JOURNAL OF BACTERIOLOGY
Vol. 88, No. 2, p. 322-328 August, 1964


# TRANSFER OF DRUG RESISTANCE BETWEEN ENTERIC BACTERIA INDUCED IN THE MOUSE INTESTINE

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Received for publication 10 March 1964

ABSTRACT

KASUYA, MORIMASA (Nagoya University School of Medicine, Nagoya, Japan). Transfer of drug resistance between enteric bacteria induced in the mouse intestine. J. Bacteriol. **88**:322-328. 1964.—Transfer of multiple drug resistance in the intestines of germ-free and conventional mice was studied with strains of *Shigella*, *Escherichia*, and *Klebsiella*. The transfer experiment was carried out under antibiotic-free conditions to eliminate the production of drug-resistant bacteria by antibiotics. All resistance factors (chloramphenicol, streptomycin, tetracycline, and sulfathiazole) were transferred with ease in the intestinal tracts of mice, when donors and recipients multiplied freely, and acquired resistance was further transferred to other sensitive enteric bacteria in the intestinal tract. Bacteria to which resistance factors were transferred showed, in most of the experiments, exactly the same level and pattern of resistance as the donors. Based on the above, a hypothesis that the same process may possibly occur in the human intestine is presented.

In Japan, information on the transfer of drug resistance in enteric bacteria has accumulated since Ochiai et al. (1959) and Akiba et al. (1960) reported investigations of the possible transfer of multiple drug resistance by mixed cultivation.

Most of these studies were carried out in vitro, with the exception of in vivo experiments done by Kagiwada et al. (1960) with human volunteers and by Akiba et al. (1961) with a volunteer and animals. In both cases, however, the effects of administered antibiotics upon the origin or selection of drug-resistant bacteria were not considered at all.

In studies of this nature, in vivo difficulties usually arise in eliminating the occurrence of bacterial resistance to the antibiotics administered, and in conducting experiments with only the "infecting strains" without contamination by other bacteria. In this study, therefore, attention was directed to the above, resulting in satisfactory infection of mice by oral administration of *Shigella*, *Klebsiella*, and *Escherichia* under antibiotic-free conditions. A germ-free mouse, which should theoretically be most suited for such an experiment, was fortunately available, and was tested in parallel.

The present communication deals with a study on the transfer of drug resistance in vivo among the above-mentioned bacteria. A preliminary report on some phases of this study was already published (Kasuya, 1962).

## MATERIALS AND METHODS

*Animals.* One germ-free female mouse weighing 18 g (supplied by M. Miyagawa, originally from the University of Notre Dame) and 11 conventional male mice weighing approximately 25 g were used. Six series of experiments were carried out with two animals each.

*Organisms. S. flexneri* 3a strain Kurano (supplied by K. Ochiai) and *E. coli* strain LNH (isolated from a mouse) were used as originally resistant donors. Both are resistant to chloramphenicol, streptomycin, tetracycline, and sulfathiazole. Strain Kurano is a erythromycin-resistant mutant selected from the parent strain by the gradient broth method. As sensitive recipients, *K. pneumoniae* strain Kasuya (isolated from a human being), *E. coli* strain M1 (isolated from a mouse), *S. flexneri* 2a strain K (from stock culture), and *S. flexneri* 2a strain Yamada (supplied by S. Naito) were used.

*Drugs and media.* Chloramphenicol powder (Parke, Davis & Co., Detroit, Mich.), dihydrostreptomycin sulfate (Takeda Chemical Industries, Ltd., Osaka, Japan), tetracycline hydrochloride powder (Lederle Laboratories, Pearl River, N.Y.), sulfathiazole (Takeda), and erythromycin lactobionate (Abbott Laboratories, North Chicago, Ill.) were used. For selection of drug-resistant colonies (strains), SS Agar and Desoxycholate Agar (Nihoneiyokagaku Co., Ltd., Tokyo, Japan) containing 12.5 μg of chlor-

amphenicol, 12.5 μg of streptomycin, 12.5 μg of tetracycline, or 1 mg of sulfathiazole per ml, as well as the four drugs combined, were prepared and used. Multiplication of the administered bacteria in intestinal tracts and contamination of mice with other bacteria were determined by fecal cultures with SS Agar, Desoxycholate Agar, EMB Agar (Nihoneiyokagaku Co., Ltd.), Enterococcus Confirmatory Agar and Broth (Difco), and beef extract-peptone-agar.

