NAFCILLIN AND OXACILLIN COMPARATIVE ANTISTAPHYLOCOCCAL ACTIVITY IN MICE

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(Received for publication January 19, 1976)

The therapeutic properties of nafcillin, oxacillin and erythromycin were determined in mice infected with a strain (Evans) of *Staphylococcus aureus* shown to be tolerant to the bactericidal action of penicillinase-resistant derivatives of penicillin. The therapeutic activity of these agents was also correlated with the extent of colonization of kidneys by resistant clones of *S. aureus* Evans. The CD_{50} values or potency ratios proved that nafcillin was highly active against this organism, whereas oxacillin and erythromycin were capable of protecting the animals to a lesser degree. Of the agents studied, only nafcillin was capable of preventing or interfering with the colonization of the kidneys by *S. aureus* Evans. Although the exact interpretation and application of these data in the management of clinical problems remains to be determined, the observations suggest important differences between nafcillin and oxacillin *in vivo* and that it is difficult to predict the antibacterial efficacy of these drugs solely from MIC and MBC data.

Staphylococcus aureus Evans, has been shown by BEST *et al.*[1] to contain a population of cells with different degrees of sensitivity to oxacillin. The resistant cells may be detected visually after prolonged incubation or when tubes showing no growth are subcultured on solid medium. The survival of these resistant clones in the presence of high concentrations of oxacillin has generated some questions as to their role and importance in human infections. For obvious reasons, the fate of strain Evans interacting *in vivo* with a β-lactam antibiotic can only be determined by using a suitable animal model.

This report examines the therapeutic properties of a representative number of antibacterial agents in CD-1 mice infected with *S. aureus* Evans. In addition, infected animals treated with nafcillin or oxacillin were studied to determine if their activity could be correlated with the extent of colonization of kidneys by clones showing tolerance for anti-staph semisynthetic penicillins. Erythromycin was included as a reference standard to determine the response of the experimentally infected mice to a bacteriostatic drug.

Materials and Methods

Microorganism

Staphylococcus aureus Evans was kindly provided by Dr. G. K. BEST, Medical College of Georgia, Augusta, Georgia. This strain was isolated from a patient who died with a brain abscess of staphylococcal etiology. The organism was propagated in brain heart infusion broth (Difco). *Staphylococcus* Media No. 110 (Difco) was used to isolate and enumerate the number of colony forming units (c.f.u.) from mouse kidney homogenates.

Antibiotics

The antibiotics employed included potassium penicillin G, nafcillin, dicloxacillin and erythromycin stearate (supplied by Wyeth Laboratories, Inc.); oxacillin and cloxacillin (supplied

by Bristol Laboratories); and cephalexin and cefazolin (supplied by Eli Lilly Co.).

Procedure

The animals used in this study were CD-1 strain male albino mice weighing 18±1 g which were purchased from the Charles River Breeding Labs., Inc., Wilmington, Mass. The animals were pre-weighed, pooled, infected at random, and distributed in groups of 10 each (per dosage level). The antibiotics were prepared in sterile distilled water immediately before use and administered subcutaneously in 0.5 ml volumes. The curative dose (CD_{50}) of each antibiotic was determined from the activity of a single dose administered 6 hours after infection. Appropriate numbers of infected, untreated controls, amounting to at least 10 % of the total number of animals used, were included in each trial.

In Vitro Studies

Sterile flasks containing 1 ml of 100 mg oxacillin were seeded with 99 ml of brain heart infusion (BHI) broth containing 1×10^6 cells per ml of *S. aureus* Evans using a Cornwall automatic syringe. The flasks were incubated at 37°C and examined visually for presence or absence of growth at 24 hours, 48 hours and 7 days. On days 1, 2 and 7, 1.5 ml samples were removed for plate counts. Tenfold dilutions were made in cold BHI and two plates were made for each dilution, using 0.5 ml in each plate. BHI agar was used as the medium and the colonies were counted after 24 hours incubation at 37°C using a Quebec counter. Calculations were made by adding the number of colonies in each 0.5 ml plate and multiplying by the dilution factor.

In Vivo Studies

Previous reports have shown that preservation of virulent bacterial cells at −70°C provides stable populations giving reproducible mortality rates in mice.[2,3] *S. aureus* Evans was therefore maintained as concentrated stock pools in a dry ice chest. When required, the cells were thawed and diluted with a modified RINGER-LOCKE's solution containing serum albumin and dextrose to a predetermined 4 LD_{95} infective dose. An equal volume of 10 % gastric mucin (aqueous, pH 7.2) was added and the cell-mucin suspension ($LD_{95}\pm5$ %) was injected intraperitoneally (0.5 ml per mouse) using a 2-ml Cornwall syringe; the $LD_{95}\pm5$ % infective dose contained 3.3×10^5 c.f.u. The number of c.f.u. counted 6 hours after infection in all the vital organs (pooled) except the intestinal tract was 4.1×10^8 per g of homogenized tissue (wet weight).

