MODE OF ACTION OF STREPTOMYCIN ON TYPE b H. INFLUENZAE

I. Origin of Resistant Organisms*

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(Received for publication, December 7, 1946)

After exposure to streptomycin a number of different organisms have been shown to decrease in their sensitivity to this antibiotic (1–5). This change, which can be demonstrated with regularity *in vitro*, has been considered to be responsible for therapeutic failure in a significant number of patients (6–11). Investigation of this phenomenon in *H. influenzae* infections has suggested that when streptomycin is used as the only therapeutic agent, emergence of resistance is conditioned by the severity of infection and therefore presumably by the size of the bacterial population in the patient (8). Of fourteen patients treated with streptomycin alone ten recovered without amplification of the therapeutic program; but in three of the four remaining cases failure was proved to be due to a change in the sensitivity of the organisms. Growth of the strains cultivated from these patients before treatment was completely prevented by a concentration of streptomycin varying between 1 and 7 units per cc., whereas strains cultivated from the same patients after a period of treatment grew luxuriantly in concentrations of streptomycin as high as 1000 units per cc.

Detailed investigations on the organisms grown from one of these patients, No. 1, during treatment provided evidence of importance in our understanding of the development of strain resistance.

A 2 year old boy had been well and running about only 24 hours before admission to hospital. He had received no specific treatment. When first examined he was semicomatose and presented signs of meningeal irritation but no evidence of localized cerebral involvement. Examination of the spinal fluid confirmed the clinical impression that the infection had progressed to a severe degree within a 24 hour period. The spinal fluid showed numerous type b *H. influenzae* on stained smear and the sugar concentration was 6 mg. per cent. The patient appeared to offer an ideal case of severe meningitis on which to test the influence of streptomycin because he had received no other therapy and because treatment could be applied early in the course of the disease. Streptomycin was used according to the standard method already described (8 *b*). Within 24 hours he became oriented, his temperature was lower, and the neurologic signs had stopped progressing. This state continued for the next 2 days. The spinal fluid showed marked improvement within 24 hours; no organisms

* The work reported in this communication was supported by grants from the Commonwealth Fund.

were seen on microscopic examination of stained smears of sediment, and the concentration of sugar was normal. On the 3rd day, however, the culture of the spinal fluid withdrawn 24 hours after institution of streptomycin therapy showed growth of *H. influenzae* which thrived in 1000 units of streptomycin per cc. In spite of the intrathecal use of streptomycin twice daily for the next 2 days, rapid growth of *H. influenzae* occurred from all samples of spinal fluid and the sugar concentration decreased. Clinically the child became rapidly worse; convulsions of the entire right side appeared and continued intermittently for several days. Signs of hemiparesis became obvious. Streptomycin was discontinued on the 4th day of treatment and type-specific rabbit antiserum and sulfadiazine were administered. Rapid improvement followed; signs of infection cleared by the 3rd day and by the end of the 4th week the patient used all extremities normally. All cultures of *H. influenzae* isolated from this child's cerebrospinal fluid after the start of streptomycin treatment were resistant to 1000 units per cc.; this was true of 100 per cent of the bacterial population, not only when first isolated but also after subculturing for 3 months in the absence of streptomycin. Moreover, the *H. influenzae* strain isolated from the nasopharynx after the start of treatment exhibited a similar degree of resistance. Nasopharyngeal cultures, 1 year after the child's recovery, continue to show type b *H. influenzae* resistant to 1000 units of streptomycin per cc.

The susceptibility of pathogenic organisms to critical concentrations of streptomycin in their fluid environment obviously lies at the root of successful therapy with this antibiotic; and the development of strain resistance in some patients during treatment constitutes a phenomenon of prime importance. Study of resistance of a number of organisms to other antibacterial agents has led to two schools of thought. One maintains that the metabolism of the bacteria is changed as a result of exposure to the agent and the other that resistant members are present in the bacterial population from the start of the infection.

