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Transfer of Antibiotic Resistance (R factor) in the Mouse Intestine* (33020)

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In *E. coli* and other species of the family *Enterobacteriacae* the genes responsible for antibiotic resistance are generally part of extrachromosomal elements (episomes) known as R factors (1–5). During conjugation R factors are transmitted to susceptible organisms with a frequency varying from about 10^{-2} to less than 10^{-7}/hour per donor cell (6). However, bacteria recently acquiring an R factor can transmit with a frequency greater than 1/hour per donor cell (6). In a given instance, the frequency of transfer depends upon the specific R factor, the specific mating pair, the growth medium, and the conditions of growth. However, R factors replicate faster than the bacterial chromosome and, *in vitro,* it is not unusual for a high proportion of a population of susceptible bacterial cells to acquire drug resistance when grown under suitable conditions with an appropriate donor.

The widespread prevalence of antibiotic resistant *Enterobacteriacae* in man and domestic animals results, primarily, from the selective pressure of antibiotic therapy in these species. However, the infectivity of R factors, their capacity for autonomous replication, and the lack of any known adverse effect upon bacterial cells, suggest that they can survive and spread among bacteria in nature without the selective pressure of antibiotic therapy.

The normal habitat of most species of *Enterobacteriacae* is the mammalian intestinal tract and several strains can be found in the same gut. Although there is epidemiologic (6) and experimental (7,8) evidence that R factor transfer can occur in the intestine, little is known of the rate of spread of R factors among populations of susceptible bacteria.

For conjugation to occur in the intestinal tract efficiently, the animal must necessarily be colonized by large numbers of donor and recipient bacteria. It has proven difficult to experimentally colonize animals with most nonenteropathic strains of *Enterobacteriacae* unless the animals' indigenous flora has first been suppressed by oral antibiotics. Moreover, heavy colonization is usually not long lasting; presumably the normal inhabitants of the intestinal flora grow back and again repress the implanted strains. The antibiotics may also favor the growth of resistant organisms and therefore the proportion of resistant bacteria in the flora may not be a measure of the efficiency of R factor transfer alone.

This study presents results derived from the purposeful colonization of germ-free mice. Obviously, bacterial antagonism from indi-

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genous organisms was not a problem and the mice were easily colonized by both the potential donor and recipient bacteria, without resort to antibiotic treatment.

Methods. E. coli K12, F^- was the recipient strain. To allow selective plating, a variant resistant to nalidixic acid was used. The donor, strain AK, was a *Klebsiella pneumoniae* isolated from a human infection. It was susceptible to nalidixic acid but resistant to ampicillin, tetracycline, streptomycin, chloramphenicol, and kanamycin. Resistance to the latter four drugs was transmitted as a unit but resistance to ampicillin was not transmitted at all.

In mixed culture in brain heart infusion broth the frequency of transfer was 2×10^{-4}/hour per donor cell (9). When K12 and AK were grown together for 18 hours, 1.6% of the K12 acquired drug resistance. The K12 that had recently acquired the R factor from AK donated resistance with a frequency of up to 1/hour per donor cell when strain PA201a, an autotrophic variant of K12, was the recipient (9).

Eight germ-free mice (20 gm female) were studied. They were housed in a single flexible film isolator (10). The mice were maintained on distilled water and autoclaved Purina chow no. 5010C. The manufacturer has assured us that this feed is free of antibiotics. After confirming bacterial, fungal, and parasitic sterility, the animals were colonized with K12 by placing 10^9 organisms of an overnight culture in 200 ml of drinking water. Six days later, the mice were fed an overnight culture of strain AK in fresh drinking water.

Feces were collected from the mice 3 days and 1 day prior to feeding AK and 1, 4, 8, 15, 22, and 29 days afterwards. One or two fecal pellets (estimated wet wt. 50–100 mg) were collected in sterile test tubes and emulsified in 1 ml of 0.85% NaCl. Dilutions were made in cold saline and portions corresponding to 10^{-2} to 10^{-7} ml were plated with calibrated loops on trypticase soy agar. Agar containing 20 μg/ml of nalidixic acid was used to count K12. The K12 that had acquired the R factor ($K12^{AK}$) was counted on agar containing nalidixic acid and either 20 μg/ml of tetracycline, chloramphenicol, or kanamycin.

