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Development of Resistance to Penicillin by Pneumococci.

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The primary object of this study was to determine whether pneumococci develop resistance to penicillin when exposed to this anti-bacterial agent in the infected animal. As corollaries of the finding that such resistance may develop, observations were also made on (a) the capacities of different strains of pneumococcus to acquire resistance, (b) the retention of resistance when contact between organism and penicillin was discontinued, (c) the change in *in vitro* sensitivity accompanying the development of resistance *in vivo*, and (d) the response of *penicillin-resistant* pneumococci to sulfonamides. The results of these studies are presented here.

Experimental. A. *Development of resistance to penicillin in vivo.* The parent strains of pneumococcus used in this experiment were the type I McGovern and the type III CHA strains, used previously in many of our sulfonamide studies. These pneumococci were exposed to penicillin by serial passage through 7 groups of mice treated with this drug. Briefly, the procedure was as follows. In the initial experiments, 4 groups of 10 white mice (males weighing 16 to 18 g) were infected intraperitoneally, each mouse receiving 10^{-6} cc of a 12- to 14-hour blood broth culture of the desired parent strain. One group of 10 animals served as untreated controls. The animals in the remaining 3 groups were treated with 500, 1000 and 2000 Oxford unit doses of penicillin, these amounts being administered by stomach tube 2, 8 and 14 hours after infection and every 8 hours thereafter for 5 additional days.* Cultures of heart blood were prepared from 2 to 6 treated mice dying either during therapy or shortly thereafter. These cultures were incubated at 37.5°C for 12 to 14 hours and then pooled. Five-tenths cc of this pooled culture was injected intraperitoneally into an untreated mouse; a 12- to 14-hour blood broth culture prepared from the heart blood of this animal was used to infect a second lot of 40 mice, which received the same treatments as the first. This procedure was repeated for 6 consecutive passages. At the end of these transfers the effectiveness of penicillin therapy against infections with the parent strains was redetermined in order to make certain that the activity of our preparation had not gradually deteriorated during the passage period.

The results of the experiment with type III pneumococcus, CHA, (Table I) show that this strain developed considerable resistance to penicillin. Thus when mice infected with the parent organisms were treated with 1000-unit doses of penicillin, 60% recovered and those that died lived an average of 95 hours (Experiment A). In contrast to this, similar treatment afforded little if any protection to mice infected with organisms obtained from the sixth serial passage (Experiment G); none of these animals recovered and their average survival time, 32 hours, was only 4 hours longer than that of untreated controls. Even 2000-unit doses, which afforded complete protection against infections with the parent strain, gave little protection against the passaged strain (*cf.* Experiments A and G).

The type I McGovern strain also developed resistance to penicillin (Table II). Eighty per cent of the mice infected with the parent strain survived when treated with 1000-unit doses of penicillin, and the average survival time of the animals that died was 127 hours (Experiment A). In contrast to this, only 10% of the mice infected with the organisms from the sixth serial passage survived when treated similarly (Experiment G); in this case, the average survival time of the animals that died

* The above doses represented 2.5, 5 and 10 mg of the penicillin preparation supplied us through the courtesy of the Lilly Research Laboratories, Indianapolis, Indiana. These amounts were administered in solution in 0.2 cc of 1% sodium bicarbonate.

TABLE I.

Development of Resistance to Penicillin by Type III Pneumococcus, Strain CHA: 10 Mice Infected in Each Group.

