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Hemolytic Mutants of Group A *Streptococcus pyogenes*

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Hemolytic mutants of Lancefield strain SS-95 and ATCC 19615 *Streptococcus pyogenes* were produced by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. These mutants contained the same levels of streptolysin O, nicotinamide adenine dinucleotidase, deoxyribonuclease, and hyaluronidase. The mutants were deficient in streptolysin S, as was the naturally occurring nonhemolytic Lowry strain. The mutants retained their pathogenicity for mice and, when reisolated from the dead animals, produced the mutant hemolytic pattern.

Beta hemolysis is one of the characteristics commonly used by diagnostic laboratories to identify and characterize group A streptococci. Whereas it is true that most group A streptococci produce beta hemolysis, atypical strains do occur.

In 1971 James and McFarland (8) reported a completely nonhemolytic or gamma-hemolytic strain of *Streptococcus pyogenes* isolated from an outbreak of rheumatic fever at Lowry Air Force Base, Colo. Upon initial isolation this organism was totally gamma- or nonhemolytic and appeared as watery, mucoid, droplike colonies among the other organisms. Eight serial subcultures on sheep blood agar were required before alpha hemolysis occurred.

Another unusual group A streptococcus was isolated from the blood of a patient with endocarditis (3). This isolate produced only alpha hemolysis on several types of blood and under varying conditions. The isolate was also resistant to bacitracin.

More recently, a clindamycin-resistant strain of group A streptococcus has been isolated from patients with bacteremic infections (4). These isolates were not beta-hemolytic under standard conditions.

We noted that isolates from patients with septic sore throat and various skin lesions produced a mixture of beta- and alpha-hemolytic colonies on a primary blood agar plate. In several instances, the alpha-hemolytic colonies were unstable and readily reverted to beta hemolysis during the first subculture on blood agar. The study described herein was undertaken to produce stable hemolytic mutants and to then compare their properties to the beta-hemolytic parent. The origin of those unusual strains that occur naturally is unknown. However, by making a comparison of their characteristics to known parent and mutant strains, some insight as to how they originated may be possible.

MATERIALS AND METHODS

Organisms. The organisms used for production of mutants were Lancefield strain SS-95 (M type 12, T type 12) and ATCC strain 19615 of group A beta-hemolytic *Streptococcus pyogenes*. The Lowry strain of *S. pyogenes*, SS-1043, was also used as the standard naturally occurring atypical organism. All strains were a gift from Richard Facklam, Chief, Staphylococcal and Streptococcal Branch, Center for Disease Control, Atlanta, Ga.

Media. Cultures were maintained at 4 to 6°C on blood agar slants prepared from Trypticase soy agar (BBL) with 5% defibrinated sheep erythrocytes added. Plating medium contained the same formulation as the slants.

Growth curves, routine cultivation, and enzyme studies used Todd-Hewitt broth prepared according to the manufacturer's specifications. Brewers' thioglycolate (BBL) was used in mutation experiments and also for stabilization of the mutants.

Mutation. Several chemical mutagens were used in an attempt to obtain hemolytic mutants. A standard procedure was developed for all mutation experiments. One-half milliliter of a suspension (optical density at 600 nm = 0.3) of the organism to be mutated was added to 9.9-ml tubes of Brewers' thioglycolate broth containing the desired concentration of mutagen and incubated for 3 h at 37°C. After incubation, 2 loopfuls, approximately 0.1 ml, from each tube were plated on blood agar. The plates were streaked for isolation and incubated in a CO_2 incubator, a GasPak, or a candle jar for 24 h. The number of hemolytic mutants was determined, and they were isolated for future studies.

The mutants were unstable and reverted to beta hemolysis production upon subculture on blood agar. The mutation was stabilized by selecting suspect hemolytic colonies and reexposing them to nitrosoguanidine at the same concentration and under the same conditions originally used. Representatives of the nonhemolytic colonies produced were picked and subcultured by at least four transfers in thioglycolate in the

absence of blood. This resulted in stable mutants that produced gamma hemolysis on streak plates and either gamma or alpha hemolysis in stab cultures.

Mutagens used included: proflavin at concentrations of 50, 10, 5, 1, 0.5, 0.1, 0.05, and 0.01 μg/ml. Acridine orange was used at the same concentrations as proflavin. Penicillin at concentrations of 125, 12.5, 6.25, and 0.625 μg/ml and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5, and 10 μg/ml were used. Mitomycin C at concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5, 10, and 20 μg/ml was also used. Cells were treated with ultraviolet light by exposure to a General Electric germicidal lamp (2,200 to 2,600 Å [220 to 260 nm]) for 5-, 10-, 15-, 20-, 30-, 40-, 50-, and 60-s intervals at a distance of 50 cm.

