Development of Chloramphenicol-Resistant and Chloramphenicol-Dependent Variants of a Strain of *Klebsiella pneumoniae*.* (18059)

THOMAS M. GOCKE AND MAXWELL FINLAND

From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital and the Department of Medicine, Harvard Medical School, Boston, Mass.

During the course of attempts to enhance the resistance of several pathogenic bacteria to some of the new antibiotics a variant of one of the strains was encountered which failed to grow in the absence of chloramphenicol. This paper deals chiefly with the details of the emergence and isolation of this "chloramphenicol-dependent" strain.

Source of the strain. The organism used at the beginning of this study was a strain of *Klebsiella pneumoniae,* type A, which was originally isolated from the urine of a patient with pyelonephritis and meningitis. As far as could be determined, the patient had not previously received any antibiotic therapy. At the time when the study was begun, the strain had been subcultured only a few times on the surface of agar plates and grew profusely in large mucoid colonies.

Materials. Brain heart infusion broth (Difco) pH 7.4, was the liquid medium used for suspending or growing the organisms and for making serial dilutions of cultures or antibiotic. The solid medium for surface growth was heart-infusion agar (Difco), pH 7.4. Crystalline chloramphenicol (Chloromycetin) was supplied by Dr. E. A. Sharp of Parke, Davis & Co. Initial solutions of the drug were made in distilled water and all subsequent dilutions were made in broth prior to their incorporation in proper amounts in the media to be used.

Methods. A series of agar plates containing 2-fold dilutions of chloramphenicol was freshly prepared each time before use. Plain agar plates without antibiotic were prepared at the same time. The inoculum for subcultures was prepared by suspending a 5 mm loopful of a surface growth in 0.4 ml of broth; a 2 mm loopful of this suspension was then streaked on a sector of each of a number of agar plates containing various concentrations of the antibiotic in an appropriate range and also on a plate containing no antibiotic. The plates were incubated at 37°C for 48 hours and then inspected for growth with the naked eye and with the aid of an engraver's glass (3x). The growth was recorded as follows: O = no discernible growth; +++ = full growth, comparable to that of a control culture not previously exposed to drug; ++ and + = intermediate growth, and ± = pin-point colonies seen with the aid of the lens. If only a few colonies developed, their number was recorded. In the attempt to enhance the resistance of the strain, the inoculum for each successive subculture was prepared from the plate containing the highest concentration of chloramphenicol on which there was moderate but incomplete growth (+ or ++).

Results. The minimum concentrations of chloramphenicol which completely inhibited growth and the minimum concentrations which produced partial inhibition (++ or less) in 30 successive subcultures made in this manner are shown graphically in Fig. 1. In the lower portion of this figure there is also indicated the degree of growth which occurred when an inoculum from the same suspension was streaked on the surface of antibiotic-free agar at the time of each of the successive transfers from the agar which contained the antibiotic.

There was a more or less progressive increase in the resistance of the strain to chloramphenicol in successive subcultures between the sixth and twenty-fourth transfers. At the time of the first 6 exposures of the original strain to chloramphenicol its growth was completely inhibited by 3.1 μg per ml and partially inhibited by 1.6 μg per ml of agar, with some variations that were within the limits of error of the method. Beginning

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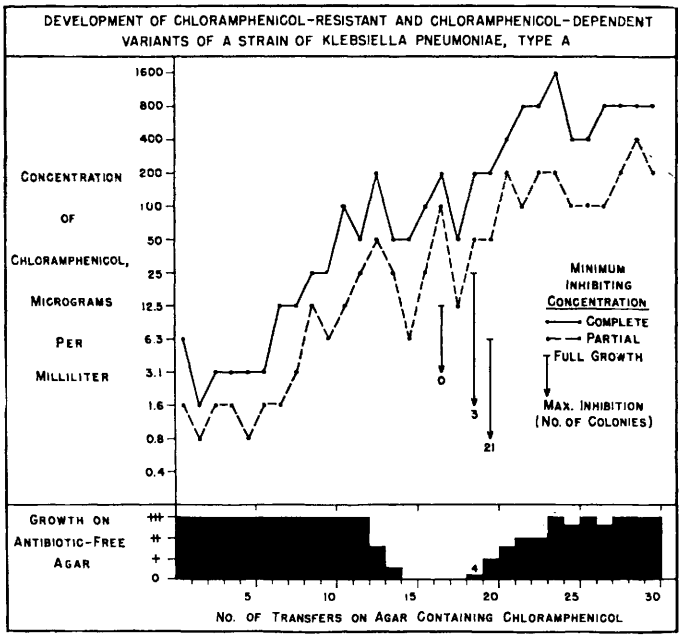

