Penicillin Tolerance among Beta-hemolytic Streptococci and Production of the Group Carbohydrates, Hemolysins, Hyaluronidases and Deoxyribonucleases

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Penicillin tolerance among 67 strains of beta-hemolytic streptococci was examined by determining the ratio of the minimal bactericidal concentration to the minimal inhibitory concentration as 32 or greater. Tolerance was demonstrated in 15 group A strains and in 11,7, and 4 of groups B, C and G, respectively. Thereafter the effects of a subminimal inhibitory concentration (1/2 MIC) of penicillin on the bacterial products of four tolerant and four nontolerant strains (two of each Lancefield group) were analyzed and compared. The antibiotic caused a marked increase in the expression of the group carbohydrates for strains of group B. Penicillin was found to reduce the cell-bound hemolysin activities of the four tolerant strains and to increase the activity of the other (free) form of nontolerant groups A, C and G hemolysins. Penicillin caused an increase in the extracellular hyaluronidase activities of one group A and groups B, C and G streptococci. With added antibiotic the production of deoxyribonuclease by tolerant groups A, C and G was greatly enhanced and that of the group B streptococcus was arrested.

Key words: streptococci - penicillin - tolerance - enzymes - toxins

Penicillin is the drug of choice the world over for the treatment of infections due to beta-hemolytic streptococci, specially those of the Lancefield group A. Groups B, C and G streptococci remain very sensitive although minimal inhibitory concentrations (MICs) for GBS are often higher than for the other groups (Allen & Sprunt 1978). No resistant strain of group A streptococci has ever been reported (Allen & Sprunt 1978, Kim & Kaplan 1985). Occasionally, streptococci have a minimal bactericidal concentration (MBC) of 32 times or more than the MIC and are said to be tolerant (Amsterdam 1991). Penicillin tolerance was first described in pneumococci (Tomasz et al. 1970) and thereafter, demonstrated in a wide spectrum of important human pathogenic bacteria (Rolston et al. 1982, Slater & Greenwood 1983, Voorn et al. 1994 , Brett 1994).

Antibiotics in subMICs have been the purpose of many investigations and reviews during the past quarter of century, because of the morphological, ultrastructural and biochemical changes that they produce in bacteria (Gemmell & Abdul-Amir 1979, Lorian & Gemmell 1991). With regards to streptococci of groups A, B, C and G exposed to subMICs of penicillin during growth *in vitro*, the changes observed in the elaboration of bacterial antigens, such as enzymes, toxins and cellular components varied with the serological group tested, the antimicrobial agent and its concentration and have been documented (Benchetrit et al. 1984, Figueiredo & Benchetrit 1989). However, the subinhibitory antibacterial effects of penicillin on the biology of streptococci tolerant to the antibiotic remained to be determined.

We report here the influence of a subMIC of penicillin on cell-bound substances and extracellular products of strains of serological groups A, B, C and G streptococci tolerant to the antibiotic. We also examined changes in the elaboration of the "C" carbohydrat and assayed the rhamnose content of antigenic extracts. The results are compared to those obtained with nontolerant strains.

MATERIALS AND METHODS

Strains - Sixty seven beta-hemolytic strains were used in this study. Twenty eight were group A, 14 group B, 15 group C and 10 group G and were clinical isolates from our laboratory (Benchetrit et al. 1984, Ferreira et al. 1992). Twenty seven strains of group A streptococci were obtained from individuals with streptococcal pharyngitis and one strain was isolated from the infected skin lesion of patient with pyoderma. Seven group B streptococci were from the perinatal period (vagina, cervix and anus) and the other strains re-

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presented diverse human sources such as throat, blood, cerebrospinal fluid, skin lesion and human lung necropsy. Twelve group C isolates were from throat specimens, one from a blood culture and two from animal (horse) sources. The ten group G strains were from patients with streptococcal pharyngitis. The group A strain, designated K443, was received from KS Kim and used as a standard microorganism to determine tolerance to penicillin.

Grouping of the streptococci was carried according to the nitrous acid procedure described by El Kholy et al. (1974). Streptococcal grouping antisera were produced in our laboratory by immunizing rabbits with vaccine strains of the four Lancefield groups and the precipitin reactions were carried out in capillary tubes (Lancefield 1933). Stock cultures were stored in sheep blood in the lyophylized state.

Media - Plates containing tryptose blood agar base and 5% sheep blood were used for isolation and counting the beta-hemolytic streptococci. Broth for bacterial growth was the Todd-Hewitt medium. Culture media were from the Difco Laboratories (Benchetrit et al. 1984, Figueiredo & Benchetrit 1989, Ferreira et al. 1992).

