Inactivation of Cephalothin and Cephaloridine by *Staphylococcus aureus*

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ABSTRACT

BENNER, ERNEST J. (University of Washington School of Medicine, Seattle), JOHN V. BENNETT, JEAN L. BRODIE, AND WILLIAM M. M. KIRBY. Inactivation of cephalothin and cephaloridine by *Staphylococcus aureus*. J. Bacteriol. **90**:1599-1604. 1965.—Marked differences were observed in the susceptibility of penicillinase-producing staphylococci to cephalothin and cephaloridine. All of 100 strains of penicillin G-resistant *Staphylococcus aureus*, with the use of a large inoculum, were found to be susceptible to 2 μ g/ml of cephalothin, whereas only 50% were susceptible to this concentration of cephaloridine, and 15% required 15 μ g/ml or more for inhibition. In contrast, penicillin G-sensitive strains were more susceptible to cephaloridine and did not show the marked inoculum effect observed with the cephaloridine-resistant strains. These differences were due to a much greater destruction of cephaloridine than of cephalothin by staphylococcal penicillinase. Cephaloridine-resistant staphylococci were stronger penicillinase producers than were susceptible strains, and the resistant strains were found to inactivate cephaloridine by hydrolysis of the β -lactam ring. In population studies, cephaloridine-resistant cells differed from methicillin-resistant cells in that they decreased in numbers as the drug concentration was increased, and the survivors in higher drug concentrations were no more resistant than was the parent strain. Treatment with acriflavine eliminated resistance of the cells to both penicillin G and cephaloridine. It was concluded that cephaloridine resistance was due to hydrolysis by penicillinase, and that this was related to the pyridine ring substitution in the cephalosporanic acid nucleus.

Cephaloridine, a new cephalosporin antibiotic quite different from cephalothin in structure (Fig. 1), yet similar in antibacterial spectrum, has been reported to be as active as cephalothin against staphylococci, and to be effective in treating staphylococcal infections (Muggleton, O'Callaghan, and Stevens, 1964; Murdoch et al., 1964; Stewart and Holt, 1964). We have observed that cephaloridine is more active than cephalothin against penicillinase-nonproducing coagulase-positive staphylococci, but about 15% of the penicillinase producers, when a large inoculum is tested, require much larger amounts of cephaloridine than of cephalothin for inhibition of growth. Slow inactivation of cephalothin by penicillinase-producing staphylococci has been reported (Ayliffe and Barber, 1963; Knox and Smith, 1963; Eriksen and Erickson, 1964; Gravenkemper, Brodie, and Kirby, 1965), and one group has presented evidence suggesting elaboration of a "cephalosporinase" by 63% of the strains tested (Chang and Weinstein, 1964).

However, coagulase-positive staphylococci are uniformly susceptible with tube dilution tests to 2 μ g/ml or less of cephalothin, and therapeutic results in severe staphylococcal infections have been good. These seeming contradictions prompted us to re-explore the whole problem of the inactivation of cephalosporin antibiotics by penicillinase and cephalosporinase. Our studies indicate that the mechanism of inactivation is quite different with cephaloridine-resistant than with methicillin-resistant staphylococci, and the nature of the inactivation of cephaloridine has been worked out in detail.

MATERIALS AND METHODS

Susceptibility, inoculum effect, and population characteristics. All of the staphylococci studied were coagulase-positive strains (102 penicillinase producers and 18 penicillin G-sensitive strains) recently isolated from patients by the clinical microbiology laboratory of the University Hospital, and were tested for penicillin G sensitivity by the single disc method (Bauer, Perry, and Kirby,

FIG. 1. Chemical structures of cephalothin and cephaloridine.

1959). Two strains were methicillin-resistant and their characteristics have been described in detail (Gravenkemper et al., 1965). Minimal inhibitory concentrations (MIC) were determined in freshly prepared solutions of cephalothin and cephaloridine. Standard powders were kindly supplied by Eli Lilly & Co., Indianapolis, Ind. Population characteristics were determined by picking a single colony of each strain, subculturing it overnight in broth, and then adding 10^{-1} , 10^{-2} , and 10^{-3} dilutions to nutrient agar pour plates with appropriate amounts of either cephalothin or cephaloridine to give final concentrations of 2.5, 5, 10, 25, or 50 $\mu\text{g}/\text{ml}$ of antibiotic in the plate. Control plates with no antibiotic furnished an estimate of the inoculum size.

