

INHIBITION OF A STAPHYLOCOCCAL HEMOLYSIN BY A SOLUBLE SUBSTANCE PRODUCED BY A NONHEMOLYTIC MICROCOCCUS SPECIES

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Received for publication June 2, 1954

Inhibition of the activity of a bacterial toxin by antitoxic immune sera or by chemicals such as formalin is commonly observed. The inhibition caused by metabolic products of other types of bacteria, however, has never been described. The present communication describes a preliminary observation of one such phenomenon.

EXPERIMENTAL METHODS AND RESULTS

A colony of a yellow pigment forming, non-hemolytic *Micrococcus* species (tentatively identified as *Micrococcus luteus*) was found on a human blood agar plate close to a hemolytic staphylococcal colony. It was also noted that the zone of hemolysis of this Staphylococcus (the term *Micrococcus* is not used here for this organism to avoid confusion with the other) was slightly but definitely indented on the side near the colony of the micrococcus. This suggested that the hemolysis was inhibited by some soluble substances produced by the nonhemolytic micrococcus.

Observation of hemolysis on blood agar plate. Since both organisms started growing at the same time on this plate, it was understandable that the inhibition of hemolysis was not marked because once hemolysis occurs, the effect of antihemolysis will never appear. It was decided to see the effect of this antihemolytic activity by allowing the micrococcus to grow for 24 hours in advance on blood agar plate and then streaking the hemolytic staphylococcus around it. Figure 1 shows one such culture. The horizontal line is the micrococcus grown for 24 hours at 37 C in advance, and the staphylococcus was then streaked vertically on both sides of the former. The plate was incubated for another 24 hours. It can be noted that the portions of both staphylococcal colonies near the line of the micrococcus do not exhibit any hemolysis although the growth of the organism itself was never inhibited. The staphylococcus

usually grew into the line of micrococcus. The picture resembles those in an article published by Elek and Levy (1950). The inhibition of hemolysis in their cases, however, was caused by specific antihemolytic sera. In order to avoid the confusion with specific antisera which are often called "antihemolysin", the soluble substance produced by the micrococcus will be referred to as "hemolysin inhibiting substance" (HIS).

Identification of the hemolysin inhibited by hemolysin inhibiting substance. It is well known that staphylococci from various sources produce several different types of hemolysins (Glenny and Stevens, 1935; Smith and Price, 1938; Williams and Harper, 1947). It was necessary to identify the type of hemolysin concerned in this phenomenon.

The staphylococcus was isolated from an urethral culture. It was coagulase negative, mannitol negative, produced white pigment, and appeared to be a *Staphylococcus albus* which is commonly found on human skin. It produced a definite and wide zone of hemolysis on human, rabbit, and sheep blood agar plate. When a 24 hour old culture plate was placed in an ice chest (4 C), no phenomenon resembling so-called "hot-cold" lysis was observed.

The *alpha*-lysin is active against rabbit red cells but not against human cells (Parker, 1924; Glenny and Stevens, 1935). The hemolysin concerned here was, therefore, not an *alpha*-lysin. It is not a *beta*-lysin because it did not show so-called "hot-cold" lysis (Smith and Price, 1938). The probable identity of the third type of lysin, the *gamma*-lysin described by Smith and Price (1938), with the fourth type, the *delta*-lysin described by Williams and Harper (1947), was pointed out by Elek and Levy (1950). The *delta*-lysin was studied in detail by Marks and Vaughan (1950) who stated that "complete lysis produced by staphylococcal cultures or extracts in human blood agar is due to *delta*-lysin". The

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hemolysin concerned here was identical with the *delta*-lysin studied by these workers in its activity on various types of red cells, optimal conditions for its production, the length of time in which the hemolysin production reached its peak, non-filtrability through Seitz filter, and inhibition of hemolytic activity by various types of normal animal sera. The amount of normal human serum necessary to inhibit one unit of this hemolysin was also equal to that stated by Marks and Vaughan (1950), i.e., 0.05 ml inhibiting one hemolytic unit. It was, therefore, identified as *delta*-lysin. Several strains of coagulase positive *Staphylococcus aureus* were also examined, and their hemolysis was inhibited by this micrococcus on human blood agar plate but not on rabbit blood agar. This observation further supports the conclusion that the hemolysin involved was a *delta*-lysin because most of these strains produced both *alpha* and *delta*-lysins and the *alpha*-lysin is not active on human cells. The hemolysis on human blood produced by these coagulase positive strains is, therefore, caused solely by the *delta*-lysin while that on rabbit blood agar was caused by both types of hemolysins.

Elek and Levy (1950) postulated the existence of another lysin designated as *epsilon* by them, but since these workers used only rabbit and sheep cells and not human cells, it was impossible to compare the results obtained here with their observations.