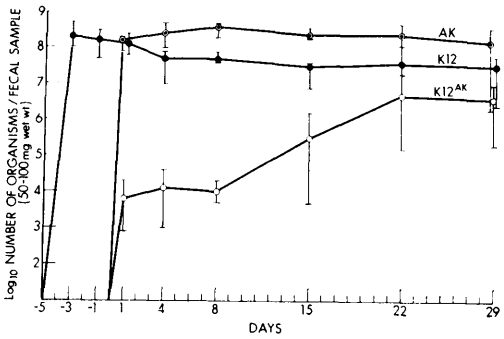

FIG. 1. Number of AK, K12, and $K12^{AK}$ found in fecal samples. The range of values includes all eight mice. The K12 was fed on day −5 and AK on day 0.

The AK was counted on agar without antibiotics; since colonies of the organisms were quite mucoid, they were easily distinguished from K12. All plates were incubated at 37°C overnight.

At days 22 and 29, when plate counts indicated that the number of $K12^{AK}$ approached the total number of K12, numerous colonies of K12 were selected from the plates containing nalidixic acid alone and their antibiotic susceptibility was determined. Single colonies were picked with a sterile toothpick and serially spotted on trypticase soy agar containing 20 μg/ml of either tetracycline, chloramphenicol, or kanamycin, or 10 μg/ml of streptomycin. Antibiotic susceptibility of AK colonies was determined by the same technique.

Results. The data are summarized in Fig. 1. Prior to feeding AK, no $K12^{AK}$ (less than 10^1) were found in any fecal sample. Eighteen hours after feeding AK, 6×10^3 (range 8×10^2–2×10^4) $K12^{AK}$ were found in each stool sample. Thereafter the number of $K12^{AK}$ increased progressively and by day 29, 21% of all colonies of K12 had acquired multiple drug resistance.

During the four weeks the mice were followed, 196 colonies of AK were tested for antibiotic susceptibility. Only one segregant was found. This colony had become susceptible to tetracycline, chloramphenicol, streptomycin, and kanamycin but remained resistant to ampicillin. Of several hundred colonies of $K12^{AK}$ tested for resistance to the multiple

antibodies only one segregant was found. This was susceptible to chloramphenicol and streptomycin but was resistant to tetracycline and kanamycin.

Discussion. It is impossible to give a precise rate for the transfer of the R factor but clearly it was able to spread epidemically and, eventually, infect a high proportion of K12. This occurred without the selective pressure of antibiotic treatment. However, the rate of spread was much slower than *in vitro*. Eighteen hours after feeding AK to the mice, only 0.005% of K12 had acquired antibiotic resistance. *In vitro* 1.6% acquired drug resistance in 18 hours. Only after more than 2 weeks did more than 1% of the K12 in the gut acquire the R factor. Nonetheless, the rate of acquisition of the R factor was much higher than the rate of segregation.

It is uncertain to what extent we may extrapolate these findings to conventional animals. There are many differences between germ-free and conventional animals. We cannot be certain that observation in mice contaminated only with *Enterobacteriacae* would be similar in conventional mice who are contaminated with many species of bacteria. For example, the bacterial density of *Enterobacteriacae* at the sites in the gut where the milieu is suitable for R factor transfer may be much different in mono-contaminated germ-free mice and in conventional animals. Also, subtle differences in the physical and chemical makeup of intestinal contents could profoundly affect the efficiency of R factor transfer. *In vitro*, R factor transfer is greatly inhibited by anaerobic growth and by inhibitors of oxidative phosphorylation (6). If the intestinal contents of mono-contaminated germ-free mice were less anaerobic than the gut contents of conventional mice, the rate of transfer of the R factor would, in all probability, be greater. Further work is required to explore these factors.

Summary. An antibiotic resistant *Klebsiella pneumoniae* containing an R factor was fed to germ-free mice previously colonized with an antibiotic susceptible *E. coli*. Within a month, 21% of the *E. coli* acquired multiple drug resistance without the selective pressure of antibiotic therapy. However, the rate of spread of the R factor was significantly slower than *in vitro*.

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An Induced Bactericidin in the Spiny Lobster, *Panulirus argus** (33021)

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Studies on the evolution of immunity (1) have shown that processes long recognized in mammals are characteristic of most vertebrates. One aspect of vertebrate immunity not yet convincingly demonstrated among the invertebrates is the synthesis of specific immu-

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