Production of Double Zones of Hemolysis by Certain Strains of Hemolytic Streptococci of Groups A, B, C, and G on Heart Infusion Agar

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Patterns of hemolysis as described by Brown (2) have, in general, been adopted as an initial step in screening for Lancefield group A streptococci. Often the observation of these hemolytic patterns is made on streak blood-agar plates, whereas Brown's description of the patterns was intended to describe subsurface growth in pour plates. Beta-hemolytic streptococcal variants with absent or incomplete hemolytic patterns have been described previously around surface colonies on sheep blood-agar plates (2, 5-7, 9, 11, 16, 17, 19). The present report describes an additional variation in the pattern of hemolysis around surface colonies of Lancefield group A, B, C, and G on heart infusion-blood-agar plates and suggests a reason for its appearance.

MATERIALS AND METHODS

Organism. Several strains of streptococci were examined for their hemolytic patterns. Many of the serological group A and all of the serological groups B, C, and G were fresh isolates from the Stanford Medical Center bacteriology laboratory. A number of group A, M type 12 strains were donated by Richard Hacklam of the Center for Disease Control, Atlanta, Ga. W. R. Maxted of the Central Public Health Laboratory, Colindale, England, donated another group of type 12 streptococci including strain no. R53/1077. K. L. Vosti of Stanford donated the strain B12/0, a type 12, and VT3, a type 3, group A streptococcus. Rebecca Lancefield of the Rockefeller University donated two group A, M type 3 strains, D5BX/11/3, 9/20/62, and F208 (16 October 1963).

Media. The following commercial solid media (Difco) were used in preparing blood-agar plates: Heart Infusion agar, Heart Infusion agar with 2% neopeptone, tryptic soy agar, nutrient agar, proteose no. 3 agar, and Tryptose blood agar base. The agar plates were supplemented with either sheep, horse, rabbit, or human sterile defibrinated blood. The concentration of defibrinated blood in the plates was 5% except in a few instances when 10% was used. Brain Heart Infusion broth (Difco) was used for growth in grouping and typing the streptococci.

Hemolysis. The criteria of Brown were followed in observing subsurface hemolysis (2).

Differing growth conditions. Streak or pour plates of the test cultures were normally carried out under aerobic conditions at 37 C for at least 48 hr. The inocula were normally made from a 5% sheep blood-Heart Infusion agar (SBHIA) plate. The anaerobic growth was investigated in some cultures by incubation in 10% CO2-90% N2 gas exchange jars and the "Gaspak" system (BBL; reference 1).

The effect of refrigeration on some strains was tested by incubation of the cultures at 37 C for 18 hr followed by refrigeration for 24 hr and then by incubation for another 18 hr. In some instances, this cycle was repeated.

Catalase experiments. Crude sterile solution of beef liver catalase (Sigma) was incorporated into 5% SBHIA plates (1,660 units/ml) just before the addition of the sheep cells. Similar plates were prepared with heat-inactivated catalase. The plates were streaked with the test organism, incubated aerobically for 48 hr at 37 C, and examined for their hemolytic patterns.

Peroxide experiments. Hydrogen peroxide (3%) was diluted 1:10 in normal saline, and 3.0 ml was overlaid on SBHIA plates for 5 min. A plate overlaid with normal saline served as a control. The plates were drained, dried in an incubator for 30 min, and then streaked with a known beta-hemolytic group A streptococcus. The plates were examined at 24 and 48 hr after incubation at 37 C.
Glucose determinations. The concentration of reducing sugars was determined by the ferrocyanate oxidation method adopted to a Technicon autoanalyzer (13). Glucose oxidase (Boehringer-Mannheim, San Francisco) determinations were performed by a method adapted from Huggett (14).

