

Penicillin Tolerance of Human Isolates of Group C Streptococci

DAVID PORTNOY,* JOHN PRENTIS, AND GEOFFREY K. RICHARDS

Department of Microbiology and Infectious Disease, Montreal General Hospital, Montreal, Quebec H3G 1A4, Canada

Received 29 December 1980/Accepted 19 May 1981

Seventeen clinical isolates of group C streptococci were tested for penicillin tolerance. Sixteen of the strains showed penicillin tolerance with a 32-fold or greater difference between the minimal inhibitory concentration and the minimal bactericidal concentration. Synergism was demonstrated with a combination of penicillin and gentamicin for all 17 strains tested. The rate of antibiotic killing was measured for five of the streptococcal strains by using the combination of penicillin and gentamicin. All isolates were killed within 5 h with the combination, but viable organisms were recovered after 48 h when either drug was used alone. Our study suggests that penicillin tolerance with group C streptococci may occur frequently and may account for the poor outcome of serious group C streptococcal infections tested with penicillin alone.

Although strains of group C streptococci are commonly associated with animal infections, the organism has been recovered from the skin, nose, throat, and vagina of healthy people (3, 16) and has been associated with puerperal, skin, and wound infections (6, 7), tonsillitis (1), glomerulonephritis (11), cellulitis (12), pulmonary infection, urinary tract infection, bacteremia (5), endocarditis (13), and meningitis (9, 10, 18) in humans. Recently, we reported the first case of penicillin-tolerant group C streptococcal endocarditis (13). Because published reports, including our own, suggest that the morbidity and mortality of serious group C streptococcal infections such as endocarditis and meningitis are excessively high despite seemingly adequate therapy (10, 13), we studied other clinical isolates of this organism to see whether penicillin tolerance was a common occurrence and thus could account for the poor outcome of serious infections caused by group C streptococci.

MATERIALS AND METHODS

Isolates. Seventeen strains of group C streptococci were isolated from different patients. There were two blood isolates, two cervical isolates, nine throat isolates, one nasal isolate, and three isolates from infected leg ulcers. The organisms were identified by the Lancefield hot acid antigen extraction technique, followed by tube precipitation grouping with Lancefield group C antiserum.

Studies of antibiotic action. (i) Choice of medium. Of the 17 strains tested, 12 grew well in tryptone soya broth (Oxoid Ltd.) and Mueller-Hinton broth (Difco Laboratories), with some variation in opacity

after overnight incubation. Five of the strains grew poorly in both media tested, with minimal and irregular opacity after overnight incubation. Both media were judged to be inadequate for assays of kinetics of antibiotic killing.

A search for a medium giving equal and adequate growth for all 17 strains was undertaken; nutrient broth (Oxoid) supplemented with 20% horse serum (HSNB) satisfied the growth requirements.

A comparison of the minimal inhibitory concentration (MIC) determination for penicillin for the 12 strains that grew well in tryptone soya broth with corresponding MIC determinations conducted in HSNB after an 18-h incubation showed either identical results or, occasionally, a one-dilution difference.

(ii) Duration of incubation. Five of the isolates which grew uniformly in both tryptone soya broth and HSNB were used to assess the effect on the MIC of penicillin of changing the duration of incubation from 18 to 24 h. When tryptone soya broth was used as the growth medium, the MIC and minimal bactericidal concentration (MBC) determinations showed considerable variation for the endpoints, depending on whether an 18- or a 24-h incubation was used.

When HSNB was used as the growth medium, it was found that there was no change in either the MIC or the MBC endpoint readings.

The determination of the MIC and the MBC of penicillin reported in this article was conducted at both 18- and 24-h subcultures, and no difference was found for any of the 17 strains tested.

(iii) MICs and MBCs. The MICs of penicillin and gentamicin for all 17 isolates were determined with the doubling-dilution tube technique. HSNB, which supported adequate growth of all the isolates, was used as the diluent. Stock solutions of penicillin (100 U/ml; Glaxo) and gentamicin (50 µg/ml; Schering Corp.) were prepared from a reference powder of

known activity provided by the manufacturer. Samples of 10 ml in HSNB were stored at -20°C until needed. Doubling dilutions in 1-ml volumes commenced for the penicillin series at 10 U/ml and for the gentamicin series at 20 $\mu\text{g}/\text{ml}$. An inoculum of the streptococcal isolate in the log phase of growth was added to each tube to achieve a final concentration of $10^5 (\pm 10^{0.3})$ colony-forming units per ml. The endpoint (least concentration of antibiotic required to inhibit growth) was read after 18 h of incubation at 37°C .

