Resistance of Dissociants of *Gaffkya tetragena* to Penicillin and Streptomycin.* (23035)

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The ability of bacteria to become resistant to antimicrobials presumably rests on a genetic basis. Current views on the matter are (a) a few originally resistant bacteria exist as variants in any bacterial population and (b) dissociation toward increased resistance is induced by exposure to antimicrobials. In both circumstances the parent population dies and the resistant forms live to perpetuate growth in the presence of the drug. A third view (c) assumes that a gradual adaptive change occurs among bacteria whose progeny are more resistant than the parent strains.

A convenient way to test these views is provided by *Gaffkya tetragena* whose numerous dissociants or variants are distinguishable by their colonial color and texture. In previous studies, a strain derived from a patient grew in its usual S-form white colonies on agar. After many months of observation, its variant S-yellow, S-brown, S-pink and S-pink-yellow types, their respective M and R phases and a colorless translucent form were isolated. Reversions between types occurred and immunologic relationships were demonstrated. Variation apparently developed by chance and could not be forced in any desired direction(1,2).

Methods. In preparation for this study, the same strain preserved for 20 years as *6007* in the American Type Culture Collection, was restudied. By applying methods previously used(1) only the mucoid-white and mucoid-pink colony type appeared after 6 months of observation. Strain *10875* A.T.C.C. then was obtained and in the course of aging for 3 to 6 months at room temperature in broth and on solid agar, the S-yellow, S-brown, and a translucent form were isolated. For unknown reasons, variant colonies appeared among the S-white colonies and not as daughter colonies nor in sectors as previously observed. Small colonies often appeared as usual among the usual large ones of all 6 forms. Subcultures of the large ones reproduced their kind; those of the small ones produced both large and small ones.

Both stock strains seldom grew in concentrations greater than .002 unit/ml penicillin, and of 0.3 unit/ml of streptomycin in broth. Growth in slightly higher concentrations or inhibition of growth by lower concentrations occurred at times, probably due to variations in density of the inoculum, age of the culture seeded, slight differences in dilution in media, in temperature and other variables.

Serial subcultures were made at 24 or 48 hour intervals in broth containing subsuppressive amounts (.001 unit/ml) of penicillin and of (.12 unit/ml) streptomycin. Because of poor growth in broth, passages then were made on agar-plates containing the same amounts respectively. The 6 types were seeded separately on sectors and transferred daily in heavy inoculums from plate to plate. Plates were incubated at 37°C for 12 hours and at room temperature for 3 to 6 days and examined for evidence of visible variation.

Results. Penicillin. During 10 transfers of both stock strains on penicillin-agar, no dissociation was detected. Cocci grown in penicillin-broth or on penicillin-agar often were much larger than those in plain media. There also was no detectable dissociation or reversion, except of one type, among the variants separately transferred 10 times on penicillin-agar nor any evidence of increased resistance. Frequent appearance of a few white colonies among the brown ones indicated a reversion. In several resistance tests of the tenth passage cultures, growth of the white, brown and translucent colonies was suppressed by concentrations higher than .001 unit/ml and of the yellow and mucoid-white colonies, by .002 unit/ml which was identical with the toler-

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TABLE I. Increase of Resistance of Variants of *G. tetragena* to Streptomycin after Growth on Streptomycin-Agar.

		Streptomycin, U/ml							
		10	5	2.5	1.2	.6	.3	.15	Control
Stock strain variants	W		—	—	—	—	±	+	3+
	Y		—	—	—	—	±	+	3+
	B		—	—	—	—	±	+	3+
	M-W		—	—	—	—	±	+	3+
	M-P		—	—	—	—	—	+	3+
	T		—	—	—	—	±	+	3+
After 9 × on streptomycin	W	—	—	—	—	+	+		
	Y	—	—	±	±	+	+		
	B	—	—	—	—	±	+		
	M-W	—	±	+	+	+	+		
	M-P	±	+	+	+	+	2+		
	T	—	—	—	—	+	+		
After 18 × on streptomycin	W	—	—	+	+	2+			
	Y	+	+	+	2+	3+			
	B	—	—	—	±	+			
	M-W	—	+	2+	3+	3+			
	M-P	±	+	+	3+	3+			

3+ to + indicates density of growth on agar plates after 12 hr at 37°C and 5 days at room temp. ± indicates growth of a few colonies only.

ance of the unpassaged cultures. In other words, resistance did not increase.

