Inducible Resistance to Tetracycline in *Staphylococcus aureus*

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SUMMARY

When staphylococci, resistant to 4 µg. tetracycline/ml., were grown in nutrient media at subinhibitory levels of the drug, phenotypical resistance increased until the cocci grew with 160 µg. tetracycline/ml. Resistance increased most rapidly at the highest concentration of tetracycline which did not significantly inhibit growth. Increase in resistance was also obtained by pre-incubation with β-apo-5-oxy-tetracycline. Increase in resistance could be prevented by chloramphenicol and actinomycin D, but not by nalidixic acid. When a highly resistant culture was transferred to tetracycline-free medium, phenotypical resistance decreased gradually; after four transfers on nutrient agar it returned entirely to the original level.

INTRODUCTION

Williams (1967) has drawn attention to the resistance to mercury salts (Moore, 1960) of tetracycline-resistant strains of *Staphylococcus aureus*. Resistance to tetracycline was in general correlated with resistance to mercury; in mercury-sensitive strains, however, high-level resistance to tetracycline could be obtained by 'training', i.e. growth in peptone water containing 3 µg. antibiotic/ml.

METHODS

Bacterial strains. The strain studied, *Staphylococcus aureus* 111 (Sompolinsky, Yiflah & Aboud, 1968), was susceptible to mercury salts (Moore, 1960) and resistant to tetracycline (Tc) by virtue of a plasmid that could be eliminated by growth at 44° (May, Houghton & Perret, 1964). The eliminated susceptible strain grew on nutrient agar containing 0.2 µg. Tc/ml.; on agar containing 0.4 µg. Tc/ml., colonies did not develop from an inoculum of 10⁸ staphylococci. With the uneliminated culture, identical colony counts were obtained on plain agar and on agar containing 4.0 µg. Tc/ml. At concentrations of up to 160 µg. Tc/ml., some colonies developed but growth did not occur on nutrient agar containing 320 µg. Tc/ml. Three types of resistant cultures have been used: (a) a culture transferred daily on plain nutrient agar; only 5 to 10% of the viable cocci from this culture produced colonies on agar containing 10 µg. Tc/ml.; (b) a highly resistant culture subcultured daily on nutrient agar containing 160 µg. Tc/ml.; and (c) cultures with intermediate levels of resistance obtained by growth of type (a) culture for a short period in nutrient broth containing tetracycline at different concentrations.

Chemicals. Tetracycline hydrochloride was a gift from Lederle Laboratories, American Cyanamid Co., Pearl River, N.Y.; β -apo-5-oxy-tetracycline was from Chas. Pfizer & Co., Groton, Conn., U.S.A.; chloramphenicol from Abic Ltd, Ramat Gan, Israel; nalidixic acid from Sterling-Winthrop Research Institute, Rensselaer, New York, U.S.A., through the courtesy of Dr Nachod; actinomycin D (Dactinomycin) was obtained from Merck Sharp & Dohme, West Point, Pa, U.S.A.

Saline buffer was prepared as described by Sompolinsky, Ernst-Geller & Segal (1967).

Culture media. Difco nutrient broth (NB) and nutrient agar (NA) were used. All cultures were incubated at 37°, NB cultures were grown in a water bath and aerated by shaking.

Viable counts were determined by plating appropriate dilutions of the bacterial suspension on NA; four replicate plates were prepared for each dilution and a total of 100 to 400 colonies were recorded on each plate.

Turbidity of bacterial cultures was determined with a Klett-Summerson photo-electrical colorimeter, using a red filter (660 nm.) for NB cultures. Five hundred Klett units = extinction (E) of 1.00.

RESULTS

When an exponentially growing culture of *Staphylococcus aureus* 111 was inoculated into nutrient broth to give an initial turbidity of 0.032 E , growth continued at an exponential rate; addition of 10 μ g. Tc/ml. caused a temporary decrease in the growth rate which then recovered to that of the antibiotic-free control. When 40 μ g. Tc/ml. was added with the inoculum, no increase in turbidity occurred for 1 h., after which growth occurred at a rate slightly less than that of the control (Fig. 1). This type of growth curve in which the lag period varies with the drug concentration is seen when an inhibiting drug is inactivated by the culture (Sompolinsky & Samra, 1968). However, no clear evidence for inactivation was found experimentally (Sompolinsky, Zaidenzaig, Ziegler-Schlomowitz & Abramova, 1970).