The MBC was determined by subculturing 0.1-ml volumes in triplicate from each tube failing to show growth onto the surface of blood-agar (5% horse blood-Columbia agar; Oxoid CM 331) incubated aerobically for 18 h at 37°C . The MBC was defined as the least amount of antibiotic which resulted in 99.9% kill.

(iv) **Bactericidal synergy.** The isolate in the log phase of growth was added to each of four tubes containing, respectively, penicillin (at a concentration of one-fourth of the MBC for the isolate), gentamicin (at a concentration of one-fourth of the MBC), a combination of both antibiotics, and no antibiotic. The latter tube served as the growth control. The final density of organisms was adjusted to $10^5 (\pm 10^{0.3})$ colony-forming units per ml. After incubation for 18 h at 37°C , 0.1-ml subcultures from each tube were made in triplicate onto the surface of blood-agar plates which were incubated for a further 18 h at 37°C . Bacterial synergy was regarded as demonstrated if there was an absence of growth only in the subcultures of those tubes containing the antibiotic combination.

A synergistic effect was therefore defined under the conditions of this test as a complete kill resulting from the combination of the antibiotics at a concentration of one-fourth of the minimum required to kill the organism when the antibiotic was used alone (14, 17).

(v) **Kinetics of bactericidal action.** Timed-killing assays were performed on five of the streptococcal isolates by using penicillin and gentamicin, singly and in combination, at serum-achievable levels. Gentamicin was chosen for this purpose, as it is the most frequently used aminoglycoside in our hospital.

Technique. To each of four tubes of 20 ml of HSNB containing, respectively, 10 U of penicillin per ml, 5 μg of gentamicin per ml, the combination of the two antibiotics, and no antibiotics (growth control), an inoculum of streptococci in the log phase of growth (4-h culture) was added to achieve a final density of $10^{6.9} (\pm 10^{0.2})$ colony-forming units per ml.

The tubes were incubated aerobically at 37°C for 48 h. At 0, 1, 3, 5, 6, 24, and 48 h, 0.5-ml volumes were withdrawn, and the bacterial numbers were estimated by the Miles and Misra surface viable counting technique (8). Each sample was decimally diluted sixfold in HSNB (dilution range, 10^{-1} to 10^{-6} of the original sample). Each decimal dilution was further subcultured in volumes of 0.1 ml plated in triplicate onto the surface of blood-agar plates, which were then incubated aerobically for a further 18 h at 37°C . Counts were made from those plates in which colony numbers fell in the range of 30 to 200 per plate. At 0, 1, 3, 5, 6, 24, and 48 h, 0.1-ml volumes were obtained in duplicate from the original 4-h tube cultures. One sample was plated onto the surface of blood agar, and the other sample was inoculated into 25 ml of HSNB. Killing

was defined as present if both subcultures showed <10 colony-forming units per ml after 18 h of incubation.

RESULTS

MICs and MBCs. The MICs of penicillin for all 17 strains tested fell in the range of 0.02 to 0.15 U/ml. The MBCs of penicillin were in the range of 0.6 to 10 U/ml (Table 1).

The MBC/MIC ratio, with one exception (strain 15), ranged from 32- to 512-fold, clearly demonstrating penicillin tolerance for 16 of the 17 strains tested (15).

Bactericidal synergy. The incubation of each strain in concentrations of penicillin or gentamicin equivalent to one-fourth of their respective MBCs resulted in bacterial growth. When the identical concentrations of both antibiotics were used in combination, there was no bacterial growth in any of the 17 strains of group C streptococci tested, clearly demonstrating synergism of penicillin and gentamicin.