RESULTS

Hemolysis. Figure 1 shows the fully developed double-zone (DZ) hemolytic pattern on SBHIA produced by surface growth of the B12/0 strain incubated aerobically. The sequence of development of this pattern was as follows. At the end of 18 hr, only a narrow zone of complete hemolysis of the sheep cells surrounded and was beneath the colony. At the end of 48 hr, the colony had grown larger, partially obscured the early hemolysis, and had formed a second zone of hemolysis outside the first. A zone of unhemolyzed red cells occasionally having a greenish tinge in the agar appeared between the two zones of hemolysis. Often the first zone of hemolysis was not apparent on direct observation but could frequently be demonstrated by pushing the colony to one side. In Fig. 1, this is contrasted with an alpha-hemolytic streptococcus which formed no zone of hemolysis beneath the colony. The DZ pattern was also seen with many strains of group A and certain strains of groups B, C, and G on both 5 and 10% SBHIA. Often the entire DZ pattern was visible within 18 hr. Wide variations in the hemolytic pattern were observed (Fig. 2). Often closely approximated colonies could be differentiated as to their hemolytic pattern (Fig. 3). However, as the colony density increased on the agar surface, the DZ phenomenon disappeared.

A collection of 42 strains of group A type 12 streptococci were examined on surface streaks of SBHIA. Of the 42 strains, 34 exhibited DZ hemolysis either alone or mixed with beta hemolysis. From the diagnostic laboratory, 11 bacitracin-sensitive (Taxos) streptococcal strains were selected at random and examined for DZ hemolysis on surface-streaked SBHIA plates. Ten of the 11 strains demonstrated the DZ hemolytic pattern.

Serial passage. Three beta-hemolytic group A, M type 3 cultures (VT3, D5BX, and F208) and the DZ hemolytic culture of B12/0 maintained their respective hemolytic patterns throughout eight passages on SBHIA. However, beta-hemolytic colonies picked from the mixed beta- and DZ hemolytic B12/0 culture produced more than half DZ hemolytic colonies in well-separated areas on the SBHIA plate with each transfer of a beta-hemolytic colony.

Differing growth conditions. The B12/0 strain was selected to study the effect of various media
on the pattern of hemolysis because it contained colonies producing both DZ and beta hemolysis. Table 1 summarizes the surface hemolytic patterns observed on different media containing 5% blood from different species. DZ hemolysis occurred primarily on Heart Infusion agar and Heart Infusion agar plus 2% neopeptone. The type of blood did not appear to affect the DZ hemolysis. With the exception of one colony on each plate, DZ hemolysis did not appear on the tryptic soy or nutrient agars. Pour plates of the B12/0 with the same conditions revealed uniform beta hemolysis of the subsurface colonies.

The B12/0 strain was tested additionally on proteose no. 3 and Tryptose blood agar base with 5% sheep blood only. The DZ hemolytic pattern was not seen on these media. Also selected streptococci of groups B, C, and G were examined by pour plate and surface streak on SBHIA and gave hemolytic patterns indistinguishable from those formed by strains of group A streptococci.

Oxygen concentration markedly influenced the hemolytic pattern of the B12/0 strain on surface growth. Beta hemolysis appeared uniformly in the anaerobic jars, whereas both beta hemolysis and DZ hemolysis were present in the 10% CO₂-90% N₂ gas exchange jars and the aerobic cultures. Refrigeration would usually delay the appearance of the second zone of hemolysis and would occasionally produce an additional zone of hemolysis if the culture underwent further refrigeration and incubation (Fig. 4 and 5). Surface growth at room temperature produced

| TABLE 1. Surface streaked B12/0 mixed beta- and double-zone (DZ) hemolytic colonies |
|--------------------------------------|-----------------|-----------------|-----------------|
| **5% Blood** | **Heart Infusion agar** | **Heart Infusion agar +2% neopeptone** | **Tryptic soy agar** | **Nutrient agar** |
| Human | Beta + DZ | DZ | Beta | Beta |
| Sheep | DZ | DZ | Beta | Beta |
| Rabbit | Beta + DZ | Beta + DZ | Beta | Beta |
| Horse | DZ | DZ | Beta | Beta |

* Also one colony of DZ.
only beta hemolysis on 5% SBHIA plates. However, incubation at 37 C of beta-hemolytic colonies picked from this plate resulted in reversion to the DZ hemolytic pattern by the second passage on SBHIA. Despite incubation at 37 C, the B12 0 strain would often produce beta hemolysis on SBHIA plates after one broth passage. However, serial transfer of the beta-hemolytic colonies on SBHIA would result in the appearance of the DZ pattern at 37 C.