*Infection in mice.* These procedures were carried out by a modification of the technique of Freter (1956), Cooper (1959), and Cooper and Pillow (1959). With two polyvinyl chloride stomach tubes, a germ-free mouse was fed 0.1 ml of a 24-hr broth culture of the donor and later of the recipient without preliminary treatment with antibiotics. In conventional mice, 2 days before administration of the donor, 10 mg of streptomycin and 2 mg of erythromycin in 0.3 ml of water were first given by a stomach tube; later, tap water containing 1.5 mg of streptomycin and 0.025 mg of erythromycin per ml was given ad libitum. At the end of the 2 days, mice were again given 0.3 ml of a solution containing 10 mg of streptomycin and 2 mg of erythromycin by a stomach tube, and with another stomach tube the donor was then given to each animal. The mice were returned to their respective cages, and given food and water containing streptomycin and erythromycin ad libitum, up to 48 hr before administration of the recipient. For some days afterward, fecal cultures of the mice were carried out every other day, to determine that the donors had multiplied and that no contamination had occurred. The animals were then transferred to new cages, and were fed with sterilized food and boiled tap water free from antibiotics. After 2 days, the recipients were given, and fecal cultures were carried out every day for the succeeding 10 days.

*Fecal culture.* A stool pellet obtained directly from each animal was emulsified with a pestle in a mortar containing 1 ml of sterile saline, and 0.1 ml of the emulsion was spread evenly over the surface of drug-containing and drug-free agar plates by means of bent glass rods. The plates were then incubated at 37 C for 24 hr, and were examined for counts of colonies of the recipients. Five colonies from each plate containing the drugs were then selected at random, and were isolated and examined for resistance to drugs by the disk method (Eiken). When the colonies were fewer than five in number, all were examined for their drug resistance. The identities of the colonies examined were determined by biochemical reactions and slide agglutinations with specific antisera.

When the recipient was a *Shigella*, SS Agar was used as the drug-containing medium; in the case of *Escherichia* or *Klebsiella*, Desoxycholate Agar was used.

*Breeding environment.* The animals were bred as germ-free as possible to prevent contamination. For drinking water they were given tap water sterilized in an autoclave at 121 C for 15 min, and for food, germ-free solid food (supplied by Germfree Life Research Laboratory, Nagoya University School of Medicine) or solid food sterilized in a hot-air oven for 2 hr. The cages, water bottles, etc., were also sterilized, at 121 C for 15 min or at 80 to 100 C for 1 hr. The mice were kept isolated in their own cages with a wire-mesh bottom, under conditions that could minimize, if not prevent, coprophagy, and the cages were placed in a box with commercial lamps which radiated rays of 2,537 A.

## RESULTS

In preliminary experiments, a loopful of a 24-hr broth culture of the recipient was streaked on four plates, each containing a different type of drug, and on one containing all four drugs. When the plates were incubated at 37 C for 24 hr, the recipients did not grow. In addition, a loopful of a 1:1 mixture of a 24-hr broth culture of donor and recipient was streaked similarly on the five drug-containing plates. When the plates were incubated, the donor grew abundantly, but the recipient did not, as was expected. From the above, it was concluded that the recipients were sensitive to the drugs, and transfer of drug resistance between the experimented strains did not take place on the plates.

