15640

Development of Streptomycin Resistance of Gram-Negative Bacilli *In vitro* and During Treatment.*

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In the course of studying a group of cases treated with streptomycin for infections with Gram-negative bacilli, therapeutic failures were found to be associated with the development of extreme resistance to that agent.[1] An attempt was then made to produce similar resistance *in vitro* in some of the streptomycin-sensitive organisms which had been isolated before therapy was started. The strains made resistant *in vitro* were then compared with the original organisms and with the resistant strains isolated from the same patients during or after treatment.

Two series of studies were done, (1) in liquid media by transfer through increasing concentrations of streptomycin in broth and (2) on solid media containing similar concentrations of streptomycin.

Methods. The two types of media used in the present study were: "Brain Heart Infusion" (Difco), broth, pH 7.3, hereafter referred to as broth, and "Heart Infusion Agar" (Difco), pH 7.4 to be referred to as agar. While not the optimum media for streptomycin studies,[2-4] they were highly satisfactory for the purpose at hand.

Streptomycin concentrations were checked for potency against the reference organisms used in this laboratory, namely, strain T of *Klebsiella pneumoniae,* type A, which is sensitive to 0.78 unit of streptomycin. This organism has given consistent and duplicable results. Organisms isolated from patients prior to treatment were subcultured successively on agar plates 4 or more times, by streaking from a single colony on each occasion. Eventually a single such colony was picked off and used to provide the starting culture for both series of tests.

The broth transfer study was carried out by a serial dilution method. A 2-fold concentration series of streptomycin in broth was set up in 10 mm x 110 mm tubes by pipetting 0.5 ml quantities from "master" tubes prepared by serial dilution. The range of each series was chosen according to the anticipated resistance of the organism being studied. The tubes were then inoculated with 0.5 ml of a 10^{-4} dilution of a 24-hour broth culture of this organism bringing the total volume in each tube to 1.0 ml. The inoculum contained approximately 100,000 to 150,000 organisms, the actual number present being followed by pour plates; it also contained 1.0% of defibrinated horse blood to act as an indicator of bacterial growth. This amount of horse blood neither stimulates growth of the reference organisms nor inhibits streptomycin action in this medium with the size of inoculum used. Tubes were incubated and examined for visible evidence of growth after 18 to 24 hours. The lowest concentration of streptomycin in which growth failed to occur, as determined by subculture on streptomycin-free agar plates (or in streptomycin-free broth tubes in the case of *B. proteus*) was read as the minimum inhibiting concentration (M.I.C.). The tube with the highest concentration of streptomycin in which there was good bacterial growth was used as the source culture for a fresh series of tubes. This process was repeated daily, the time per transfer thus being ap-

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TABLE I.
Development of Streptomycn Resistance During Treatment and *in Vitro*.

No.	Case	Organism	M.I.C. of organism isolated before treatment, units/ml	Days treatment to isolation of strain of max. resistance	No. of transfers required to achieve max. resistance	
					In streptomycin broth	In streptomycin agar
1	A.P.	*A. aerogenes*	6.3	3	49	29
2	E.F.	" "	12.5	1	41	12
3	E.F.	" "	12.5	1	27	—
4	C.H.	*K. pneumoniæ*	12.5	3	44	19
5	C.M.	" "	6.3	1	40	6
6	W.T.	Paracolon bacillus	25	4	—	29
7	C.M.	*Ps. aeruginosa*	12.5	3	13	5
8	M.K.	*E. coli*	25	1	44	19
9	M.S.	*B. proteus*	50	*	31	*
					Avg 36	Avg 17

* Organism not recovered after treatment was started and not studied on agar.

Note. Resistant strains grew well in broth containing 50 mg (50,000 units) per ml from an inoculum of 100,000 to 150,000 organisms.