Minimal inhibitory and bactericidal concentrations and penicillin tolerance - Ten colonies of streptococci were picked from blood plates and grown for 5 hr at 37°C in 5 ml of broth in a water bath. This aerobic culture was then diluted into broth so as to contain 10^5 to 10^6 CFU/ml. The penicillin MIC was then determined by the macro broth dilution method (Amsterdam 1991). Thereafter tubes that showed no growth were vigorously shaken for 15 seconds, incubated for 4 hr and shaken again. Aliquots of this culture were diluted in physiological saline and subcultured into blood plates that were incubated for 24 hr at 37°C and colony counts were performed. The MBC was determined as the lowest penicillin concentration which killed 99.9% of the viable cells in the primary inoculum. Penicillin tolerance was recognized when ratios of MBC to the MIC were 32 or greater (Sabath et al. 1977).

Bacterial cells and culture supernatants - Cells grown in broth or in a penicillin-medium and the respective supernates were obtained as follows: five colonies of streptococci were inoculated into 50 ml of broth and bacteria were grown for 18 hr at 37°C. Penicillin was added to the medium at inoculation time in the concentration of 1/2 of the MIC. The culture was then vigorously mixed and absorption readings of the cell suspension was carried out at 540 nm. Results were expressed in optical density units (ODU). Viable counting, expressed in terms of CFU/ml was also performed. Thereafter bacterial cells were removed by centrifugation. The supernatant fluid thus obtained was sterilized by filtration through membrane filters before testing for extracellular hemolysin (streptolysin O, SLO), deoxyribonuclease (DNase; Ferreira et al. 1992) and hyaluronidase activities. The streptococci in the bacterial sediment were washed twice in physiological saline and ressuspended in 0.01 M sodium phosphate buffer at pH 7.2 (one tenth of the original volume of the culture). The cell suspension was then adjusted to an absorbance of 0.3 at 540 nm by the addition of buffer. These streptococci served as the material for quantitative studies of the group antigen and of the cell-bound (streptolysin S, SLS) activity .

Streptococcal products - Free hemolysin activity was determined by incubating the following reactions mixtures for 45 min at 37°C: culture supernatant fluid (5.6 ml), sodium phosphate buffer 0.01M at pH 7.0 (1.26 ml), 0.03 M 2-mercaptoethanol (0.14 ml) and a sheep red blood cells (SRBCs) button obtained after sedimenting 3.5 ml of a 1% suspension by centrifugation. Thereafter, the mixture was centrifuged at 500g for 20 min and optical density of the supernatant was read at 540 nm against a blank containing broth (5.6 ml), phosphate buffer (1.26 ml), mercaptoethanol (0.14 ml) and the SRBCs button. This blank also served as a negative control, that is, absence of lysis. The reaction mixture used as a positive control, that is, complete lysis contained distilled water (1.26 ml; first used to lyse the SRBCs), broth (5.6 ml), mercaptoethanol (0.14ml) and the blood cells button. Hemolysin activity was expressed in terms of ratios of optical density readings of the supernatant fluid obtained after lysis of SRBCs with whole culture supernatant/optical density readings of the bacterial growth.

Cell-bound hemolysin activity was determined by incubating the following reaction mixture for 45 min at 37°C: bacterial suspension (7 ml; A_{540} = 0.3) and a SRBCs button. Thereafter, the mixture was centrifuged and optical density readings of the supernatant was read at 540 nm against a blank containing SRBCs and phosphate buffer (7 ml; negative control). The system used as a positive control contained distilled water (7 ml) and SRBCs. Hemolysin was expressed in terms of optical density readings of the supernatants obtained after lysis of SRBCs with whole streptococci.

The method for the assay of extracellular DNase activity has been previously detailed (Ferreira et al. 1992). The technique follows the extent of degradation of DNA by observing the capability of the enzyme to decolorize a DNA-methylgreen complex included in an agarose gel. Specific activity was expressed as the number of enzyme units (U) per optical density unit (ODU) of bacterial cultures or per number of viable cells (CFU).

Quantitative determinations of the group antigen has been previously described (Figueiredo & Benchetrit 1989). Rhamnose in the antigenic extracts (El Kholy et al. 1974) was measured by using a chemical method (Dische & Shettles 1948).