Bactericidal action and drug destruction. Bactericidal activity and drug inactivation were determined by adding either cephalothin or cephaloridine (final concentration of 100 $\mu\text{g}/\text{ml}$) to a 10^{-1} dilution of an overnight broth culture (4×10^7 to 5×10^7 cells per milliliter). Duplicate tubes were incubated at 37 C, and colony counts were determined from one of the tubes while samples were removed from the other for determination of residual antibiotic. For the latter, samples were heated to 85 C and held at that temperature for 30 sec to inactivate all the enzyme; they were then Seitz-filtered and frozen until time for assay. Controls for each drug were incubated, so that spontaneous deterioration could be determined.

Penicillinase production. To measure the rate and extent of penicillin G destruction by actively growing cells, a 10^{-1} broth dilution of overnight growth was agitated for 2 hr at 37 C, and was then added to penicillin G in broth to give a final concentration of 2,000 units per ml. Pour plates were made to establish the numbers of viable bacteria. Samples were removed from each tube and from the antibiotic controls at specified times, and were heated, filtered, and frozen. Results were expressed as that amount of penicillin G inactivated by 10^9 organisms per milliliter per minute. Each strain was also induced to produce

penicillinase by overnight incubation in a concentration of 0.25 $\mu\text{g}/\text{ml}$ of methicillin or cephalothin, and penicillin G destruction was determined in the same manner.

β -Lactamase activity and induction. A powder with potent penicillinase activity against penicillin G was extracted from penicillin G-resistant strains of *Staphylococcus aureus* (Kirby, 1944). Each strain was grown in four batches: one each with 0.25 $\mu\text{g}/\text{ml}$ of methicillin, cephalothin, and cephaloridine, and one with no antibiotic. For comparison, a penicillinase-nonproducing strain was also incubated with subinhibitory amounts of each of the antibiotics. After production of the powders, suspensions were prepared in distilled water to correspond to 5×10^6 organisms per milliliter, as determined by pour plates made at the end point of the induction process. These powders were then tested for their ability to hydrolyze penicillin G, cephaloridine, and cephalothin. The hydroxylamine method (Ford, 1947) with standard curves constructed from optical density readings on a Coleman Junior spectrophotometer set at 515 $m\mu$ was used to measure nonhydrolyzed antibiotic. Residual drug was also determined microbiologically by cup-plate assay.

Elimination of penicillin resistance and cephalosporinase screening. Penicillin resistance was eliminated from the *S. aureus* strains by treating thrice-seeded single-colony isolates of each strain with acriflavine (Hashimoto, Kono, and Mitsuhashi, 1964), and then testing penicillin G sensitivity on pour plates. Loss of drug resistance was verified by tube dilution MIC determinations for penicillin G and cephaloridine. The modification of the Haight-Finland method used by Chang and Weinstein (1964) in screening for cephalosporinase was used to test both penicillin G-sensitive and penicillin G-resistant strains for the presence of this enzyme.

Antibiotic assay method. The microbiological activity of residual antibiotic was assayed by a modified cup-plate method (Bennett et al., submitted for publication). The assays were performed with standards prepared in solutions appropriate for each experiment. In determining inactivation of cephalothin, cephaloridine, or penicillin G by growing cultures of staphylococci, standards were made in sterile filtrate of staphylococcal broth growth. In studies with the enzyme-rich powders, standards were made in distilled water, the fluid used to suspend or dissolve the powders and antibiotics. Standards were made at the beginning of each experiment, and were then frozen until used. Large glass plates with a uniform agar depth of 2.8 mm with 81 agar wells of 4.5-mm diameter enabled us to make five to seven replications of each standard and each sample from all experiments. Standards over the range of 0.65 to 16 $\mu\text{g}/\text{ml}$ gave values represented by a parabolic curve when zone size was plotted against the log of the drug concentration. Assay by this method yielded results with $\pm 10\%$ accuracy at the 95% confidence limit.