M.I.C. = Minimum inhibiting concentration giving no growth on transfer to streptomycin-free broth.

proximately 24 hours. On occasions when it was necessary to miss a day the tubes were refrigerated and no significant changes in the general trend were noted. Concentrations of 2,000, 5,000, 10,000 and 50,000 units per ml were found to be convenient and satisfactory when resistance had been raised to the 1,000-unit level.

In the second series of studies agar plates were prepared containing increasing 2-fold concentrations of streptomycin by mixing 1.0 ml of broth containing the required amount of streptomycin with 9.0 ml of melted agar. These plates, together with a control containing no streptomycin were each streaked with a 2 mm loopful of an undiluted 24-hour broth culture of the organisms. It was possible to accommodate several tests on each plate. The plates were examined for growth after 24 hours incubation and the lowest concentration of streptomycin showing no growth was read as the M.I.C. Material for transfer to the next series was taken directly from the surface of the plate with the highest concentration of streptomycin on which there was good growth. A 2.0 mm loopful of the surface growth was used for each inoculation. Transfers were made daily. Occasionally when a delay was necessary, transfers were made through streptomycin-free plates to avoid contact of the organisms with streptomycin for longer than 24 hours per transfer. It was found impractical to use concentrations of over 6,400 units per ml in agar. In practice all the organisms studied became highly resistant before this value was reached. When it became evident that an organism had become highly resistant, *e.g.*, when improved growth was noted on concentrations which previously supported only poor growth or when there was a sudden rise in resistance, subcultures were made to streptomycin-free broth and the sensitivity was then determined by the usual serial dilution method. The final M.I.C. values reported for the 2 series of experiments were, therefore, all determined by the serial dilution method in broth and so are comparable.

The streptomycin dilutions used in both series of studies were checked daily against the reference organism. This allowed corrections to be applied for loss of potency of stock streptomycin solutions.

Nine strains were studied, as shown in Table I. Eight of them were from 6 patients who later yielded highly resistant but otherwise indistinguishable strains during or after treatment with streptomycin. One strain of *B. proteus* from a case which responded to therapy was included in the broth dilution series, but not with the surface plate series because of its spreading properties. Strain No. 1 was obtained from the blood culture of a patient who died of bacterial endocarditis;

