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Transfer of Infectious Drug Resistance in Microbially Defined Mice

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Germ-free mice were intentionally associated with drug-resistant donor strains of *Escherichia coli* known to carry *R* factors and with drug-sensitive recipient strains. In vivo transfer of R factors was observed in all experiments, involving five different donor-recipient combinations. The number of converted recipients varied, depending upon the donor-recipient combination, but in all cases it was restricted by limiting numbers of either recipient or donor strains in the digestive tract of the microbially defined mice. Converted recipients were detected in fecal material as early as 5.5 hr after mice were associated with donor and recipient bacteria. Donors, recipients, and converted recipients were detectable in the stomach, small intestine, cecum, and large intestine of the microbially defined mice and their suckling young.

Infectious drug resistance was first recognized in Japan in 1959, when it was shown that multiple drug resistance is transferred between strains of *Shigella* and *Escherichia coli.* Subsequent investigations in many countries have indicated that infectious drug resistance in bacteria is widespread and involves an impressive number of antimicrobial agents and bacterial species (18, 20). Much work has been done on the mechanism of in vitro transfer of multiple drug resistance, but little information is available concerning in vivo transfer. In vivo transfer of infectious drug resistance was initially demonstrated in the intestinal tract of human volunteers, dogs, and mice (19), and it was concluded that transfer is less readily achieved than by in vitro methods (18). In later experiments (4, 6), antibiotics were used to reduce the intestinal flora of mice and to favor the establishment of the experimental strains. Although antibiotic administration does favor the establishment of experimental strains, the procedure also selects for antibiotic-resistant bacteria. The isolation of R factor-bearing coliforms from young chicks and laboratory mice after treatment of these animals with antibiotics only (13) points out a serious problem in experiments designed to demonstrate in vivo transfer of infectious drug resistance in conventionally reared animals. Walton (17) fed donor and recipient bacteria to newly hatched chicks and demonstrated in vivo transfer of infectious drug resistance. The success of this method was due to the ability of experimental strains of bacteria to establish themselves in the intestinal tract of the newly hatched chick in the absence of the inhibitory effect of a normal intestinal flora. These results suggested that germ-free (GF) animals, intentionally associated with donor and recipient bacteria, might serve as a convenient system for studies on in vivo transfer of infectious drug resistance.

In this paper, we show that in vivo transfer of infectious drug resistance can occur in microbially defined (MD) mice.

MATERIALS AND METHODS

Mice. Mice of the CF 1 strain were used throughout this study. GF CF 1 mice were originally obtained from Carworth, Inc., New City, N.Y., and have been maintained by breeding in a GF environment in our laboratory.

Gnotobiotic methods. GF mice were housed in plastic cages in Trexler flexible film isolators (14) and were maintained by routine gnotobiotic procedures selected for use in this laboratory (8). All mice received sterile water and sterile Wayne Sterilizable Lab-Blox ad lib. GF mice were examined weekly by established procedures (16) for microorganisms and macroparasites. We do not claim that these mice are virus-free; however, serum samples were assayed for antibodies to 12 common murine viruses and antibodies were not detected. All attempts to isolate *Mycoplasma* from these mice have yielded negative results.

To associate GF mice with drug-resistant donor or drug-sensitive recipient bacteria, we added growth from an overnight Brain Heart Infusion slant culture to 150 ml of sterile drinking water. To avoid possible interaction of donor and recipient bacteria in the drinking-water bottle, we gave the bacterial strains to the mice sequentially. After both strains were estab-

lished, the MD mice were given sterile water for the duration of the experiment.

To reduce cross-contamination between cages within the flexible film isolators, we housed experimental mice in plastic cages with individual filter tops (Isocage system, Carworth, Inc.).

Bacterial strains. The recipient used in most experiments was *E. coli* K-12 F⁻ Nal-R Trp⁻ (K-12; obtained through the courtesy of Sidney Cohen, Michael Reese Hospital, Chicago, Ill.). In two experiments, *E. coli* 100-I, isolated by us from a human urinary tract infection (12), was employed as a recipient.

Donor cultures used were *E. coli* E124 Str-R Tet-R Chm-R Sul-R (E124; kindly made available to us by David H. Smith, Harvard Medical School), *E. coli* SW Str-R Tet-R Chm-R Sul-R (SW; courtesy of Sidney Cohen), *E. coli* 20-IR Str-R Tet-R Sul-R Suc⁻, and *E. coli* K-12(20-IR) Nal-R Str-R Tet-R Sul-R Trp⁻. *E. coli* 20-IR was isolated by us from a human urinary tract infection (12). *E. coli* K-12(20-IR) was obtained as a converted recipient after mating *E. coli* K-12 with *E. coli* 20-IR in vitro (12). All donor cultures were shown, by in vitro tests (12), to contain R factors. R factors were not detectable in recipient cultures. The following abbreviations are used for phenotypic traits: Nal-R, resistant to nalidixic acid (NA); Str-R, resistant to streptomycin; Tet-R, resistant to tetracycline; Chm-R, resistant to chloramphenicol; Sul-R, resistant to sulfamethizole; Trp⁻, tryptophan-dependent; Suc⁻, unable to ferment sucrose.