RESULTS

MIC values and inoculum effect. With an inoculum of approximately 10^6 cells per milliliter, all 100 strains of penicillin G-resistant staphylococci were inhibited by 2 $\mu\text{g}/\text{ml}$ or less of cephalothin, whereas with cephaloridine only 50% were inhibited by this concentration, and 15 strains had MIC values of 15 $\mu\text{g}/\text{ml}$ or higher (Fig. 2). Furthermore, the much greater influence of inoculum size on cephaloridine MIC values by the penicillinase-producing strains is shown in Table 1. There was a greater than 100-fold increase with some strains, as opposed to a maximal increase of 10-fold with cephalothin.

FIG. 2. Comparative susceptibility to cephalothin and cephaloridine of 100 penicillin G-resistant strains of *Staphylococcus aureus*.

The penicillin G-sensitive strains were more susceptible to cephaloridine and showed no inoculum effect.

Population characteristics. Six strains of *S. aureus* requiring 15 $\mu\text{g}/\text{ml}$ or more of cephaloridine for inhibition were found to contain a small number of cells (0.003 to 0.1%) which survived in pour plates containing 25 to 50 $\mu\text{g}/\text{ml}$ of the antibiotic. The percentage of surviving cells decreased progressively as the concentration of drug in the plates was increased. When the survivors were recovered from the pour plates, grown overnight in broth, and adjusted to an inoculum of 2×10^6 cells per milliliter, their susceptibility was the same as the parent strains. With the two strains of methicillin-resistant staphylococci, on the other hand, the small percentage of resistant cells (about 1%) did not decrease appreciably in numbers in pour plates containing 12.5, 25, or 50 $\mu\text{g}/\text{ml}$ of methicillin. When the methicillin survivors were grown overnight in broth and retested, the resistance was much greater than with the parent strain. Similar observations were made by Sutherland and Rolinson (1964). The cephaloridine-sensitive penicillinase-producing strains, as well as the penicillin G-susceptible strains, consisted of a uniform population of cells killed in pour plates containing 2.5 $\mu\text{g}/\text{ml}$ of cephaloridine. When tested with cephalothin, all cells in all the strains were killed by 5 $\mu\text{g}/\text{ml}$ or less.

Bactericidal action correlated with drug inactivation. A large inoculum of five strains of cephaloridine-resistant *S. aureus* (10^{-1} dilution of overnight growth) underwent killing for a few hours in the presence of a high concentration (100

TABLE 1. Comparison of cephalothin and cephaloridine activity against penicillin G-resistant and -susceptible strains of *Staphylococcus aureus**

Type of strain	No. of strain	Cephalothin			Cephaloridine		
		10^4	10^6	5×10^7	10^4	10^6	5×10^7
Resistant	19	0.2	0.2	1.0	0.1	0.4	20.0
	20	0.2	0.4	0.8	0.1	0.2	10.0
	21	0.4	0.6	2.0	0.2	8.0	15.0
	22	0.4	0.4	2.0	0.2	2.0	30.0
	23	0.2	0.4	1.0	0.2	1.0	8.0
	24	0.2	0.4	2.0	0.1	0.8	2.0
Susceptible	1	0.2	0.2	0.4	0.05	0.1	0.1
	2	0.2	0.2	0.2	0.05	0.1	0.1
	3	0.2	0.2	0.2	0.05	0.1	0.1
	4	0.1	0.2	0.2	0.05	0.05	0.1
	5	0.1	0.2	0.2	0.05	0.05	0.1
	6	0.4	0.4	0.4	0.1	0.1	0.2

* Both drugs were tested against inocula of 10^4 , 10^6 , and 5×10^7 organisms of six resistant and six susceptible strains. Drug concentrations are expressed in micrograms per milliliter.

FIG. 3. Growth characteristics and cephaloridine inactivation with five cephaloridine-resistant and three cephaloridine-sensitive strains of staphylococci.

$\mu\text{g/ml}$) of cephaloridine (broken lines, Fig. 3), but thereafter multiplied, reaching control levels at 24 hr. There was rapid destruction of cephaloridine by these strains during the first few hours, and the cephaloridine was completely inactive after 24 hr. Three cephaloridine-sensitive strains (solid lines, Fig. 3) underwent progressive killing during the 24-hr incubation period, and no drug was inactivated; the slight inactivation shown was the same as in control tubes with no bacteria.