Other possible explanations for this pattern of growth in 40 μ g. Tc/ml. NB might be 'adaptation' to the drug during the lag period (Williams, 1967) or outgrowth of a few bacteria with initial resistance to 40 μ g. Tc/ml. To examine these possibilities, further growth studies were performed by determining viable counts simultaneously on NA and on 40 μ g. Tc/ml. NA (Fig. 2). The rate of growth demonstrated by counts on 40 μ g. Tc/ml. NA soon approximated to that shown by viable counts on NA from cultures in NB and 10 μ g. Tc/ml. NB. Furthermore, after 3 to 5 h. the difference between the number of colony-forming units on NA and 40 μ g. Tc/ml. NA was relatively constant, corresponding approximately to the original inoculum size (3×10^7 cocci/ml.). These data might suggest that the population was initially heterogeneous in ability to grow on medium containing Tc, that only a few organisms (4×10^6 out of 3×10^7) were able to grow at 40 μ g. Tc/ml. and that these eventually outnumbered the sensitive non-proliferating organisms.

However, selection from a heterogeneous bacterial population was not the only cause of development of tetracycline resistance. After growth in non-inhibitory concentrations of Tc (0.1 or 1.0 μ g./ml.), significant increase in the proportions of cocci growing on 10 to 40 μ g. Tc/ml. was observed (Table 1). Resistance was also produced (Table 2) by growth with 10 μ g. apo-oxy-tetracycline/ml. which is virtually non-inhibitory. In these experiments, practically the whole bacterial population increased

in resistance through contact with Tc. The apparent discrepancy between these and earlier results could be explained by the assumption that resistance depends on the synthesis of a specific protein which is induced by Tc at a rate dependent on the concentration of antibiotic. Apo-oxy-tetracycline would, according to the results in Table 2, be a less effective inducer. Development of resistance was inhibited by chloramphenicol as would be expected for such induction.

Fig. 1

Fig. 2

Fig. 1. Growth of *Staphylococcus aureus* 111 in nutrient broth (NB) (O—O); tetracycline (Tc) 10 µg./ml. NB (●—●); and 40 µg. Tc/ml. NB (▲—▲). Determined by measurement of extinction (*E*) at 660 nm.

Fig. 2. Growth of *Staphylococcus aureus* 111 in nutrient broth (NB) (O—O); 10 µg. tetracycline (Tc)/ml. NB (●—●); and 40 µg. Tc/ml. NB (▲—▲). Determined by viable counts on nutrient agar (NA). The culture in 40 µg. Tc/ml. NB was also counted on 40 µg. Tc/ml. NA. (▲---▲).

When an inhibitor of protein synthesis is also an inducer, no simple kinetics of induction can be expected (Shaw & Brodsky, 1968). Figure 3*a* shows that growth of *Staphylococcus aureus* 111 in NB+Tc was independent of the drug concentration in the range 5 to 10 µg./ml. and that growth after 4 h. was little influenced by 20 µg./ml., but considerably by 40 µg./ml. If Tc is an inducer and induction is concentration-dependent, it would be expected that the highest concentration of the drug with no

Table 1. *Increase in phenotypical resistance to tetracycline in Staphylococcus aureus III during growth in nutrient broth with subinhibitory concentrations of the drug*

Growth in Tc-free NB							
Time (h.)	Viable cocci on plain NA	Cocci resistant to ($\mu\text{g. Tc/ml.}$)					
		5	10	20	40		
0	2.0×10^7	84.7*	79.2	3.1	nt		
2	9.5×10^7	66.5	25.1	0	nt		
4	3.3×10^8	58.5	9.9	0	nt		

Growth in 0.1 $\mu\text{g. Tc/ml. NB}$							
Time (h.)	Viable cocci on plain NA	Cocci resistant to ($\mu\text{g. Tc/ml.}$)					
		5	10	20	40	80	160
0	2.0×10^7	84.7	79.2	3.1	nt	nt	nt
2	9.6×10^7	88.2	77.8	12.9	0.9	0	0
4	3.1×10^8	104.8	99.2	21.0	0	0	0

Growth in 1.0 $\mu\text{g. Tc/ml. NB}$							
Time (h.)	Viable cocci on plain NA	Cocci resistant to ($\mu\text{g. Tc/ml.}$)					
		5	10	20	40	80	160
0	2.0×10^7	84.7	79.2	3.1	nt	nt	nt
2	9.2×10^7	89.0	80.8	58.3	9.2	1.6	0
4	2.9×10^8	99.6	101.2	100.0	24.6	10.6	0

Abbreviations: NA = nutrient agar; NB = nutrient broth; Tc = tetracycline; nt = not tested.

* Resistant cocci, determined by viable count on NA + indicated concentrations of Tc, expressed as % of viable count on NA.