According to the latter theory, which is well supported by experimental data from a number of investigators (12–17) and which conforms also to our own results, the emergence of resistance represents a selective process. The usual *in vitro* sensitivity tests, involving relatively small numbers of organisms and thus failing at times to include samples of the comparatively rare resistant members of the initial bacterial population, reflect a conventional degree of sensitivity of the strain under investigation to the antibiotic used. However, in the course of treatment of the patient, the more sensitive members of the bacterial population are killed off and subsequent tests of sensitivity reflect survival of resistant forms. Selective survival alone explains the rapid changes in susceptibility often found. For example, in the patient (No. 1) described in detail, resistance of the strain to more than 1000 units of streptomycin per cc. was found within 24 hours of exposure to streptomycin; in fact, careful colony counts showed that 100 per cent of the bacterial population resisted this concentration, and that all sensitive organisms had been killed. It is hard to believe that so short a period would permit a fundamental change to occur in the metabolism of the surviving organisms as to enable them to thrive in streptomycin. The facts are more in keeping with the view that

streptomycin has a rapid bactericidal action which promptly kills enormous numbers of sensitive organisms (18). The latter comprise virtually the entire population, but there is present initially a minute number of organisms which differ from the rest of the population in that they can thrive in a high concentration of streptomycin, in the example cited, over 1000 units per cc.

This hypothesis was examined by experimental methods which studied three aspects of the problem: influence of the size of the bacterial population on the minimal effective concentration of streptomycin, examination of a number of different strains, before any exposure to streptomycin, for the presence of organisms resistant to 1000 units per cc., and comparison of strains from patients in whom streptomycin failed with those isolated from the group successfully treated.

Two *in vitro* procedures were used for these purposes. One tested the influence of concentration of streptomycin ranging from 1 to 13 units per cc. on a relatively small inoculum (varying from 1 million to 1700 million organisms);[1] the other examined large bacterial populations, 142 billion to 522 billion organisms, of ten of these strains for presence of members which are resistant to 1000 units of streptomycin per cc.

Comparison of Strains by in Vitro Sensitivity Tests Using Inoculum of 1 Million to 1700 Million Organisms

Strains of *H. influenzae* cultivated before and after start of treatment in all patients were tested for sensitivity to streptomycin by the same test. The method (5) determines the minimal concentration of streptomycin which when incorporated in Levinthal agar completely prevents growth of an inoculum varying from 1 million to 1700 million organisms after an incubation period of 48 hours; this value is termed the minimal effective concentration or M.E.C. The results obtained on the strains isolated *before treatment* are listed in Table I. Patients 1, 2, and 3 represent streptomycin failures; the corresponding strains developed resistance to streptomycin during the course of treatment. Strains 5 to 13 were cultivated from patients in whom streptomycin therapy was successful. The spinal fluid of patient 4 cleared rapidly but death occurred on the 5th day; an enormous subarachnoid abscess was found postmortem. It is seen that the M.E.C. of strains varied from test to test and also depended upon whether the source of inoculum was a 6 hour Levinthal broth culture or a 6 hour growth on Levinthal agar, a 2 mm. loop being used in each instance; the size of the bacterial population transferred from agar plates averaged approximately 700 times that from broth tubes. The mean M.E.C. and standard deviations have been computed for two purposes: to determine whether the larger inoculum, from Levinthal agar source, averaging 700,000,000 organisms, requires significantly higher concentrations of streptomycin to com-

[1] See footnotes, Table I.

pletely prevent growth under conditions of experiment, and whether the M.E.C. of strains cultivated from patients in whom streptomycin treatment failed, differs significantly from the M.E.C. of strains from patients in whom

TABLE I

In Vitro Sensitivity Tests on Levinthal Agar Containing Concentrations of Streptomycin Varying from 0.5 to 13 Units per Cc.

