EFFECT OF SUBLETHAL CONCENTRATIONS OF PENICILLIN ON THE VIRULENCE AND ANTIGENIC COMPOSITION OF GROUP A STREPTOCOCCI

J. GABRIEL MICHAEL,[1] BENEDICT F. MASSELL, AND ROBERT E. PERKINS

House of the Good Samaritan, Children's Hospital Medical Center, and Department of Pediatrics, Harvard Medical School, Boston, Massachusetts

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ABSTRACT

MICHAEL, J. GABRIEL (House of the Good Samaritan, Children's Hospital Medical Center, Boston, Mass.), BENEDICT F. MASSELL, AND ROBERT E. PERKINS. Effect of sublethal concentrations of penicillin on the virulence and antigenic composition of group A streptococci. J. Bacteriol. **85**:1280–1287. 1963.—Virulent strains of group A streptococci were grown in sublethal concentrations of penicillin and tested for possible changes in colonial morphology, virulence, and antigenic composition. After overnight growth in broth containing penicillin, there was a marked reduction in precipitable group A and M protein content of the bacteria. Upon transfer to broth without antibiotic, the streptococci regained their ability to produce these substances. After multiple transfers in broth containing nonbactericidal levels of penicillin, variants developed which lacked group and type substances and which were also relatively resistant to penicillin. These variants were avirulent for mice and susceptible to the bactericidal action of normal human blood. Two of three strains tested, when grown on blood agar, failed to produce beta hemolysis. The mutants produced by multiple transfers in broth containing small amounts of penicillin, when passed through mice, regained all of their original properties.

It is widely accepted that the antimicrobial activity of penicillin is due to interference with the formation of the cell wall (Park and Strominger, 1956). This interference produces morphological changes, such as spheroplast formation, and lysis, and has been observed in experiments with both gram-positive and gram-negative bacteria (Lederberg, 1957; Michael and Braun, 1958; Ciak and Hahn, 1962). All studies concerned with these effects have utilized bactericidal concentrations of the antibiotic.

In the present study, we attempted to determine the possible effects of sublethal concentrations of penicillin on streptococci. Amounts of penicillin were used which did not interfere appreciably with the growth of the bacteria. Since C carbohydrate and M protein are well defined antigenic components of the streptococcal cell wall, particular attention was given to possible changes in these antigens and to the effect of such changes on virulence.

MATERIALS AND METHODS

Bacteria. Three strains of *Streptococcus pyogenes* group A were used in this study. Two of these, which had previously been passed through mice, were obtained from Rebecca Lancefield. They were type 3 strain B930 and type 6 strain S43. The third strain, Z361 type 5, was isolated from a clinic patient.

Penicillin-sensitivity test. Serial dilutions of penicillin G were prepared in brain heart broth (Difco). Tubes containing 2 ml of media and varying amounts of penicillin were inoculated with 3×10^3 bacteria in 0.1 ml. The tubes were incubated overnight at 37 C, and sensitivity of the inoculated streptococcal strain was determined by observing the lowest concentration of penicillin that prevented visible growth.

Multiple transfers in penicillin. The original strains were grown in 5 ml of Todd-Hewitt broth, containing 0.005 unit of penicillin G per ml. After overnight growth, 0.1 ml of this streptococcal culture, containing approximately 10^7 bacteria, was transferred to another tube containing the same amount of broth and penicillin. This process of daily transfer was repeated six to eight times. The variants resulting from transfer of streptococci in the presence of penicillin are des-

[1] Fellow of the Medical Foundation, Inc., Boston, Mass.

ignated as follows: B930/P8, S43/P6, and Z361/P6.

Preparation of extracts for serological grouping and typing. The bacteria to be extracted were grown overnight in Todd-Hewitt broth, and 0.5 ml of the resulting growth, containing approximately 5×10^7 bacteria, was inoculated into 40 ml of fresh Todd-Hewitt broth. The latter was then incubated for 18 hr at 37 C, and the bacterial sediment obtained by centrifugation was extracted by boiling in 0.2 N hydrochloric acid according to the method described by Lancefield (1943). When required by the experimental conditions, penicillin was incorporated in the 40-ml volume of broth prior to inoculation. Because of the relatively heavy inoculum, abundant growth of streptococci for extraction was obtained even in the presence of penicillin concentrations up to 0.025 unit per ml.