Kinetics of bactericidal action (killing kinetics). Timed-killing assays were performed on five isolates of the group C streptococci (Table 2). Killing was complete within 5 h when a combination of penicillin (10 U/ml) and gentamicin (5 $\mu\text{g}/\text{ml}$) was used, but viable organisms were recovered in all samples after an incubation of up to 48 h when these drugs were used individually. All five strains tested had similar killing curves, and a combined curve showing the reduction in bacterial numbers with respect to time is shown in Fig. 1.

DISCUSSION

Group C streptococcus is a rare cause of seri-

TABLE 1. MIC and MBC of penicillin G for 17 strains of group C streptococci

Strain no.	Penicillin (G U/ml)		MBC/MIC ratio	Gentamicin ($\mu\text{g}/\text{ml}$)	
	MIC	MBC		MIC	MBC
1	0.04	10	256	5	5
2	0.04	10	256	5	5
3	0.04	10	256	5	5
4	0.08	10	128	5	5
5	0.02	0.6	32	10	20
6	0.08	10	128	5	5
7	0.04	10	256	5	5
8	0.04	10	256	5	5
9	0.04	10	256	5	10
10	0.15	10	64	5	10
11	0.04	5	125	5	5
12	0.02	10	512	2.5	10
13	0.04	2.5	64	5	5
14	0.08	10	128	5	5
15	0.15	0.6	4	5	10
16	0.15	10	64	5	10
17	0.04	2.5	64	10	10

TABLE 2. Averaged timed-killing assays for five strains (2, 4, 11, 13, and 16) of group C streptococci in the presence of penicillin G, gentamicin, and a combination of antibiotics

Incubation time (h)	No. of bacteria (logs/ml)					
	Penicillin G (10 U/ml)		Gentamicin (5 µg/ml)		Combination	
	Avg	Range	Avg	Range	Avg	Range
0	6.9	±0.2	6.9	±0.2	6.9	±0.2
1	6.1	±1.0	6.6	±0.6	5.4	±0.5
3	5.7	±0.6	5.7	±1.1	2.3	±2.3
5	5.4	±1.1	4.5	±1.8	0	
6	5.1	±1.0	3	±1.7	0	
24	1.7	±0.3	2.9	±1.6	0	
48	1.0	±0.05	3.1	±2.0	0	

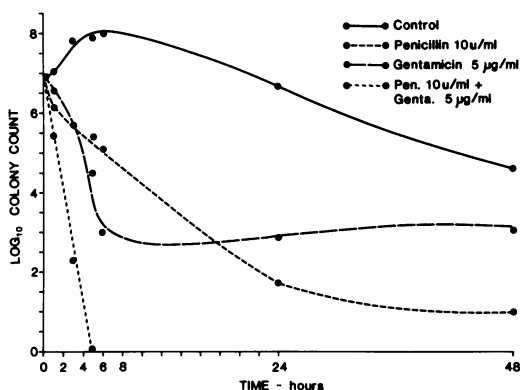


FIG. 1. Averaged timed-killed curves of five strains of group C streptococci in the presence of penicillin G, gentamicin, and a combination of penicillin and gentamicin.

ous infection in humans. To date, there have been 12 reported cases of endocarditis (13) and 3 cases of meningitis (10, 18) due to this organism. Despite seemingly adequate antibiotic therapy, group C streptococcal endocarditis usually follows an acute destructive course with a high morbidity and mortality. Similarly, group C streptococci have been responsible for severe cases of meningitis with various neurological complications and sequelae. In most of these case reports, treatment was based on the results of disk sensitivity testing alone or MIC determinations. Sabbath and colleagues (15) described a new type of penicillin resistance of *Staphylococcus aureus*. These organisms were defined as having a low MIC, but an MBC of 32-fold or greater than the MIC. In 16 of the 17 strains of group C streptococci tested, the MBC was at least 32-fold or greater than the MIC of penicillin. A synergistic effect of penicillin with gentamicin was demonstrated in all of the strains

tested. Killing kinetic studies showed rapid total killing of the organisms tested within 5 h when both antibiotics were used together, as compared with a survival time of the streptococci of 48 h when each antibiotic was used alone (Table 2).