In the study designed to determine whether nafcillin, oxacillin or erythromycin was capable of preventing the colonization of CD-1 mouse kidneys by *S. aureus* Evans the processing of the animals and their treatment was identical to that described above except that larger numbers of animals were used in the treatment groups. These consisted of 40 animals per dosage level segregated into groups of 10 each. Three, seven and fourteen days after treatment 5 animals from each group were sacrificed for the kidney studies; the remaining animals monitored the therapeutic activity of the agents tested. The kidneys were removed aseptically, homogenized in cold brain heart infusion broth (50 % by weight), and diluted in cold broth. The number of colong forming units (c.f.u.) was determined using the agar pour plate method. The volumes screened for c.f.u. were 0.1 ml portions of undiluted kidney broth homogenates and dilutions ranging from 1:10 through 1:10,000; two plates were poured for each dilution tested. The paired kidneys from each animal were cultured individually after their removal from mice killed at random. All animals were observed for 14 days; deaths were recorded daily and the CD_{50} values were calculated by the method of REED and MUENCH.[4]

Results

Table 1 summarizes the *in vitro* susceptibility of *S. aureus* Evans to oxacillin. This study demonstrated that flasks which were visually negative contained viable cells when subcultured on solid medium.

Table 2 shows the relative activity of a number of therapeutic agents in CD-1 mice infected with *S. aureus* Evans. According to the CD_{50} values or potency ratios nafcillin proved highly

Table 1. *In vitro* susceptibility of *Staphylococcus aureus* Evans to oxacillin.

Oxacillin (μg/ml)	1 Day		2 Days		7 Days	
	Visual growth	Subculture (c.f.u./ml)	Visual growth	Subculture (c.f.u./ml)	Visual growth	Subculture (c.f.u./ml)
250.0	—	0.0	—	0.0	—	1.7×10^{4}
62.5	—	4.0×10^{1}	—	0.0	—	5.9×10^{8}
15.6	—	1.5×10^{2}	—	2.5×10^{1}	—	3.0×10^{4}
3.9	—	6.0×10^{2}	—	2.3×10^{8}	4+	1.0×10^{9}
0.975	—	6.0×10^{2}	3+	4.3×10^{8}	4+	2.1×10^{8}
0.0	4+	3.7×10^{7}	4+	4.7×10^{8}	4+	2.9×10^{8}

active against this organism. Potassium penicillin G was inactive; the remaining agents were capable of protecting the animals, but to a lesser degree than nafcillin.

The results in Table 3 compare the effectiveness of nafcillin, oxacillin and erythromycin as therapeutic agents and concomitantly their ability to interfere with colonization of CD-1 mouse kidneys by *S. aureus* Evans. In accordance with the CD_{50} values and associated kidney counts (c.f.u.), nafcillin proved to be more active than oxacillin or erythromycin by both criteria.

Table 2. Oral CD_{50} of antibiotic agents in mice infected with *Staphylococcus aureus* Evans*

Agent	CD_{50} (mg/kg) (mean±S.D.)	Potency ratio
Nafcillin	88.4± 2.3	1.00
K Penicillin G	>800.0	<0.11
Methicillin	320.6±103.3	0.28
Oxacillin	187.2± 13.9	0.47
Cloxacillin	282.0	0.31
Dicloxacillin	168.9± 31.1	0.52
Erythromycin	178.8	0.49
Cephalexin	245.6	0.36
Cefazolin	176.1± 23.1	0.50

* Percent survivors among infected controls: 3.9

The majority of the paired kidneys homogenized and cultured separately on day 3 after infection and treatment were positive for strain Evans irrespective of the treatment dose or agent used. The numbers of c.f.u. counted, in descending order of concentration, were dose dependent. The mean percentages of positive kidneys within the treatment group for nafcillin, oxacillin and erythromycin were respectively 88, 93 and 85 % (Table 4).

These results also show that the counts for erythromycin (day 3) tended to be lower than those noted for nafcillin or oxacillin (Table 3). However, with a few exceptions, when the counts for oxacillin or erythromycin were compared, taking into consideration the effect of concentration against time, the c.f.u. isolated from the kidneys were fairly constant, indicating some order of stabilization.

