Resistance of Klebsiella and Enterobacter to the Penicillins* (32834)

STEPHEN J. SELIGMAN
Department of Medicine, UCLA School of Medicine, Los Angeles, California 90024

Both penicillinase activity and intrinsic resistance (resistance of the individual organism) contribute to penicillin resistance. Use of a dilute inoculum reduces the effect of penicillinase activity and permits evaluation of intrinsic resistance. Penicillinase activity is completely eliminated in penicillinase-negative variants (1,2). No one has reported penicillinase-negative variants of gram-negative bacteria. Penicillinase-resistant penicillins, however, may diminish penicillinase activity upon penicillinase-susceptible penicillins (3–5) and consequently might aid in the study of intrinsic resistance of large inocula. This report concerns the mechanisms of resistance in Klebsiella and Enterobacter both to benzylpencillin and to hetacillin with and without a subinhibitory concentration of the penicillinase-resistant dicloxacillin. Although hetacillin is frequently a more potent inhibitor of gram-negative bacteria than benzylpenicillin, both are penicillinase-susceptible. In aqueous media hetacillin is rapidly hydrolyzed to ampicillin which is probably responsible for its antibacterial effect (6).

Materials and Methods. Trypticase soy agar (TSA) and broth (TSB) were used throughout. Antibiotic activities of sodium benzylpenicillin, sodium hetacillin, and potassium dicloxacillin were 90–93% of total weight as assayed by the manufacturers. Species identification was done by the methods of Edwards and Ewing (7) with the substitution of the genus name Enterobacter for Aerobacter. Antibiotic agar was made with twofold dilutions of either benzylpenicillin or hetacillin with and without 100 μg of dicloxacillin/ml. After overnight incubation at 37°C to dry the surface of the medium, the antibiotic agar was streaked with 0.05 ml of an undiluted and a 10^{-6} dilution of an overnight broth culture. Readings are reported after 24 hours and did not show significant change after 48 hours. All suspected mutants were restreaked from isolated colonies on TSA at least 3 times prior to testing. For reaction broths, 180 ml of TSB, 10 ml of a benzylpenicillin solution, 10 ml of overnight culture, and 10 ml of 0.2 *M* phosphate buffer pH 7.4 or 2 mg of dicloxacillin/ml in buffer were incubated at 37°C. Samples were removed at periodic intervals for viable count, wet mount, Gram stain, and estimation of residual benzylpenicillin. Residual benzylpenicillin was estimated by iodometric titration (8). *Bacillus cereus* penicillinase in a final concentration of 10^5 Kersey units/ml was added for 10 min to 5-ml samples. Residual benzylpenicillin was calculated from the difference in titration between samples treated with *B. cereus* penicillinase and untreated samples. Neither *B. cereus* penicillinase nor penicillinase of the study strains inactivated a significant amount of dicloxacillin. Thin-layer chromatography was performed using silica gel G prepared with 0.1 *M* phosphate buffer, pH 6.0, (to reduce tailing). Five μliter of an overnight reaction mixture originally containing 500 μg of benzylpenicillin and 5×10^8 organisms/ml were pipetted next to equimolar amounts of benzylpenicillin, 6-aminopenicillanic acid (6-APA) and their corresponding penicilloic acids. Chromatography was done in the ammonium sulfate-N-butanol–ethanol solvent system of Tardrew and Johnson (9) for 4 hours at room temperature. Spraying with a freshly prepared solution containing 2% starch, 0.2 *M* phosphate buffer pH 7.4, 0.4 *M* of I in 0.3 *M* KI, *B. cereus* penicillinase 10^6 Kersey units/ml (20:20:1:4) revealed benzylpenicillin and related compounds (10,11).

Results. All but one Klebsiella strain of 11 tested and all 4 Enterobacter strains grew when undiluted overnight cultures were inoculated onto antibiotic agar containing 1 mg of benzylpenicillin or hetacillin/ml. Dilute inocula were usually inhibited at considerably lower antibiotic concentrations. Dilute inocula

* This investigation was done during the tenure of a USPHS Research Career Development Award and was supported in part by a grant from Bristol Laboratories, Syracuse, New York.

TABLE I. Comparison of Small and Large Inocula of Klebsiella Strain No. 81 on Penicillin, Penicillin-Dicloxacillin, and Dicloxacillin Agar.

(no. of colonies or description of growth)

			Penicillin + 100 μg of dicloxacillin/ml	
Penicillin (μg/ml)	Undiluted[a]	10^{-6}[b]	Undiluted	10^{-6}
0	Confluent	41	Confluent	60
16	Confluent	25	Confluent	24
32	Confluent	0	Confluent	0
64	Confluent	0	50	0
256	Confluent	0	3	0
512	TNTC[c]	0	1	0
2048	Ca. 100	0	0	0
4096	0	0	0	0
Dicloxacillin (μg/ml)				
0	Confluent	72		
800	Confluent	38		
1600	43	0		
3200	0	0		

[a] A 0.05-ml sample of an overnight culture streaked onto agar.