The clinical significance of penicillin tolerance is still debatable (4). Denny and colleagues (2) noted that, in serious staphylococcal infections, 4 of 10 patients treated with bacteriostatic antibiotics died, and cultures remained positive for a mean of 6.1 days, as compared with no death and sterile cultures after a mean of 1.3 days in patients treated with bactericidal antibiotics.

Our study suggests that penicillin tolerance with group C streptococci occurs commonly and may explain the poor outcome of serious group C streptococcal infections treated with penicillin alone. Penicillin-gentamicin synergism and the accelerated killing of the streptococci in vitro with this antibiotic combination may have clinical relevance. We therefore recommend that, for serious group C streptococcal infections, the MBC for penicillin be determined and that, for initial therapy of serious beta-hemolytic streptococcal infections, the use of gentamicin in addition to penicillin be considered pending appropriate in vitro susceptibility results.

LITERATURE CITED

- Benjamin, J. I., and V. A. Perriello. 1976. Pharyngitis due to group C hemolytic streptococci in children. *J. Pediatr.* 89:254-256.
- Denny, A. E., L. R. Peterson, D. N. Gerding, and W. H. Hall. 1979. Serious staphylococcal infections with strains tolerant to bactericidal antibiotics. *Arch. Intern. Med.* 139:1026-1031.
- Duma, R. J., A. N. Weinberg, T. F. Medrek, and L. J. Kunz. 1969. Streptococcal infections: a bacteriologic and clinical study of streptococcal bacteremia. *Medicine (Baltimore)* 48:87-127.
- Editorial. 1980. Penicillin-tolerant bacteria. *Lancet* i:856-857.
- Feingold, D. S., N. L. Stagg, and L. J. Kunz. 1966. Extracerebral streptococcal infections. Importance of the various serologic groups. *N. Engl. J. Med.* 275:356-361.
- Hiu, A. M., and H. M. Butler. 1940. Haemolytic streptococcal infection following childbirth and abortion. *Med. J. Aust.* 1:293.
- Hutchison, R. I. 1946. Pathogenicity of group C (Lancefield) hemolytic streptococcus. *Br. Med. J.* 2:575.
- Miles, A. A., and S. S. Misra. 1938. The estimation of the bactericidal power of the blood. *J. Hyg.* 38:372.
- Mohr, D. N., D. J. Feist, J. A. Washington II, and P. E. Hermans. 1978. Meningitis due to group C streptococci in an adult. *Mayo Clin. Proc.* 53:529-532.
- Mohr, D. N., D. J. Feist, J. A. Washington II, and P. E. Hermans. 1979. Infections due to group C streptococci in man. *Am. J. Med.* 66:450-456.
- Poon-King, T., I. Mohammed, R. Cox, E. V. Potter, N. M. Simon, A. C. Siegel, and D. P. Earle. 1967. Recurrent epidemic nephritis in South Trinidad. *N. Engl. J. Med.* 277:728-733.
- Portnoy, B., and R. Reitler. 1944. Cellulitis due to a haemolytic streptococcus type C. *Lancet* ii:597-598.
- Portnoy, D., I. Wink, G. K. Richards, and M. Z. Blanc.

1980. Bacterial endocarditis due to penicillin-tolerant group C streptococcus. *Can. Med. Assoc. J.* **122**:69-75.
14. **Rahal, J. J.** 1978. Antibiotic combination: the clinical relevance of synergy and antagonism. *Medicine (Baltimore)* **57**:179-195.
15. **Sabath, L. D., N. Wheeler, M. Laverdiere, D. Blazevic, and B. J. Wilkinson.** 1977. A new type of penicillin resistance of *Staphylococcus aureus*. *Lancet* **i**:443-447.
16. **Sanders, V.** 1963. Bacterial endocarditis due to a group C beta hemolytic streptococcus. *Ann. Intern. Med.* **58**: 856-861.
17. **Schauf, V., A. Develkis, L. Riff, and A. Serota.** 1976. Antibiotic killing kinetics of group B streptococci. *J. Pediatr.* **89**:194-198.
18. **Stewardson-Krieger, P., and S. P. Gotoff.** 1977. Neonatal meningitis due to a group C beta-hemolytic streptococcus. *J. Pediatr.* **90**:103-104.