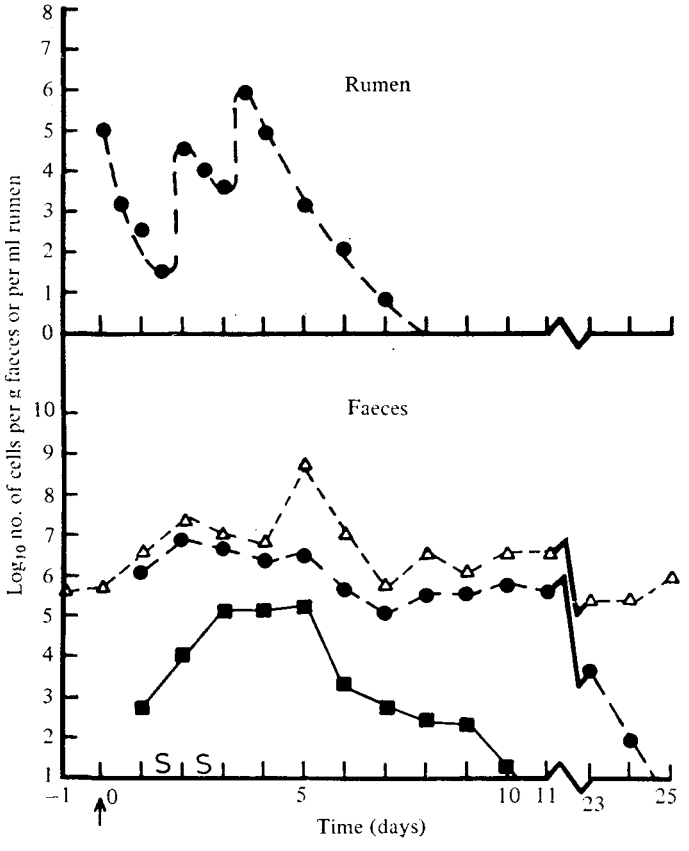

Fig. 2. Number of exconjugant cells (■) detected in the faeces of a sheep fed for 1 day and then starved for 2 days (S) after *ca.* $2{\cdot}4 \times 10^8$ cells donor *E. coli* (●) were introduced into the rumen (↑). The recipient organisms (△) were nalidixic acid resistant *E. coli* implanted in the lower gastro-intestinal tract.

Transfer of an R factor in vivo *to implanted* E. coli *in animals fed for* 1 *day and then starved for* 2 *days*

Two animals, each of which was continuously excreting large numbers of *E. coli* resistant to nalidixic acid (10^5–10^8 cells/g faeces) were used. None of these implanted organisms were detected in the rumen of either animal at any stage during the experiments. The results obtained with one animal are represented in Fig. 2. After the donor organisms ($2{\cdot}4 \times 10^8$ cells) were introduced into the rumen the animal was fed for 1 day. Food was then withheld for 2 days. The introduced *E. coli* organisms decreased in the rumen for about 30 h, multiplied rapidly for a few hours, then again decreased. On refeeding the host animal, there was another short period of rapid growth. As the animal continued to feed, organisms in the rumen containing the R factor again decreased and were no longer detectable 5 days later.

Coliform organisms resistant to streptomycin and sulphadimidine were detected in the faeces 24 h after the *E. coli* cells containing the R factor were introduced into the rumen. Exconjugant organisms, resistant to the three antibiotics, were

