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Mutator Bacteria as a Risk Factor in Treatment of Infectious Diseases†

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We show in a gnotobiotic mouse model that, in addition to direct selection of antibiotic-resistant bacteria, some antibiotic treatments also select for mutator alleles. Because of these mutator alleles' high mutation rates, the initial treatment failure increases the probability of failures in subsequent treatments with other drugs.

The emergence of antibiotic resistance during therapy can increase the rate of secondary bacteremia, hospitalization costs, and mortality (3). Bacterial resistance can be acquired either by chromosomal mutations or by horizontal transfer of plasmid-borne resistance genes. In sensitive, strictly clonal bacterial populations (plasmid bearing or not), the generation of antibiotic resistance depends on the rate of emergence of resistant mutants, i.e., on the bacterial mutation rate (8, 9). A correlation between high mutation rate and antibiotic resistance has been reported in the case of *Pseudomonas aeruginosa* isolated from the lungs of cystic fibrosis patients (11). We used a model of bacterial colonization of germfree mice to assess in vivo the risk of emergence of antibiotic-resistant mutants due to mutator bacteria in the course of antibiotic treatment.

Animals and bacteria. Adult germfree mice (C3H/He/Jax./Jouy-en-Josas) were reared in isolators (La Calhène, Vélizy, France). Inoculation was performed per os with 0.5 ml of an inoculum containing wild-type strain *Escherichia coli* MG1655 (2) or its isogenic mutator variant (6) that bears a mutation in the *mutS* gene that inactivates the methyl-directed mismatch repair (MMR) system (5) and that leads to a 100-fold increase in the mutation rate.

Antibiotic treatment design and bacterial count. On the first day of treatment, 0.5 ml of an antibiotic(s) solution was administered per os to mice. Their drinking water was then supplemented with the antibiotic(s) until the end of the experiment. Concentrations were 30 μg/ml for fosfomycin (FOF), 80 μg/ml for spectinomycin (SPT), 100 μg/ml for streptomycin (STR), and 500 μg/ml each for nalidixic acid (NAL) and ampicillin (AMP). The evolution of the bacterial population was monitored before and during the treatment by plating dilutions of feces on Luria-Bertani plates supplemented with antibiotics, when needed (STR at 100 μg/ml, NAL at 40 μg/ml, SPT at 80 μg/ml, FOF at 30 μg/ml, AMP at 500 μg/ml, and rifampin at 100 μg/ml). Because rifampin was not used in the treatments, the frequency of spontaneously occurring rifampin-resistant mutants [f(Rif^r)] reflects the bacterial mutation rate (6).

Emerging mutator characterization. f(Rif^r) was obtained in three independent measurements for at least seven clones randomly isolated from fecal populations from each mouse. Plasmids (19) carrying the wild-type alleles of the different genes of the MMR system were used to electrotransform one clone from each population exhibiting a high mutation rate. Transformants were tested for a reduction in f(Rif^r).

We inoculated the guts of germfree mice with wild-type strain *E. coli* MG1655 (2) or its isogenic mutator variant (6). Such mutator bacteria have been found at high frequencies among different pathologies (7, 10, 11, 13). A few days after inoculation, the bacterial population size reached 10^{10} bacteria per gram of feces, the maximum population size reached when *E. coli* is inoculated into axenic mice. In this animal model, the bacterial population is clonal and readily reaches a high density (6), mimicking the infection conditions of normally sterile sites (e.g., urinary tract, the lungs, and surgical sites). After a delay of 13 days to allow the establishment of the inoculated strain, the bacterial population was challenged with various antibiotic treatments (Table 1). The elimination (reduction below the detection limit, e.g., 10^3 bacteria per of feces) of the bacterial population from the gastrointestinal tract (decontamination) or the maintenance of an antibiotic-resistant bacterial population was monitored as an indicator of the efficiency of the treatment.

The administration of FOF and SPT at a 1-day interval (FOF then SPT) successfully eliminated the bacteria in four of the six mice inoculated with the wild-type strain. The treatment was unsuccessful in the other two mice (mice M1 and M2; Table 1), in which bacteria resistant to both antibiotics reached the maximum population size soon after the beginning of the treatment. The same protocol failed to decontaminate the digestive tract of each of the six mice inoculated with the mutator strain (Table 1).

When mice received FOF and SPT simultaneously, the bacterial population was eliminated in 10 of 12 mice inoculated with the wild-type strain (Table 1). In the other two mice (mice M3 and M4), the bacterial population sizes were only tran-

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† Dedicated to the memory of J. Bjorkman.

