CHLORAMPHENICOL-FASTNESS: DEVELOPMENT *IN VIVO* AND EXPERIMENTAL PRODUCTION *IN VITRO*¹

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The development of bacterial resistance to chloramphenicol was observed during a study of patients who were being treated with this drug for infections of the urinary tract (1). Nine of 33 strains of gram-negative bacilli isolated from the urine of 24 patients prior to therapy demonstrated during treatment a decrease in susceptibility to chloramphenicol. The development of resistance to the drug by some of these organisms was associated with therapeutic failures in five of these subjects. Similar observations had not been reported. Because of the potential clinical importance of these findings the results of a bacteriologic study of the strains which developed fastness to chloramphenicol *in vivo*, and quantitative observations on the origin of this phenomenon made *in vitro* are presented.

METHODS

The strains of organisms that were studied were recovered from the urine of patients before, during, and after treatment with chloramphenicol. A description of cases and the schedules of treatment used in the clinical study have been noted elsewhere (1). Specimens of urine were obtained from females by catheterization and from males by careful local preparation prior to voiding. Ten cc. samples of urine were centrifuged and the sediment was stained for bacteria and cultured on MacConkey agar and on tryptose phosphate agar containing 5 per cent human blood. The various organisms that appeared were isolated in pure culture and were stored on tryptose phosphate agar slants at -20° C. In several instances agar pour plates of 10-fold dilutions of urine were made to determine the numbers of bacteria present. All strains of bacteria recovered from a single patient during all periods of observation were compared *at the same time* as regards their morphology, cultural and biochemical

characteristics, and susceptibility to chloramphenicol and streptomycin. The *in vitro* sensitivities of these organisms to the drugs were determined by a serial dilution method in agar (2). Inocula for a test of susceptibility to a drug were taken from 24-hour broth cultures of similar optical density as measured by a Coleman Junior Spectrophotometer. Tests of susceptibility to streptomycin were performed on all organisms to serve as one possible method for differentiating a particular strain throughout the period of observation.

Concentrations of active chloramphenicol in samples of urine collected during treatment in one patient (*e.g.*, Table II) were measured by a turbidimetric microbiologic assay (3).

Studies *in vitro* on the origin of chloramphenicol-fastness were made on a mucoid strain of *Klebsiella pneumoniae*, type B, that was highly susceptible to both chloramphenicol and streptomycin. This parent organism originally had been isolated from the spinal fluid of a patient with meningitis prior to the administration of a chemotherapeutic agent. A stock culture was purified by repeated single colony transfers on agar media and stored on slants at -20° C. These slants served as the source of the inoculum for all experiments.

Bacterial variants in cultures of the stock strain, that exhibited different degrees of resistance to chloramphenicol *in vitro* were demonstrated by exposing 10-fold dilutions of a 24-hour broth culture to varying concentrations of the drug in agar pour plates. Further details of the quantitative techniques used to isolate and study the drug-fast bacterial variants have been described previously (4).

Chloramphenicol-fastness in vivo

The results of tests of susceptibility to chloramphenicol performed on successive cultures of nine strains of bacteria that developed increased degrees of resistance to this drug during treatment are presented in Table I (six patients with infection caused by a single organism) and Table II (one patient with a mixed bacterial infection). This phenomenon developed in one or more steps of two- to eight-fold increments and occurred at unpredictable intervals during the first week of treatment. A decrease in the number of bacteria in the urine was noted during the first day of

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therapy in all of the seven patients. When the decrease was marked (greater than 99.999 per cent), even though drug-fastness developed, continued treatment with chloramphenicol usually eliminated the infecting organism. The patient represented in Table II is of interest in this regard. Pretreatment cultures showed a heavy growth of *Aerobacter aerogenes*. Concomitant with a decrease in the population of this organism during the first 24 hours of therapy, chloramphenicol-susceptible strains of *Escherichia coli* and *Proteus vulgaris* appeared in the urine. During subsequent periods of treatment these latter two species persisted in small numbers whenever they were cultured. All three of these organisms developed degrees of fastness to chloramphenicol which were greater than the average levels of active drug re-

covered from the urine. Despite a decrease in the dosage of chloramphenicol two of the three species subsequently were eliminated during a period of two weeks, while therapy was continued.