Distribution of hemolysin inhibiting substance among yellow pigment forming Micrococcus species. Five strains of similar yellow pigment forming *Micrococcus* species were isolated from clinical materials and tested for hemolysin inhibiting substance activity. They all exhibited similar inhibitory effect on *delta*-lysin on human blood agar plate. Twenty strains of coagulase negative *Staphylococcus albus* and ten strains of coagulase positive *Staphylococcus aureus* were tested and their hemolysis on human blood agar plate was inhibited by the above mentioned strains of *Micrococcus* species.

The phenomenon described here, therefore, is of a fairly common occurrence.

Production of delta hemolysin. It has been a common practice since Burnet's work (1929) to produce staphylococcal hemolysin in a semisolid agar incubated with high concentration of CO₂. A better technique, however, has been described by Marks and Vaughan (1950). The superiority

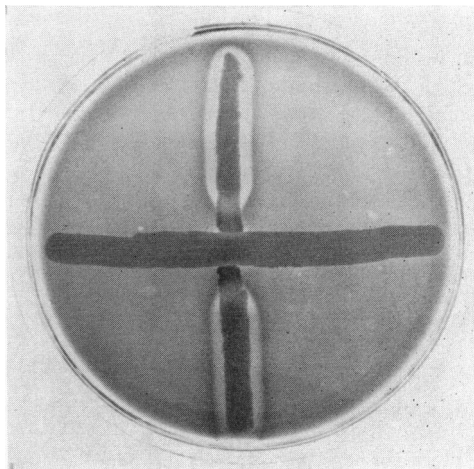


Figure 1. Inhibition of the hemolysis of a *Staphylococcus albus* on human blood agar plate by a nonhemolytic yellow pigment forming micrococcus species. The horizontal line is the micrococcus grown for 24 hours at 37 C in advance, and the staphylococcus was then streaked vertically and, separately on both sides of the micrococcus colony. The plate was incubated for another 24 hours. Note the absence of hemolysis of staphylococcal colony near the line of the micrococcus.

of this technique to the old one has been confirmed in the present study. It is to grow the staphylococcus on the surface of a nutrient agar plate covered by cellophane. The nutrition from the agar will pass through the cellophane to support the growth of the organism while the hemolysin does not diffuse through this substance. A high concentration of hemolysin can thus be obtained by washing off the growth with sterile saline and centrifuging the suspension. No significant difference in the titer of hemolysin was observed by using various types of nutrient agar, and the blood agar base (Difco) was used in most of these experiments. An ordinary wrapping cellophane was cut to fit the petri dishes, sterilized at 15 lb, for 10 minutes with moisture. These sterilized sheets were placed aseptically on freshly poured, undried agar plates and inoculated with 3 drops of 18 hour old broth culture of the organism. The inoculum was distributed over the whole surface of the plate by a sterile cotton swab, and the plates were then incubated with 10 per cent CO₂ at 37 C. It is important that the cellophane sheets be free from wrinkles because this will reduce the area of attachment of the cellophane to the agar and also reduce con-

siderably the amount of hemolysin harvested from a plate. The hemolysin production usually reached the maximum between 24–30 hours of incubation, and harvests were made at these time intervals. Three ml of saline were used for each plate to wash off the growth, and the suspensions were allowed to stand for 1 hour at 4 C before centrifugation.

Marks and Vaughan (1950) defined one unit of *delta*-lysin as that amount which caused 50 per cent lysis of 0.025 ml of 20 per cent human red cell suspension. The same amount of red cells was used, but for the sake of convenience, 0.5 ml of 1 per cent suspension was employed in most of these experiments. The reading was made after one hour incubation at 37 C. Complete hemolysis instead of 50 per cent lysis was taken as one hemolytic unit because 50 per cent lysis was difficult to evaluate in cases of hemolysin inhibiting test by hemolysin inhibiting substance which will be described later. The hemolytic unit used here was, therefore, twice as large as that employed by Marks and Vaughan (1950). The above mentioned technique usually yielded a hemolytic extract with about 64 to 128 units per ml. No attempt was made to improve the medium to obtain a higher titer of hemolysin because it was not the purpose of this study.

Production of "hemolysin inhibiting substance" (HIS). The optimal condition for production of hemolysin inhibiting substance by the micrococcus was found to be identical with that for hemolysin production. The hemolysin inhibiting substance production was practically nil in broth culture throughout the entire period of examination which lasted from 12 hours to 5 days. Semisolid agar-CO₂ technique yielded some hemolysin inhibiting substance, but the best yield was obtained on the cellophane plate described for hemolysin production. The peak of hemolysin inhibiting substance production also reached maximum around 24 hours of incubation. The only difference was that the production of hemolysin inhibiting substance does not require a high concentration of CO₂ which is essential for hemolysin production. One unit of hemolysin inhibiting substance was arbitrarily defined as that amount which completely inhibits one unit of *delta*-hemolysin following 30 minutes' incubation of the mixture of both substances at 37 C. The combination of the *delta*-lysin and hemolysin inhibiting substance seemed to occur immediately

when they were mixed because the titer of hemolysin inhibiting substance was practically the same when the red cell was added immediately after these substances were mixed (i.e., without 30 minutes' incubation of the mixture at 37 C).