*Experiment 1.* In experiment 1, transfer of resistance from *S. flexneri* 3a to *K. pneumoniae* was determined (Table 1). (In this experiment, a germ-free mouse was used.) Throughout the experiment, both donor and recipient grew abundantly on SS and Desoxycholate Agar free from antibiotics. Two or three colonies of the recipient were first found to grow on Desoxycholate Agar plates containing chloramphenicol, tetracycline, sulfathiazole, or the four drugs, on the second day after administration of the recipient. Thereafter, there was a tendency for

TABLE 1. *Number of colonies of recipient and donor developing on drug-containing and drug-free media after oral administration to a germ-free mouse of resistant Shigella flexneri 3a strain Kurano and sensitive Klebsiella pneumoniae strain Kasuya**

| Organism | Medium | Drug† | Day after challenge | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *S. flexneri* 3a | SS Agar | Free‡ | +3 | +3 | +3 | +3 | +3 | +3 | +3 | +3 | +3 | +3 |
| *K. pneumoniae* | Desoxycholate Agar | Free‡ | +3 | +4 | +4 | +4 | +3 | +4 | +4 | +3 | +3 | +3 |
| | | CM | 0 | 3 | 16 | 20 | 1 | 77 | 95 | 16 | 13 | 9 |
| | | TC | 0 | 3 | 31 | 37 | 0 | 78 | 72 | 19 | 10 | 12 |
| | | SM | 0 | 0 | 17 | 31 | 0 | 61 | 61 | 15 | 8 | 14 |
| | | ST | 0 | 2 | 22 | 45 | 1 | 72 | 39 | 19 | 1 | 9 |
| | | CM, TC, SM, and ST | 0 | 3 | 16 | 39 | 0 | 74 | 59 | 7 | 13 | 13 |

* Number of visible colonies developing on a plate spread with 0.1 ml of an emulsified stool after incubation at 37 C for 24 hr.

† CM = chloramphenicol; TC = tetracycline; SM = streptomycin; ST = sulfathiazole. Amount of CM, TC, and SM in the medium was 12.5 μg/ml, and that of ST was 1 mg/ml.

‡ The number of visible colonies developing on drug-free media is graded +1 to +4; +1 = <200; +2 = >200; +3 = >+2 plus adherent colonies; +4 = >+3 with most colonies adherent.

TABLE 2. *Number of colonies of recipient and donor developing on drug-containing and drug-free media after oral administration to a conventional mouse of resistant Shigella flexneri 3a strain Kurano and sensitive Escherichia coli strain M1**

| Organism | Medium | Drug | Day after challenge | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *S. flexneri* 3a | SS Agar | Free | +3 | +3 | 0 | +2 | +2 | +1 | +1 | +1 | +1 | +1 |
| *E. coli* | Desoxycholate Agar | Free | +3 | +3 | +3 | +3 | +3 | +3 | +3 | +3 | +3 | +3 |
| | | CM | 0 | 0 | 0 | 1 | 4 | 98 | 81 | 222 | $10^3$ | 227 |
| | | TC | 0 | 0 | 0 | 0 | 12 | 96 | 56 | 218 | $10^3$ | 237 |
| | | SM | 0 | 0 | 0 | 0 | 9 | 96 | 81 | 212 | $10^3$ | 226 |
| | | ST | 0 | 0 | 0 | 0 | 5 | 88 | 73 | 209 | $10^3$ | 199 |
| | | CM, TC, SM, and ST | 0 | 0 | 0 | 0 | 5 | 98 | 83 | 182 | $10^3$ | 248 |

* See footnotes to Table 1.

increase in number with prolongation of culture, but a decrease in number was also seen on certain days of the 8-day period of observation; the numbers of the colonies on the five drug-containing plates of a definite day were approximately equal. The five colonies selected at random from each plate were examined for their sensitivities to the drugs, and it was found that all colonies were resistant to the four drugs at an equal level and in the same pattern.

A control experiment, with administration of *K. pneumoniae* alone and no donor, failed to show the growth of resistant colonies.