Exp.	Infecting Organisms: Source	Infecting Organisms: No. in infecting dose	Units P‡ per dose*	No. of deaths, Days after infection: 1	2	3	4	5	6-10	Avg hrs survival of mice that died	30-day survivors: No.	30-day survivors: %
A	Parent strain†	400	500	2	3	2	2	0	0	50	1	10
			1000	0	0	1	1	1	1	95	6	60
			2000	0	0	0	0	0	0	—	10	100
			0	2	8	0	0	0	0	28	0	0
B	2 mice of Exp. A treated with 500 units of P; 2 mice with 1000 units	640	500	1	8	1	0	0	0	35	0	0
			1000	0	4	3	0	0	1	67	2	20
			2000	0	0	0	0	0	1	143	9	90
			0	0	10	0	0	0	0	30	0	0
C	5 mice of Exp. B treated with 1000 units of P	280	500	1	9	0	0	0	0	31	0	0
			1000	0	2	3	1	0	0	57	4	40
			2000	0	0	1	0	0	0	69	9	90
			0	1	9	0	0	0	0	28	0	0
D	5 mice of Exp. C treated with 1000 units of P; 1 mouse with 2000 units	490	1000	0	6	4	0	0	0	45	0	0
			2000	0	1	2	3	1	1	82	2	20
			0	0	10	0	0	0	0	33	0	0
E	4 mice of Exp. D treated with 1000 units of P; 2 mice with 2000 units	970	1000	1	9	0	0	0	0	33	0	0
			2000	0	1	2	2	0	0	65	5	50
			0	5	5	0	0	0	0	29	0	0
F	4 mice of Exp. E treated with 2000 units of P	270	1000	2	7	1	0	0	0	34	0	0
			2000	0	3	4	1	1	0	58	1	10
			0	5	5	0	0	0	0	28	0	0
G	6 mice of Exp. F treated with 2000 units of P	1070	1000	0	10	0	0	0	0	32	0	0
			2000	0	5	2	1	0	0	50	2	20
			0	2	8	0	0	0	0	28	0	0
Control	Parent strain	600	500	0	5	1	1	1	0	54	2	20
			1000	0	0	3	1	0	0	88	6	60
			2000	0	0	0	0	0	0	—	10	100
			0	6	4	0	0	0	0	26	0	0

* Doses of penicillin administered 2, 8 and 14 hours after infection and at 8-hour intervals thereafter for 5 additional days.

† This parent strain was of maximum virulence and invasiveness, having been passed through normal mice either daily or on alternate days for more than 4 years.

‡ P = penicillin.

was only 51 hours. Although this comparison shows that strain McGovern acquired considerable resistance to penicillin, this organism did not develop this property as rapidly as did strain CHA (compare Experiments D in Tables I and II), nor did it develop as great a degree of resistance as did the CHA strain (compare Experiments G in Tables I and II).

B. *Retention of Resistance to Penicillin.* Cultures of the resistant CHA strain of pneumococcus, prepared from 4 mice of Experiment G (Table I), were pooled and passed daily through normal mice, the organisms isolated from the 30th serial passage being tested for their response to penicillin. In this test, groups of 10 mice were infected as described above. One group received 1000-unit doses of penicillin as indicated previously; a second group received 2000-unit doses, and a third group served as untreated controls. All 10 control mice died, the average survival time being 27 hours. All 10 mice treated with 1000-unit doses of penicillin died, their average survival time being 40 hours. Two of 10 mice receiv-

TABLE II.

Development of Resistance to Penicillin by Type I Pneumococcus, Strain McGovern: 10 Mice Infected in Each Group.

Exp.	Infecting Organisms: Source	Infecting Organisms: No. in infecting dose	Units P‡ per dose*	No. of deaths Days after infection: 1	2	3	4	5	6-10	Avg hrs survival of mice that died	30-day survivors: No.	%
A	Parent strain†	460	500	0	6	3	0	0	1	62	0	0
			1000	0	0	0	0	0	2	127	8	80
			2000	0	0	0	0	0	1	157	9	90
			0	1	9	0	0	0	0	35	0	0
B	5 mice of Exp. A treated with 500 units of P	970	500	0	3	2	2	0	1	70	2	20
			1000	0	1	0	0	0	3	129	6	60
			2000	0	0	0	0	0	0	—	10	100
			0	3	7	0	0	0	0	31	0	0
C	5 mice of Exp. B treated with 500 units of P	29	500	0	6	2	0	0	1	57	1	10
			1000	0	0	1	4	0	1	100	4	40
			2000	0	0	0	0	0	0	—	10	100
			0	1	9	0	0	0	0	35	0	0
D	4 mice of Exp. C treated with 1000 units of P	620	500	6	0	2	0	0	1	55	1	10
			1000	0	0	2	2	4	2	106	0	0
			2000	0	0	0	0	0	0	—	10	100
			0	0	9	1	0	0	0	38	0	0
E	5 mice of Exp. D treated with 1000 units of P	510	1000	1	6	1	1	0	0	43	1	10
			2000	0	0	0	0	0	0	—	10	100
			0	3	7	0	0	0	0	25	0	0
F	5 mice of Exp. E treated with 1000 units of P	890	1000	0	1	5	0	2	0	74	2	20
			2000	0	0	0	0	0	2	161	8	80
			0	1	9	0	0	0	0	32	0	0
G	2 mice of Exp. F treated with 2000 units of P	980	1000	0	5	3	1	0	0	51	1	10
			2000	0	2	0	0	1	2	105	5	50
			0	1	9	0	0	0	0	32	0	0
Control	Parent strain	1630	500	0	2	1	2	1	1	80	3	30
			1000	0	0	1	0	0	1	106	8	80
			0	2	8	0	0	0	0	31	0	0

* Doses of penicillin administered 2, 8 and 14 hours after infection and at 8-hour intervals thereafter for 5 additional days.