Discussion

Previous studies from this laboratory demonstrated that nafcillin has profound effects on the cell wall of *Staphylococcus aureus*.[5~7] These effects include an alteration or disorganization of the wall and an increased sensitivity of the cell to enzymic lysis. Other penicillins differ widely in ability to influence lysis. Moreover, it has been demonstrated that lysis is not conditioned by the *in vitro* activity of the penicillin; nafcillin and oxacillin had the same *in vitro* antibacterial activity againts several strains of staphylococci, yet nafcillin produced a significantly greater rate and extent of lysis.[6] The published findings, as described, suggested that various penicillins may produce their antibacterial effects by different mechanisms or primary sites of action and

Table 3. Comparative therapeutic activity and colonization of kidneys in CD-1 mice infected with *Staphylococcus aureus* Evans

Agent	Dose (mg/kg)	c.f.u./ml Kidney homogenate*			CD_{50} (mg/kg)(1)	CD_{50} (mean±S.D.) (mg/kg)(2)
		3 days	7 days	14 days		
Nafcillin	800	3.1×10^{4}	0	0	101.1	94.8± 6.3
	400	2.5×10^{5}	0	0		
	200	9.6×10^{6}	1.9×10^{4}	0		
	100	5.7×10^{6}	5.7×10^{3}	0		
	50	nd	nd	7.0×10^{1}		
Oxacillin	800	7.6×10^{4}	3.6×10^{6}	1.0×10^{1}	253.3	220.3±33.1
	400	7.2×10^{4}	2.2×10^{4}	2.3×10^{4}		
	200	1.4×10^{5}	3.5×10^{4}	5.0×10^{2}		
	100	1.6×10^{7}	4.1×10^{4}	1.7×10^{4}		
	50	nd	nd	1.6×10^{3}		
Erythromycin	800	4.5×10^{3}	4.5×10^{4}	4.4×10^{3}	198.3	188.6± 9.8
	400	1.6×10^{3}	2.3×10^{3}	2.5×10^{3}		
	200	1.6×10^{4}	2.1×10^{5}	3.0×10^{1}		
	100	2.0×10^{6}	1.1×10^{5}	3.0×10^{3}		
	50	nd	nd	1.2×10^{3}		

* Negative kidneys excluded from calculations.
(1) Values determined in conjunction with kidney studies.
(2) Includes data from Table 2.

Table 4. Percent kidneys positive with *Staphylococcus aureus* Evans on day sacrificed

Agent	Dose (mg/kg)	Total no. paired kidneys tested(1)	Percent kidneys positive on culture		
			3 Days	7 Days	14 Days
Nafcillin	800	15	100	0	0
	400	30	70	0	0
	200	30	100	30	0
	100	25	80	10	0
	50	5	nd	nd	80
Oxacillin	800	15	100	20	20
	400	30	100	80	100
	200	30	70	80	30
	100	25	100	80	40
	50	5	nd	nd	100
Erythromycin	800	15	100	40	20
	400	30	80	80	80
	200	30	100	70	10
	100	25	60	80	50
	50	5	nd	nd	100
Evans Controls		—	—	—	50(2)

(1) Five mice sacrificed/trial/dose/time interval.
(2) Controls, percent survivors —3.6%.

that these effects may be independent of the action on cell wall synthesis. It is noteworthy that a similar study showed that incubation of penicillin-resistant staphylococci with nafcillin at a subinhibitory concentration increased the susceptibility of the bacteria to subsequent phagocytosis *in vitro* by mouse peritoneal exudate cells and that comparable concentrations of oxacillin did not significantly affect subsequent phagocytosis by such cells.[8] In more recent publications[9,10] a remarkable enhancement of killing of clinical isolates of *S. aureus* (endocarditis patients) occurred with a combination of nafcillin-gentamicin. Oxacillin and methicillin when combined with gentamicin showed enhancement of activity against a significantly smaller number of isolates and were clearly much less effective in killing *S. aureus* than nafcillin.

In the current study it was shown, as was also described by BEST *et al.*,[1] that *S. aureus* manifests a tolerance to oxacillin, as evidenced by a markedly reduced bactericidal activity. The existence of cells tolerant to oxacillin and their rate of multiplication within the 7 day period (Table 1) suggests that resistance (tolerance) by this strain of staphylococci proceeded slowly and may be attributable to slowly growing mutants.

At this juncture it is impossible to include additional statements about this interesting strain, since this would entail special attention to population and biochemical studies. We refer the reader to the original paper by BEST *et al.*[1] for their personal interpretation as to why strain Evans develops high tolerance for the penicillins.

In substantiating the *in vitro* data reported earlier by BEST *et al.*,[1] we were convinced that an *in vivo* study would give a similar response in animals infected with Evans and treated with antibiotics, *i.e.*, the presence and multiplication of β-lactam resistant clones would radically interfere with antibacterial activity. The results noted for nafcillin, as opposed to oxacillin and erythromycin, were therefore unexpected and are, currently, unexplainable.