Hyaluronidase activity in the supernate fluids was measured by following the extent of degradation of hyaluronic acid of a substrate-bovine serum albumin complex included in an agarose gel (Smith & Willett 1968). A mixture containing 0.02% hyaluronic acid (grade III, human umbilical cord, Sigma Chemical Co), 1% albumin, 1.5% agarose and 50 mM sodium phosphate buffer (pH 7.2) was poured into 100mm Petri dishes (12 µl per dish). Wells of 3mm diameter were cut and filled with 10µl of test samples. Substrate degradation was then allowed to occur at 37°C for 20 hr. The presence of the enzyme in the sample was indicated by a clear zone surrounding the wells against an opaque background of precipitated substrate-protein complex when the plates were flooded with 2N acetic acid. The zone diameters, in millimeters, were then measured and plotted on a standard straight line previously constructed with known enzyme (bovine testes, type IS, Sigma) quantities within the range (0.3 to 7.0 µg) of the assay. Correlation between the hyaluronidase amounts and clear zone diameters was assessed by calculating the coefficients of correlation and regression. Thus unknown enzyme concentrations could be calculated from the straight line. Specific activity of the enzyme was expressed as the number of enzyme units (U) per optical density unit (ODU) or per viable cell (CFU) of bacterial cultures.

RESULTS

Tolerance was demonstrated in 15 group A strains and in 11, 7 and 4 of groups B, C and G, respectively. Thereafter two strains of each of the four Lancefield groups, one penicillin-tolerant isolate and one nontolerant organism were used for the studies which results are described below.

Exposure of the tolerant group A streptococcus to the antibiotic (1/2 MIC) resulted in a decreasc in the amount of antigen-antibody complex, as there was a precipitin reaction after dilution (1:4) of the extract but not after a 1:8 dilution (Table). In contrast, with cells of the tolerant groups B and C strains grown in the presence of penicillin there was still a precipitin reaction after a 1:32 dilution of the extract. The tolerant group G streptococcus was not affected by the subMIC of penicillin as antigenic extracts obtained from drug-treated cells and control (no added antibiotic) bacteria reacted at the same (1:2) dilution. The rhamnose contents of the various nitrous acid extracts were slightly affected.

Penicillin inhibited the production of cell-bound hemolysins of tolerant and nontolerant strains of the four groups as well as that of extracellular lysins of tolerant groups C and G streptococci, although to a lesser extent (Table). Levels of free hemolysins increased in cultures of the tolerant group A and nontolerant groups A, C and G strains. Growth of the two group B streptococci in the penicillin-medium resulted in significant decreases in the activities of cell-bound streptolysins.

The effects of penicillin on hyaluronidase and deoxyribonuclease production by the streptococci are shown in Table. There was an increse in the specific activity of the two enzymes for all but two strains as the nontolerant group A streptococcus did not elaborate hyaluronidase in the presence of penicillin and a similar observation was made with regards to the production of deoxyribonuclease by the tolerant group B isolate.

DISCUSSION

The emergence of bacterial strains tolerant to antibacterial agents has become the subject of many reports dealing with its detection although the clinical significance of the laboratory and epidemiological observations remain to be determined (Kim & Kaplan 1985, Voorn et al. 1994). Among the various genera and species are streptococci in which penicillin can cause a whole spectrum of different responses, i.e., streptococci can be killed and lysed (Horne & Tomasz 1977), killed but not lysed (Horne & Tomasz 1977, Mc Dowell & Reed 1989) and neither killed nor lysed (Horne & Tomasz 1977). In addition, streptococcal strains of Lancefield groups A, B, C and G do not show resistance to the antibiotic (Allen & Sprunt 1978, Kim & Kaplan 1985).

The present investigation employing the MBC: MIC ratio estimation has provided the first information on the penicillin-tolerant response in streptococcal strains isolated in Brazil, mainly in the large urban area of Rio de Janeiro. Thirty seven of 67 strains were shown to be tolerant to the antibiotic. It should be made clear that this study, the first in a large developing country, does not differ from other reports in several respects. The prevalence rate is similar to that of previous surveys where authors have used the same criteria for inclusion as a tolerant strain (Slater & Greenwood 1983, Van Asselt & Mouton 1993). Percentages are not even much different from those determined in studies in which a number of factors, such as geographical considerations (most previous studies were from countries of the northern hemisphere) and laboratory techniques and variables may influence the frequency of streptococcal strains reported as tolerant (Amsterdam 1991). It seems evident that tolerance to penicillin by groups A,

TABLE

Effects of penicillin on cell-bound substances and extracellular products of streptococci