† This parent strain was of maximum virulence and invasiveness, having been passed through normal mice either daily or on alternate days for more than 4 years.

‡P = penicillin.

ing 2000-unit doses recovered; those that died survived 59 hours on the average. From a comparison of these results with those of Experiment G (Table I), it is clear that the resistance to penicillin developed by strain CHA was not significantly impaired by as many as 30 consecutive passages through normal mice. Whether a larger number of passages would alter this characteristic has not been determined.

C. *Comparison of the* in vitro *sensitivity of the parent and resistant strains to penicillin.* Quantities of 9 cc of basal medium, containing 2% defibrinated rabbit blood and various concentrations of penicillin,† were inoculated with 1 cc quantities of 10^{-5} dilutions of 12-hour blood broth cultures of the respective parent

† The basal medium was a beef heart infusion broth (pH 7.8) containing 2% neopeptone and 0.5% sodium chloride. Various amounts of penicillin, dissolved in 5% $NaHCO_3$ (and sterilized by filtration through sintered glass), and 2% defibrinated rabbit blood were added just before use. The amounts of penicillin added were such that the final concentrations (after addition of inoculum) were 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 Oxford units per cc of medium.

TABLE III.
Comparison of Growth of Parent and Resistant Strains of Pneumococcus in Media Containing Penicillin.

Strain	Inoculum: No. of pneumococci per cc culture	Hrs incubated	Growth in medium containing penicillin Units per cc					
			0	0.2	0.4	0.8	1.6	3.2
III	730	12	+	+	+	—	—	—
CHA		24	+	+	+	—	—	—
Parent		48	+	+	+	—	—	—
III	530	12	+	+	+	+	?	—
CHA		24	+	+	+	+	+	—
Resistant		48	+	+	+	+	+	—
I	750	12	+	+	+	—	—	—
McGovern		24	+	+	+	—	—	—
Parent		48	+	+	+	—	—	—
I	830	12	+	+	+	+	—	—
McGovern		24	+	+	+	+	+	—
Resistant		48	+	+	+	+	+	—

and resistant organisms.‡ The resulting cultures, incubated at 37.5°, were examined visually for growth at 12, 24 and 48 hours. The observations of growth, recorded in Table III, show that the strains of pneumococcus that had developed resistance to penicillin *in vivo* were significantly less sensitive to the *in vitro* action of this anti-bacterial agent than were the parent strains. The highest concentration of penicillin permitting visible growth of either parent strain was 0.4 unit per cc of medium. Comparable growth of either resistant strain occurred even when the penicillin concentration was 1.6 units per cc.

D. *Response of* penicillin-resistant *pneumococci to sulfapyridine.* A group of 25 mice was infected as described above with the *penicillin-resistant* McGovern organisms isolated from the mice of Experiment G (Table II). Ten of these animals served as untreated controls; the remaining mice received 10 mg doses of sulfapyridine, suspended in acacia and administered by stomach tube 2, 8 and 14 hours after infection and every 8 hours thereafter for 5 additional days. A second group of 25 mice, infected with the parent McGovern organisms, was treated in an identical manner.

The results of this experiment showed that infections with penicillin-resistant pneumococci responded to sulfapyridine at least as well as infections with the parent strain. All 15 mice infected with the resistant strain recovered under sulfapyridine therapy, as compared with recovery of 10 of the 15 mice infected with the parent strain. All control animals of both groups were dead within 36 hours after infection.

Discussion. It is now well established that pneumococci and other microörganisms may acquire resistance to the sulfonamides upon prolonged exposure to these drugs. This reaction occurs in the treated patient[1-4] as well as in experimental animals and culture media. In treated patients, the reaction does not commonly occur during ordinary short-term usage of the sulfonamides.[4] It does occur frequently, however, during prolonged administration of these drugs,[4] and definitely militates against successful therapy of diseases which require long-term therapy, such as pneumococcal endocarditis, unresolved pneumonia

‡ The resistant organisms used in this test were the strains isolated from mice of Experiments G (Tables I and II).