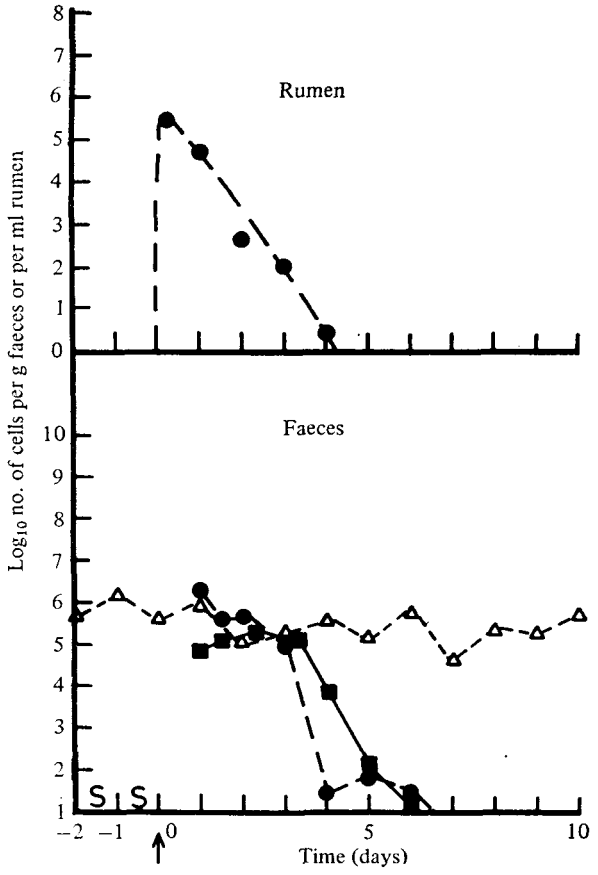

Fig. 3. Number of exconjugant cells (■), resistant to streptomycin and sulphadimidine and able to ferment lactose, in the faeces of a sheep when the animal was re-fed after being deprived of food for 2 days (S) and *ca.* $2{\cdot}0 \times 10^3$ cells of a non-lactose fermenting donor *E. coli* (●) were inoculated into the rumen (↑). The recipient cells (△) were the lactose-positive resident coliform strains normally present in the lower gastro-intestinal tract of the animal.

also detected in the faeces at this time. Much higher numbers of exconjugant organisms were detected in the faeces for a much longer time than in the experiments in which no starvation period was imposed on the inoculated animals.

Exconjugant organisms were continuously detected in the faeces of one animal for 10 days (Fig. 2) and in the second animal for 8 days. Faecal coliforms resistant to streptomycin and sulphadimidine were detected for much longer periods – 24 and 65 days respectively. In each case the prospective recipient *E. coli* organisms, resistant to nalidixic acid, were continuously excreted in large numbers (10^5–10^8 cells/g).

Transfer of an R factor to the resident coliform microflora in the lower gastro-intestinal tract of adult sheep

The five wethers known to harbour resident *E. coli* in the lower gastro-intestinal tract capable of accepting an R factor from a non-lactose fermenting donor *in vitro*

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were held without food for 2 days before *ca.* 10^3–10^4 cells of the donor organisms were introduced into the rumen. Samples taken 30 min later showed the volume of rumen fluid had diluted these organisms to less than one cell/ml. The animals were then re-fed. Unfortunately, lactose-fermenting coliforms were found to be present in the rumen of three of these animals either at the time of inoculation or were detected soon afterwards during the phase of rapid growth. Therefore, these three animals were eliminated from the experiment. However, no lactose-fermenting organisms capable of growth on MacConkey agar plates were detected in the rumen of the other two animals at any time during the experiment. The growth of the inoculated donor organisms and transfer of the R factor to resident lactose-fermenting coliforms in the lower gastro-intestinal tract was similar in both of these animals and the results obtained with one are shown in Fig. 3.

In neither animal were antibiotic resistant organisms detected in the faeces for more than 6 days.

DISCUSSION

The implantation of a known recipient *E. coli* resistant to nalidixic acid in the lower gastro-intestinal tract of sheep, although difficult, was accomplished in several animals. The longest period such organisms subsequently survived in a host animal was 105 days. Somewhat similar results were found by Cooke, Hettiaratchy & Buck (1972) in their attempts to implant different *E. coli* serotypes in the alimentary tract of human volunteers.

When donor *E. coli* were introduced into the rumen of animals carrying the implanted recipient *E. coli*, exconjugant organisms resistant to the three antibiotics were isolated from the faeces 24 h later (Figs. 1, 2). As none of the implanted nalidixic acid resistant recipients were detected in the rumen of these animals at any stage during the experiments, the transfer of the R factor from donor to recipient organisms must have occurred post rumen in the lower gastro-intestinal tract.

In previous experiments (Smith, 1975) in which the same donor and recipient organisms used in the present experiments were introduced into the rumen of sheep fed daily, no exconjugant organisms were detected in the faeces. However, in the present experiments, exconjugant organisms were detected in the faeces of such animals for 2 days (Fig. 1). The difference may be due to the fact that in the present experiments the recipient organism was a 'resident' strain. Why some strains of *E. coli* predominate for periods in the gut of animals does not appear to be well understood (Sears *et al.* 1956; Robinet, 1962) but they may become closely associated with the epithelial mucosa of the colon (Savage, Dubos & Schaedler, 1968). If this is the case then perhaps transient *E. coli* are more likely to make cell-to-cell contact with organisms of the resident microflora and hence transfer of the R factor can occur more readily.