**Effect of catalase.** Streptococcal colonies of groups A, B, C, and G known to produce DZ hemolysis produced only beta hemolysis on catalase-impregnated SBHIA plates. The DZ hemolysis appeared on the plates containing heat-inactivated catalase.

**Effect of peroxide.** The surfaces of SBHIA plates were exposed to either normal saline or hydrogen peroxide. After surface streaks of a group A beta-hemolytic strain, beta-hemolysis surrounded colonies only on the control plates. Incomplete hemolysis surrounded the colonies on the peroxide-treated plates, and with a low power magnification red cells could be seen in the agar matrix. Figure 6 shows the hemolysis surrounding the surface colonies of both test and control colonies.

**Sugar determinations.** The glucose and reducing sugar contents of the various agar solutions were measured before autoclaving. In addition, the serum of a specimen of refrigerated, defibrinated sheep blood was analyzed for its glucose content (Table 2).

**DISCUSSION**

This report describes and illustrates a pattern of incomplete DZ hemolysis seen surrounding surface colonies of streptococci on sheep blood-Heart Infusion agar. Similar pathogenic streptococci have been reported earlier to show incomplete or nonhemolytic surface growth hemolysis (2, 5–7, 9, 11, 16, 17, 19). Although most of these reports preceded the ability to group or type streptococci serologically, the organisms noted by Coburn and Pauli, Dillon, Fuller and Maxted, and Rantz and Jewell were of serological group A (6, 9, 11, 16).

DZ hemolysis consists of a zone of hemolysis around the surface colony surrounded by a zone
of intact red cells which is enclosed by an outer zone of hemolysis. This should not be confused with either the alpha prime hemolysis which is a subsurface phenomenon with "fixed corpuscles next to the colony" (2) or the subsurface DZ hemolysis described by Brown for group B organisms (3, 4). This subsurface pattern usually requires refrigeration for its development. The "reverse alpha zone" pattern of hemolysis also described by Brown for Strep. Brig. III αα (mucosus) mimics the DZ pattern (2). However, the organism is in other respects alpha-hemolytic, and the pattern is seen only with anaerobic incubation and refrigeration of subsurface colonies. One instance of another pattern similar to the DZ pattern was described by Brown with streptococcus B-7α. This pattern also occurred with aerobic surface incubation; however, in pour plates, it was clear that the organism was alpha-hemolytic in character. The subsurface hemolysis separates this pattern from the DZ hemolytic patterns described in the present report, since strains with the latter pattern reverted to beta hemolysis on subsurface growth in pour plates.

The ability of sugar to suppress hemolysis on blood plates has been known for a long time. Ruediger in 1906 noted that the addition of glucose at 1,000 mg/100 ml to agar previously free from glucose would abolish beta hemolysis and resulted in a green coloration surrounding the streptococcal colony (17). Fuller and Maxted have shown that group A, type 3 streptococci do not produce peroxide (12), and these organisms also do not exhibit DZ hemolysis on Heart Infusion agar. In another study, the same authors examined nonhemolytic variants of group A streptococci (11). The beta-hemolytic pattern of their organisms was also restored by catalase incorporation and oxygen exclusion. In addition in their nonhemolytic greening variants, the peroxide production of the streptococci preceded the hemolysis production by the organisms. Other studies in broth of hemolysin and peroxide production also indicate that the production of these materials does not appear to be simultaneous (19).