Selective media. In experiments with *E. coli* K-12 as recipient, Mueller Hinton Broth (Difco) containing 2% agar (BBL) was supplemented with 100 μg of NA (Sterling-Winthrop) per ml, 25 μg of tetracycline hydrochloride (T; Lederle) per ml, or both NA and T in the above concentrations (NAT). In these experiments, the recipients grew on NA, the donors grew on T, and the converted recipients grew on NA, T, and NAT.

In an experiment involving *E. coli* K-12(20-IR) as donor and *E. coli* 100-I as recipient, minimal medium (1) was supplemented with 0.2% glucose (MG); 0.2% glucose and 25 μg of T per ml (MGT); or 0.2% glucose, 100 μg of tryptophan per ml, and 100 μg of NA per ml (MNA). K-12(20-IR) grew on MNA, and 100-I grew on MG. Converted recipients grew on MG and were the only organisms involved which could grow on MGT.

In an experiment involving *E. coli* 20-IR as donor and *E. coli* 100-I as recipient, minimal medium (1) was supplemented with 0.2% sucrose (S), 0.2% glucose and 25 μg of T per ml (GT), or 0.2% sucrose and 25 μg of T per ml (ST). Strain 20-IR grew on GT, and 100-I grew on S. Converted recipients grew on GT and S and were the only organisms involved which could grow on ST. Thus, in all experiments, it was possible to determine the number of donor, recipient, and converted recipient cells.

Assay procedure. Fresh fecal specimens were collected in sterile screw-cap tubes, at various times, from mice intentionally associated with donor or recipient bacterial strains, or both. After removal from the microbially defined unit, one fecal pellet from each mouse was macerated, by using a blunt glass rod, in 4.0 ml of Mueller Hinton Broth. Portions (0.2 ml) of such suspensions and various dilutions of the suspensions were spread on selective media. Plates were incubated at 37 C until colonies were suitable for counting. Counts are expressed as the number of viable cells per milliliter of fecal homogenate.

It is possible, although unlikely, that mating between donor and recipient cells might occur during the assay procedure, during maceration and dilution of the fecal specimen, or on the selective media during the incubation period. Thus, the following control was included. A fecal pellet from a mouse associated with donor only and a pellet from a mouse associated with recipient only were macerated in the same tube, and material from this suspension was plated as described above. If mating were to occur during the assay procedure, one would expect converted recipients to appear in this control. In addition, drinking water bottles were routinely tested for the presence of converted recipients. Converted recipients were never detected in these controls, nor in mice associated with donors or recipients only. The mice associated with donors or recipients only also served as controls for spontaneous mutations involving drug resistance and fermentation patterns.

Drug resistance patterns of donors, recipients, and converted recipients were determined by replica plating onto Mueller Hinton Agar containing, singly, the following chemotherapeutic agents: T, 25 μg/ml; sulfamethizole, 100 μg/ml; streptomycin (Squibb), 10 μg/ml; and NA, 100 μg/ml. Growth requirements were determined by replica plating onto minimal medium (1) supplemented with appropriate combinations of glucose (0.2%), sucrose (0.2%), and tryptophan (100 μg/ml).

RESULTS

In vivo mating of E. coli 20-IR and E. coli K-12. Ten GF mice were associated with 20-IR. The mice were associated with K-12 9 days later. Fecal specimens from these MD mice were collected and examined at frequent intervals, during a 31-day observation period, for the presence of donors (20-IR), recipients (K-12), and converted recipients [K-12(20-IR)].

The MD mice were sampled immediately before (day 0) and 24 hr after (day 1) adding K-12 to the water supply. Only donors were detected on day 0. Donors, recipients, and converted recipients were present in large numbers in samples collected on day 1 and in all subsequent samples (Fig. 1). Thus, transfer of the R factor did occur, and the converted recipients persisted without the selective pressure of antibiotics. The number of converted recipients obtained was limited because the recipient population was always considerably smaller than the donor population.