Passage of streptococci through mice. The streptococcal variants obtained by multiple transfers of the bacteria in penicillin-containing broth were passed through mice in the following manner: 1.0 ml of tenfold dilutions of overnight culture in Todd-Hewitt medium were injected intraperitoneally into groups of mice, and 18 hr later the survivors were killed by ether. The spleens of these animals were removed under sterile conditions and spread on blood agar plates. The plates were incubated overnight at 37 C, colonies of streptococci were identified and isolated, and the bacteria were regrown in Todd-Hewitt broth. This procedure was then repeated until the various strains of streptococci were subjected to two to six mouse passages. The strains obtained after mouse passage are designated B930/M6, S43/M4, and Z361/M2.

Preparation of concentrate of growth medium. Overnight growth of bacteria was removed from the broth by high-speed centrifugation. The supernatant was then dialyzed overnight against cold running water, and the dialyzed portion was finally concentrated to a dry powder by freeze-drying. To reduce the amount of cross-reacting substances, the concentrate was boiled with 0.2 N hydrochloric acid and then adjusted with sodium hydroxide to pH 7.2 in the manner suggested by Lancefield (1943) for the preparation of extracts of bacterial sediments.

Bactericidal test. The method used is essentially the same as that described by Rothbard (1945). A small inoculum of streptococci (50 to 200 chains in 0.1 ml) prepared from an overnight culture was mixed with 0.3 ml of fresh heparinized human blood, with or without a supplement of 0.1 ml of type-specific rabbit antiserum, and the mixture was rotated at a speed of 6 rev/min for 3 hr in an incubator at 37 C. A sample of 0.1 ml was removed after incubation and incorporated in a pour plate. The number of surviving streptococci was determined by colony counts made after overnight incubation.

Mouse virulence tests. Female white Swiss mice, 6 weeks old and weighing about 16 to 20 g, were obtained from the Harvard Animal Farm. Groups of animals were injected intraperitoneally with a tenfold dilution of the strain to be tested. The number of survivors was determined at the end of 1 week when the experiment was terminated.

RESULTS

Penicillin sensitivity. Tests for penicillin sensitivity, using serial dilutions of the antibiotic, were done with all three strains and their corresponding variants. All three of the original strains were highly sensitive, no growth being visible in tubes containing 0.04 unit or more of penicillin per ml (Table 1). Variants of strains S43 and Z361, obtained by six serial transfers in sublethal concentrations of penicillin, became resistant to as much as 0.33 unit of penicillin per ml. Strain B930, even after eight transfers in sublethal concentrations of penicillin, became only slightly resistant, and its variant produced visible growth in 0.08 unit of penicillin per ml. When the variants resulting from transfers in penicillin were then passed through mice, all three strains became as sensitive to penicillin as the original streptococcal strains. The number of mouse passages required for the streptococci to regain penicillin sensitivity was two for strain Z361, four for strain S43, and six for strain B930.

Cellular and colonial morphology. Since bactericidal concentrations of penicillin are known to affect the bacterial cell wall, it was of interest to determine whether sublethal amounts of the antibiotic caused any obvious changes in cellular or colonial morphology of streptococci. When preparations from broth cultures were made, all three of the original streptococcal strains were found to be strongly gram-positive and to grow in moderately long chains. The bacteria which were kept in low concentrations of penicillin grew in rather short chains of about five to six cocci

TABLE 1. *Penicillin sensitivity of group A streptococci**

Strain tested†	Penicillin concn‡ (units/ml)					
	0.7	0.33	0.16	0.08	0.04	0.02
Type 3						
B930	0	0	0	0	0	++
B930/P8	0	0	0	++	+++	+++
B930/M6	0	0	0	0	0	++
Type 6						
S43	0	0	0	0	0	++
S43/P6	±	++	+++	+++	+++	+++
S43/M4	0	0	0	0	±	+++
Type 5						
Z361	0	0	0	0	0	++
Z361/P6	0	++	+++	+++	+++	+++
Z361/M2	0	0	0	0	0	++

* No visible growth shown by 0; degree of visible growth by + to +++; questionable growth by ±. Size of inoculum: 3×10^3 bacteria per tube.