Table 2. *Influence of pre-incubation with tetracycline or apo-oxy-tetracycline on resistance of Staphylococcus aureus III to tetracycline*

Pre-incubation conditions (3 h.)	Cocci resistant to tetracycline ($\mu\text{g./ml.}$)			
	20	40	80	160
No Tc	33.4*	0	nt	nt
Tc 10 $\mu\text{g./ml. NB}$	99.6	79.0	66.8	56.8
apo-oxy-Tc 10 $\mu\text{g./ml. NB}$	65.8	32.3	4.9	0
Tc 10 $\mu\text{g./ml.}$ + chloramphenicol 200 $\mu\text{g./ml. NB}$	6.7	0	0	0

Abbreviations: Tc = tetracycline; NB = nutrient broth; nt = not tested.

* Resistant cocci, determined by viable count on NA + indicated concentrations of Tc, expressed as % of viable count on NA.

significant inhibitory effect would be most rapidly effective in induction. This was confirmed by the experiments reported in Fig. 3*b*.

In a similar experiment, the appearance of cocci resistant to 160 $\mu\text{g. Tc/ml.}$ during growth in 20 $\mu\text{g. Tc/ml. NB}$ was depicted at shorter time intervals (Fig. 4); at the time of positive growth acceleration there was a rapid increase in the proportion of highly

resistant bacteria from 8 to 60% of the whole population. Development of phenotypic high-level resistance seemed therefore to be associated with other vital metabolic activities of the organisms. Further experiments (Tables 2, 3; Fig. 5*a* to *c*) indicated that protein synthesis was involved, since no highly resistant cocci developed in the presence of chloramphenicol, and substitution of NB by saline buffer caused a striking delay in the appearance of high-level resistance. This is in accordance with the observation that inhibitory concentrations of Tc inhibit induction (Fig. 1).

Fig. 3*a*. Growth of *Staphylococcus aureus* 111 in nutrient broth (NB) with 5 µg. tetracycline (Tc)/ml. (○—○); 10 µg. Tc/ml. NB (●—●); 20 µg. Tc/ml. NB (▲—▲); and 40 µg. Tc/ml. NB (■—■). Determined by viable counts on nutrient agar (NA).

Fig. 3*b*. Change in frequency of bacteria resistant to 160 µg. Tc/ml. in the cultures of Fig. 3*a*. Determined by viable counts on 160 µg. Tc/ml. NA and calculated as % of viable count on NA.

In a Tc-susceptible (eliminated) culture of *Staphylococcus aureus* 111, growth in 0.05 µg. Tc/ml. NB had no influence on resistance; the strain remained resistant to 0.2 µg. Tc/ml. and susceptible to 0.4 µg. Tc/ml.

The increased resistance of the non-eliminated cultures achieved by incubation with subinhibitory concentrations of Tc might be due either to induction of resistance through derepression of the synthesis of a specific 'resistance protein' or to the development of a higher multiplicity of 'resistance genes' (plasmids) per coccus. In the latter case, induction of increased resistance would require DNA synthesis, and therefore be susceptible to nalidixic acid, whereas induction by repressor inactivation would be susceptible only to drugs that inhibit transcription (actinomycin D) or translation (chloramphenicol) of the resistance gene. In experiments with nalidixic acid at 200 µg./ml., significant increase in viable count of a culture of *Staphylococcus aureus* 111

Fig. 4. Kinetics of increase in resistance of *Staphylococcus aureus* 111, growing in nutrient broth (NB) containing 20 µg. tetracycline (Tc)/ml. Growth curve of broth cultures by viable counts on nutrient agar (NA) (●—●), and % resistant cocci by viable count on 160 µg. Tc/ml. NA as percentage of viable count on NA. (○—○).

Fig. 5. Frequency of colony-forming staphylococci (*Staphylococcus aureus* 111) from nutrient broth (NB) culture, developing on 20 µg. tetracycline (Tc)/ml. nutrient agar (NA) (●—●); 40 µg. Tc/ml. NA (○—○); and 80 µg. Tc/ml. NA (△—△); as percentage of viable count on NA. Cultures incubated during the time indicated, in (a) 20 µg. Tc/ml. NB, (b) 20 µg. Tc/ml. NB + 200 µg. chloramphenicol/ml., (c) 20 µg. Tc/ml. saline buffer. Inoculum size was in all cases 1.6×10^7 bacteria/ml. After 24 h., viable counts on plain NA were (a) 3.9×10^8 /ml., (b) 2.3×10^8 /ml. and (c) 3.2×10^8 /ml. The bacteria incubated with chloramphenicol were washed twice in saline buffer before plating.

during 2 h. incubation, was prevented, whereas 256 µg./ml. caused an extensive decrease in the number of colony-forming units. Table 3 shows that 1.0 µg. Tc/ml. caused a rapid increase in resistance of a previously entirely uninduced culture, in

Table 3. *Induction of tetracycline resistance in Staphylococcus aureus 111 in presence of nalidixic acid*

An exponential-phase culture of *S. aureus* 111, in nutrient broth (NB) (O.D. = 0.100), was diluted in NB + nalidixic acid to a calculated O.D. of 0.008; the other indicated drugs were added and the cultures incubated with aeration. Samples of the cultures were centrifuged, the organisms washed once in saline buffer, suspended and diluted in saline buffer and viable counts determined on NA and NA + Tc.