Converted recipients were analyzed for drug resistance pattern and for tryptophan require-

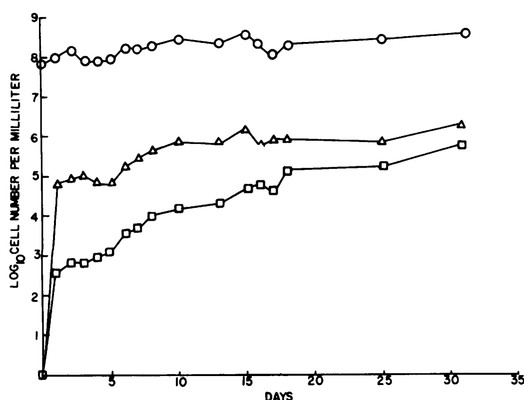

FIG. 1. *Number of donors (20-IR, ○), recipients (K-12, △), and converted recipients [K-12(20-IR), □] per milliliter of fecal homogenate. Strain 20-IR was added to the water supply on day 9, and K-12 was added on day 0. Points through day 13 are the average from 10 mice; points at later times are the average from 4 mice.*

ment. All converted recipients required tryptophan. Segregation of drug resistance markers was not observed; all of 955 converted recipient cultures examined were resistant to tetracycline, sulfamethizole, and streptomycin, the drug resistances previously shown to be transferred in vitro from 20-IR to K-12 (12).

During the course of this experiment, two of the MD females bore young. Of these young mice, four were sacrificed at weaning, and fecal specimens and contents of the stomach, small intestine, cecum, and large intestine were diluted and plated on selective media. Donors, recipients, and converted recipients were found in moderate numbers in the stomach and small intestine and in large numbers in the cecum, large intestine, and in fecal material. The number of donor cells was greater in all locations than the number of recipient and converted recipient cells.

In an attempt to increase the number of converted recipients obtained, we conducted a second experiment in which the order of association of GF mice with donor and recipient cultures was reversed. In this experiment, K-12 was added to the water supply on day −5 and 20-IR was added on day 0. The number of K-12 rapidly decreased and the number of 20-IR rapidly increased (Fig. 2) after we added 20-IR. Thus, the number of converted recipients obtained was again limited by the number of recipient cells. In this experiment, converted recipients were detected in fecal specimens from two of six mice at the first sampling, 5.5 hr after adding 20-IR to the water supply. Converted recipients were detected in all mice at all subsequent samplings (Fig. 2).

At the termination of the second experiment (day 37), four of the MD mice were sacrificed, and the contents of the stomach, small intestine, cecum, and large intestine were diluted and plated on selective media. Donors, recipients, and converted recipients were detected throughout the gastrointestinal tract. Again, the number of donor cells always exceeded the number of recipient and converted recipient cells.

Other in vivo matings. Five additional in vivo matings were performed, and transfer of drug resistance was observed in all cases (Table 1). As in the two experiments previously described, the number of converted recipients was limited in all matings because of limiting numbers of either donor or recipient cells. Thus, in matings involving *E. coli* E124 and *E. coli* SW as donors and *E. coli* K-12 as recipient, E124 and SW were present in fecal homogenates in numbers 100-fold greater than the number of K-12. Similarly, K-12(20-IR) and 20-IR were outgrown by 100-I (Table 1).

DISCUSSION

In spite of a wealth of knowledge concerning the mechanisms of genetic exchange in bacterial cells, the significance of such exchange in producing new phenotypes in nature has not been determined. Limited direct experimentation suggests that such genetic exchange may be significant in nature. Thus, Velaudapillai (15) first demonstrated transduction of bacteria in vivo by showing that transduction of factors controlling the synthesis of flagellar antigens could

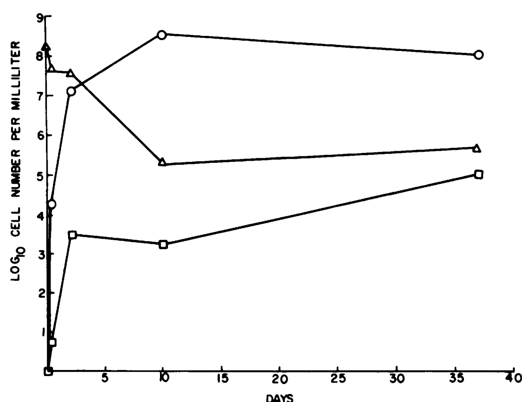

FIG. 2. *Number of donors (20-IR, ○), recipients (K-12, △), and converted recipients [K-12(20-IR), □] per milliliter of fecal homogenate. K-12 was added to the water supply on day 5 and 20-IR was added on day 0. Points are the average from four mice.*

