

Streptomycin Resistant Variants Obtained from Recently Isolated Cultures of Tubercle Bacilli.*

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One of the most important limitations upon the use of streptomycin in the treatment of tuberculosis is the rapidity with which tubercle bacilli which have become resistant to bacteriostatic action of streptomycin develop in many patients undergoing treatment with the drug. The development of such drug-resistant forms of bacteria is generally believed to be brought about by the selection of resistant mutants present in the original bacterial population.

Pyle¹ has shown that the tubercle bacilli isolated directly from patients prior to the institution of streptomycin therapy are composed of bacterial cells which do not have a uniform susceptibility to the bacteriostatic action of streptomycin. According to Pyle, the streptomycin resistant population of tubercle bacilli in patients undergoing treatment with streptomycin gradually increases as administration with streptomycin continues. Apparently, the more susceptible tubercle bacilli are retarded in their growth, whereas the more resistant ones are not. Once a predominantly resistant population develops, the tuberculous process is refractory to streptomycin therapy.

Youmans, Williston, Feldman and Hinshaw,² Middlebrook and Yegian³ and Williston and Youmans⁴ have shown that cultures of virulent tubercle bacilli, including the H37Rv strain, will become resistant to the bacteriostatic action of streptomycin following serial transfer in media containing small amounts of streptomycin.

Vennesland, Ebert, and Bloch⁵ later reported the isolation of streptomycin resistant variants of the virulent laboratory strain of *M. tuberculosis* H37Rv after a single exposure of large numbers of tubercle bacilli to high concentrations of streptomycin. These variants were resistant to more than 10,000 times the concentration of streptomycin necessary to inhibit the growth of the original culture. There is a possibility that these streptomycin resistant variants may play a large role in the development of the high degree of resistance frequently observed in cultures obtained from patients under treatment with streptomycin. The demonstration that recently isolated strains of tubercle bacilli would produce these variants, would tend to strengthen this possibility.

Methods. Streptomycin in the following concentrations, 1000.0, 500.0, 100.0, 50.0, 25.0, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19, and 0.095 μ g per ml, was incorporated in Herrold's glycerin egg agar medium⁶ cooled to 45°C. The medium was then tubed in 20 by 125 mm screw top test tubes, and was allowed to harden in the form of slants. Fine suspensions of the recently isolated cultures of tubercle bacilli were prepared by grinding, as previously described,⁷ and a slant of medium of each streptomycin concentration was inoculated by pipet with 0.1 to 0.25 mg wet weight of tubercle bacilli. In some cases inoculation was made directly from cultures

* This investigation was supported (in part) by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

¹ Pyle, M. M., *Proc. Staff Meetings, Mayo Clinic*, 1947, **22**, 465.

² Youmans, G. P., Williston, E. H., Feldman, W. H., and Hinshaw, H. C., *Proc. Staff Meetings, Mayo Clinic*, 1946, **21**, 126.

³ Middlebrook, G., and Yegian, D., *Am. Rev. Tuberc.*, 1946, **53**, 553.

⁴ Williston, E. H., and Youmans, G. P., *Am. Rev. Tuberc.*, 1947, **55**, 536.

⁵ Vennesland, K., Ebert, R. H., and Bloch, R. G., *Science*, 1947, **106**, 476.

grown in Dubos medium.⁷ Following inoculation, the cultures were incubated at 37°C for a period of 6 weeks. At the end of 2 weeks, a reading was made of the least amount of streptomycin which completely inhibited the growth of the culture. This value was recorded as the sensitivity of the culture as a whole to streptomycin. At weekly intervals thereafter, the tubes were examined for the presence of colonies of tubercle bacilli that may have grown out on the higher concentrations of streptomycin. When found, these were transferred to fresh medium and, in most cases, retested for their sensitivity to streptomycin both on solid and liquid medium. Again the least amount of streptomycin which completely inhibited growth was recorded as the sensitivity of these cultures to streptomycin.

The 57 recently isolated cultures used in this study were obtained from various hospitals and sanatoria in the Middle West, and came from patients either under streptomycin treatment or from patients for whom streptomycin therapy was contemplated.

Results. All the cultures of tubercle bacilli showed profuse growth within two weeks on the Herrold's control medium which did not contain streptomycin. At this time, depending upon the sensitivity of the culture to streptomycin, there was also growth on some of the tubes of medium containing the lower concentrations of streptomycin. As the time of incubation lengthened, growth would usually appear on tubes containing the next one or two higher concentrations of streptomycin. This later growth usually consisted of only a few scattered colonies. These, by test, were found to be organisms of slightly greater resistance to the bacteriostatic action of streptomycin than the majority of the cells in the original culture. These findings confirmed the report of Pyle¹ that cultures of tubercle bacilli are not composed of cells having a uniform sensitivity to streptomycin.