Strains exhibiting increased degrees of resistance to chloramphenicol *in vitro* (eight- to 64-fold as compared with pre-treatment cultures tested simultaneously) persisted in the urine throughout the period of treatment or recurred after therapy in five of the seven patients. In two instances (cases 24 and 13), a chloramphenicol-susceptible strain reappeared in the urine one and five weeks after treatment was ended.

All strains of a given species that were recovered during and after therapy were compared with their respective pretreatment cultures with regard to cultural characteristics, definitive biochemical

TABLE I
Development of bacterial resistance to chloramphenicol during treatment with this drug of urinary tract infections caused by a single organism

PATIENT	ORGANISM	DAY OF OBSERVATION																		
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	21	28	35	
2	E COLI	MIC	CL	6.2	6.2	25	S	S	S						S			S		
		8/CC	SM	12.5	12.5	12.5											S			
		THERAPY CHLORAMPHENICOL 2.5 GM/DAY																		
4	A AEROGENES	MIC	CL	3.1	S	S	S	S	S	12.5	S						S	S	S	
		8/CC	SM	>100							>100							S	S	S
		THERAPY CHLORAMPHENICOL 2.5 GM/DAY																		
7	A AEROGENES	MIC	CL	3.1	3.1	12.5	S	S	S	S	S						25	50	50	
		8/CC	SM	>100	>100	>100											>100	>100	>100	
		THERAPY CHLORAMPHENICOL 2.5 GM/DAY																		
13	A AEROGENES	MIC	CL	6.2	6.2	25	S	S	S	S	S						50	50	6.2	
		8/CC	SM	>100	>100	>100											>100	>100	>100	
		THERAPY CHLORAMPHENICOL 2.5 GM/DAY																		
22	PS. AERUGINOSA	MIC	CL	12.5	12.5	100	200	200		200	200					200				
		8/CC	SM	>100	>100	>100	>100	>100		>100	>100					>100				
		THERAPY CHLORAMPHENICOL 2.5 GM/DAY																		
24	PS. AERUGINOSA	MIC	CL	25	200										200			25	25	
		8/CC	SM	>100	>100												>100	>100	>100	
		THERAPY CHLORAMPHENICOL 3.5 GM/DAY																		

} = RIGHT KIDNEY AUTOPSY

CL = chloramphenicol; SM = streptomycin; MIC = minimum inhibiting concentration of antibiotic *in vitro*; S = sterile culture.

TABLE II
Development of bacterial resistance to chloramphenicol during treatment with this drug of a mixed infection of the urinary tract

ORGANISM	DAY OF OBSERVATION												
			1	2	3	4	5	6	7	8		22	36
E. COLI	MIC γ/CC	CL	S	3.1	1.5	1.5	S	S	100	S		S	S
		SM		>100	>100	>100			>100				
A. AEROGENES		CL	3.1	25	12.5	12.5	50	50	200	200			
		SM	>100	>100	>100	>100	>100	>100	>100	>100		S	S
P. VULGARIS		CL	S	6.2	25	25	25	25	25	25		25	50
		SM			>100	>100	>100	>100	>100	>100		>100	>100
CHLORAM- PHENICOL	DOSE	2.5 GM / DAY										0.5 GM / DAY	1 GM / DAY
	URINE γ/CC (ACTIVE)	140 18 15 9 11 15											

ical reactions, and growth rates in broth as measured turbidimetrically. No significant differences were noted.