[b] A 10^{-6} dilution of the above overnight culture similarly streaked.

[c] Too numerous to count.

of Klebsiella strain no. 81 failed to grow on more than 16 μg of benzylpenicillin/ml, but an undiluted inoculum produced colonies on agar originally containing 2048 μg/ml (Table I). Intrinsic resistance, defined as the highest antibiotic concentration permitting growth of more than 10% of the number of colonies of a dilute inoculum, was not lowered by the addition of 100 μg dicloxacillin/ml. This concentration of dicloxacillin was one eighth the intrinsic resistance to dicloxacillin, yet it reduced the concentration of benzylpenicillin permitting confluent growth of undiluted inocula from 256 μg/ml to 32 μg/ml. The sole colony formed after inoculation of the undiluted streak on 512 μg benzylpenicillin–100 μg dicloxacillin agar was isolated and had increased intrinsic resistance to 512 μg benzyl penicillin/ml, hence it was a mutant with a large one-step increase in resistance.

Comparison of intrinsic resistances. Intrinsic resistance to benzylpenicillin varied from 8 to 4000 μg/ml and was usually lower in Klebsiella strains than in Enterobacter strains (Table II). In Enterobacter strains, but not in Klebsiella, intrinsic resistance was distinctly lower to hetacillin than to benzylpenicillin. A subinhibitory concentration of dicloxacillin lowered the intrinsic resistance of Enterobacter both to benzylpenicillin and to hetacillin. This synergistic effect of dicloxacillin upon intrinsic resistance was not a consequence of inhibition of penicillinase activity since the synergistic effect in benzylpenicillin–dicloxacillin broth occurred prior to any significant decrease in benzylpenicillin in reaction flasks which did not contain dicloxacillin.

Penicillinase activity. Thin-layer chromatography revealed that all strains had penicillin-β-lactamase activity since benzylpenicillin was converted to benzylpenicilloic acid. Neither 6-APA nor the penicilloic acid of 6-APA appeared as expected from penicillin acylase (amidase) activity or combined β-lactamase and acylase activity respectively.

Overnight cultures were incubated with a benzylpenicillin concentration exceeding the intrinsic resistance but insufficient to prevent confluent growth on solid media (Fig. 1). Both benzylpenicillin and viable count declined initially. Reduction in benzylpenicillin did not occur in controls during this interval. Wet mounts revealed conversion of rod forms

TABLE II. Intrinsic Resistance of Klebsiella and Enterobacter.
[highest antibiotic concentration (μg/ml) in which number of colonies was >10% of control][a]

Klebsiella (type)	Strain no.	Benzylpenicillin	Benzylpenicillin + 100 μg dicloxacillin/ml	Hetacillin	Hetacillin + 100 μg dicloxacillin/ml	Dicloxacillin
39	76	8	8	4	4	800
Nontypable	74	16	8	8	8	400
8	73	16	16	8	8	100
16	81	16	16	8	8	800
71	97	16	16	8	8	800
47	415	32	32	16	16	800
47	83	32	32	16	16	800
13	85	32	32	32	32	800
39	43	128	128	64	64	≧1600
19	71	128	128	128	128	800
54	366	2000	2000	2000	2000	800
Enterobacter						
E. aerogenes	96	256	8	16	2	400
	72	512	16	64	4	400
E. cloacae	22	2000	128	512	8	800
	75	4000	256	512	32	800

[a] A 0.05 ml sample of 10^{-6} dilution of an overnight culture streaked onto TSA.

to spheroplasts and subsequent spheroplast lysis. Bacterial growth occurred following benzylpenicillin destruction. In contemporaneous experiments, 100 μg of dicloxacillin/ml delayed benzylpenicillin inactivation. Preliminary experiments with a system in which bacterial growth and additional penicillinase formation were prevented by the addition of 8-hydroxyquinoline (12) indicated that dicloxacillin is a competitive inhibitor of penicillin-β-lactamase.

Mutations to increased resistance. Addition of 100 μg of dicloxacillin/ml to benzylpenicillin or to hetacillin decreased the concentrations of the latter antibiotics which permitted confluent growth of undiluted inocula in the most sensitive 8 of 11 Klebsiella strains and in all the Enterobacter strains. A few isolated colonies were frequently observed in lieu of confluent growth. When tested in parallel with their parent strains, such colonies characteristically showed a 16–32-fold increase in intrinsic resistance to benzylpenicillin and to hetacillin. Relative selection of such mutants also occurred in antibiotic-containing broth (Fig. 1).