In simultaneous studies with cephalothin, the five cephaloridine-resistant and the three cephaloridine-sensitive strains underwent progressive killing during the 24-hr period. Cephalothin was much less stable in solution than was cephaloridine, losing 40 to 60% of its activity in the control tubes in 24 hr. Slight additional inactivation (10 to 15%) was caused by the five cephaloridine-resistant strains, whereas the three cephaloridine-sensitive strains caused no breakdown of this antibiotic.

Penicillinase production by cephaloridine-resistant and cephaloridine-sensitive staphylococci. Most cephaloridine-resistant strains hydrolyzed penicillin G at two to three times the rate of the cephaloridine-sensitive strains when actively growing cultures were compared (Table 2). When hydrolysis was allowed to continue for 1 hr, the resistant strains could destroy as much as 2,000 units of penicillin G per 10^9 organisms per milliliter, whereas the sensitive strains did not destroy more than 1,000 units. After induction by methicillin or cephalothin, cultures of both the

TABLE 2. Penicillinase activity (units of penicillin G destroyed per minute by 10^9 organisms/ml) for cephaloridine-sensitive and cephaloridine-resistant staphylococci

Type of strain	Strain no.	With no induction	After 18 hr of induction	
			Methicillin	Cephalothin
Cephaloridine-resistant (MIC, 15 $\mu\text{g/ml}$)	1	28	1,120	940
	3	41	680	940
	6	16	780	910
	28	20	840	805
	29	22	980	1,160
	35	24	622	820
Cephaloridine-sensitive (MIC, 0.4 $\mu\text{g/ml}$)	4	12	730	490
	5	10	422	312
	9	9	222	264

cephaloridine-resistant and -sensitive strains had a sharp increase in penicillinase activity, with penicillin G hydrolysis still usually much greater by the resistant strains.

Nature of cephaloridine inactivation and the influence of induction. To determine whether inactivation of cephaloridine by staphylococci was due to hydrolysis of the β -lactam ring, highly potent powders of two penicillin G-resistant strains of *S. aureus* were prepared with the objective of giving sufficient drug inactivation to permit accurate determinations with the hydroxylamine test. With these powders, both penicillin G and cephaloridine were inactivated quite rapidly (see Fig. 4), and the hydroxylamine test clearly showed that the inactivated drug no longer contained an intact β -lactam ring. Simultaneous tests for microbiological activity gave values within 10% of those obtained by the hydroxylamine method for both penicillin G and cephaloridine. Powders from penicillinase-non-producing strains of *S. aureus* caused no drug inactivation.

Studies were then undertaken to see whether methicillin, cephalothin, or cephaloridine would induce enzymes with differing abilities to inactivate penicillin G and cephaloridine when exposed to the two strains of staphylococci. As shown in Fig. 4, inactivation of both antibiotics occurred much more rapidly with the induced strain, and the increased rate of inactivation was identical no matter which of the three drugs was used as an inducer. Inactivation was so rapid that these relationships do not show up too clearly in Fig. 4. These results are better illustrated in Fig. 5 where, after induction, the powders were diluted 1:10 before testing. Again, drug destruction was measured by the hydroxylamine method, and the

FIG. 4. Destruction of penicillin G and cephaloridine by induced and noninduced enzyme-rich penicillinase powders extracted from *Staphylococcus aureus*. β -Lactum ring hydrolysis of both antibiotics was detected by the hydroxylamine method. Microbiological inactivation (not shown) agreed closely with results obtained with the hydroxylamine method.

FIG. 5. Destruction of penicillin G and cephaloridine by a 1:10 dilution of the enzyme-rich powders shown in Fig. 4, permitting a better comparison of enzyme induction.

rates of inactivation were identical, regardless of which drug was used to induce the enzyme. If a cephalosporinase had been induced, inactivation of cephalothin and cephaloridine should have occurred much more rapidly than with the preparation induced by methicillin, since the activity of cephalosporinase is 200 times greater against the cephalosporins than against penicillin G (Fleming, Goldner, and Glass, 1963; Chang and Weinstein, 1964). Simultaneous tests of microbiological activity again gave results corresponding closely with those obtained by the hydroxylamine method.