† Number of penicillin transfers indicated by P; number of mouse transfers by M.

‡ Penicillin concentrations of 0.01 and 0.005 units/ml resulted in a high degree of visible growth in all strains tested.

each, and the cocci appeared faintly gram-negative. As expected, the original strains grown on blood agar formed matt colonies surrounded by a wide zone of beta hemolysis. The penicillin-induced variants of all three strains produced glossy colonies. The colonies of strain B930/P8 were surrounded by narrow zones of beta hemolysis. However, colonies of the other two strains (S43/P6 and Z361/P6) were surrounded only by narrow zones of alpha hemolysis. In other words, cultures of these last two strains on sheep blood agar could no longer be recognized as beta-hemolytic streptococci. After mouse passage, all three strains regained their original cellular and colonial morphology and produced wide zones of hemolysis on blood agar.

Effect of penicillin on C carbohydrate and M antigen. Acid extracts of the bacteria were prepared according to the Lancefield (1943) method. A uniform amount of bacterial sediment was assured by first adjusting the bacterial suspensions to the same turbidity in a Coleman Junior photometer. In these experiments, the following variants of the three serological types of bacteria were tested: (i) the three original strains (B930, S43, and Z361), (ii) the same three strains after overnight growth in varying sublethal concentrations of penicillin, (iii) bacteria which had been transferred a number of times in broth containing 0.005 unit of penicillin per ml (strains B930/P8, S43/P6, and Z361/P6), and (iv) the preceding strains after they were subsequently passed through mice (B930/M6, S43/M4, and Z361/M2).

The results with all three serological types of streptococci showed a rather similar pattern (Table 2). Extracts of the original strains and of the corresponding strains that had been passed through mice reacted strongly with group A antiserum and with homologous type-specific antiserum. When the bacteria were grown overnight in the presence of increasing amounts of penicillin, a gradual loss of reactivity of the ex-

TABLE 2. *Effect of penicillin on group A and type substances in streptococci as shown by precipitin reaction**

Strain tested†	Penicillin concn‡ (units/ml)					
	None		0.006		0.0125	
	Group	Type	Group	Type	Group	Type
Type 3						
B930	+++	+++	+++	+++	++	0
B930/P8	++	0				
B930/M6	+++	+++				
Type 6						
S43	+++	+++	+++	+++	+	0
S43/P6	0	0				
S43/M4	+++	+++				
Type 5						
Z361	+++	+++	+++	+++	0	0
Z361/P6	0	0				
Z361/M2	+++	+++				

* Results expressed as amount of group and type substances detected after growth of streptococci in the indicated concentrations of penicillin. No precipitate indicated by 0; amount of precipitate by + to +++.

† Number of penicillin transfers indicated by P; number of mouse transfers by M.

‡ With a penicillin concentration of 0.025 units/ml, no precipitate was detected for either group or type substances in any of the strains tested.

tracts with their respective antisera was observed, and in the presence of 0.025 unit of penicillin per ml these bacteria failed to produce any precipitable substance. Streptococci transferred a number of times in penicillin also lost their ability to produce precipitating group A and M protein antigens, with the exception of strain B930/P8. The extract of this last strain reacted with group A antiserum but not with its homologous type 3 antiserum. An estimate of the amounts of antigens present in the various bacterial sediments was obtained by testing serial dilutions of the extracts with appropriate antisera. The extracts of all three original strains, which gave strong reactions with both group and type antisera, produced a visible precipitate with group A antisera, even when the extracts were diluted as much as 1:100, and with their homologous type-specific antisera when the extracts were diluted 1:8. Extracts of sediments which gave only weak reactions when undiluted no longer precipitated when they were diluted beyond 1:2 or 1:4. Those extracts which failed to react when undiluted also failed to precipitate with the appropriate sera when the extracts were diluted.