TABLE 1. Impact of bacterial mutation rate on the result of antibiotic therapies[a]

Treatment	No. of treatment failures (no. of treatment failures due to EM)/total no. of mice inoculated with:		*P* value[b]	
	Wild-type strain	Mutator strain	A	B
FOF and then SPT at a 1-day interval	2 (1)/6	6/6	0.03	0.01
FOF + SPT	2 (2)/12	10/13	0.004	0.0001
NAL + STR	ND[c]	7/9		
NAL + STR	0/6[d]	6/6[e]	0.001	
FOF + SPT + STR	ND	0/12		
AMP	ND	0/6		

[a] Germfree mice were inoculated per os with the wild-type *E. coli* MG1655 strain or MG1655 *mutS3* mutator which is the *mutS* mutator mutant derivative of the ancestral strain, except as indicated in footnotes *d* and *e*.
[b] The differences between the failures occurring in the mutator (A) or the mutator plus the emerging mutator (EM) (B) and the wild-type populations were tested by the Fisher exact test (one tailed).
[c] ND, not determined.
[d] The mice were inoculated with one clone isolated from mouse M1.
[e] The mice were inoculated with one clone isolated from mouse M2.

siently diminished by the treatment. The same protocol failed in 10 of 13 mice inoculated with the mutator strain (Table 1). However, the simultaneous use of FOF, SPT, and STR efficiently eliminated the mutator populations from all of the mice (Table 1). Interestingly, the single use of AMP at a high concentration efficiently eliminated the mutator bacteria (Table 1), presumably because, in the strain used, no simple mutational events can generate resistance to a high AMP concentration. However, it was previously shown in vitro that high-level resistance could be obtained in a mutator background if the AMP concentration gradually increases (17).

Clones were isolated from the fecal populations of mice M1, M2, M3, and M4, in which the treatments failed, to measure f(Rif^r). The clones from mouse M1 did not show a significant difference in f(Rif^r) from that of the wild-type ancestor ($P = 0.3$ by the *t* test). The clones from mice M2, M3, and M4 had significantly higher f(Rif^r) values (on average, 200-, 200-, and 400-fold increases, respectively; $P \leq 0.008$ by the *t* test), suggesting that strains with high mutation rates (emerging mutators [EMs]) were selected from the original populations with low mutation rates. The high mutation rates of one EM clone from mouse M3 and one EM clone from mouse M4 were lowered when the bacteria were transformed with a plasmid bearing a wild-type *mutS* allele, suggesting that these EMs carry a defect in the MMR system.

One clone isolated from the wild-type population that had survived the treatment with FOF and SPT at a 1-day interval in mouse M1 and one EM clone isolated from the population that survived the same treatment in mouse M2 were each inoculated separately into a new group of six germfree mice. Animals were then treated with STR and NAL. This treatment failed to decontaminate all mice inoculated with the EM strain isolated from mouse M2, whereas it was successful against the nonmutator bacterial populations originating from mouse M1 (Table 1). In mice inoculated with the genetically constructed mutator strain, the same treatment failed in seven of nine mice (Table 1).

Our results show that some antibiotic treatments can select for mutator bacteria present at low frequencies among all wild-type populations. Actually, by selecting for a resistance allele, the antibiotic selective pressure also selected for a mutator allele as the mechanism that generated the resistance. Moreover, mutators could also facilitate the modification of the active sites of detoxification enzymes to shift the resistance from resistance to a low dose to resistance to a high dose (17) and extend their resistance spectra (12). It could also rapidly accumulate compensatory mutations that limit the cost to the bacteria associated with the resistance alleles (1). Mutator bacteria can be considered risk markers for antibiotic therapy. If corroborated by epidemiological data, our results would suggest that, in the case of a first therapeutic failure and if time allows, a diagnostic assay for the presence of mutators should determine the next therapeutic step. If a drug is available for which mutational events can very rarely generate antibiotic resistance, such as AMP at a high concentration used against the bacteria in the present study, then it is the first choice for use against the mutators. Otherwise, the use of a combination therapy seems to be the best alternative. As the selection of mutator bacteria is favored by several bacterial and environmental factors (18), some conditions (e.g., a large bacterial population) allow the selection of such strains more than others. In these cases, if possible, therapies should be initiated directly with a protocol that limits the risk of selection of mutators. For example, the use of antibiotics that inhibit a single enzyme should probably be set aside, even when used in combination therapies, as we did in the present work.

Due to their increased genetic adaptability, the rate of selection of mutator variants among populations undergoing multiple adaptive steps should be increased (6, 8, 14, 16, 18). For this reason, some pathology might allow the emergence and fixation of mutator alleles more frequently than others (4). In the case of chronic infections, the size of the infecting population combined with the duration of infection, the reiterated challenges imposed by antibiotic therapies, and the host's immune response should provide conditions prone to the fixation of mutator alleles. Numerous mutator *E. coli*, *Salmonella enterica*, and *P. aeruginosa* isolates from patients and other natural environments have defective MMR systems (6, 7, 10, 11), similar to the emerging mutator strains isolated in the present work. Given the abundance of mutators among bacterial pathogens, viruses, and tumors (15), the concept that one

failed therapy is a potential risk factor for the next therapy might be relevant to other therapeutic strategies.

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