⁶ Herrold, R. D., *J. Infect. Dis.*, 1931, **48**, 236.

⁷ Youmans, G. P., and Karlson, A. G., *Am. Rev. Tuberc.*, 1947, **55**, 529.

⁸ Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1936, **83**, 409.

TABLE I.
Streptomycin Resistant Variants Obtained from Cultures Isolated from Patients Who Had Not Received Streptomycin Therapy.

Strain No.	Streptomycin sensitivity of culture in Herrold's Medium	Conc. of streptomycin on which streptomycin resistant variant was found
H37Rv	1.56	12.5
H37Rv	1.56	6.25
H37Rv	1.56	50.0
1	1.56	6.25
2	1.56	6.25
3	6.25	50.0
4	1.56	25.0
4	1.56	12.5
5	1.56	1000.0
5	1.56	500.0
5	1.56	100.0
5	1.56	50.0
6	1.56	12.5
7	3.12	12.5
8	3.12	100.0
9	3.12	25.0

The sensitivity of these organisms, however, was no more than 2 to 4 times less than that of the majority of the cells in the culture. Patients being treated with streptomycin, however, are known to discharge tubercle bacilli that are several thousand fold more resistant to the bacteriostatic action of streptomycin.

Examination of the tubes after 4 weeks of incubation frequently revealed single isolated colonies of tubercle bacilli growing on much greater concentrations of streptomycin than the rest of the culture. These colonies seldom appeared within less than 4 weeks of incubation and were usually quite small; the colonial morphology, however, was similar to that of the rest of the culture.

Table I shows the number of these streptomycin resistant variants isolated from 9 out of 14 (64.3%) cultures of tubercle bacilli obtained from patients who had never received streptomycin. Also included is the virulent laboratory strain, H37Rv. The majority of these variants, after subculture, were retested for their streptomycin sensitivity; in all cases they were resistant to a concentration of streptomycin which was equal to or greater than the concentration in the agar medium upon which they were originally isolated.

TABLE II.
Streptomycin Resistant Variants Obtained from
Cultures Isolated from Patients Who Had Re-
ceived Streptomycin Therapy.

Strain No.	Streptomycin sensitivity of culture in Herrold's Medium	Conc. of streptomycin on which streptomycin resistant variant was found
10	1.56	50.0
10	1.56	12.5
11	1.56	12.5
12	1.56	12.5
13	3.12	50.0
13	3.12	25.0
14	1.56	500.0
14	1.56	100.0
15	6.25	50.0
15	6.25	25.0
16	6.25	100.0
17	6.25	100.0
17	6.25	50.0
18	3.12	50.0
18	3.12	12.5
19	3.12	500.0
19	3.12	100.0
19	3.12	50.0
19	3.12	25.0
19	3.12	12.5
20	1.56	50.0
20	1.56	12.5
21	1.56	50.0
22	12.50	100.0
23	1.56	25.0
24	1.56	25.0
24	1.56	12.5
25	3.12	1000.0

Of the 43 cultures obtained from patients following the institution of streptomycin therapy, streptomycin resistant variants were isolated from 16 (37.1%). These are shown in Table II. All of these cultures, with the exception of number 22 would be considered relatively sensitive strains on the basis of conventional streptomycin sensitivity tests. All of the streptomycin resistant variants which were retested for sensitivity to streptomycin

were again found to be resistant to a concentration of streptomycin which was equal to or greater than the concentration contained in the medium upon which they were originally isolated. Frequently they were considerably more resistant. For example, 5 streptomycin resistant variants of strain No. 19 were found upon the following concentrations of streptomycin: 500.0, 100.0, 50.0, 25.0, and 12.5 μ g per ml. When retested, these variants were found to be sensitive to the following concentrations of streptomycin, respectively; greater than 1000.0, greater than 1000.0, 100.0, 500.0, and 12.5 μ g per ml.

Discussion. The presence of streptomycin resistant variants of tubercle bacilli in such a high proportion of recently isolated strains of tubercle bacilli, suggests strongly that these variants may play a role in the development of the streptomycin resistance which occurs in cultures of tubercle bacilli isolated from patients having had prolonged treatment with streptomycin. As yet, sufficient data are not available to determine whether those patients, whose cultures which were isolated prior to the administration of streptomycin and which subsequently showed the presence of streptomycin-resistant variants, will be more likely to develop resistant cultures upon streptomycin therapy. This may be of importance because if such a correlation does exist, it might provide an indication, before the beginning of therapy or early during therapy, as to which patients are likely to develop resistant strains.

Conclusions. Streptomycin resistant variants were obtained from 25 out of 57 (43.5%) recently isolated strains of tubercle bacilli.