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Activity of Mitomycin C, Other Antibiotics, and Serum Against Lysogenic Bacteria

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ABSTRACT

MUSCHEL, LOUIS H. (University of Minnesota, Minneapolis), AND KAREN SCHMOKER. Activity of mitomycin C, other antibiotics, and serum against lysogenic bacteria. J. Bacteriol. **92**:967–971. 1966.—Lysogenic bacteria were found to be more sensitive to the bactericidal action of mitomycin C or streptonigrin than the corresponding sensitive or indicator strains. This result may be attributed to the induction of phage production in lysogenic cells by these antibiotics. Lysogenic and sensitive bacteria were, however, equally sensitive to chloramphenicol, streptomycin, and polymyxin B. In contrast to their greater sensitivity to certain phage-inducing antibiotics, the lysogenic state resulted in greater resistance to the bactericidal reaction of serum mediated by the complement system. In general, therefore, the lysogenic state may result in either decreased or increased sensitivity to various antimicrobial agents.

Preliminary experiments indicated that the antibiotic mitomycin C, which acts as an inducer of phage production in lysogenic bacteria, exerted greater bactericidal activity against strains of *Escherichia coli* K-12 lysogenic for lambda phage [K-12(λ)] than against *E. coli* K-12, the indicator organism sensitive to lambda (Muschel, Bacteriol. Proc., p. 65, 1965). Further investigations were made of the comparative sensitivity of different lysogenic bacteria and the corresponding indicator strains to streptonigrin, an antibiotic that induces phage production in lysogenic bacteria and that is similar in many other ways to mitomycin C (3), to other unrelated antibiotics, and to the bactericidal action of normal serum mediated by the complement system.

It is well established that certain changes may be observed in host properties as a consequence of lysogenization. These include the production of toxin by *Corynebacterium diphtheriae*, colonial morphology in *Bacillus megaterium*, synthesis of antigens in *Salmonella*, or capacity to reproduce various unrelated phages (9). It seemed of significance, therefore, to determine whether lysogeny might result in greater resistance or susceptibility to different antimicrobial substances.

MATERIALS AND METHODS

Cultures. The strains of K-12(λ) and K-12 were obtained from L. C. McLaren and S. G. Bradley of our department, and also from G. R. L. Worthington, Naval Biological Laboratory, Oakland, Calif. *E. coli* C-16 (P2) and *E. coli* C-1 (indicator for P2 phage) were obtained from L. C. McLaren; S. *typhimurium* LT-2 (P22) and *S. typhimurium* LT-2, indicator for P22, from M. Levine, University of Michigan Medical School, Ann Arbor; and K-12 strain W1485 and K-12 strain W1485(φ80), from L. S. Baron, Walter Reed Army Institute of Research.

Antibiotics. Mitomycin C was purchased from Sigma Chemical Co., St. Louis, Mo.; streptonigrin was obtained through the courtesy of T. J. McBride, Chas. Pfizer & Co., Inc., Maywood, N.J.; and polymyxin B sulfate (USP) B grade was purchased from Calbiochem. Standard preparations of chloramphenicol and streptomycin were kindly supplied by the Pure Food and Drug Administration.

Serum. Lyophilized guinea pig serum was purchased from the Texas Biological Laboratories, and reconstituted with water prior to use.

Bactericidal assays. The bactericidal assays for antibiotics and serum have been described previously (11). The bacterial growth was suspended in broth, and a measured amount of the suspension containing about 3×10^7 organisms was added to tubes containing appropriate amounts of the antibiotic or serum. For the assay of serum bactericidal *antibody*, in contrast to the determination of whole serum bactericidal activity, excess of guinea pig serum was used as a complement source with different amounts of the antiserum being assayed (5). The tubes containing the mixtures were incubated at 37 C for 60 min. Assays of the organisms surviving the antibiotics or serum substances during this reaction period were made by the addition

of 2.5 volumes of broth to each tube. Upon continued incubation, when a suitable reading range was obtained with the control tubes still in the log phase of growth, the optical densities of the tubes were determined. The ratio of the density in the test and control tubes, subtracted from 1.0 and multiplied by 100, yielded the percentage of killed organisms. When the percentages killed, converted to probits, were plotted against the logarithm of the serum amounts, a linear representation of the data resulted. The amount of antibiotic or serum required for 50% reduction in the bacterial population was read off by interpolation. For the bactericidal antibody determinations, the reciprocal of the 50% end point of the antiserum amount represented the titer. In addition to the advantage of a linear plot, such graphs also give a new parameter, the slope, which assigns a numerical value to the dosage-response relationship. Different slopes indicate a different mechanism of action when comparing two bactericidal substances; equal slopes suggest similar mechanisms, although the equality may be fortuitous (10).