Another possible explanation is that the transfer occurred in the faecal suspension before plating, but this seems unlikely. The faeces were blended at 20 000 rev./min for 30 s which would have sheared off the conjugative pili on the donor organisms, and platings on MacConkey agar were made 3–5 min later. This short interval was

necessary to allow the faecal suspension to separate out slightly so that the liquid phase could be pipetted. The experiments of Hayes (1957), Watanabe & Fukasawa (1961), and Brinton (1965) indicate that a substantially longer period (at least 8 min even under optimal conditions) is required for the sex pili to be resynthesized, specific pairs formed between conjugative organisms, and the transfer of an R factor or other plasmid into a recipient cell. In experiments carried out previously (Smith, 1975) when the same strains of donor and prospective recipient *E. coli* were introduced into the rumen of animals fed daily, no exconjugant organisms were detected in the faeces even though large numbers of each of the introduced organisms appeared in the faeces after 24 h. Therefore, the evidence strongly implies that in the present experiments the R factor was actually transferred *in vivo* in the lower gut and not within the faecal suspension afterwards. This would indicate that transfer of an R factor can take place more readily from transient to resident organisms in the lower gastro-intestinal tract of sheep than between two transient organisms. Similarly, Anderson (1975) found a low level of transfer of an R factor to the resident coliform microflora of a normal adult human being after a large number of prospective donor *E. coli* K12 (10^{10} cells) were administered by mouth.

A short perod of starvation (24–48 h) imposed on the experimental sheep after the donor *E. coli* were introduced into the rumen led to the detection of much higher numbers of exconjugant organisms in the faecal material (Fig. 2). Once more, exconjugant organisms were detected in the faeces 24 h after the prospective donor cells were introduced into the rumen and before the starvation period was imposed. The numbers of donor *E. coli* in the rumen were still declining at this time. However, during the starvation period, when the donor organisms grew rapidly in the rumen for a short time, and again when the host animal was re-fed and there was another brief period of growth, much larger numbers of exconjugant organisms were detected in the faeces.

Organisms harbouring the R factor persisted much longer in the gut of animals after a short starvation period but neither of the sheep used in this experiment became a prolonged carrier and organisms containing the streptomycin and sulphonamide resistance determinants could not be detected in the faeces after 24 days in one case and after 65 days in the other. Large numbers of prospective recipient *E. coli* were still present in both.

Experiments were also undertaken to transfer an R factor from a specially prepared, non-lactose fermenting donor strain of *E. coli* to the resident 'wild-type' lactose-positive coliforms carried in the lower gastro-intestinal tracts of two animals (Fig. 3). The results demonstrate that on re-feeding sheep after a period of starvation transfer appears to occur *in vivo* from even quite low numbers of transient donor organisms (10^3–10^4 cells) introduced into the rumen, to the resident microflora maintained within the lower gut. Once again, however, the R factor did not become established in the resident microflora of either of these experimental animals and was eliminated from both in 7 days.

In other experiments where donor *E. coli* were introduced into the rumen of sheep which were subjected to a short starvation period and then re-fed, coliforms con-

taining the streptomycin and sulphonamide resistance determinants were shed continuously by several animals for over 12 months afterwards (unpublished work). However, none of these animals were also carriers of the nalidixic acid resistant recipient *E. coli*.

As no antibiotics were administered to any of the animals during these experiments there should have been no selective pressure favouring growth or survival of the resistant bacteria. Thus, it is possible that under similar conditions other plasmids, and not only those containing antibiotic resistance determinants, could also be transferred between coliform organisms in the lower gut of sheep.

If R factors and perhaps other plasmids can be transferred readily between bacteria in the lower gastro-intestinal tract of sheep, such transfer probably occurs between suitable organisms in the gut of other animal species as well. It would be expected that other ruminants would behave similarly to sheep and, as antibiotic resistant organisms are widespread in non-ruminant species (Smith, 1966), it is likely that R factors can also be transferred readily in the gut of monogastric animals. Whether this occurs under conditions similar to those affecting sheep must still be determined.

There is little doubt that within the alimentary tract of animals there is not only a complex milieu of different micro-organisms, some of which are 'resident' strains with some 'transient' cells probably often present as well, but, at least under some conditions, plasmids can be transferred between them and sometimes persist in the 'resident' microflora without any apparent advantage to the new host cells. Of course, if a selective pressure is imposed, such as the administration of an antibiotic to an animal harbouring organisms containing an R factor which confers resistance to that antibiotic, these organisms would have a considerable survival advantage and either rapidly outgrow the sensitive cells or perhaps transfer the R factor to them *in vivo*. In either case the number of resistant cells within the animal would increase. As more resistant organisms would now be excreted by the animal, there is a greater possibility that other animals may ingest some. Again, under the right conditions, which may not be uncommon in nature, the R factor could be transferred to the 'resident' microflora in a new animal host. It is important to realize that the antibiotic resistant 'transient' organisms need not become implanted in the second animal, although this could happen, but only that the plasmid is transferred *in vivo* and survives in the 'resident' microflora. Thus, an R factor may rapidly become established in the gut microflora of other individuals and perhaps even of other species which have never been directly exposed to the antibiotic in question. Also, by analogy, other plasmids not containing antibiotic resistance determinants and influenced by other selective pressures could be spread in the same way.

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