FIG. 1.

with the twenty-second transfer the strain was completely inhibited in most instances by 800 μg/ml and partially inhibited by 200 μg/ml.

On the antibiotic-free agar plates, the growth of the successive subcultures from the chloramphenicol agar plates appeared maximal for the first 12 transfers. The 13th and 14th transfers from drug-containing to drug-free agar showed progressively diminishing growth and the 15th through the 18th of these transfers yielded no discernible growth on the antibiotic-free agar. The 19th similar transfer yielded only 4 colonies on drug-free agar and subsequent subcultures from the chloramphenicol-agar plates produced progressively increasing growth on the antibiotic-free agar to the 24th transfer; after that the growth on antibiotic-free agar was at or near maximum.

Growth was maximum or nearly so throughout the early transfers to the plates which contained chloramphenicol in lower concentration than those which are indicated in Fig. 1 as showing partial inhibition. Up to the 17th transfer, however, only 2 or 3 dilutions were included beyond the first one which permitted maximal growth. Subsequent transfers included lower dilutions down to concentrations of 1.6 or 0.4 μg of chloramphenicol per ml. In each of 3 of these transfers, namely the 17th, 19th and 20th, there was a progressive decrease in the amount of growth in decreasing concentrations of chloramphenicol until little or no growth occurred. This is indicated in Fig. 1 by the arrows which begin at the level of the least concentration that yielded maximum growth and point at the greatest concentration in which maximum inhibition of growth again occurred.

These findings suggested that the suspension of organisms made from the 14th to the 17th subcultures on chloramphenicol-agar consisted largely, if not wholly, of organisms which required chloramphenicol for growth. In other words, these organisms were "chloramphenicol-dependent." It was also evident that (1) a minimum of 3.1 μg or more of chloramphenicol per ml of agar was usually required to permit any appreciable amount of growth of these dependent organisms, (2) optimum growth occurred on agar containing 12.5 or 25 μg per ml, (3) higher concentrations of the antibiotic again inhibited growth and (4) it was nevertheless possible to continue to

TABLE I.
Some Additional Transfers of the Chloramphenicol-Dependent Culture.

Conc. of chloramphenicol in agar, μg/ml	Growth of subcultures				
	Series A Regular series, 17th transfer*	Series B Growth from Plate *a* of series A	Series C Growth from Plate *b* of series A	Series D Growth from Plate *c* of series C	Series E Growth from Plate *d* of series D
400	0	0	0	0	0
200	0	0	±	0	±
100	+a	0	±	0	±
50	+++	0	+++c	0	+++
25	+++	±	+++	±	+++e
12.5	+++	++	+++	++	+++
6.3	±	++	++	++d	++
3.1	0	±	±	±	±
1.6	±b	0	±	0	0
0.8	0	0	0	0	1
None	0	0	0	0	1

+++ = full growth; ++, + ± = diminishing growth, and numerals indicate number of colonies where growth was minimal.

* From agar plate containing chloramphenicol, 25μg/ml.

The growth from "e" was used for subcultures in broth (Table II).

enhance the resistance of the strain by additional subcultures in progressively higher concentrations of antibiotic, using as the inoculum for each subculture organisms which had survived exposure to the highest concentration that was partially inhibitory.