Not shown in the tables are the results of control experiments in which the three original strains of streptococci were transferred serially in plain broth six to eight times. After this procedure, extracts of the bacterial sediments continued to give good precipitin reactions with group A antiserum and with homologous type-specific antisera.

When the streptococcal strains produced by overnight exposure to penicillin were regrown in plain broth, they immediately regained the characteristics of the original parent strains. On the other hand, the variants resulting from multiple transfers in penicillin-containing broth apparently were stable; even after eight transfers in plain broth they retained their altered antigenic properties.

Release of group A and M antigens into growth medium. In view of the observation that streptococci grown in the presence of increasing concentrations of penicillin seemed to contain decreasing amounts of group and type antigenic substances, we considered the possibility that the effect of penicillin was on the release of the antigens into the media during bacterial growth rather than on the actual production of the antigens. To investigate this problem, samples of broth containing varying amounts of penicillin in which bacteria were grown overnight were dialyzed against running water and then freeze-dried. Concentrates of the growth medium prepared in this way reacted nonspecifically with most group and type antisera, but the nonspecific reactions could be eliminated to a large extent by boiling the concentrates in the presence of 0.2 N hydrochloric acid. As shown in Table 3, the hydrochloric acid-treated concentrates of media in which streptococci had grown in the absence of penicillin gave strong precipitin reactions with group A antiserum. When increasing amounts of penicillin were incorporated into the broth, the concentrates showed decreasing reactivity, and the concentrates made from broth containing 0.025 unit of penicillin per ml did not form any visible precipitate. This pattern of results resembles that observed with extracts of the bacterial cells (Table 2). Type-specific antisera did not react well with any of the hydrochloric acid-treated concentrates (Table 3), suggesting that only small amounts of M protein were released into the growth media.

Susceptibility of the streptococci to the bactericidal action of human blood. It is known that, whereas virulent streptococci will succumb to phagocytosis by human leukocytes only in the presence of type-specific antiserum, the avirulent strains lacking in M protein are destroyed by normal

TABLE 3. *Effect of penicillin on release of streptococcal group A and type substances into growth medium**

Strains tested	Penicillin concn (units/ml)							
	None		0.006		0.0125		0.025	
	Group	Type	Group	Type	Group	Type	Group	Type
Type 3 B930	+++	±	+++	0	±	0	0	0
Type 6 S43	+++	+	++	+	+	0	0	0
Type 5 Z361	+++	+	+++	+	+	+	±	0

* Results expressed as amount of group and type substances detected after growth of streptococci in the indicated concentrations of penicillin. No precipitate indicated by 0; questionable precipitate by ±; amount of precipitate by + to +++.

TABLE 4. *Effect of exposure to penicillin on susceptibility of group A streptococci to the bactericidal action of human blood**

Strain tested	Penicillin concn (units/ml)											
	None			0.003			0.006			0.012		
	Broth	Whole blood	Blood and serum†	Broth	Whole blood	Blood and serum†	Broth	Whole blood	Blood and serum†	Broth	Whole blood	Blood and serum†
Type 3												
B930	N	N	85	N	175	90	N	140	30	N	45	8
B930/P8	N	0	0									
Type 6												
S43	N	N	80	N	620	32	N	105	9	N	10	0
S43/P6	N	10	3									
Type 5												
Z361	N	N	190	N	350	100	N	40	0	N	0	0
Z361/P6	N	0	0									

* Results expressed as the number of colonies in 0.1 ml of mixture after 3 hr of incubation. N indicates numerous streptococci with confluent growth on the plate.