*Experiment 2.* Transfer of resistance from *S. flexneri* 3a to *E. coli* was determined in experiment 2. (In experiments 2 to 6, conventional

TABLE 3. *Number of colonies of recipient and donor developing on drug-containing and drug-free media after oral administration to a conventional mouse of resistant Escherichia coli strain LNH and sensitive Klebsiella pneumoniae strain Kasuya**

| Organism | Medium | Drug | Day after challenge | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *E. coli* | SS and Desoxycholate Agar | Free | +3 | +3 | +3 | +3 | +3 | +3 | +3 | +3 | +3 | +3 |
| *K. pneumoniae* | Desoxycholate Agar | Free | +3 | +3 | +3 | +3 | +3 | +3 | +2 | +2 | +1 | 0 |
| | | CM | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | TC | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | SM | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | ST | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | | CM, TC, SM, and ST | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

* See footnotes to Table 1.

mice were used.) As shown in Table 2, large amounts of both donor and recipient were continuously excreted. One colony of the recipient was first found to grow on the Desoxycholate Agar plate containing chloramphenicol on the fourth day after challenge with the recipient. Thereafter, the colonies of the recipient increased in number, and colonies developing on the five drug-containing plates were also found to be almost equal in number. Examination of their sensitivities to the drugs revealed all the colonies to be resistant to the four drugs to an equal level.

When *E. coli* M1 alone was given, no resistant colony was found to develop.

*Experiment 3.* Transfer of resistance from *E. coli* to *K. pneumoniae* was determined in experiment 3 (Table 3). Lactose-nonfermenting *E. coli* LNH was used as donor. Excretion of the donor and recipient continued, but recipient colonies developing on the drug-containing plates numbered only two during the 10 days of observation, probably owing to the transfer of resistance factors being less frequent here than in the preceding cases. One of the two colonies was resistant to the four drugs, and the other was resistant only to three (streptomycin, tetracycline, and sulfathiazole).

In the case of administration of the recipient alone, no resistant colony was found to develop.

*Experiment 4.* Retransfer of resistance from *K. pneumoniae* to *S. flexneri* 2a was attempted in experiment 4. A resistant *K. pneumoniae* strain Kasuya 4R6-1, isolated in experiment 1 from the Desoxycholate Agar plate containing the four drugs on the sixth day after the administration of recipient, was used as donor, and further transfer of the acquired resistance was attempted. The recipient used was *S. flexneri* 2a strain K.

Excretion of the donor occurred abundantly, but on a drug-free SS Agar plate the recipient numbered only about 200. The colonies of the recipient developing on the plates containing drugs totaled only two, one developing on the 10th day and the other on the 15th day after administration of the recipient. These results indicate probably that the transmission frequency between the two strains is less than $10^{-2}$ per recipient cell. The two colonies were found to be resistant to all four drugs.

In the case of administration of the recipient alone, no resistant colony of the recipient was found to develop during the 15 days after administration of the recipient.

*Experiment 5.* Retransfer of resistance from *E. coli* to *S. flexneri* 2a was attempted in experiment 5. A resistant *E. coli* strain M1 4R5-1 $A^r$, isolated in experiment 2 from the Desoxycholate Agar plate containing the four drugs on the fifth day after the administration of recipient, was used as donor. The recipient used was *S. flexneri* 2a strain K.

Excretion of the donor occurred abundantly, but the recipient was excreted poorly and rapidly disappeared from the feces. Therefore, the re-

TABLE 4. *Number of colonies of recipient and donor developing on drug-containing and drug-free media after oral administration to a conventional mouse of resistant Escherichia coli strain M1 4R5-1 A$^r$ and sensitive Shigella flexneri 2a strain Yamada**

| Organism | Medium | Drug | Day after challenge | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *E. coli* | Desoxycholate Agar | Free | +2 | +2 | +1 | +3 | +2 | +2 | +3 | +3 | +3 | +3 |
| *S. flexneri* 2a | SS Agar | Free | +2 | +2 | +2 | +3 | +2 | +3 | +3 | +2 | +3 | +2 |
| | | CM | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 5 |
| | | TC | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 3 |
| | | SM | 10 | 500 | 160 | 464 | $10^3$ | $10^3$ | $10^3$ | $10^3$ | $10^3$ | 872 |
| | | ST | 105 | 400 | 300 | $10^3$ | $10^3$ | $10^3$ | $10^3$ | $10^3$ | $10^3$ | $10^3$ |
| | | CM, TC, SM, and ST | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

* See footnotes to Table 1.

cipient was administered again to the same mouse on the fifth day after the first administration of recipient. However, excretion of the recipient was poor, and the colonies of the recipient failed to develop on SS Agar plates containing drugs during the 6 days of observation after reinfection.