Induction of phage production in lysogenic bacteria. Log-phase lysogenic cells in concentrations of about 5×10^7 per milliliter were incubated at 37 C for 20 min with an amount of the antibiotic sufficing to kill 50% of those cells in 1 hr, as determined in the previous section. The suspensions were centrifuged, and the supernatant fluid containing almost all of the antibiotic was removed. A 5-ml amount of broth was then added to the sediment, and, after incubation for 2 hr at 37 C, the organisms were removed from the supernatant fluid by centrifugation. The supernatant fluid was treated with 0.3 ml of chloroform to kill any viable bacteria, and, after mechanical mixing, it was recentrifuged, appropriately diluted, and assayed for its phage content. A 0.1-ml amount of each dilution was added to 2 ml of soft agar (at 45 C; 0.65% agar in tryptone broth with 0.01 M $MgSO_4$) seeded with 0.2 ml of an overnight culture of the sensitive indicator strain. The entire contents of each tube were poured on the surface of tryptone agar plates, allowed to solidify, and then incubated overnight at 37 C. Plaques of surviving phage were then counted.

Production of lysogenic bacteria from uninfected bacteria. Subsequent to a high multiplicity of infection with λ phage, certain cells of K-12 became lysogenized rather than lysed. On plates seeded with *E. coli* K-12, λ phage produced plaques with turbid centers containing the K-12 organisms that were lysogenized rather than lysed by the phage. These cultures were transferred several times. They liberated λ phage spontaneously, and were immune to infection with that phage.

RESULTS

Sensitivity of lysogenic bacteria to mitomycin C and streptonigrin. Lysogenic bacteria were compared with corresponding indicator strains for their sensitivity to the bactericidal action of mitomycin C and streptonigrin. The bactericidal action of mitomycin C against K-12(λ) was characterized by a dose-response relationship

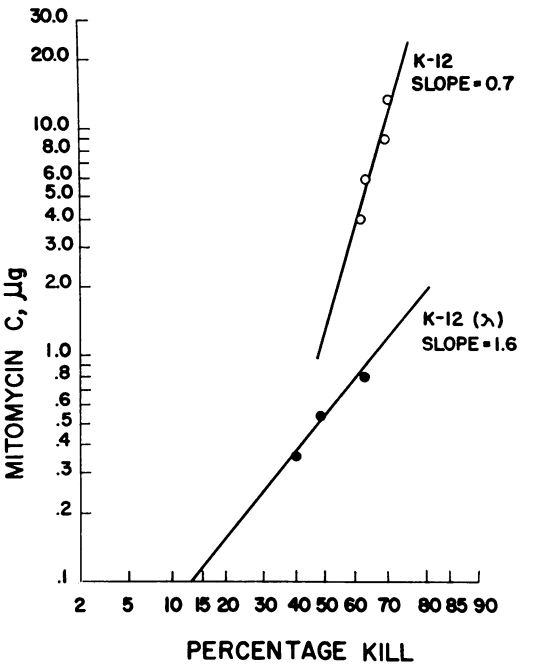

FIG. 1. *Dose-response relationship between different amounts of mitomycin C and the percentage killing of K-12(λ) and K-12.*

different from that against the indicator strain K-12, in that the slope was equal to 1.6 with the former and 0.7 with the latter (Fig. 1). This difference in slope indicated that the concentration of mitomycin C increased disproportionately greater against K-12, in comparison with K-12(λ), to effect further equal increases in bactericidal effect. It suggested further that a different mechanism of action may be operative against the two organisms, and that the greater slope may be associated with phage induction of K-12(λ). Similarly, with streptonigrin, the slope was 2.8 and 1.9, respectively, against K-12(λ) and K-12. The 50% end points of the two antibiotics against these strains, as well as against two other pairs of lysogenic and sensitive organisms, are given in Table 1. In contrast to the experimental results with K-12(λ) and K-12, LT-2 (P22) and LT-2 were equally sensitive to streptonigrin, although the former lysogenic strain was slightly more sensitive to mitomycin C. C-16 (lysogenic for P2 phage) and C-1 (indicator for P2) were equally sensitive to mitomycin C and streptonigrin.