Streptomycin. In similar serial passages of the stock strains and of their separate variant forms, now including the mucoid-pink type, on agar containing .12 then .25 unit/ml, no variation or reversion was detected, except for the usual appearance of a few white colonies among the brown. A test after the ninth transfer indicated increased resistance of all 6 types as shown in the table. The translucent form was least resistant and died out later; the S-yellow, mucoid-white and mucoid-pink types acquired the highest resistance. In previous studies the S-yellow type was more resistant than the others to adversity(2).

Transfers were continued for 9 more passages on agar containing 0.5 unit/ml. Cultures from the eighteenth passage revealed further increase in resistance of all forms except the brown, and especially of the yellow, mucoid-white and mucoid-pink. Small white, yellow, brown, mucoid-white and mucoid-pink colonies continually appeared among the respective usual large ones, and their tolerance, surprisingly, was slightly greater by one-step higher concentration. In general, growth on agar containing subsuppressive amounts of streptomycin increased the resistance of all types but not equally from about 0.3 unit/ml to 0.6 to 10 units/ml.

Comment. The limit of growth tolerance of *G. tetragena* strain *6007,* to penicillin in amounts more than .002 unit/ml in our hands was much lower than that reported by Mock and Wynne who obtained growth on agar containing 2 units/ml(3). Resistance of a different strain to 125 units/ml was reported in clinical studies(4). In other tests, the tolerance of different strains ranged from .016 to .06 unit ml(5.) Evidently, the resistance of strains of *G. tetragena,* like certain other bacteria, varies greatly and perhaps differences in technic may account for some of the wide range. Mock and Wynne failed to induce increased resistance to penicillin, and succeeded to do so against streptomycin from 0.5 unit/ml, initially to as much as 1000 units/ml after 4 serial transfers. In our experiments, induced resistance rose at most from 0.3 unit/ml to about 10 units/ml and to that degree only with the yellow and mucoid-pink variants.

We also were unable to increase the resistance to penicillin of the white type or of any of the variants derived from the 2 strains tested. After 9 and 18 passages on streptomycin-agar, resistance of each variant increased but to different degrees, greatest with

the yellow, mucoid-white and mucoid-pink types, least with the white and brown. There was no evidence of spontaneous or induced variation to account for significantly increased resistance or for the survival of one variant over another. However, Leidy and Alexander found that the incidence of resistant variants in cultures of *H. influenzae* varied from 1 to 1 billion to 1 to 14 billions organisms(6). It is possible in our experiments that some cocci were missed which might have had or developed resistance to penicillin or to streptomycin as great as that reported by others.

Our observations lend support to views (a) and (b) mentioned previously to the extent that variant forms of the strains of *G. tetragena* tested can acquire more resistance to an antimicrobial, streptomycin in this case, than the parent white type. There was, however, no evidence that exposure to antimicrobials induced variation any more than other adverse growth conditions did(2). The ability of *G. tetragena* to dissociate, apparently by chance, may indeed provide means for its survival *in vitro* if any new variant emerges and is able to grow better than the original form in the presence of an antimicrobial.

The results of the tests favor view (c) as well. A gradual adaptation, without visible colonial changes, also took place allowing the development of unequal grades of resistance of each variant independently.

In general, the observations agreed with those previously reported wherein dissociation, more or less by chance, provided variants hardier than the parent cocci(2). In addition, the gradual adaptive ability demonstrated against streptomycin suggests that either or both changes may enable *G. tetragena* to continue growth in certain unfavorable conditions. The magnitude of resistance developed by either method may be of therapeutic significance. So far as is known, infection in man is caused by *G. tetragena* in its S-white colony type. The white type as noted here acquired resistance without evident variation and this may account for the development of resistance as observed clinically(4). Type or phase variation rarely is detected during disease(7).

Conclusion. *G. tetragena,* strains *6007* and *10875,* failed to acquire resistance to penicillin after repeated transfers in or on media containing it, nor did the procedure induce visible variation or reversion of the usual dissociant forms. Growth on streptomycin-agar increased the tolerance of each variant to 5 to 30 times the original amount of streptomycin which may be of clinical significance. There was no evidence that variant forms better fitted than the original one to grow under new adverse circumstances were either induced or purposefully developed. The change seemed to be a gradual adaptive one.

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