Size of inoculum 1 million to 1700 million

Patient and strain		Source of inoculum: Levinthal broth* No. of tests 1§	2	3	4	Levinthal agar‡ No. of tests 1	2	3	4	Clinical results	
		M.E.C. *units per cc.*				M.E.C. *units per cc.*					
M. = 2.07 S.D. = 1.18	1	1.1	1.1	1.6		4.4	2.8	4.4		Failure	M. = 3.8 S.D. = 1.45
	2	0.8	1.1	2.8	3.5	2.5	2.7	7.5	4.4	"	
	3	2.0	5.0	3.0	3.0	3.0	5.0	5.0	3.0	"	
	4	0.8	1.1	1.6	2.6	1.6	2.6	4.4		"	
M. = 1.87 S.D. = 0.74	5	1.6	2.8			7.5	10.7‖			Recovered	M. = 6.42 S.D. = 3.18
	6	1.6	1.6			4.4	4.4			"	
	7	0.8	2.8			1.6	7.5			"	
	8	1.6	2.8			7.5	13.0‖			"	
	9	2.8				7.5	7.5			"	
	10	1.2				7.5				"	
	11	0.8				2.7				"	
	12	1.6				3.6				"	
	13	1.6				7.7‖				"	
	14	1.1	2.5	2.8		2.5	2.7	10.8		"	
		M. = 1.97 S.D. = 0.97				M. = 5.31 S.D. = 2.86					

M.E.C., minimal effective concentration.
M. = mean.
S.D. = standard deviation.
* 2 mm. loop inoculum averaged 1,000,000 organisms.
‡ 2 mm. loop inoculum averaged 700,000,000 organisms.
§ Numbers indicate number of tests carried out.
‖ 1 to 2 colonies grew in this dilution—higher concentration not used.

this therapy was successful. It is seen that there is no significant difference between the sensitivity of organisms isolated from the unsuccessful group and those cultivated from the successful group. Moreover the difference in inoculum size between broth and agar source is not great enough to influence the M.E.C. significantly. There occurred occasionally on the plates of Levinthal agar containing 7 to 13 units of streptomycin per cc. a spotty distribution

of resistant colonies, small in number and usually less than 5, which varied in incidence in repeated tests on the same strain, suggesting a chance phenomenon. Nonetheless it can be seen that the M.E.C. of all strains falls within the range of 1 unit per cc. to 13 units per cc. with the one exception noted. The values for the first three strains, which emerged resistant during streptomycin treatment, are not significantly different from those of the other strains listed. These *in vitro* results correlated well with those obtained *in vivo*. Mouse protection tests showed a striking difference between the sensitivity of the strains before and after emergence of resistance as judged by *in vitro* tests (5, 8). The original cultures, which *in vitro* showed an M.E.C. of 1 to 13 units per cc., had an M.E.D. (the minimal effective dose of streptomycin which protected 50 per cent of mice against 1,000,000 M.L.D.) between 19 and 78 units per mouse. After treatment with streptomycin the strains which thrived in the presence of 1000 units per cc. *in vitro* were so resistant in mice that 5000 units per mouse failed to protect.

After exposure to streptomycin during treatment, strains from patients 1, 2, and 3 exhibited resistance to 1000 units of streptomycin per cc. No. 1 emerged resistant within 24 hours after the start of treatment. No. 2, first retested after 21 days of therapy, and No. 3, when first retested on the 3rd day, both resisted 1000 units of streptomycin per cc. at these times. In all other patients spinal fluid cultures were sterile within 48 hours. The strains from four of these successfully treated patients, isolated 24 hours after beginning streptomycin, all showed an M.E.C. within the range of 1 and 7 units per cc. Likewise the organisms isolated from the nasopharynx of several patients during and following successful treatment exhibited comparable sensitivity. Therefore the routine *in vitro* test, which failed to differentiate in advance those strains which developed resistance during treatment, provided a satisfactory index of efficacy of streptomycin in a given patient when applied to strains cultivated after exposure to the antibiotic.

Comparison of Large Bacterial Populations (142 to 522 Billion) for Incidence of Organisms, in the Original Cultures, Which Resist 1000 Units of Streptomycin per Cc.

In this phase of the work large samples of the original strains, prior to therapeutic use of streptomycin, were searched for the possible inclusion among them of resistant forms. Certain factors simplified the problem. When emergence of resistance was responsible for therapeutic failure, the organisms grew in or on Levinthal agar containing 1000 units per cc. of streptomycin. This concentration was so rapidly bactericidal for sensitive organisms that it was possible to inoculate 15 cc. of melted Levinthal agar at 40°C., containing 1000 units of streptomycin per cc., with 15 to 30 billion organisms in a poured plate preparation without inhibiting growth as a result of accumulation of

end products of bacterial metabolism; sensitive organisms were killed before they had a chance to reproduce. All colonies growing under these circumstances were resistant to 1000 units of streptomycin per cc., and the resistance of propagated scions has not changed after a number of subcultures in streptomycin-free medium.