Nafcillin and oxacillin are two members of the semi-synthetic penicillins of β-lactam group. Although they are comparable to some extent in their antibacterial properties, they differ to some degree in serum levels and serum binding[11] and to an appreciable degree in their resistance to both inoculum effect and the production of staphylococcal β-lactamase.[12] This study has also recorded another major difference between nafcillin and oxacillin.

According to the CD_{50} values or potency ratios, nafcillin was 2~4 times more effective than the other agents listed. A qualitative study of the relative therapeutic properties revealed further differences. These results show that in addition to a lower CD_{50} value, only nafcillin was capable of preventing or interfering with the colonization of the kidneys by strain Evans. More interesting is the fact that a concentration of nafcillin roughly equivalent to the calculated CD_{50} value prevented the invasion of kidney tissue. It will be noted that there was a satisfactory correlation between effectiveness and concentration (dose-response effect).

The results for oxacillin and erythromycin are self-explanatory. Qualitatively, neither proved capable of preventing the establishment of kidney infections. Initially, the counts for erythromycin were lower than those noted for nafcillin or oxacillin. However, when the counts for oxacillin or erythromycin were compared against concentration and time the numbers of c.f.u. isolated from the kidneys were fairly constant. This suggests some order of stabilization between the animals and/or the kidneys and the invading cells. That the kidneys (which on direct inspection showed no obvious gross pathology) continued to function despite the invading cells suggests that the kidneys and infecting organisms may have accomodated each other and thus co-exist in a state of equilibrium or tolerance. The surviving animals, whether treated with nafcillin, oxacillin or erythromycin, appeared healthy, gained weight and continued to live for several weeks after termination of the study.

Although the exact interpretation and application of these data in the management of clinical problems remains to be determined, such observations, along with previous ones, suggest important differences between nafcillin and oxacillin *in vivo* and indicate the basis for the difficulty in predicting the antibacterial efficacy of these drugs solely from MIC and MBC data.

References

1) BEST, G.K.; N. H. BEST & A.V. KOVAL: Evidence for participation of autolysins in bactericidal action of oxacillin on *Staphylococcus aureus*. Antimicr. Agents & Chemother. 6: 825~830, 1974
2) YURCHENCO, J. A.; C. R. PIEPOLI & M. C. YURCHENCO: Low temperature storage for maintaining stable infectious pools. Appl. Microbiol. 2: 53~55, 1954
3) YURCHENCO, J. A.; M.W. HOPPER & G. H. WARREN: Therapeutic activity of semisynthetic penicillin Wy-3277 in experimental infections in mice. Antibiot. & Chemoth. 12: 534~544, 1962
4) REED, L. J. & H. MUENCH: A simple method of estimating fifty-percent end-points. Am. J. Hyg. 27: 493~497, 1938
5) WARREN, G. H. & J. GRAY: Production of a polysaccharide by *Staphylococcus aureus*. III. Action of penicillins and polysaccharides on enzymic lysis. Proc. Soc. Exptl. Biol. Med. 116: 317~323, 1964
6) WARREN, G. H. & J. GRAY: Effect of sublethal concentrations of penicillins on the lysis of bacteria by lysozyme and trypsin. Proc. Soc. Exptl. Biol. Med. 120: 504~511, 1965
7) WARREN, G. H. & J. GRAY: Influence of nafcillin on the enzymic lysis of *Staphylococcus aureus*. Canad. J. Microbiol. 13: 321~328, 1967
8) FRIEDMAN, H. & G. H. WARREN: Enhanced susceptibility of penicillin-resistant staphylococci to phagocytosis after *in vitro* incubation with low doses of nafcillin. Proc. Soc. Exptl. Biol. Med. 146: 707~711, 1974
9) WATANAKUNAKORN, C. & C. GLOTZBECKER: Enhancement of the effects of anti-staphylococcal antibiotics by aminoglycosides. Antimicr. Agents & Chemother. 6: 802~806, 1974
10) GLEW, R. H.; R. C. MOELLERING, Jr. & C. WENNERSTEN: Comparative synergistic activity of nafcillin, oxacillin, and methicillin in combination with gentamicin against enterococci. Antimicr. Agents & Chemother. 7: 828~832, 1975
11) WARREN, G. H.: The prognostic significance of penicillin serum levels and protein binding in clinical medicine. A review of current studies. Chemotherapia 10: 339~358, 1965/66
12) SABATH, L. D.; C. GARNER, C. WILCOX & M. FINLAND: Effect of inoculum and of beta-lactamase on the anti-staphylococcal activity of thirteen penicillins and cephalosporins. Antimicr. Agents & Chemother. 8: 344~349, 1975