The two colonies of the recipient recovered on a drug-free SS Agar plate on the sixth day after reinfection were therefore isolated and examined for their sensitivities to drugs; both were found to be resistant to streptomycin and sulfathiazole.

When the recipient alone was given, no resistant colony was found to develop.

*Experiment 6.* Retransfer of resistance from *E. coli* to *S. flexneri* 2a was attempted in experiment 6. Excretion of *S. flexneri* 2a strain K was found to be poor in experiments 4 and 5; hence, five sensitive strains of *S. flexneri* (2a, 2a, 2b, 2b, and 3a) were examined for possible multiplication in the mouse intestine. Two of the five strains, 2a and 2b, were abundantly recovered from the feces of infected animals pretreated with antibiotics. One of these two strains, *S. flexneri* 2a strain Yamada, was therefore used as recipient in this experiment. As donor, *E. coli* strain M1 4R5-1 A$^r$ was again used.

Large amounts of both donor and recipient were excreted constantly during the course of the experiment, and a large number of resistant colonies of the recipient were recovered (Table 4). Most of these resistant colonies were repeatedly found to grow on SS Agar containing streptomycin or sulfathiazole, and some were found to develop first on SS Agar containing chloramphenicol or tetracycline on the ninth and tenth days after administration of the recipient. The former group of colonies was resistant to two drugs (streptomycin and sulfathiazole), and the latter, to the four drugs.

When the recipient alone was given, no resistant colony was found to grow.

## DISCUSSION

The preliminary finding that drug resistance cannot be transferred on SS and Desoxycholate Agar plates was confirmed. It was also proven that resistant recipients obtained in the present experiments were neither spontaneous mutants nor contaminants, from the control experiments on administration of recipients alone.

In the experiment with a germ-free mouse, the effect of antibiotics in producing drug-resistant bacteria may be ignored, as no drugs were given. Therefore, the fact that generation of resistant recipients in the intestinal tract occurred after administration of a resistant donor and a sensitive recipient indicates clearly the possible transfer of drug resistance in vivo between a previously existing donor and a recipient entering subsequently. In experiments with conventional mice, an antibiotic-free state was produced 2 days before administration of the recipient. Therefore, the results obtained from these cases also indicate the possible transfer of drug resistance in vivo.

TABLE 5. *Patterns of acquired resistance**

| Expt no. | Donor | | Recipient | |
|---|---|---|---|---|
| | Organism | Resistant to | Organism | Resistance acquired |
| 1 | *Shigella flexneri* 3a strain Kurano | CM, TC, SM, ST | *K. pneumoniae* strain Kasuya | CM, TC, SM, ST |
| 2 | *S. flexneri* 3a strain Kurano | CM, TC, SM, ST | *E. coli* strain M1 | CM, TC, SM, ST |
| 3 | *Escherichia coli* strain LNH | CM, TC, SM, ST | *K. pneumoniae* strain Kasuya | (1) TC, SM, ST<br>(2) CM, TC, SM, ST |
| 4 | *Klebsiella pneumoniae* strain Kasuya 4R6-1 | CM, TC, SM, ST | *S. flexneri* 2a strain K | CM, TC, SM, ST |
| 5 | *E. coli* strain M1 4R5-1 $A^r$ | CM, TC, SM, ST | *S. flexneri* 2a strain K | SM, ST |
| 6 | *E. coli* strain M1 4R5-1 $A^r$ | CM, TC, SM, ST | *S. flexneri* 2a strain Yamada | (1) SM, ST<br>(2) CM, TC, SM, ST |

* CM = chloramphenicol; TC = tetracycline; SM = streptomycin; ST = sulfathiazole.