† Dilution 1:100 of rabbit antiserum corresponding to the streptococcal type.

human blood in the absence of antibody. Since streptococci grown overnight in the presence of penicillin and streptococci transferred a number of times in penicillin-containing broth seemed to have reduced amount of M antigen, it was of interest to test the susceptibility of such strains to the bactericidal action of normal human blood. The results are presented in Table 4. As would be expected, the original virulent strains of all three types resisted the bactericidal activity of normal blood and were destroyed only when homologous type-specific immune serum was added. On the other hand, when these bacteria were grown overnight in penicillin-containing broth before their exposure to blood, they behaved like strains lacking M protein, and they were destroyed by normal human blood alone. The streptococcal variants produced by multiple transfers in broth containing sublethal concentrations of penicillin were also killed by normal human blood in the absence of type-specific antiserum.

It should be noted that in preparation of the inoculum for the bactericidal test, streptococcal cultures were diluted 1:100,000 in plain broth. Thus, in tests involving streptococci grown in sublethal concentrations of penicillin, the amount of penicillin actually present in the reaction mixture for the bactericidal test was extremely small. However, to be sure that such small amounts of penicillin had no direct effect on the results, control experiments were done with comparable small amounts of penicillin mixed with virulent streptococci and normal human blood. In these control tests, the virulent streptococci multiplied profusely. The streptococcal variants produced by multiple transfers in penicillin were grown in plain broth prior to their use in the bactericidal test.

Virulence for mice. The foregoing experiments with precipitin and bactericidal tests suggest that exposure to penicillin greatly reduces or eliminates M antigen from group A streptococci. It was shown previously by Lancefield (1962) that M antigen plays an important role in the virulence of streptococci for animals. Therefore, the loss of M antigen from the streptococcal variants produced in the present study was also investigated by mouse virulence tests.

Observations were made with the original streptococcal strains (B930, S43, and Z361), the variants produced by multiple transfers in penicillin-containing broth (B930/P8, S43/P6, and Z361/P6), and the strains obtained by mouse passage (B930/M6, S43/M4, and Z361/M2). Serial dilutions of these various strains were injected intraperitoneally into groups of 6-week-old mice, and the number of survivors was determined 1 week

after challenge. As shown in Table 5, the original streptococcal strains of the three different serological types varied in virulence. However, for each serological type, the original strain and the strain resulting from mouse passage exhibited about the same degree of virulence. In contrast, the variants of all three types produced by multiple transfers in penicillin-containing broth were avirulent for mice.

TABLE 5. *Effect of exposure to penicillin on virulence of group A streptococci for mice**

Strain tested	No. of bacteria injected ip per animal							
	10	10^2	10^3	10^4	10^5	10^6	10^7	10^8
Type 3								
B930	9/9	2/9	0/9	0/9				
B930/P8					9/9	9/9	8/9	3/9
B930/M6	9/9	6/9	2/9	0/9				
Type 6								
S43			9/9	9/9	3/9	0/9		
S43/P6					9/9	9/9	9/9	8/9
S43/M4			9/9	9/9	5/9	1/9		
Type 5								
Z361				9/9	9/9	4/9	1/9	
Z361/P6					9/9	9/9	9/9	9/9
Z361/M2				9/9	9/9	7/9	2/9	

* Results expressed as number of survivors/number tested, 1 week after challenge.

Discussion

The data presented demonstrate that sublethal amounts of penicillin may have profound effects on group A streptococci. These effects, which are brought about more or less concurrently, include loss of group-specific carbohydrate and type-specific M protein, alteration in colonial appearance, decrease in production of hemolysin, decrease in virulence for mice, increase in susceptibility to the bactericidal effect of normal human blood, and increase in resistance to penicillin.

The results of extensive studies by many investigators on the mode of action of penicillin indicate that the antibiotic exerts its bactericidal effect through the inhibition of cell-wall synthesis (Park and Strominger, 1956). For example, Sharp, Hijmans, and Dienes (1956), using high concentrations of penicillin, produced L forms of streptococci lacking cell walls. Since the sublethal concentrations of penicillin used in our study influence the production of type-specific M protein and group-specific carbohydrate and since these antigens are known to be components of the cell wall, it would appear that penicillin may affect the cell wall of streptococci even when it is present in amounts that are inadequate to interfere appreciably with growth of the organism.