Titration of streptolysin O. Streptolysin O concentration was determined by the method outlined by Campbell et al. (2).

Streptolysin S determination. Streptolysin S was assayed as cell-bound hemolysin according to the method of Ginsberg et al. (5). Antistreptolysin O (200 Todd units/ml) was used to inactivate streptolysin O instead of cholesterol.

Enzyme determinations. Nicotinamide adenine dinucleotidase was determined by the method set forth in the *Worthington Enzyme Manual* (10).

Deoxyribonuclease activity was determined by the method of McCarty (9).

Hyaluronidase comparison was made by using a modification of the procedure outlined in the *Worthington Enzyme Manual* (10). One-half milliliter of the supernatant was added to a tube containing 1.0 ml of the buffer. The mixture was heated in boiling water for 5 min and cooled in cold water, and 9 ml of acid albumin reagent was added. After 10 min the tube was read at 540 nm. A control tube was used for comparison of changes in turbidity.

Antibiotic sensitivities. Penicillin, ampicillin, bacitracin, chloramphenicol, erythromycin, gentamicin, kanamycin, cephalothin, lincomycin, novobiocin, neomycin, streptomycin, and tetracycline were tested on the parent and mutant strains, using the Kirby-Bauer technique for sensitivity testing.

Pathogenicity studies. Mature Swiss-Webster mice were injected intraperitoneally with 0.5 ml of 7.5×10^7, 7.5×10^6, or 7.5×10^5 parent or mutant cells per ml. A control group of mice was injected by the same route with 0.5 ml of physiological saline. All mice were observed, and the number of deaths was recorded.

Dead mice were autopsied, and sample cultures were taken from the spleen, heart, blood, and peritoneum. Samples were streaked for isolation on sheep blood agar, incubated, and observed for hemolysis.

Chemicals. Proflavin, mitomycin C, catalase, and acridine orange were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was acquired from Aldrich Chemical, Inc., Milwaukee, Wis. Antibiotic disks were obtained from Difco Laboratories, Detroit, Mich. Penicillin, nicotinamide adenine dinucleotide, hyaluronidase, and deoxyribonuclease were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Known strains, Lancefield SS-95 and ATCC 19615, of group A *S. pyogenes* were treated with several chemical mutagens in an attempt to produce stable hemolytic mutants. Proflavin, acridine orange, mitomycin C, and ultraviolet light did not alter the type of hemolysin produced by the organisms. Penicillin and nitrosoguanidine were both capable of producing atypical hemolytic cell types. The greatest number of mutants occurred after treatment with 5 to 10 μg of nitrosoguanidine per ml (Fig. 1), and it was chosen for all future mutation experiments. These mutants were unstable after primary isolation and reverted to beta hemolysis after the first or second subculture on blood agar. The mutation was stabilized by reexposure of unstable mutants to the same concentration of nitrosoguanidine followed by several successive transfers in thioglycolate broth. This technique produced large numbers of stable gamma-hemolytic colonies on surface cultures and either gamma or alpha hemolysis in subsurface cultures. Narrow microscopic zones of beta hemolysis appeared around the colonies in pour plates, as it did with naturally occurring atypical Lowry strain.

The hemolytic mutation produced from both parent strains could not be reversed by the addition of catalase to the growth medium or by cultivation in a GasPak under anaerobic conditions, suggesting that peroxide formation is not responsible for loss of beta hemolysis.

No detectable differences between parent and mutant strains in streptolysin O, hyaluronidase, nicotinamide adenine dinucleotidase, or deoxyribonuclease levels were noted. The Lowry

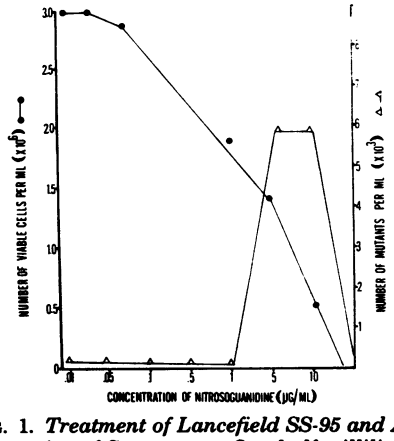

FIG. 1. *Treatment of Lancefield SS-95 and ATCC 19615 strains of S. pyogenes. One-half milliliter of a suspension (optical density at 600 nm = 0.3) of organisms was treated with varying concentrations of N-methyl-N'-nitro-N-nitrosoguanidine in Brewers' thioglycolate medium for 3 h at 37°C. Duplicate plates of blood agar were streaked with 0.1 ml from each tube. Plates were counted for both total viable cells and hemolytic variants. Symbols:* (●) *viable cell counts;* (△) *number of mutants.*

strain did produce significantly higher levels of streptolysin O, deoxyribonuclease, and hyaluronidase than either the parent or the mutant strain.