Chloramphenicol-fastness in vitro

Chloramphenicol-fast variants: Though the stock strain of *K. pneumoniae* had not been ex-

posed to chloramphenicol prior to testing, repeated examinations showed that a small number of cells (approximately 0.0001 per cent) in six independent cultures exhibited low degrees of fastness to this drug (survival curves B, Figure 1). Substrains derived from colonies of bacteria that were resistant to 7 micrograms per cc. of chlor-

FIG. 1. SURVIVAL CURVES OF *K. PNEUMONIAE* AND SOME OF ITS CHLORAMPHENICOL-FAST VARIANTS IN AGAR CONTAINING VARIOUS CONCENTRATIONS OF THIS DRUG

Curves B = independent cultures of the stock organism. Curves B/CL 7 = variant substrains originating from colonies that had survived a single exposure of 7 micrograms of chloramphenicol per cc. in agar media.

FIG. 2. THE FLUCTUATION TEST AS APPLIED TO THE VARIANTS OF *K. PNEUMONIAE* THAT SURVIVED IN SOLID MEDIA CONTAINING 10 (B/CL10) AND 15 (B/CL15) MICROGRAMS OF CHLORAMPHENICOL PER CC.

amphenicol contained cells with higher degrees of fastness than did the stock culture (survival curves B/CL7, Figure 1). These substrains are analogous to others exhibiting specific resistance to low concentrations of streptomycin previously described (4).

In two experiments, very large populations of the stock culture (totalling approximately 7×10^{11} organisms) were spread on the surface of agar plates containing 50 and 100 micrograms per cc. of chloramphenicol. This method failed to expose variants exhibiting resistance to these large concentrations of the drug. Using inocula of the same size from the same stock strain and similar levels of streptomycin in agar media, cells with high degrees of fastness to this latter antibiotic (resistant to greater than 1,000 micrograms per cc.) were readily demonstrated in small numbers (0.5 to 5.3 in 10^{10} organisms).

The property of fastness to chloramphenicol was inheritable, *in vitro*. Five variant substrains were established from colonies that appeared on solid media containing 10 micrograms per cc. of chlor-

amphenicol. After 11 consecutive transfers in broth without this drug, quantitative tests showed that all cells in each strain remained resistant to 10 micrograms per cc. of chloramphenicol. Mutation back toward chloramphenicol-susceptibility could not be demonstrated under these experimental conditions.

The property of chloramphenicol-fastness in bacteria appeared to be drug-specific. Strains of bacteria originating from variants resistant to 15 micrograms per cc. of this drug exhibited no change from the parent cultures as regards susceptibility to streptomycin or polymyxin B. Survival curves of two variant strains of *K. pneumoniae* that were susceptible to chloramphenicol but highly resistant to streptomycin and polymyxin B were similar in all respects to the chloramphenicol survival curves of the stock culture (curve B, Figure 1).

The origin of chloramphenicol-fast variants: The property of resistance to chloramphenicol in the rare bacterial variants isolated from cultures of the stock organism could have originated spon-

taneously (by mutation) or could have been induced by interaction between the antibiotic and the cell (physiologic adaptation). Luria and Delbrück (5) have developed a statistical method for distinguishing between these two possibilities. Their Fluctuation Test is based on the frequency distribution of mutants in multiple cultures that have been initiated by inocula from a common source. By the use of this method, variants in the stock strain of *K. pneumoniae* that were resistant to 10 and 15 micrograms per cc. of chloramphenicol were shown to have arisen spontaneously prior to their contact with this drug. Independent cultures were initiated with very small inocula (approximately 20 to 50 organisms). After an incubation period of 48 hours at 37° C. all cultures contained a similar number of viable organisms. Large and highly significant variations were noted in the numbers of B/CL10 and B/CL15 variants isolated from separate cultures when compared with the error of sampling determined by replicate assay of a single culture (Figure 2). The presence of wide fluctuations in the number of variants appearing among the independent cultures indicates a spontaneous origin of those that were resistant to 10 or 15 micrograms per cc. of chloramphenicol. This observation is explained by the occurrence of mutations which result in drug fastness in a small number of bacteria at unpredictable periods during the growth of each culture. Mutations that occur early in the growth of a culture result in a larger number of drug-fast progeny than those that occur when the culture is approaching its maximum number of viable organisms.