A number of separate subcultures of the chloramphenicol-dependent organisms were also made from plates containing various concentrations of chloramphenicol to further series of plates containing serial dilutions of the antibiotic. The results of some of these subcultures are shown in Table I. Within rather wide limits, the concentration of the drug to which the chloramphenicol-dependent organisms were last exposed did not greatly affect the range of concentrations which would support their growth on the next subculture.

Attempts to quantitate the growth of the chloramphenicol-dependent organisms were somewhat hampered by difficulties in obtaining good growth on subculture from agar plates to broth containing the antibiotic. The growth on the plate which contained 25 μg of chloramphenicol per ml in Series E (designated as "e" in Table I) was suspended in broth and aliquots of this suspension were inoculated into tubes of broth containing the antibiotic in concentrations of 6.25, 12.5, 25 and 50 μg per ml, respectively. Almost no visible growth resulted at first but serial subcultures were nevertheless made at 48 hour intervals from each of these tubes to others containing the same amount of drug. The fifth subculture showed moderate growth in the broth containing 6.25 and 12.5 μg per ml, barely visible growth in the one containing 25 μg and no discernible growth in the tube containing 50 μg of antibiotic per ml of broth.

The fifth subculture in 12.5 μg of chloramphenicol per ml of broth was chosen for further quantitative studies. Serial 100-fold dilutions of this culture were made and 2 series of agar plates were poured with 1 ml of each dilution of organisms; one set of plates contained 25 μg of chloramphenicol per ml and a parallel series contained no antibiotic. The number of colonies that developed in each of these plates is shown in Table II. These results suggested that all but a small proportion of the organisms originally present in the broth culture were chloramphenicol-dependent and that probably all of the organisms represented in the 14 colonies which developed from the 10^{-6} dilution of this culture in agar containing 25 μg of antibiotic per ml were chloramphenicol-dependent.

A single colony from the latter plate (designated as "f" in Table II) was then transferred to broth containing 12.5 μg of chloram-

TABLE II.
Growth of Chloramphenicol-Dependent Strain After 5 Subcultures in Broth Containing 12.5 μg of Chloramphenicol per ml.

Dilution of broth culture	No. of colonies per ml	
	In agar containing chloramphenicol, 25 μg/ml	In antibiotic-free agar
10^{-2}	+++	4
10^{-4}	145	1
10^{-6}	14f	0
10^{-8}	0	0

f—One of these colonies was used for further subcultures (Table III).

TABLE III.
Growth of Chloramphenicol-Dependent Strain in Agar Containing Various Amounts of the Antibiotic.

Conc. of chloramphenicol, μg per ml	No. of colonies*
400	0
200	0
100	3 g
50	1426 h,i,j,k
25	560 l,m,n,o
12.5	235
6.3	19
3.1	1
1.6	16
0.8	15 p
0.4	20 q
0	0
0	3r

* From 1 ml of a 10^{-4} dilution of a 72-hour culture (in broth containing 12.5 μg of chloromycetin per ml) of one of the colonies (f) indicated in Table II. These plates were read after 72 hr incubation. The letters signify isolated colonies which were subcultured, as indicated in Table IV.

phenicol per ml. Moderate growth occurred after incubation for 72 hours. This growth was then diluted 1:10,000, and 1 ml of this dilution of culture was incorporated into each of a series of agar plates containing graded concentrations of chloramphenicol. The plates were incubated for 72 hours and the number of colonies which developed in each of the plates is shown in Table III. A few colonies developed in plates containing only small amounts of chloramphenicol and a total of 3 colonies developed in 2 plates of antibiotic-free agar. This suggested that a few organisms had now emerged which were no longer chloramphenicol-dependent.