The two parent organisms, strain SS-95 and ATCC 19615, contained appreciable levels of streptolysin S. Both mutants and the Lowry strain, all of which are nonhemolytic on surface colonies, were deficient in streptolysin S, and it was undetectable by methods used in this investigation.

Parent and mutant strains exhibited sensitivity patterns identical to those antibiotics tested.

Lancefield parent strain SS-95 was pathogenic for mice when injected intraperitoneally and offered a model system for comparison of virulence of parent and mutant (Table 1), killing all but one mouse at employed concentrations in 48 h. The SS-95 mutant also killed at the highest cell concentrations. Nonlethal doses of the parent and mutant strains produced similar symptoms in the mice, observed as ruffling of fur and inactivity followed by recovery. Where recovery occurred, it was complete without notable permanent damage.

Parent and mutant strains of ATCC 19615 were not pathogenic for mice under the conditions used. The Lowry strain was extremely pathogenic for mice; however, there is no "parent" organism for its comparison. Extensive tests to determine the extent of its lethality were not performed.

Autopsy was performed on mice that died from the injection of the SS-95 mutant. Streaks and stabs of blood agar were made of samples taken from the peritoneum, blood, heart, liver, and lung tissue. All samples showed the typical mutant hemolytic pattern, with no reversion of hemolysis occurring from passage through mice.

Ten successive transfers of the gamma-hemolytic mutants on blood agar resulted in reversion to predominately alpha-hemolytic stab and surface colonies. At seventeen successive subcultures, the stabs of both mutants showed beta hemolysis, whereas surface colonies remained alpha hemolytic. After 33 continual transfers on blood agar, the organism showed beta-hemolytic stabs with borderline beta hemolysis on surface colonies.

The mutant organisms do not appear to be producing an inhibitor that blocks complete lysis of the blood cells by these strains. The spent growth medium used to grow the mutants was tested for inhibitory activity and proved incapable of inhibition of beta hemolysis by the parent strains. Cell sonic extracts also did not exhibit inhibitory action on beta-hemolytic strains, thus eliminating some internal inhibitor produced by the mutant strains. Reciprocal experiments using the spent growth medium from the parent did not cause reversion of the mutants to beta hemolysis production. These data do not rule out the loss or inactivity of some essential activator such as that which occurs in *S. faecalis* subsp. *zymogenes* (7).

Lancefield strain SS-95, ATCC 19615, and the mutant of ATCC 19615 reached stationary growth phase after approximately 10.5 h of growth. The mutant of Lancefield strain SS-95 and Lowry strain SS-1043 grew slower, exhibited a longer log phase, and did not reach maximum stationary phase until after 14.5 h of growth (Fig. 2).

DISCUSSION

The mutant produced from Lancefield strain

TABLE 1. *Effect of Lancefield SS-95 parent and mutant strains on mice injected intraperitoneally*[a]

Time after injection (h)	Effect after injection of:			
	7.5×10^7 cells/ml	7.5×10^6 cells/ml	7.5×10^5 cells/ml	7.5×10^4 cells/ml
SS-95 parent				
10	All dead	4/5 dead	2 dead 3 sick	0 dead All sick
20	All dead	4/5 dead	All dead	2 dead 3 sick
48	All dead	4/5 dead	All dead	All dead
SS-95 mutant				
10	1 dead 4 sick	0 dead 5/5 sick	No effect	No effect
20	2 dead 3 sick	5/5 recovered	No effect	No effect
48	All dead	5/5 recovered	No effect	No effect
64	All dead	5/5 recovered	No effect	No effect

[a] Five mice were injected during each experiment. Table represents average of three experiments.