TABLE 1. *Summary of R factor transfers in MD mice*

Donor[a]	Recipient[a]	No. of transfers observed[b]	Avg no.[c] of donor, recipient, and converted recipient cells at termination of expt			
			Day of termination	Donor	Recipient	Converted recipient
20-IR (1)	K-12 (2)	10/10	31	3.9×10^8	2.1×10^6	6.5×10^5
20-IR (2)	K-12 (1)	6/6	37	9.8×10^7	3.8×10^5	9.2×10^4
E124 (1)	K-12 (2)	1/3	3	1.3×10^8	4.7×10^4	0
E124 (2)	K-12 (1)	1/1	2	9.0×10^7	5.5×10^5	10
SW (2)	K-12 (1)	2/2	8	3.2×10^8	3.4×10^6	8.8×10^2
K-12(20-IR) (1)	100-I (2)	5/6	21	7.9×10^4	8.6×10^6	37
20-IR (2)	100-I (1)	5/5	22	1.3×10^4	1.8×10^8	19

[a] The numbers in parentheses after strain designations indicate the order the strains were fed to mice.
[b] Numerators are the number of mice in which transfer of drug resistance was observed at some time during the course of the experiment; denominators are the number of mice used in the experiment.
[c] Expressed as number of viable cells per milliliter of fecal homogenate.

occur between strains of *Salmonella* species in live chick embryos and in mice. Other investigators (5, 9) have shown that transduction of *Staphylococcus aureus* to drug resistance can occur in vivo. The work of Novick and Morse (9) is especially relevant. In their experiments, mixed kidney infections were produced in mice by using lysogenic strains of donor and recipient bacteria which differed in drug resistance patterns. In each instance in which heavy infection was established, recombinants were isolated. Recombinants were present in the mice in small numbers, but increased to very large numbers when selected by the administration of a combination of drugs which would kill the parental strains. In their system, transduction of plasmids, with genes determining drug resistance, occurred without the use of exogenously administered bacteriophage.

Conjugation has also been shown to occur in vivo. Schneider and co-workers (11) fed mice, pretreated with antibiotics, an Hfr culture of *E. coli* and later an F⁻ culture of *Salmonella typhimurium*. Hybrids were recovered from the feces of the mice within 24 hr. Significantly, hybrids were recovered which had not been observed in single matings performed in vitro. Conjugation and formation of recombinants between two strains of *E. coli* have been reported to occur in the digestive tract of MD mice (2).

In the present work, we have shown that transfer of R factors from drug-resistant to drug-sensitive bacteria can occur in the digestive tract of MD mice. The converted recipients obtained were indistinguishable from those derived from in vitro matings. These results confirm and extend the observations of Salzman and Klemm (10). Converted recipients were not detected in mice associated with donors or recipients only, nor were they detected in water bottles or in experiments designed to test the in vitro assay procedures (*see above*). Thus, the converted recipients must have originated in the digestive tract of the MD mice.

The limited number of converted recipient cells obtained in these experiments requires comment, since, in the in vivo experiments of Schneider and co-workers (11), the number of hybrid cells was frequently larger than the number of donor or recipient cells. In our experiments, converted recipients were always outnumbered by donor cells and never exceeded the number of recipient cells (Fig. 1 and 2, Table 1). This discrepancy is most probably the result of two factors. At any given time, most of the Hfr donor cells would be competent donors. In contrast, except for high-frequency donor activity of cells newly infected with R factors, the R factor-carrying donors used in our studies were self-repressed, and only an occasional cell was capable of mating at any given time. In addition, in our experiments, donor and recipient strains were never established in the intestinal tract in similar numbers; thus, one requirement for optimal transfer of R factors was not obtained. High frequency transfer by recently infected cells was limited by the small recipient population. Repetition of these experiments with donor and recipient strains which establish in the gut in similar numbers would probably yield a larger number of converted recipients. The reasons for differences in growth rates of donor and recipient strains are not apparent to us, although, in some instances, similar differences have been noted in vitro.

Converted recipients could be detected in

fecal material as early as 5.5 hr after associating mice with donor and recipient bacteria (Fig. 2). Donors, recipients, and converted recipients were distrubuted throughout the digestive tracts of mice intentionally associated with donor and recipient strains and in the suckling young of these mice. In some crosses, converted recipients persisted in large numbers for long periods of time (Fig. 1 and 2; Table 1); in other crosses, the converted recipients failed to persist. The factors determining persistance have not been discovered.

There are differences between the gastrointestinal tracts of GF and conventionally reared animals (3); thus, we may not extrapolate our findings in MD mice to conventional animals. However, we believe that the MD mouse is a well-defined, convenient biological system which will be useful in studies designed to determine the effects of other microorganisms, antibiotics, and chemicals (7) on the in vivo transfer of R factors.

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