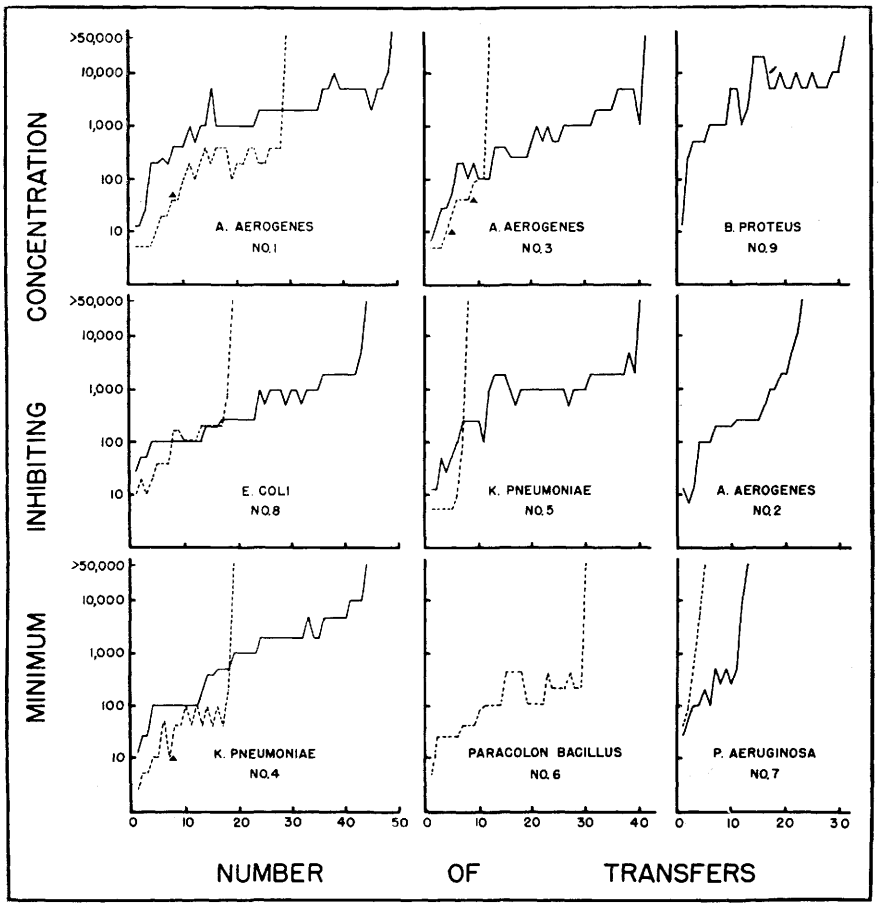

FIG. 1.

Development of resistance in Gram-negative bacilli by transfers through streptomycin-containing broth (solid lines) and agar (broken lines). The solid triangles indicate points at which high resistance developed suddenly in an isolated colony. The minimum inhibiting concentration is given in units (micrograms) per ml.

the others were from cultures of urine in cases of urinary tract infection. Two strains of *Aerobacter aerogenes,* No. 2 and No. 3, obtained from the same patient at different times before treatment, were run through the broth series for comparison and No. 3 was also run on surface plates. Strains 5 and 7 were different organisms from the same patient and both had become resistant during treatment.

Results. Composite results are shown in Table I and Fig. 1. It was possible to raise the resistance of these strains to high values, both in broth and on surface plates. All of the organisms acquired resistance so that they grew well in a concentration of 50,000 units of streptomycin per ml. Varying numbers of transfers were required to accomplish this, ranging from 5 for *Pseudomonas aeruginosa* on surface plates to 49 for a strain of *A. aerogenes* in the broth transfers. Initial sensitivities as indicated by the plate method were lower than those shown in broth by the same strains except in the case of No. 7, a strain of *Ps. aeruginosa,* which developed resistance more rapidly than the other strains.

In the course of the surface plate study a few isolated colonies were noted growing on a concentration of streptomycin higher than the M.I.C. for the rest of the bacterial population of the same culture. Such colonies were picked off, grown in streptomycin-free broth and checked for sensitivity by the broth dilution method. Four of them, shown

by solid triangles in Fig. 1, were found to be resistant to over 50,000 units of streptomycin per ml.

There did not seem to be any definite correlation between the rate at which resistance developed *in vitro* and in the patient (Table I). Nor was there any relation between the nature of the organism or its initial sensitivity and the rate of development of resistance *in vitro*. The number of observations, however, are too few to permit any final deductions in this regard.

Organisms in which resistance to streptomycin had been induced as described above were examined and compared, with respect to cultural characteristics and biochemical reactions, with the original sensitive strains and with corresponding resistant strains which were isolated from the patients during the course of treatment. It was previously shown[1] that the original strain and the strain isolated from the patient after therapy were identical morphologically and in their cultural and biochemical characteristics. All organisms which had been passed through streptomycin broth showed some degree of pleomorphism and grew poorly on the usual culture media. The strain of *Escherichia coli* was rough, but was identical in its biochemical reactions with the original sensitive strain and with the corresponding *in vivo* resistant strain. Both of the strains of *K. pneumoniae* grew very poorly in most of the test media used. They failed to ferment saccharose, to produce a Voges-Proskauer reaction or to grow on citrate. Strain No. 5 reacquired the property of fermenting saccharose after 9 passages through broth without loss of resistance to streptomycin but No. 4 failed to do so after 15 such transfers. The remainder of the strains which developed resistance on broth transfer did not appear to differ from the original sensitive strain or from the corresponding *in vivo* resistant strains. Where resistance was acquired on surface plates either by repeated transfer or suddenly in relatively low concentrations of streptomycin, there was less pleomorphism, and, with the exception of the *E. coli* strain, which was rough, could not be distinguished culturally or by biochemical reactions from the corresponding starting or *in vivo* resistant strains. The broth cultures of No. 6 became contaminated during the course of its many transfers and the results are not included.