1 Lowell, F. C., Strauss, E., and Finland, M., *Ann. Int. Med.*, 1940, **14**, 1001.

2 Frisch, A. W., *Am. J. Clin. Path.*, 1941, **11**, 797.

3 Hamburger, M., Jr., Schmidt, L. H., Ruegsegger, J. M., Sesler, C. L., and Grupen, E. S., *J. A. M. A.*, 1942, **119**, 409.

4 Hamburger, M., Jr., Schmidt, L. H., Sesler, C. L., Ruegsegger, J. M., and Grupen, E. S., in press.

and, in some instances, meningitis. Obviously, in such conditions it would be highly desirable to have available a therapeutic agent to which organisms did not develop resistance as readily as to the sulfonamides.

From the results of the present study it is apparent that pneumococci may develop resistance to penicillin upon continued exposure to this anti-biotic agent in the treated animal. McKee and Rake[5] observed a slight increase in the resistance of pneumococci to penicillin following serial passage in culture media containing this drug. Rammelkamp and Maxon[6] and Abraham and co-workers[7] also found that staphylococci acquired resistance to penicillin *in vitro*. Superficially, at least, these observations indicate that prolonged penicillin therapy suffers from the same defect as extended sulfonamide treatment. This suggestion is borne out by the apparent clinical failure of penicillin therapy[8] in subacute bacterial endocarditis caused by *Streptococcus viridans*, a disease invariably requiring long-term treatment.

The present work has also shown that development of penicillin resistance does not alter the sulfonamide sensitivity of pneumococci. Powell and Jamieson[9] and McKee and Rake[5] have previously shown that the converse is also true; namely, that development of sulfonamide resistance does not alter sensitivity of pneumococci to penicillin. These observations are of theoretical interest in that they indicate that the fundamental actions of these two agents are distinctly different. Practically, they offer the hope that alternate short-term treatment with these substances might succeed in curing those infections which because of development of resistant organisms have hitherto resisted treatment with either agent alone.

Finally, it has been shown that the two strains of pneumococcus used in this study differed significantly in the rates at which they acquired resistance to penicillin, the CHA strain becoming resistant more rapidly than the McGovern strain. It is noteworthy that these strains developed resistance to sulfapyridine at different rates,[10,11] the CHA strain also acquiring resistance to this drug more rapidly than the McGovern strain. A study of the composition of the parent strains showed that the organisms composing the CHA strain were more variable and unstable in respect to sulfonamide sensitivity than were the organisms of the McGovern strain.[12] The present observations suggest that the same variability and instability characterize the reactions of the CHA strain to penicillin.

Summary. The present study has shown the following: (1) Two strains of pneumococcus developed resistance to penicillin as a result of serial passage through mice treated with this drug. (2) The rate at which resistance developed and the degree of resistance acquired varied significantly with the different strains. (3) The resistance of the one strain tested was not impaired by 30 serial passages through normal mice, indicating that, once established, resistance to penicillin is retained for a considerable period. (4) The development of resistance to penicillin *in vivo* was accompanied by an increase in resistance to this drug *in vitro*. (5) The response of the pneumococci to sulfonamides was not altered by the development of resistance to penicillin.

[5] McKee, C. M., and Rake, G., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 275.

[6] Rammelkamp, C. M., and Maxon, T., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 386.

[7] Abraham, E. P., Chain, E., Fletcher, C. M., Florey, H. W., Gardner, A. D., Heatley, N. G., and Jennings, M. A., *Lancet*, 1941, **2**, 177.

[8] Editorial, *New Eng. J. Med.*, 1943, **228**, 295.

[9] Powell, H. M., and Jamieson, W. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 387.

[10] Schmidt, L. H., Sesler, C., and Dettwiler, H. A., *J. Pharm. and Exp. Therap.*, 1942, **74**, 175.

[11] Sesler, C. L., and Schmidt, L. H., *J. Pharm. and Exp. Therap.*, 1942, **75**, 356.

[12] Schmidt, L. H., and Sesler, C. L., *J. Pharm. and Exp. Therap.*, 1943, **77**, 165.