Serological group	Strain number	MIC (μg/ml)	Tolerance [a]	Antibiotic level in cultures	Cell-bound substances			Extracellular products					
					Precipitin reaction	Rhamnose (μg/ml)	Hemolysin activity	Hemolysin		Hyaluronidase		Deoxyribonuclease	
								CFU/mlx10^{-8}	Activity	U/ODU	U/CFU x 10^{-5}	U/ODU	U/CFU x 10^{-6}
A	K443 [b]	0.02	+	0	8 [c]	7.5 [d]	0.27 [e]	1.2	0.87 [f]	128.7	0.07	16.3	0.09
				1/2 MIC	4	5.5	0.23	0.1	1.43	281.7	0.21	33.8	0.25
	89644	0.02	-	0	8	7.0	0.23	1.3	0.76	19.6	0.02	598.8	7.18
				1/2 MIC	8	6.0	0.18	0.12	1.87			453.2	38.52
B	89646	0.04	+	0	4	8.5	0.10	5.5		320.5	0.23	9.5	0.07
				1/2 MIC	32	7.5	0.03	0.48		372.9	3.17		
	88611	0.04	-	0	4	8.0	0.08	1.5		158.5	0.31	6.8	0.13
				1/2 MIC	16	7.0	0 01	0.55		178.2	2.31	10.46	1.36
C	89632	0.01	+	0	16	7.5	0.30	1.2	1.03	382.5	0.37	68.5	0.66
				1/2 MIC	32	8.5	0.12	0.19	0.96	422.5	2.03	60.3	2.89
	79194	0.01	-	0	32	9.5	0.28	1.1	0.85	304.1	0.39	54.5	0.70
				1/2 MIC	4	6.5	0.25	0.47	1.20	260.8	2.53	54.1	5.09
G	89151	0.01	+	0	2	7 5	0.24	1.3	1.10	284.3	0.38	47.4	0.64
				1/2 MIC	2	8.0	0.12	0.15	0.85	275.6	0.63	55.3	1.27
	89267	0.02	-	0	8	7.0	0.30	1.2	0.86	158.5	0.74	41.1	1.93
				1/2 MIC	32	7.0	0.23	0.15	1.39	141.5	2.75	33.6	6.53

[a]: the two GBS isolates are of serotype III and one was from a vaginal culture (Ferreira et al. 1992). The other seven streptococcal strains were from throat specimens

[b]: MBC : MIC as 32 or greater

[c]: the nitrous acid extracts were serially diluted in sodium phosphate buffer and precipitin tests were performed in agarose. The numbers represent the dilution factors of the highest antigen dilutions with specific precipitin reactions

[d]: rhamnose concentrations were determined in undiluted antigenic extracts

[e]: optical density readings (540 nm) of the supernates obtained after lysis of sheep red blood cells with whole bacterial cells

[f]: ratio:optical density readings (540 nm) of the supernates obtained after lysis of sheep red blood cells with whole culture supernatant fluids/optical density readings of bacterial growth

B, C and G streptococci is common in Rio de Janeiro where streptococcal infections and rheumatic fever remain major public health problems and the risk of recurrence are relatively great (Kim & Kaplan 1985).

Studies from our and various other laboratories have described the effects of subMICs of antimicrobials on bacterial morphology, cell wall components and extracellular products (Gemmell & Mc Leod 1992, Braga & Piatti 1993). It would even seem reasonable to assume that among these numerous strains studied, in previous studies, some were actually tolerant. Since no attention has been paid to the ability of subMICs of antibiotics to interfere with the physiology and biochemistry of tolerant bacteria,we considered this aspect of the streptococcal biology worthy of investigation.

We determined significantly increased production of streptococcal substances as well as decreases and even arrests in the elaboration of the substances. The changes observed in the production of the "C" carbohydrate, SLS, SLO, hyaluronidases and deoxyribonucleases were greatly or sometimes barely affected according to the streptococcal serological group, the penicillin-tolerant response and the presence of the subMIC of the antibiotic (Table). With added penicillin the production of hyaluronidase by the non tolerant group A strains was arrested and that of the tolerant streptococcus was increased. The group B deoxyribonuclease activity of the non tolerant strain was stimulated in the presence of penicillin. However, when the antibiotic was added to cultures of the tolerant isolate, the enzyme activity was inhibited. In respect of free hemolysin activity, we observed that the tolerant group B streptococcus cell exposed to penicillin did not produce the toxin. Neither this tolerant strain nor the one used in a previous investigation (Merquior & Benchetrit 1989) elaborated the free hemolysin. In addition, growth of the tolerant and non tolerant GBS isolates in the presence of penicillin caused a decrease in the cell-bound hemolytic activity of both cultures, a phenomenon already observed in one of our previous reports (Merquior & Benchetrit 1989).

The results assume particular relevance as they indicate that the *in vitro* synthesis (or secretion) of enzymes by tolerant streptococci can be differently affected by low doses of a beta-lactam antibiotic. Thus, according to observations made in earlier reports (Gemmell & Abdul-Amir 1979, Michel et al. 1982), in our previous surveys (Benchetrit et al. 1981, Figueiredo & Benchetrit 1989) and according to the present investigation, it is each time more evident that the production of enzymes, toxins and cellular components by beta-hemolytic streptococci of groups A, B, C and G cannot be readily predicted. This would seem to indicate that further efforts may be justified in determining the effects of low doses of an antibiotic on the biology of streptococci tolerant to the same antibacterial agent and, generally speaking, of antibiotics on tolerant bacteria.

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