Cultures exposed to benzylpenicillin alone had an increase in the relative proportion of mutants from $3/10^7$ to $1/10^4$. The increase resulted from growth of the mutants during that period in which benzylpenicillin had been reduced to concentrations inhibitory to the parent strain but not to the mutant. With more prolonged incubation, all benzylpenicillin was destroyed and the mutants lost their selective advantage. In reaction flasks inoculated with both benzylpenicillin and dicloxacillin, growth frequently did not occur until the second day. Mutants with increased resistance predominated.

Most mutant colonies were normal-sized after overnight growth. *Enterobacter cloacae* strain no. 22, however, characteristically produced mutants with diminished colonial size. As with small colony variants from heteroresistant (methicillin resistant) staphylococci, revertants producing normal-sized colonies were frequent (2). An interesting although unexplained property of mutants from strain no. 22 was increased motility as estimated from wet mounts.

Discussion. The present studies suggest the usefulness of a measurement of "intrinsic resistance" in evaluating resistance to penicillins. Strains highly resistant in large inocula had markedly different intrinsic re-

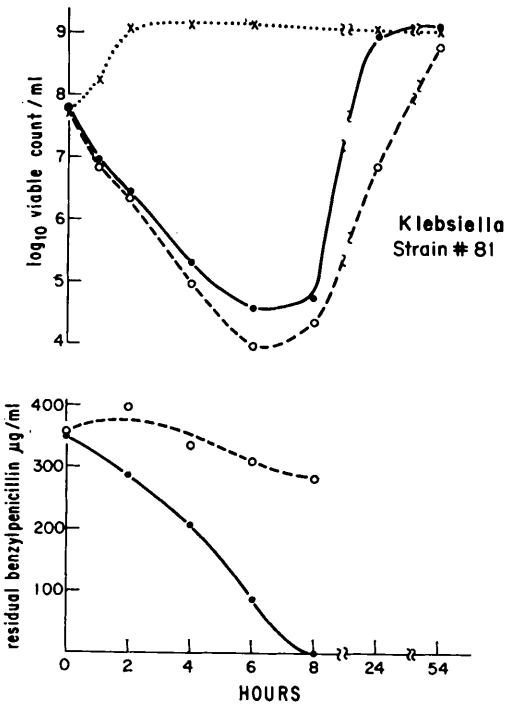

FIG. 1. Colony count and residual benzylpenicillin in antibiotic broth. At 54 hours the ratio, mutants with increased intrinsic resistance/parent strain, was $3/10^7$ in the control, $1/10^4$ in the benzylpenicillin flask, and 4/1 in the benzylpenicillin–dicloxacillin flask. (●) Initial benzylpenicillin, 383 µg/ml by weight; (○) initial benzylpenicillin, 383 µg/ml, plus dicloxacillin, 100 µg/ml; (×) control.

sistances. In contrast to staphylococci (13), penicillinase activity probably does not contribute significantly to intrinsic resistance in most gram-negative bacteria. Dicloxacillin in a concentration sufficient to diminish penicillinase activity of large Klebsiella inocula did not decrease intrinsic resistance. In Enterobacter, dicloxacillin-mediated decrease in intrinsic resistance did not depend upon inhibition of penicillinase activity.

Penicillinase activity contributed significantly to resistance of a large inoculum and permitted confluent growth on agar initially containing penicillin in concentrations considerably higher than the intrinsic resistance. Since the clinical significance of penicillinase activity in gram-negative bacteria is uncertain, the clinical significance of the synergistic effect of the dicloxacillin-mediated decrease in penicillinase activity is also uncertain.

Dicloxacillin suppressed confluent growth on high concentrations of penicillinase-susceptible penicillins thereby revealing isolated colonies of mutants with large one-step increases in intrinsic resistance reminiscent of highly resistant mutants produced by heteroresistant (methicillin resistant) staphylococci (2,13). Large increases in penicillin resistance contrast with small multistep increases previously considered characteristic of penicillin resistance (14).

Penicillin inhibits cell wall synthesis by preventing a cross-linking transpeptidation reaction, the last step in peptidoglycan synthesis (15,16). In some organisms, a stable penicilloyl-transpeptidase complex is probably formed (17). Hydrolysis of the complex would liberate penicilloic acid and hence result in penicillin-β-lactamase activity. Consequently, cell-associated penicillinase in gram-negative bacteria may be identical with the penicillin-susceptible enzymes involved in peptidoglycan synthesis. The modest increases in penicillinase-activity found in resistant *E. coli* mutants (18–21) may result from increased transpeptidase activity but reduction in penicillin concentration need not be responsible for increased resistance.