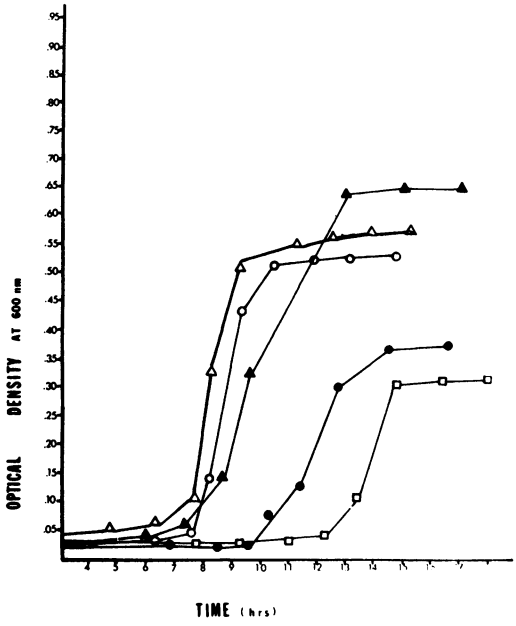

FIG. 2. *Growth curve for parent and mutant strains. Cells were grown in Todd-Hewitt broth. Samples were taken at 30-min intervals, and optical density was read at 600 nm. Symbols:* (○) *SS-95 parent;* (●) *SS-95 mutant;* (△) *ATCC 19615 parent;* (▲) *ATCC 19615 mutant;* (□) *Lowry SS-1043.*

SS-95 was sent to the Center for Disease Control, Atlanta, where it was identified as a group A M-type 12, T-type 12 streptococcus. The mutant was described as being the same as the SS-95 parent except for hemolytic pattern. The hemolytic pattern of this mutant is the same as that of Lowry strain SS-1043. Obviously a change in hemolytic pattern did not alter the group-specific carbohydrate, the T antigen, or the M-protein type. The mutant produced from ATCC 19615 was assigned the same characteristics as the SS-95 mutant. It is, however, non-M and -T typable.

These mutants have a number of properties that are very similar or identical to the beta-hemolytic parents from which they were derived. All of the mutants, as well as the atypical naturally occurring Lowry strain, were deficient in streptolysin S. It is commonly accepted that in deep stab or pour plate colonies both streptolysin S and streptolysin O are responsible for hemolysis (6). In our studies, however, it was noted that the mutants and the naturally occurring Lowry strain, which give high titers of streptolysin O, produce very little hemolysis even in pour plate and stab cultures. The Lowry strain, as reported by the Center for Disease Control, shows at best only tiny zones of beta hemolysis in pour plates, and this strain had the highest titer of those tested for streptolysin O. These findings would suggest that beta hemolysis is primarily dependent on streptolysin S, with streptolysin O making a minimal contribution, regardless of whether it is a streak plate, a stab culture, or pour plate culture. The very small, sometimes microscopic, zones of beta hemolysis by these atypical strains do, however, appear to be dependent on the available streptolysin O. These findings further support the need for pour plate cultures, as suggested by the Center for Disease Control, when at all possible. Atypical variants of the type described here would be overlooked on streak plate or in stab cultures.

The similarity among properties of the artificially described nonhemolytic mutants and the naturally occurring nonhemolytic Lowry strain tend to suggest that these naturally occurring atypical variants may have developed by mutation similar to that described here. Certainly, the presence of mutagenic nitroso compounds in natural environments is well documented. The fact that the mutants produced in this study were still pathogenic for mice lends further support to this possibility. However, there is not enough experimental evidence available at present to prove that the naturally occurring atypical strains arose in this manner.

Table 1 indicates that the SS-95 mutant was virulent for mice, as was the parent. The virulence for mice was obviously decreased over that of the parent despite the fact that these mutants still contain M protein. The factors responsible for the virulence expressed is unknown at present. The actual concentrations of M protein were not compared in this study. It is possible that the decreased virulence is the result of a decrease in the level of M protein produced by the mutant. Becker et al. (1) reported observations suggesting that M protein and capsule are not the only determinants of virulence of group A streptococci for mice. Their studies indicate that other unidentified factors, in the presence of M protein, are necessary for virulence and that passage of the organism through mice favors selection of streptococci containing these factors.

Hemolytic variants were noted when beta-hemolytic parent cells were treated with specific concentrations of penicillin. These variants were not characterized; however, they are resistant to penicillin (10 μg/ml). In view of bacteremic infections noted recently (4) caused by antibiotic-resistant, non-beta-hemolytic strains of group A *S. pyogenes,* these mutants may be more significant than originally thought. Prolonged antibiotic therapy could lead to alteration in the hemolysis produced and give misleading results

concerning the presence of the causative organism.

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