Although DZ hemolysis may be caused by nonsynchronous production of the hemolysin and peroxide, the inner ring of unhemolyzed corpuscles requires further explanation. It is possible that peroxide affects the red cell in such a way as to prevent the action of the hemolysin. Brown has noted that corpuscles which surround alpha-hemolytic streptococci and which have acquired a greenish or brownish discoloration are no longer subject to hemolysis by beta-hemolytic streptococci (2). Before the full development of the DZ hemolytic pattern, the inner hemolytic zone is often surrounded by an outer zone of greenish discolored corpuscles. This discolored zone becomes the inner ring of unhemolyzed corpuscles and its final width often decides whether its greenish discoloration remains. The capacity of a beta-hemolytic, group A streptococcus to pro-

### Table 2. Glucose and reducing sugar contents of various agar solutions before autoclaving

| Agar solution       | Reducing sugar | Glucose | Type 12, group A sheep blood surface hemolysis | Other components (8)       |
|---------------------|----------------|---------|-----------------------------------------------|---------------------------|
| Tryptic soy         | 64a            | 0a      | B                                             | Tryptose, soytone         |
| Neopeptone          | 70             | 3.7     | DZ                                            | Beef Heart Infusion-neopeptone |
| Nutrient            | 31             | 0       | B                                             | Peptone, beef extract     |
| Heart Infusion      | 64             | 4.8     | DZ                                            | Beef Heart Infusion, Tryptose |
| Proteose no. 3      | 82             | 55      | B                                             | Peptone, dextrose         |
| Tryptose blood      | 57             | 20      | B                                             | Tryptose, beef extract    |
| Sheep blood         | 35             |         |                                               |                           |

*a Expressed as milligrams per 100 ml.
duce a green reaction has been demonstrated by Stinebring and Morton (18). With a cellophane sack technique, they demonstrated identical rates of conversion of oxyhemoglobin to methemoglobin by a group A and a Viridans streptococcus (18). In addition in the present report, dilute solutions of peroxide overlaid on SBHIA plates prevented complete hemolysis of the corpuscles surrounding the beta-hemolytic colonies. Thus, it is probable that the DZ hemolysis pattern starts with early hemolysis production which causes the clear zone surrounding the colony. Subsequent peroxide formation by the colony probably fixes the red cells at the periphery of the hemolyzed area. This in turn prevents lysis of the corpuscles when the hemolysin again diffuses outward to form the second zone of hemolysis.

The expression of the DZ pattern seems to be limited to the Heart Infusion and neopeptone agars of those agars tested. Beef heart infusion is a common ingredient of both these agars. In some of the reports previously cited, the blood-agar base was not specified, but, in many, meat infusion was a common ingredient (2, 5–7, 16). However, the medium used by Fuller contained tryptic digest of ox hearts and that used by Dillon contained a papain digest of ox liver (9, 11, 15).

Because of the wide variation in the DZ hemolytic pattern and the common occurrence of greening in the unhemolyzed zone, confusion between alpha and DZ hemolysis on surface streaks is likely to occur. Fortunately in heavy growths of DZ hemolytic organisms in groups A, B, C, and G, the densely concentrated colonies appeared beta-hemolytic and only the relatively well-separated colonies demonstrated the DZ pattern. Also alpha-hemolytic surface colonies do not typically have an inner zone of hemolysis with lysin underlying the colony. However, a characteristic shared by the surface DZ colonies and the alpha-hemolytic colonies is the formation of additional zones of hemolysis with alternate incubation and refrigeration as demonstrated by Brown (2). This DZ phenomenon has not previously been demonstrated with group A organisms.

The phenomenon of the DZ hemolytic pattern should be considered in the selection of a blood-agar base used in screening for beta hemolysis. If either Heart Infusion or neopeptone agars are to be used, reduced oxygen conditions must be provided by anaerobic jars, pour plates, or subsurface cuts into the agar as suggested by Fry (10). It should be noted that the gas exchange method did not prevent the DZ hemolysis. The use of such media without providing the corresponding reduced oxygen environment may result in confusion of the relatively nonpathogenic alpha-hemolytic streptococci with potentially pathogenic DZ hemolytic streptococci of groups A, B, C, and G.

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