Methods and Materials

All strains studied had been preserved within 5 days after isolation from the patient by drying and sealing under vacuum of 5 to 10 microns of mercury measured by a MacLeod gauge (19). The dried culture is seeded in 10 to 20 cc. of Levinthal broth in a 50 to 125 cc. flask. After 20 to 24 hours' incubation at 37.5°C. the broth culture is seeded in 0.5 cc. quantities on the surface of a series of Levinthal agar plates and incubated for 6 hours at 37.5°C.

TABLE II

Incidence of Resistant Survivals in Large Bacterial Populations Seeded in Levinthal Agar Containing 1000 Units of Streptomycin per Cc.

Patient and strain	Total organisms cultured	Organisms cultured per plate	Colonies total survival	Incidence of resistant colonies*	Clinical result
	billions	*billions*			
1	381	25.4	57	1:6.7 billion	Failure
2	142	14.2	23	1:6.2 "	"
3	301	30.1	26	1:11.5 "	"
4	423	29.2	32	1:13.2 "	"
5	253	25.3	20	1:12.6 "	Recovered
6	522	34.8	474	1:1.1 "	"
7	188	18.8	18	1:10.4 "	"
8	256	25.6	172	1:1.5 "	"
9	166	16.6	12	1:13.8 "	"
10	284	28.4	37	1:7.7 "	"

* Ratio of resistant colonies to total population cultured.

The growth is then removed by washing each plate with 2 to 4 cc. of plain broth. The yield from the plates is combined in one pool; 1 cc. is added to each of ten to fifteen poured plates, together with Levinthal agar containing 1000 units of streptomycin per cc. and the number of surviving colonies is recorded after 72 hours' incubation. The number of organisms per cubic centimeter in the pool, determined by making poured plates in Levinthal agar of appropriate dilutions of the heavy suspension, varied from 15 to 30 billion per cc. Statistical analysis showed the distribution of resistant colonies so evenly divided among individual plates and groups of five plates that five to ten plates, each seeded with an inoculum between 15 and 30 billion organisms, provided an adequate index of the ratio of resistant members to total population cultured.

RESULTS

The results are listed in Table II. The numbers used for designating the ten strains studied by this method correspond to those in Table I; as before, strains 1, 2, and 3 were isolated before streptomycin therapy from those

patients whose strains became resistant during treatment. Again it should be emphasized that the tests described were applied in all instances to cultures isolated from patients before the start of streptomycin therapy.

It is seen that all ten cultures studied contain a minute fraction of members which before exposure to streptomycin can grow in the presence of 1000 units per cc. of the antibiotic. The difference among strains in the number of resistant members present initially (column 5 of Table II) is larger than the analytical error of the method used; yet the size of this fraction seems to bear no relationship to the tendency exhibited by a strain to emerge resistant during treatment of the patient. For example the strain from patient 1 yielded one resistant organism in 6.7 billion when a total population of 381 billion was cultured by seeding each of fifteen poured plates containing 1000 units per cc. of Levinthal agar with 25 billion organisms. Strain 2 had the same proportion, whereas strain 3 showed a lower incidence, only one in 11.5 billion. It is clear from the figures that two of the strains (Nos. 6 and 8) cultivated from patients in whom streptomycin was promptly successful in eliminating the infection showed a greater incidence of resistant members than those which emerged resistant during treatment.

It is important to emphasize that in repeated experiments on the same strain, under as controlled conditions as possible, the incidence of resistant members varies. The factors responsible for this change are under investigation but the results indicate clearly that the size of the bacterial population is the most important single factor among those which determine the potentialities of a given strain to develop resistance during treatment.