Drugs in incubation medium (in µg./ml.)	Incubation (37°) in h.	Cocci resistant to tetracycline (µg./ml.)			
		10	20	40	80
Nil	0	16.2*	0.9	< 0.01	< 0.01
Nalidixic acid 200.0	1	7.8	0.3	< 0.01	< 0.01
	2	9.2	0.3	< 0.01	< 0.01
	3	nt	0.4	0.02	< 0.01
Nalidixic acid 200.0 + tetracycline 20.0	1	12.9	0.01	< 0.01	< 0.01
	2	nt	0.01	< 0.01	< 0.01
	3	12.4	0.2	< 0.01	< 0.01
Nalidixic acid 200.0 + tetracycline 5.0	3	22.5	2.8	0.7	0.2
Nalidixic acid 200.0 + tetracycline 1.0	1	106.8	60.2	5.7	< 0.01
	2	91.0	60.3	22.9	1.6
	3	92.3	68.6	28.6	1.5
Nalidixic acid 200.0 + tetracycline 0.1	3	85.6	23.6	7.0	0.6
Nalidixic acid 200.0 + chloramphenicol 200.0 + tetracycline 1.0	1	17.3	1.1	< 0.01	< 0.01
	2	12.4	2.2	0.02	< 0.01
Nalidixic acid 200.0 + actinomycin D 1.5 + tetracycline 1.0	1	13.5	7.6	0.5	0.07
	2	7.8	3.0	0.3	0.12

nt = Not tested.

* Resistant cocci, determined by viable count on NA + indicated concentrations of Tc, expressed as % of viable count on NA.

Table 4. *Loss of phenotypical high-level resistance to tetracycline after growth on drug-free nutrient agar*

Original strain	→	Fraction* of cocci resistant to 160 µg. Tc/ml. 1.1×10^{-7}
↓		
After several transfers on 160 µg. Tc/ml. NA	→	1.00
↓		
After two transfers on drug-free NA	→	4.3×10^{-5}
↓		
After two more transfers on drug-free NA	→	3.1×10^{-7}

Abbreviations: Tc = tetracycline; NA = nutrient agar.

* Determined by colony counts on plain agar and on nutrient agar containing 160 µg. Tc/ml. NA.

spite of the addition of nalidixic acid. Chloramphenicol and actinomycin D, added to nalidixic acid + Tc, strongly inhibited induction of increased resistance. In these experiments, 20 µg. Tc/ml. inhibited induction almost entirely, while 5.0 and 0.1 µg. Tc/ml. were less effective than 1.0 µg. Tc/ml.

When *Staphylococcus aureus* III was grown in NB + Tc until the whole population was phenotypically of high-level resistance, and the bacteria were subcultured on plain NB or NA, high-level resistance was gradually lost. Table 4 shows that after four subcultures the frequency of high-level resistant cocci was as in a culture that has had no previous contact with the drug. If high resistance depends on induction of synthesis of a specific protein, then return to lower levels of resistance would be explicable simply as dilution of this protein by cell division and re-repression of its synthesis *de novo*.

DISCUSSION

Resistance of staphylococci to penicillin (Richmond, 1965) and chloramphenicol (Shaw & Brodsky, 1968) is due to inactivation of the drugs by enzymes synthesized after induction, the respective drugs serving as inducers. Our results indicate that in Tc resistance, although resistance is probably not due to inactivation, an inducible genetic system is involved. This has not been shown for low-level resistance; either the coccus produces constitutively a small amount of the resistance protein, or it has sufficient time to produce such small amounts, protecting against low concentrations of Tc, before uptake inhibits further protein synthesis. High resistance of a bacterial population, however, depended under our experimental conditions on pre-incubation with subinhibitory concentrations of the drug. This induction of high resistance was inhibited by both chloramphenicol and actinomycin D, but was not dependent on growth since it occurred in cultures inhibited by nalidixic acid during the period of contact with tetracycline.

It may seem curious that an inhibitor of protein synthesis is also an inducer for the development of resistance by a procedure that depends on protein synthesis. Pre-incubation with low concentration of the drug in order to induce high-level resistance is probably an entirely artificial laboratory procedure with no biological importance. However, it is possible that in the natural environment of the staphylococcus Tc molecules might penetrate even more slowly than in laboratory media, due to differences in the metabolic state of the organism; under such conditions the organism might be induced to develop a high level of resistance before phenotypical expression is inhibited by the inducer itself.

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