Summary. Klebsiella and Enterobacter grow after exposure to large penicillin concentrations because of intrinsic resistance (resistance of the individual organism), penicillin-β-lactamase activity, or mutant overgrowth. In Klebsiella strains intrinsic resistances to benzylpenicillin and hetacillin were similar. In Enterobacter strains intrinsic resistances were distinctly lower to hetacillin than to benzylpenicillin and were reduced by a subinhibitory concentration of dicloxacillin. All strains had β-lactamase activity which was usually inhibited by dicloxacillin. Single step mutations to large increases in intrinsic resistance were common.

The author wishes to thank Mrs. Betty Corbett for technical assistance, Mr. Gene Marso for identification of strains, and Bristol Laboratories for a supply of hetacillin and dicloxacillin. Klebsiella typing was kindy performed through the courtesy of Dr. W. H. Ewing. Klebsiella strains 43, 83, 366, and 415

were kindly supplied by Dr. J. M. T. Hamilton-Miller.

1. Seligman, S. J., Nature **209**, 994 (1966).
2. Seligman, S. J., J. Gen. Microbiol. **42**, 315 (1966).
3. Hamilton-Miller, J. M. T. and Smith, J. T., Nature **201**, 999 (1964).
4. Sutherland, R. and Batchelor, F. R., Nature **201**, 868 (1964).
5. Sabath, L. D. and Abraham, E. P., Nature **204**, 1066 (1964).
6. Sutherland, R. and Robinson, O. P. W., Brit. Med. J. **2**, 804 (1967).
7. Edwards, P. R. and Ewing, W. H., "Identification of Enterobacteriaceae," 2nd ed., Burgess, Minneapolis, Minnesota, 1962.
8. Perret, C. J., Nature **174**, 1012 (1954).
9. Tardrew, P. L. and Johnson, M. J., J. Biol. Chem. **234**, 1850 (1959).
10. Thomas, R., Nature **191**, 1161 (1961).
11. Sneath, P. H. A. and Collins, J. F., Biochem. J. **79**, 512 (1961).
12. Pollock, M. R., Brit. J. Exptl. Pathol. **31**, 739 (1950).
13. Seligman, S J. and Hewitt, W. L., Antimicrobial Agents Chemotherapy **1965**, 387 (1966).
14. Demerec, M., Proc. Natl. Acad. Sci. U.S. **31**, 16 (1945).
15. Wise, E. M. and Park, J. T., Proc. Natl. Acad. Sci. U.S. **54**, 75 (1965).
16. Tipper, D. J. and Strominger, J. L., Proc. Natl. Acad. Sci. U.S. **54**, 1133 (1965).
17. Strominger, J. L. and Tipper, D. J., Am. J. Med., **39**, 708 (1965).
18. Percival, A., Brumfitt, W., and de Louvois, J., J. Gen. Microbiol. **32**, 77 (1963).
19. Kabins, S. A., Sweeney, H. M., and Cohen, S., Ann. Internal Med. **65**, 1271 (1966).
20. Smith, J. T., J. Gen. Microbiol. **30**, 299 (1963).
21. Eriksson-Greenberg, K. G., Boman, H. G., Jansson, J. A. T., and Thoren, S., J. Bacteriol. **90**, 54 (1965).

Received July 10, 1967. P.S.E.B.M., 1968, Vol. 127.

Long Term Maintenance of Cell Cultures under Agar Overlay and Development of Herpes T Plaques (32835)

M. D. DANIEL AND L. V. MELENDEZ (Introduced by B. F. Trum)
New England Regional Primate Research Center, Harvard Medical School, Southborough, Massachusetts 01772

Dulbecco's agar overlay plaque technique (1) has been widely and successfully employed in a variety of viral studies for more than a decade. None of these studies (2), however, provide enough information on long term maintenance of cell cultures under agar, period in which maximum plaque size is reached, nor as to virus recovery from the plaques after they have reached their maximum size.

During the course of studying the plaque characteristics of herpes viruses particularly that of herpes T virus (3) we were able to maintain cell cultures for extended periods of time under agar overlay. This allowed the herpes T plaques to develop to a maximum size, and to retain this characteristic for several days, with the recovery of viable virus from these plaques for as long as 53 days.

This report results from a study on the plaque characteristics of herpes viruses which is still being pursued. The purpose of this report is to describe the technique employed for the preparation and long term maintenance of various cell cultures under agar overlay, and the behavior of herpes T virus under the conditions studied.

Materials and Methods. A variety of primary cell cultures and cell lines was examined for their suitability for plaque morphology studies (Table I). The medium employed for growth of both primary cells and cell lines was Eagle's minimum essential medium supplemented with 10% fetal calf serum (MEM-10). Penicillin and streptomycin were added at a concentration of 250 units and 250 mg/ml, respectively. The agar overlay medium was Temin's modification of Eagle's medium (4,5). A commercial company prepared this medium for us as a 2× solution [Grand Island Biological Company, Buffalo, New York (GIBCO)]. The composition of Temin's