It seemed desirable, then, to determine whether the difference in susceptibility of the paired organisms to mitomycin C and streptonigrin was associated with the inducibility of the lysogenic

TABLE 1. *Fifty per cent end points for the bactericidal action of mitomycin C and streptonigrin against lysogenic and sensitive bacterial cultures*

Organism	Amt required for 50% kill	
	Mitomycin C	Streptonigrin
	μg/ml	μg/ml
Escherichia coli K-12(λ)	0.50	6.2
E. coli K-12	1.40	24.0
Salmonella typhimurium LT-2 (P22)	1.85	2.2
S. typhimurium LT-2	2.70	2.3
E. coli C-16 (P2)	0.84	2.6
E. coli C-1	0.88	2.8

TABLE 2. *Induction of phage production by action of mitomycin C and streptonigrin with amounts required for 50% killing of lysogenic bacteria*

Organism	Mitomycin C	Streptonigrin	Control
Escherichia coli K-12(λ)	140[a]	85	3
Salmonella typhimurium LT-2 (P22)	33	46	2
E. coli C-16 (P2)	2	4	3

[a] Induction was measured by number of plaque-forming units per milliliter (× 10^5) obtained by comparison with cultures not subjected to either antibiotic. All values represent the mean of three determinations.

strains by the antibiotic. The results (Table 2) indicated that K-12(λ) and LT-2 (P22) were inducible by both antibiotics, and that C-16 (P2) was not. Thus, the inducibility of K-12(λ), but not of C-16 (P2), by these antibiotics correlated nicely with the greater sensitivity of the former organism to mitomycin C and streptonigrin compared with K-12, and the comparable sensitivity of C-16 (P2) and C-1. The anomalous result with streptonigrin and the LT-2 lysogenic and sensitive strains suggested that phage induction does not play a significant part in the bactericidal action of this antibiotic against *S. typhimurium* (P22). Moreover, there was relatively little difference in the bactericidal action of mitomycin C against LT-2 (P22) and LT-2, in contrast to its almost threefold greater activity against K-12(λ) compared with K-12.

Sensitivity of K-12(λ) and K-12 to other antibiotics. To determine whether the differential sensitivity of these organisms was unique to those antibiotics with phage-inducing activity, the organisms were subjected to several other antibiotics, including chloramphenicol, streptomycin, polymyxin B, and novobiocin. The lysogenic and sensitive organisms were equally sensitive to those antibiotics, except for novobiocin (Table 3). It was learned subsequently that novobiocin induces λ phage (Worthington and Wolochow, Bacterial. Proc., p. 136, 1964).

Serum sensitivity. To ascertain whether the lysogenic state was associated with altered resistance to the serum bactericidal reaction mediated by the complement system, the appropriate cultures were tested for their susceptibility to normal guinea pig serum. The results (Fig. 2) indicated an approximately twofold difference in sensitivity between K-12, which required 0.051 ml of serum for 50% killing, and K-12(λ), which required 0.11 ml. Similarly, LT-2 (P22) was more resistant to normal guinea pig serum (50% end point, 0.4 ml) than uninfected LT-2 (50% end point, 0.08 ml), C-16 (P2) required at least twice as much serum for 50% killing as C-1, and W1485 (φ80) required about 50% more serum than W1485. Thus, whereas lysogenic bacteria

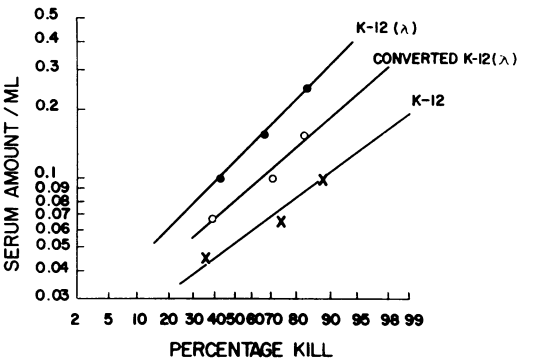

FIG. 2. *Dose-response relationship between different amounts of guinea pig serum and the percentage killing of K-12(λ), K-12, and K-12 after conversion to the lysogenic state.*

TABLE 3. *Fifty per cent end points for the bactericidal action of different antibiotics against K-12(λ) and K-12*

Antibiotic	End point (μg)	
	K-12 (λ)	K-12
Chloramphenicol	1.8	1.5
Streptomycin	1.7	1.7
Polymyxin B	2.0	2.0
Novobiocin	0.84	1.3

TABLE 4. *Bactericidal antibody titers of rabbit anti-K-12 and anti-K-12(λ) before and after absorption with K-12 and K-12(λ)*

Test organism	Sera	Absorbed with	Bactericidal antibody titer
Escherichia coli K-12	Anti-K-12(λ)	Untreated	2,500
		K-12	1,000
		K-12(λ)	1,040
	Anti-K-12	Untreated	1,190
		K-12	715
		K-12(λ)	833
E. coli K-12(λ)	Anti-K-12(λ)	Untreated	1,960
		K-12	952
		K-12(λ)	1,130
	Anti-K-12	Untreated	1,560
		K-12	1,250
		K-12(λ)	910

may be more sensitive to mitomycin C, they are more resistant to serum.