Finally, 12 individual colonies, indicated by separate letters in Table III, were picked from some of the plates. Each colony was emulsified in 0.2 ml of broth and the suspensions were used to inoculate on the surface of a new series of agar plates containing graded concentrations of chloramphenicol. The growths obtained after 48 hours are indicated in Table IV. The colonies which had developed in the antibiotic-free agar and those which were picked from the agar containing less than 1.6 μg of chloramphenicol per ml yielded growth of organisms which were essentially as sensitive to the antibiotic as a culture of *K. pneumoniae* that had not previously been exposed to the drug. They also grew profusely on antibiotic-free agar. On the other hand, the colonies that were picked from the plates containing 25, 50 and 100 μg per ml all yielded organisms that were chloramphenicol-dependent. These failed to grow in the absence of chloramphenicol or on concentrations lower than 3.1 μg per ml. Furthermore all of the latter cultures grew best on the agar which contained 25 μg of chloramphenicol per ml.

Detailed comparisons of the sensitive, resistant and dependent variants were not made but a number of observations were recorded in the course of this study. The colonies of the dependent organisms which developed on optimum concentrations of chloramphenicol were large and mucoid and resembled those which had never been exposed to the antibiotic. The colonies of the resistant variant that grew in the later transfers on both chloramphenicol-agar and on antibiotic-free agar were smooth, smaller and no longer mucoid in character. All of the variants obtained from plates on which good growth had occurred retained their microscopic morphology and staining characteristics. The chloramphenicol-resistant and chloramphenicol-dependent variants, however, had lost their serological specificity with respect to the Quellung reaction in homologous antiserum, but the same was also true of the control organisms which had been maintained by serial transfer on antibiotic-free agar.

Discussion. A number of workers, notably Miller and Bohnoff(1,2) and Paine and Fin-

TABLE IV.
Growth of Organisms from Individual Colonies Derived from a Chloramphenicol-Dependent Strain.

Colony*	Last exposure†	Concentration of chloramphenicol, μg/ml. 400	200	100	50	25	12.5	6.3	3.1	1.6	0.8	0.4	0
g	100	0	0	±	+++	+++	±	0	0	0	0		0
h	50	0	0	0	±	±	±	0	±	0	0		0
i	50	0	0	0	+	++	++	±	0	0	0		0
j	50	0	0	0	++	++	+++	±	±	0	0		0
k	50	0	0	0	++	++	+	+	0	0	0		0
l	25	0	0	0	++	+++	++	+	±	0	0		0
m	25	0	0	0	++	+++	++	+	±	0	0		0
n	25	0	0	±	++	+++	++	±	0	0	0		0
o	25	0	0	0	++	+++	++	±	0	0	0		0
p	.8	0	0	0	0	0	0	±	+	++	+++	+++	+++
q	.4	0	0	0	0	0	0	±	+	++	+++	+++	+++
r	0	0	0	0	0	±	±	+	++	++	+++	+++	+++
Control‡	0	0	0	0	0	0	0	0	++	+++	+++	+++	+++

* See Table III.
† Chloramphenicol, μg/ml.
‡ Strain of *K. pneumoniae* not previously exposed to chloramphenicol.

land(3,4) have succeeded in developing streptomycin-resistant and streptomycin-dependent variants from originally sensitive strains of a number of different organisms after exposing them to this antibiotic. This subject has been reviewed recently by Miller and Bohnhoff(5). As far as could be ascertained, organisms which are dependent for growth on any of the newer antibiotics have not previously been described.

The course of events leading to the emergence and isolation of chloramphenicol-resistant and chloramphenicol-dependent variants of a strain of *Klebsiella pneumoniae* that was originally sensitive to this antibiotic have been detailed in this paper. As was the case with the streptomycin-dependent variants previously developed in this laboratory(4) and with those described by Miller and Bohnhoff(2), there was a critical concentration of antibiotic in which the chloramphenicol-dependent variant grew most vigorously. As the concentration was increased above this level or decreased below it, the growth of the chloramphenicol-dependent variant decreased progressively until it completely failed to grow. It is of interest that the chloramphenicol-dependent variant emerged during the step-grade increase in the resistance of the strain by successive exposures to increasing concentrations of the antibiotic and it was first recognized when the resistance of the strain to chloramphenicol had attained this critical level. As variants of greater resistance appeared in later transfers in the chloramphenicol-containing medium, they gradually submerged the dependent variants.