DISCUSSION

Several other investigators have reported that chloramphenicol is a useful chemotherapeutic agent for infections of the urinary tract caused by a number of gram-negative organisms (6, 7). The development of bacterial resistance to this drug was not noted. The clinical observations presented here indicate that in certain instances the organism causing the infection may develop increased degrees of fastness to this drug during treatment and that under such circumstances therapeutic failure can result. Though the data are not numerous, they are significant because all of the cultures obtained from each patient during the

period of observation were tested at the same time and under almost identical conditions.

Bacterial resistance to chloramphenicol appeared during treatment in single or successive small steps after an initial fall in the number of infecting organisms recovered from the urine. Studies *in vitro* detected the presence of bacterial variants with low degrees of fastness to chloramphenicol in a susceptible strain of *K. pneumoniae*. These variants exhibited the characteristics of a mutation in that they appeared spontaneously in the culture in very small numbers; their property of fastness to chloramphenicol was highly specific and was inherited. Cultures of mutants which were isolated *in vitro* by drug selection (differential multiplication of nonsusceptible cells) gave rise to progeny with higher degrees of fastness than those present in the stock culture.

These data suggest that chloramphenicol-resistant strains are produced either *in vivo* or *in vitro* through a mechanism similar to that proposed by Demerec to explain the development of bacterial resistance to penicillin (8). This theory postulates that in a large population of susceptible organisms (as is usually the case in urinary tract infections) a few cells appear as a result of mutation and are resistant to low concentrations of antibiotic even though they have not had contact with it on any previous occasion. If the population is exposed to these concentrations of drug, the susceptible cells are suppressed selectively and the resistant ones actively multiply. Higher degrees of fastness may then be built up by further mutation and drug selection by a series of small steps.

Organisms with very high degrees of fastness to streptomycin may be produced *in vivo* in a step-wise fashion but more commonly this phenomenon appears suddenly in a single step. This later clinical observation has been correlated with the finding *in vitro* of rare bacterial variants (approximately 1 in 10^{10} organisms) in pretreatment cultures which demonstrate 100- to 1000-fold decreases in their susceptibility to streptomycin (4, 9). The examination of very large numbers of organisms in the stock culture of *K. pneumoniae* failed to reveal chloramphenicol-fast variants with decrease in susceptibility of greater than eight-fold. From the same cultures, mutants exhibiting greater than a 500-fold increase in resistance to streptomycin were recovered easily. This quan-

titative difference in variant types suggests that fastness to chloramphenicol may develop clinically in a stepwise fashion *only* and that in the presence of adequate therapeutic levels of the drug high degrees of resistance may occur less frequently than has been observed during treatment with streptomycin.

Though resistance to chloramphenicol developed in approximately 25 per cent of the bacterial species recovered from the urine during treatment, it is noteworthy that these variants appeared usually in small numbers. Under such circumstances the local defense mechanisms of the host would be more effective (10) and further mutation to higher degrees of resistance should occur less frequently. These factors may explain why some of the drug-fast strains were cleared from the urine during continued therapy.

SUMMARY

1. Bacterial resistance to chloramphenicol developed in patients during treatment with this drug. Fastness appeared in single or successive small steps. In most instances, it was associated with failure of treatment.

2. Small numbers of organisms exhibiting low degrees of fastness to chloramphenicol were detected in a susceptible strain of *K pneumoniae*. These bacterial variants had properties that were common to mutants and gave rise to other variants that exhibited higher degrees of drug fastness than the parent organism.

3. These clinical and laboratory observations suggest that chloramphenicol-fast strains are produced by the process of drug selection and succes-

sive mutation of rare drug-fast variants that exist in otherwise susceptible species of bacteria.

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