These experiments show quite conclusively that inactivation of cephaloridine was caused by a β -lactamase with the characteristics of staphylococcal penicillinase. The failure of the three drugs to induce enzymes at different rates further substantiates the fact that the inactivating substance is penicillinase.

Haight-Finland screening test for cephalosporinase. Chang and Weinstein (1964) modified this test, originally used to detect penicillinase, by incorporating minimal concentrations of

cephalothin into agar seeded with *Sarcina lutea*. Satellite colonies of *Sarcina* appeared near a heavy growth of staphylococci with 63% of strains tested, and this was attributed to destruction of cephalothin by a cephalosporinase. In studying this phenomenon, we observed no destruction of either cephalothin or cephaloridine (appearance of satellite colonies) when the plates had a heavy growth of 10 strains of penicillinase-nonproducing staphylococci. All of 20 penicillinase-producing strains, on the other hand, inactivated both drugs, and a very wide zone of satellite colonies was particularly prominent with strong penicillinase producers on plates containing cephaloridine. It was concluded that penicillinase, and not "cephalosporinase," was responsible for antibiotic destruction detected by this test.

Elimination of penicillin G and cephaloridine resistance. Two penicillin G-resistant strains were grown in acriflavine and then tested for elimination of penicillinase production. Penicillin G resistance was lost by 0.2% of the treated cells and MIC values decreased from greater than 1,000 to 0.02 $\mu\text{g/ml}$. The cephaloridine MIC values for these same cells decreased from 15 to 0.02 $\mu\text{g/ml}$ after penicillinase elimination. Thus, loss of the ability to produce penicillinase was accompanied by a loss of the factor responsible for resistance to cephaloridine, and it appeared likely that the same enzyme was responsible for both effects.

DISCUSSION

The marked differences in MIC values we have observed for cephalothin and cephaloridine with penicillinase-producing staphylococci have not been previously described, although a greater inoculum effect with cephaloridine has been noted (Ayliffe and Barber, 1963). Our observation of this striking difference is undoubtedly due to the larger inoculum size we routinely use for MIC values with staphylococci. In striking contrast, penicillinase-nonproducing staphylococci were actually more susceptible to cephaloridine than to cephalothin, and no inoculum effect was present, suggesting that penicillinase might be involved.

The detailed studies we have subsequently performed show conclusively that the differences between the two drugs are due to the much greater susceptibility of cephaloridine to inactivation by penicillinase. The relative rates of inactivation have been delineated, and studies with hydroxylamine have demonstrated that inactivation of cephaloridine is associated with disruption of the β -lactam ring. Furthermore,

enzyme activity and induction occurred to exactly the same degree when staphylococci were exposed to methicillin, cephalothin, and cephaloridine, indicating that a single enzyme was involved. Studies by others (Chang and Weinstein, 1964) indicating that staphylococci produce cephalosporinase have not been confirmed, and we have shown that this destruction was actually due to staphylococcal penicillinase. These studies all indicate that the chemical structure of cephaloridine, with substitution of a pyridine ring at the three position (Fig. 1), makes the molecule much more susceptible to hydrolysis by penicillinase than is cephalothin.

The mechanism responsible for resistance to cephaloridine appears to be quite different from that associated with resistance of staphylococci to methicillin. Of interest in this regard was our observation that survivors decreased progressively with increasing concentrations of cephaloridine, whereas with methicillin-resistant strains there was no decrease in survivors with increasing concentrations of methicillin. Methicillin resistance has been attributed by some to penicillinase, but such inactivation has not been directly demonstrated, and there is not even agreement as to whether methicillin is inactivated by methicillin-resistant staphylococci. In contrast, the studies reported in this paper show quite clearly that penicillinase is responsible for cephaloridine resistance, and that the resistant strains are stronger producers of penicillinase than are the susceptible strains.

The clinical implications of these observations are not clear, and a number of patients with staphylococcal infections appear to have been treated successfully with cephaloridine. However, the susceptibility of cephaloridine to destruction by penicillinase, together with the marked induction of the enzyme that occurs when staphylococci are in contact with the drug, raises the possibility that clinical efficacy may be less than optimal, as is true with penicillin G in the treatment of infections caused by penicillinase producers.

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