A VARIANT OF MYCOBACTERIUM RANAE REQUIRING STREPTOMYCIN FOR GROWTH

DIRAN YEGIAN AND VERA BUDD

Ray Brook State Tuberculosis Hospital, Ray Brook, New York

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Miller and Bohnhoff (1947) in a recent publication have described a meningococcus variant that requires streptomycin for reproduction *in vitro* and *in vivo*. A type b *Hemophilus influenzae* whose growth is favored by a medium containing streptomycin has been reported by Alexander and Leidy (1947). These differ from the usual streptomycin-resistant variants, which grow equally well in the presence or absence of the drug. The present report deals with the isolation of a streptomycin-resistant variant of *Mycobacterium ranae* that grows only in the presence of streptomycin. This variant is also resistant to the sulfonamide drugs and has other unusual characteristics. The variant was isolated in studies dealing with the incidence of spontaneously occurring resistant forms of *M. ranae* in media containing different concentrations of streptomycin.

M. ranae was chosen for the preliminary studies because it has been shown (Middlebrook and Yegian, 1946) to develop streptomycin resistance rapidly *in vitro*. It is a fast-growing, acid-fast bacillus that is nonpathogenic for mammals. When large populations of the parent susceptible strain are plated out on a medium containing streptomycin, a small percentage of resistant organisms is consistently obtained. For example, in a population of approximately 400 billion organisms, 20 colonies will grow in the presence of 1 μg per ml of streptomycin. In 10 μg per ml, 4 colonies will grow; and only 1 colony will grow out on a medium containing 100 μg per ml of the drug. Those organisms selected by 100 μg per ml were also resistant to 1,000 μg per ml. The colonies isolated on 1 μg per ml showed a wide range in their degree of resistance. When these were plated out, 5 million organisms yielded 1 colony that would grow in the presence of 100 μg per ml, whereas only 1 colony resistant to 1,000 μg per ml was isolated from a population of 200 million organisms. The resistant organisms (variant A) grow rapidly and luxuriantly on glycerol nutrient agar either in the presence or absence of streptomycin.

The possibility of isolating another variant from this resistant strain that might require streptomycin for growth was considered. The following method proved successful in isolating such a variant:

A deficient medium was prepared which did not support growth of either the parent culture or variant A, and to this was added 1,000 μg per ml of streptomycin.[1] Plates of this medium were inoculated with 0.5 ml (approximately

[1] Composed of the following: asparagine, 0.5 per cent; ammonium citrate, 0.5 per cent; potassium phosphate (dihydrogen), 0.3 per cent; sodium carbonate anhydrous, 0.3 per cent; sodium chloride, 0.2 per cent; magnesium sulfate, 0.1 per cent; ferric ammonium citrate, 0.005 per cent; agar, 1.5 per cent; and streptomycin, 1,000 μg per ml. Final pH 7.2.

25 billion organisms) of a suspension of resistant variant A. After 10 to 15 days of incubation each plate showed from 5 to 7 colonies that were 1 to 2 mm in diameter. These colonies (B) were dense and dry, and were unlike those of either the parent or variant A on glycerol agar. There was no increase in pigmentation, and the cellular morphology was not altered, as was shown by the Ziehl-Neelsen technique.

The B colonies were removed with a needle and heavily seeded on fresh deficient medium. After prolonged incubation there was evidence of slight growth but no definite gross colony formation, and repeated subcultures did not show any appreciable increase in growth. It was obvious that this variant B could not be cultured on the deficient medium. Glycerol nutrient agar to which were

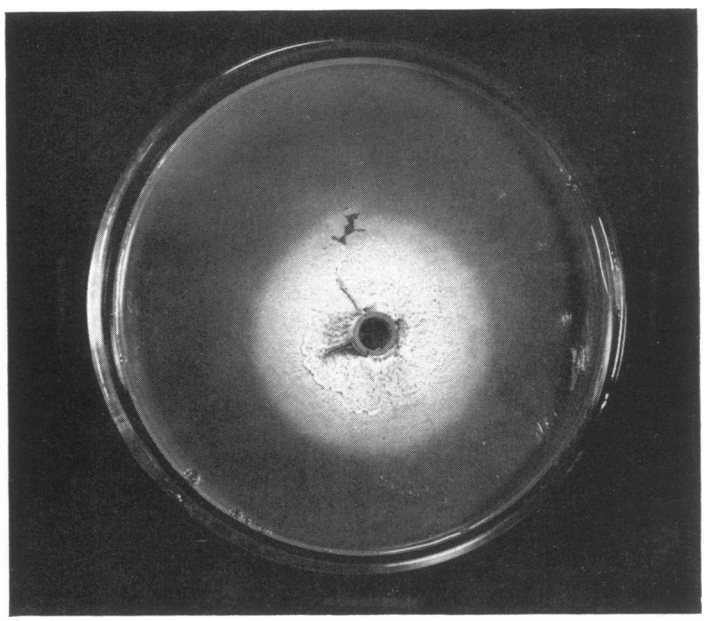

FIG. 1. SHOWING GROWTH OF M. RANAE ONLY IN THE AREA WHERE STREPTOMYCIN HAS DIFFUSED INTO THE MEDIUM AT SUFFICIENT CONCENTRATION

added 1,000 μg per ml of streptomycin yielded luxuriant growth after a few days of incubation. In the absence of the drug, the nutrient medium does not support growth of variant B even after prolonged incubation, whereas the parent and variant A show abundant growth within 2 days.

That variant B requires streptomycin for growth can be further demonstrated by the cylinder plate method used in penicillin and streptomycin assay. A drop of bacillary suspension is distributed evenly over the surface of a glycerol nutrient agar plate, and streptomycin is placed in a glass cylinder lightly imbedded in the surface of the medium. After incubation for 4 or 5 days, growth will be seen only in the area in which the drug diffused in sufficient concentration (figure 1).

Other studies with both liquid (Dubos and Davis, 1946) and solid media have shown that, whereas there is visible growth of variant B after 4 days' incubation in the presence of 50 μg per ml of streptomycin, the maximum growth is only obtained when the concentration of the drug is 100 or more μg per ml. A trace of growth in 5 μg per ml and good growth in 10 μg per ml may be secured by prolonging the incubation period 10 days. On subculture from 10 μg per ml the organisms grew rapidly in 5 μg per ml, and on further transfer good growth was obtained in as low as 1 to 2 μg per ml. The results were the same when streptomycin was used from different sources and different batches.

Variant B is also unusual with regard to sulfonamide sensitivity. The parent strain and the streptomycin-resistant variant A are inhibited by 1 mg per cent of sulfathiazole. The variant B will grow in medium containing 100 mg per cent of this drug in the presence of the streptomycin necessary for its growth. Since this culture had not previously been in contact with the sulfonamides, it would seem that in this case resistance was not the result of some action of the drug on the microorganisms.

SUMMARY

The isolation of a variant of a streptomycin-resistant nonpathogenic *Mycobacterium* which requires streptomycin for growth *in vitro* has been described. If such a variant can also develop from susceptible parent strains or streptomycin-resistant variants of cultures of pathogenic tubercle bacilli, its significance in the chemotherapy of tuberculosis is evident. Studies along this line are in progress.

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