In point of fact, it was largely a matter of chance that the dependent variant was recognized at all. Its recognition was due to the fact that the particular inoculum used for some of the subcultures consisted entirely of chloramphenicol-dependent variants so that little or no growth occurred on the antibiotic-free medium. Had there been in these inocula a sufficient proportion of chloramphenicol-resistant variants which were also capable of growing in the absence of the antibiotic, the presence of the dependent variants could readily have gone unrecognized by the method used in this study.

1. Miller, C. P., and Bohnhoff, M., *Science*, 1947, v105, 620.

2. Miller, C. P., and Bohnhoff, M., *J. Bact.*, 1947, v54, 467.

3. Paine, T. F., and Finland, M., *Science*, 1948, v107, 143.

4. Paine, T. F., Jr., and Finland, M., *J. Bact.*, 1948, v56, 207.

5. Miller, C. P., and Bohnhoff, M., Development of Streptomycin-Resistant and Streptomycin-Dependent Bacteria, Ch. 10 in Waksman, S. A., *Streptomycin: Its Nature and Practical Application*, Baltimore, Williams & Wilkins Co., 1949, pp. 158-176.

The strain used to initiate the present study was one of 14, each of a different species, which were employed in attempts to enhance the resistance of bacteria to 4 new antibiotics other than penicillin and streptomycin. In no other instance was a dependent variant recognized although each strain had been transferred 40 times in media containing graded concentrations of the respective antibiotics and from the antibiotic-containing media also to antibiotic-free media. It cannot be stated with any assurance that such dependent variants had not developed in other strains or to other antibiotics since the method used was not suitable for their recognition unless they replaced the sensitive and resistant variants completely, or nearly so, at some stage in the course of the subcultures.

No attempts have been made to study the genetic pattern of the emergence of the resistant and dependent variants and of the "back mutations" to more sensitive and more resistant variants. The possible significance of these variants with respect to the mechanism of action of antibiotics has been discussed previously in relation to the streptomycin-dependent variants(4).

Summary and conclusions. The resistance of a strain of *Klebsiella pneumoniae* to chloramphenicol was enhanced 128-fold by serial subculture on the surface of agar plates containing graded concentrations of this antibiotic.

A chloramphenicol-dependent variant also emerged during the course of these subcultures. This variant grew best in the presence of a critical concentration of chloramphenicol; as the concentration of antibiotic was either increased progressively beyond this critical level or decreased below it there was a steady decline in growth until complete inhibition of growth occurred.

The chloramphenicol-dependent variant was first recognized soon after the resistance of the strain had been enhanced to a point where it was completely inhibited by this critical concentration.

Evidence was also obtained of possible "back-mutation" to variants which were as sensitive as the parent strain.

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Hemorrhagic Skin Lesions Produced by Intradermal Meningococcus Toxin in Rabbits following Treatment with ACTH or Cortisone.*(18060)

LEWIS THOMAS AND WILLIAM J. MOGABGAB

From the Division of Infectious Disease, Department of Medicine, Tulane University School of Medicine, New Orleans, La.

Inhibition of the Shwartzman phenomenon by ACTH has been described by Soffer, Shwartzman, Schneierson, and Gabrilove(1). These authors reported that the phenomenon was prevented in 8 out of 10 rabbits when 12.5 mg of ACTH was injected intramuscularly 2 hours prior to the intravenous or "provocative" injection of meningococcus toxin. Comparable amounts of ACTH had no inhibitory effect when given on the preceding day, before the intradermal or "preparatory" injection of toxin.

In preliminary experiments, a similar result was obtained in this laboratory. When ACTH was administered to 8 rabbits in 2 separate doses of 10 mg Armour Standard ACTH, 6 and 4 hours before the intravenous injection of toxin, the gross intradermal hemorrhage which characterizes the Shwartzman phenomenon failed to occur in every case, while typical reactions were produced in each of 4 control rabbits. However, it was noted that the skin sites prepared with

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1. Soffer, L. J., Shwartzman, G., Schneierson, S. S., and Gabrilove, J. L., *Science*, 1950, v111, 303.