DISCUSSION

The demonstration of organisms initially resistant to 1000 units of streptomycin per cc. in all ten strains of type b *H. influenzae* studied, including both those from patients who responded promptly to streptomycin treatment as well as some from more refractory patients, adds convincing evidence to the data obtained from the study of patient 1; resistance of a strain after treatment is not due to action of the antibiotic upon the bacteria but reflects a selective process which by elimination of the sensitive members from a bacterial population permits the resistant ones to emerge as sole survivors. In cultures taken from patients prior to treatment, organisms with this degree of resistance can be detected with regularity only when very large populations are used. The tests which measured the influences of concentrations of streptomycin on relatively small bacterial populations, within the range of 1 million to 1700 million organisms, demonstrated members resistant to as much as 13 units per cc. in only one strain out of the fourteen taken from untreated patients. On the other hand when the few colonies which occasionally survived in media containing concentrations of 7 to 13 units per cc. were

tested for their M.E.C., significantly higher degrees of resistance were demonstrated; in all instances studied the organisms required between 75 and 1000 units per cc. to prevent growth completely. It is clear that the recovery by cultural methods of organisms with this degree of resistance is a chance phenomenon when an inoculum of this relatively small magnitude is used. It seems clear that in all populations of *H. influenzae* there exist initially organisms with many different degrees of resistance, just as has been shown by Demerec (17) from a study of the influence of penicillin on staphylococci. The number of organisms exhibiting capacity to grow in 1000 units per cc. is so small that enormous bacterial populations have to be examined to demonstrate their presence, whereas the number which can thrive in 3 units per cc. is great enough to be readily detected in much smaller populations. For example, preliminary studies showed that when approximately 50 billion organisms are seeded into melted Levinthal agar containing only 10 units of streptomycin per cc., fourteen colonies appeared with 72 hours' incubation. Nine of these, when tested after one subculture in streptomycin-free medium, showed resistance to 1000 units per cc.; growth of four of the others was completely prevented by 100 units per cc. and one by 50 units per cc. When the same inoculum was seeded into Levinthal agar containing 1000 units per cc., seven colonies grew within 72 hours. All of these grew as well as the controls in 1000 units per cc.

It was not possible with either of the two methods used to correlate emergence of resistance during treatment with differences between strains from patients successfully treated with streptomycin and those in whom it failed. This is not surprising in the test using the smaller inocula, for chance is against demonstration of organisms resistant to 1000 units per cc. However, even when bacterial populations were large enough to permit their demonstration regularly the ratio of resistant organisms to total population was no greater in those strains which emerged resistant during treatment of patients than in those from patients treated successfully with streptomycin. In fact the highest incidence of colonies resistant to 1000 units per cc. occurred in strains isolated from the group in whom streptomycin promptly eliminated the infection.

All the data obtained from these procedures indicate that emergence of resistance of a strain during treatment of a patient is conditioned not by a change in the sensitivity of the organisms as the result of streptomycin action nor by an unusually high initial incidence of organisms with resistant traits, but rather by the size of the bacterial population, which is presumably closely correlated with severity of infection.

CONCLUSIONS

1. Of fourteen patients with type b *H. influenzae* infections treated with streptomycin alone ten recovered promptly; in three of the four remaining

cases failure was proved to be due to emergence of resistance of the organisms to streptomycin.

2. The rapid development of resistance to 1000 units of streptomycin per cc. during treatment argues against metabolic adaptation of the bacteria.

3. Careful study of large samples of initial bacterial populations shows resistant members in all ten strains examined. The incidence varies from 1 in 1.1 billion to 1 in 13.8 billion organisms.

4. The proportion of resistant members is not significantly higher, before treatment, in patients who do poorly than in those who respond promptly.

5. The results indicate that emergence of resistance is the result of a selective process; after elimination of sensitive members the very few resistant ones, apparently present in all strains, make up most or all of the population in the cultures taken after commencement of treatment.

6. The survival of organisms which can grow in high concentrations of streptomycin, either in patients or *in vitro*, is influenced more by the size of the bacterial population than by any other known factor.

7. Since the bacterial population is relatively small in those patients with mild or moderately severe infections the likelihood of a significant number of very resistant members being present is remote.

8. The traits responsible for resistance of the organisms are apparently inherited: (*a*) The resistant state of one strain has been transmitted *in vitro* without change in degree through over one hundred subcultures in the absence of streptomycin. (*b*) Persistence of resistant organisms in the nasopharynx of one patient during a one year period raises an important public health problem.

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