The germ-free cages used were structurally not perfect, but were believed to prevent bacterial contamination of mice. Mice exposed to ultraviolet rays and poorly nourished were attacked with alopecia, dermatitis, and ophthalmia, and did not increase in body weight, but did not die during the course of experiments.

Experiments 4 and 5 showed failure of multiplication of *Shigella* in the intestines of mice. When the susceptibilities of mice to enteric infections were examined with five strains of *Shigella*, it was found that two strains, 2a and 2b, could continue to be recovered in large numbers from the feces, but the remaining three strains, 2a, 2b, and 3a, disappeared from the feces within 2 or 3 days. Hence, the existence of a relationship between possible multiplication of *Shigella* in the intestine of mice and their serotypes was not noted. These findings suggest that selection of a strain which may multiply in the intestinal tract of an experimental animal is necessary for the success of this type of investigation. In the other four experiments, a considerable number of recipient colonies grew on the plates and resistant colonies of the recipients could be selected and isolated, so technical difficulties did not arise.

All the results obtained are briefly summarized in Table 5. Resistance factors were transferred in all cases, and acquired resistance was further transferred in experiments 4, 5, and 6. In four of the six experiments, bacteria to which resistance had been transferred showed single patterns and levels of resistance common to each group. In the remaining two, the patterns of resistance were of two types but the levels of resistance to specific drugs were equal. It was thus found that transfer of drug resistance in vivo occurs according to a definite mechanism, and it is believed that the mechanism is cell-to-cell contact. In experiments 1 and 2, and 5 and 6, the patterns of acquired resistance were found to be exactly the same, when resistance factors were passed from the same donor to recipients of a different genus. From experiments 1 and 3, and 4 and 5, if transfer in vivo occurred between donors of a different genus and the same recipient, the patterns of acquired resistance might differ, or be the same. It seems, therefore, that the pattern of acquired resistance is determined more by the donor than by the recipient.

Watanabe and Fukasawa (1961*a*, *b*) reported that the level of the resistance transferred by mixed cultivation might differ from recipient to recipient. In the present study, however, the level did not differ from recipient to recipient, a point that needs more detailed investigation.

In two experiments, bacteria to which resistance had been transferred showed resistance to two drugs only, streptomycin and sulfathiazole. These results support the suggestion by Ochiai, Yamanaka, and Kimura (1961) that resistance factors of streptomycin and sulfathiazole are transferred together.

The numbers of colonies of the recipients which

grew on plates containing drugs were compared with those recovered from the same feces spread on drug-free plates on a definite day, and their ratios were obtained with the latter as denominator. The results were $10^{-3}$ to $10^{-5}$ per recipient cell, but in experiment 6 the ratio was $10^{-2}$. Harada et al. (1961*a*, *b*, *c*) reported that this ratio is $10^{-4.5}$ to $10^{-6}$ per recipient cell in vitro, so transfer of resistance in vivo may occur at the same frequencies as in vitro.

From the results obtained above, it may be considered that in the human intestines too, if donor and recipient multiply together, resistance factors may be transferred also. Thus, generation of drug-resistant enteric bacteria in the human intestine may occur owing to the transfer of resistance from one previously existing enteric bacteria to a newcomer, or vice versa, from a newly arriving resistant bacteria to a previously existing enteric bacteria.

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to K. Ogasawara of the Department of Bacteriology, Nagoya University School of Medicine, and to K. Ochiai of the Nagoya Municipal Higashi Shimin Hospital, for their encouragement and helpful discussions in conducting the present study; to M. Miyagawa of the Germ-free Life Research Laboratory, Nagoya University School of Medicine, for supplying germ-free mouse and food; and to S. Naito of the Nagoya Municipal Higashi Shimin Hospital for supplying *Shigella* strains.

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