Discussion. The fact that it was possible to increase the resistance of the organisms studied confirms results described by others.[5-11] Resistance to over 50,000 units of streptomycin per ml represents a figure much higher than has been reported elsewhere and approaches the limit of concentration which can be employed under the conditions of the test. Organisms which are able to grow in this concentration may be regarded as totally resistant. The highest number of transfers required for the induction of such resistance was 49, while the average was 36 for the broth transfer series and 17 for transfer on surface plates. This number, and the time involved, might possibly be reduced were the time per transfer shortened.

The difference in the rates at which resistance developed on surface plates and in broth is of interest. It may be the result of using a larger inoculum in the surface cultures thus offering a larger number of cells of varying resistance (cf. penicillin and staphylococcus[12,13]). This may have some practical implications in connection with the development of resistance by bacteria growing in pockets or walled-off spaces where the concentration of organisms is high and

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that of the antibiotic is relatively low, or in some instances where the exposure of the organism to the antibiotic may resemble that of a surface growth.

The sudden acquisition of extreme resistance by isolated colonies on surface plates suggests that in addition to the gradual increase in resistance shown by organisms exposed to streptomycin, some bacterial cells may suddenly become highly resistant while exposed to comparatively low concentrations. This phenomenon, occurring as it does in material derived from a single picked off colony might offer a partial explanation of the manner in which resistance is acquired *in vivo*, making it unnecessary to postulate the presence of initially resistant strains prior to treatment in those cases where rapid development of extreme resistance is encountered during streptomycin therapy. Failure to observe any sudden increase in resistance in the broth transfer series, however, might have been related to the smaller size of the inoculum used, as judged in terms of numbers of bacteria. The average inoculum in the broth series contained 100,000 to 150,000 organisms while a loopful of surface culture used as the inoculum in the agar plate series contained several billion.

Bacteria developing resistance after prolonged exposure to sublethal concentrations of streptomycin showed impaired growth characteristics. On the other hand, resistant strains from patients under therapy and strains which developed resistance rapidly *in vitro* could not otherwise be distinguished from the strain originally isolated from patients prior to streptomycin therapy.

Conclusions. It was possible to increase the resistance of Gram-negative bacilli to over 50,000 units per ml, in all cases studied, by passage through increasing concentrations of streptomycin in broth and on agar plates.

In most instances resistance increased gradually, the change being more rapid on surface plates than in broth.

In the course of transfers on streptomycin agar, 4 instances of sudden increase in resistance of isolated colonies from relatively low values to over 50,000 units per ml were noted.

The organisms made resistant by exposure to streptomycin in broth showed marked pleomorphism and in some instances suffered changes in their biochemical reactions. When resistance developed as a result of exposure to the agent on surface plates, however, there was less pleomorphism, and no changes in biochemical reactions were noted. Resistant organisms obtained from the patient during or after treatment showed no morphological or cultural differences from the original sensitive strains isolated from the same patient.

15641

Effects of Sodium Fluoride on Hair Growth in the Rat.

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Shour and Smith[1] have shown that intraperitoneal injections of 0.3 cc of 2.5% sodium fluoride at 48-hour intervals induced hypocalcified stripes in the enamel and dentin of rats. When the injections were given at intervals of 24 hours or the dosage was increased, cumulative effects were produced which severely disturbed the formation of enamel and dentin. The effects produced by adding NaF to the diet were essentially similar to the effects produced by injections. Since the cytoplasm of the ameloblasts showed an abnormal character and disturbance of its globules, Shour and Smith suggested that fluorine exerted a direct local action on the enamel-forming cells.

[1] Shour, I., and Smith, M. C., *University of Arizona Agr. Exp. Sta. Tech. Bull.*, 1934, **52**, 69.