Since it was not certain that the K-12 and K-12(λ) strains tested were isogenic, K-12 was converted to the lysogenic state by infection with λ phage. Its 50% end point then was 0.08 ml (Fig. 2). This result indicated that, although lysogeny resulted in a significant increase in serum resistance, one could not attribute the entire difference in serum sensitivity between K-12 and K-12(λ) to the lysogenic state. A similar experiment in which K-12 was converted to the lysogenic state with φ80 resulted also in slightly increased, but reproducible, serum resistance. The 50% end point was equal to 0.023 ml against K-12 and 0.026 ml against K-12(φ80).

Antigenic analysis of K-12(λ) and K-12. Because resistance to serum has been associated with certain antigenic structures (4), and because viral infection has been demonstrated to elicit new antigenic determinants (12), an attempt was made to detect antigenic differences between K-12 and K-12(λ). Samples of rabbit antiserum to both organisms were absorbed with the homologous organism, and other samples with the heterologous organisms. The absorbed samples were then tested and compared for remaining antibody titer by use of a quantitative bactericidal antibody assay (5). As the results indicate (Table 4), a significant differential absorptive effect was not exerted by K-12 or K-12(λ) with either antiserum, and differences in the antigenic determinants of the two organisms were, therefore, not detectable with this procedure.

DISCUSSION

The results of these experiments have indicated that the lysogenic state may result in increased susceptibility of bacteria to certain antibacterial substances and reduced susceptibility to others. At the 50% end point, K-12 was three to four times more resistant to mitomycin C and streptonigrin (Table 1) and slightly more resistant to novobiocin than K-12(λ). These antibiotics have been reported to induce phage production in lysogenic bacteria (2, 3), and that ability was demonstrated with K-12(λ) and LT-2 (P22) in these experiments (Table 2). Nonetheless, the bactericidal effect of mitomycin C against LT-2 (P22) was only slightly greater than against the indicator strain for P22 phage, and the streptonigrin was equally active against both strains (Table 1). If induction of phage production contributes to the bactericidal effect of phage-inducing antibiotics, as would seem altogether reasonable, there are marked quantitative differences, therefore, in the extent of that contribution.

With *E. coli* C-16 (P2) and *E. coli* C-1, neither mitomycin C nor streptonigrin induced phage production under the experimental conditions employed, and, accordingly, both antibiotics showed equal bactericidal activity against the lysogenic and indicator strains. Moreover, when tested against antibiotics with different mechanisms of action, such as chloramphenicol and streptomycin, which inhibit protein synthesis, and polymyxin B, which affects membrane permeability (6), K-12(λ) and K-12 were equally susceptible.

Of unusual interest was the finding that all three lysogenic strains were, in contrast to the increased sensitivity of two of them to mitomycin C, more resistant than the corresponding indicator strains to the bactericidal action of normal serum. The virulence of members of the *Enterobacteriaceae* has been associated with serum resistance in many studies (7, 8), so that the lysogenic state assumes critical significance in the host-parasite relationship. Similarly, the most dramatic effect of lysogeny involves the capacity of *Corynebacterium diphtheriae* to produce toxin only as a result of infection with a suitable bacteriophage (1). Although toxin production has no known relationship to serum resistance, it is interesting that both are related to the lysogenic state. Disease potential is closely associated, therefore, not only with a bacterial agent, but also with the virus with which it is infected.

Since the presence of certain antigenic structures is associated with resistance to normal serum mediated by the complement system, and

since the presence of certain antigens on the surface of *Salmonella* is the consequence of lysogenization with particular phage types (12), it seemed reasonable that K-12(λ) may have acquired a new antigen not present in the uninfected bacteria. The very sensitive bactericidal antibody studies performed with absorbed antisera failed, however, to reveal any significant antigenic change resulting from lysogeny.

To determine whether the difference in serum sensitivity between K-12(λ) and K-12 was associated with λ phage, the latter indicator strain was converted to the lysogenic state by exposure to λ phage. Its serum resistance was increased, though not to the extent of the original lysogenic cultures (Fig. 2). The greater serum resistance of LT-2 (P22) compared with LT-2, of C-16 (P2) compared with C-1, and of W1485 (φ80) compared with W1485 also strongly suggests that infection of an organism with a bacterial virus may lead often to a change in its serum resistance and potential virulence. The increased resistance to serum noted in these experiments was accompanied, however, by decreased resistance to those antibiotics inducing phage production from the lysogenic state, but with no detectable effect against other antibiotics incapable of such activity. The lysogenic state, therefore, may result in either increased or decreased sensitivity to various antimicrobial agents.

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