Alterations in streptococci resulting in decreased production of type-specific and group-specific antigens may be transient or permanent. The transient changes, observed when the original streptococcal strains were grown overnight in the presence of small amounts of penicillin, would appear to be due to temporary suppression of the synthetic activity of the bacterial cell, since transfer of the organisms to penicillin-free media resulted in renewed production of M protein and C carbohydrate. When the streptococci were transferred serially in broth containing low concentrations of penicillin, changes of a more stable nature developed and were retained even when the organisms were again transferred as many as eight times in plain broth. This apparent mutation is consistent with the observations of Braun et al. (1952), who noted a mutagenic effect of penicillin on *Brucella*, and with other investigators' observations made in the course of studies on the in vitro development of penicillin resistance by streptococci (Gezon, 1948; Rake et al., 1944; Rosendal, 1958).

The loss of reactivity of the streptococcal extracts with group A antisera could be due either to a marked decrease in synthesis of group A carbohydrate or to slight alteration in the chemical structure of the C carbohydrate. That slight alterations in chemical structure can occur and can affect group specificity was shown by McCarty and Lancefield (1955), who studied a group A streptococcal mutant isolated by Wilson (1945). In this A variant strain, there was a loss of the *N*-acetyl glucosamine side chain, and the marked decrease in this particular carbohydrate fraction was accompanied by a loss of reactivity with group A antisera. It would be of interest to determine whether a similar loss of *N*-acetyl glucosamine occurs under the influence of low concentrations of penicillin.

The effect of small amounts of penicillin on the production of M protein is shown not only by the loss of reactivity of bacterial extracts

with homologous type-specific antiserum but also by the decreased virulence of the bacteria for mice and by the increased susceptibility of the streptococci to the bactericidal action of normal human blood. Correlation of M protein content of streptococci with mouse virulence and with resistance to the bactericidal action of human blood has been well documented by Lancefield (1962).

A possible mechanism to be considered for the loss of cell-wall antigens under the influence of penicillin is the release of these antigens into the growth media. The results of the present study do not support this hypothesis. Although appreciable amounts of group A carbohydrate and small amounts of type-specific M protein could be found in concentrates of broth in which streptococci had grown in the absence of penicillin, the presence of these antigens could not be demonstrated when the bacteria were grown in penicillin-containing broth.

Although the changes effected in streptococci by their repeated exposure to small amounts of penicillin appeared to be rather stable, these changes could be reversed by passing the bacteria through mice. Thus, after mouse passage, the streptococci again produced wide zones of hemolysis when grown on blood agar, elaborated abundant amounts of type-specific and group A antigens, became sensitive to low concentrations of penicillin and resistant to the bactericidal action of normal human blood, and regained virulence for mice. Only a few mouse passages were required for the streptococcal variants to regain their original virulence and other characteristics. This property is in contrast to that of most freshly isolated strains of streptococci, which usually require multiple passages in mice to make them virulent for these animals.

The observations concerning the effect of penicillin in vitro on the antigenic composition and colonial morphology of group A streptococci have potential clinical implications that require consideration. Thus, when penicillin treatment seems to eradicate virulent, hemolytic group A streptococci from the throats of persons who are ill with streptococcal throat infections, one must keep in mind the theoretical possibility that in some instances the streptococci may have been altered rather than eradicated. Such streptococcal variants, especially if they no longer produce beta hemolysis when grown on blood agar, would not be recognized by the usual culture techniques. Nevertheless, it is conceivable that under appropriate conditions the organisms might revert to their original form and thereby might spread to other individuals and cause disease.

The association of alteration in antigenic composition of beta-hemolytic streptococci with the in vitro development of penicillin resistance possibly explains the fact that penicillin-resistant group A streptococci have never been isolated from human beings. Thus, if, as a result of penicillin therapy, penicillin-resistant streptococcal mutants should develop in vivo, it is likely that these bacteria also would be avirulent and lacking in group A carbohydrate.

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