The mechanism of the hemolysin inhibition. There can be two processes by which a hemolysin may be inhibited. The first is to neutralize the hemolysin itself before it combines with red cells, as is done by antihemolytic sera. The second is to block the receptors of the hemolysin on the surface of red cells. Since the hemolysins are chemical substances with definite structures, the receptors on red cells must be specific for each hemolysin. It is well known that various types of red cells vary considerably with regards to their susceptibility to the action of various types of hemolysin. This variation in susceptibility exists even among different types of staphylococcal hemolysins as mentioned above. Since the micrococcus is closely related to staphylococcus, it was thought more likely that the inhibition of the hemolysis was due to the latter process, i.e., the blocking of red cell surface by a substance structurally resembling the *delta*-lysin and thus preventing the access of the hemolysin to the red cell. An example of the procedure employed is given in table 1. As will be seen in the table, the red cell never became resistant to the action of hemolysin by combining with hemolysin inhibiting substance. When the hemolysin was mixed with varying dilutions of hemolysin inhibiting substance, its hemolytic activity was inhibited by a very high dilution of this substance. One ml of the extract produced with the micrococcus by the above mentioned technique usually yielded from 512 to 1,012 hemolysin inhibiting substance units. An example of the experimental procedure is given in table 2.

The nature of the hemolysin inhibiting substance. The hemolysin inhibiting substance has many characteristics similar to the *delta*-lysin which it inhibits. As mentioned before the optimal conditions for its production, the length of time in which its production reached peak, nondialyzability through cellophane, inhibition of its production by normal animal sera, etc., are the characteristics they have in common. However, many differences were also observed between the two substances, and these differences are summarized in table 3.

Aside from the hemolytic activity, the hemolysin inhibiting substance differs from the hemoly-

TABLE 1

The effect of the "hemolysin inhibiting substance" (HIS) on human red cells

Tube	1	2	3	4	5	6	7	8	Hemolysin Control	Red Cell Control
Saline	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0 ml
HIS	1.0	serially diluted twofold to tube 8								
1% Red cell	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5 ml

Incubation at 37 C for 1 hour with occasional stirring
Centrifuge with 3,000 rpm for 10 minutes

Discard the supernatant fluid and wash red cells three times with sterile saline

Saline	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1.5 ml
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Shake to resuspend the red cells

Hemolysin, 1 μ /ml	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	ml
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Incubation at 37 C for 1 hour with occasional stirring

Result	4	4	4	4	4	4	4	4	4	0
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Numerals in "Result" indicate degrees of hemolysis.

4 indicates complete hemolysis and 0 indicates complete absence of hemolysis.

TABLE 2

The effect of the "hemolysin inhibiting substance" (HIS) on the delta-hemolysin

Tube	1	2	3	8	9	10	Hemolysin Control	Red Cell Control	
Saline	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1.0 ml	
HIS	0.5	serially diluted twofold to tube 10								
Hemolysin, 2 μ /ml	0.5	0.5	0.5	0.5	0.5	0.5	0.5	ml	
Incubation at 37 C for 30 minutes										
1% red cell	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5 ml	
Incubation at 37 C for 1 hour with occasional stirring										
Result	0	0	0	0	1	3	4	0	

Numerals in "Result" indicate degrees of hemolysis.

4 indicates complete hemolysis and 0 indicates complete absence of hemolysis. 1 and 3 indicate degrees of hemolysis.

sin in that its production does not require a high concentration of CO₂, and the titer is not significantly reduced by the Seitz filter which reduces greatly the titer of *delta*-lysin. The hemolysin inhibiting activity of the hemolysin inhibiting substance is completely lost by heating at 100 C for 10 minutes while the hemolytic activity of the *delta*-lysin is not completely destroyed by this

treatment (Marks and Vaughan, 1950). Marks and Vaughan (1950) pointed out that *delta*-lysin has many characteristics similar to fatty acids. The hemolysin inhibiting substance resembles more closely protein in that its activity is destroyed by heat, and the extracts containing this substance exhibit strong biuret reactions.

It has already been pointed out by Marks and

TABLE 3
The differences between the delta-lysin and the
"hemolysin inhibiting substance" (HIS)

	delta-Hemolysin	HIS
Hemolysis.....	+	-
CO ₂ required for production...	Yes	No
Absorbed by Seitz filter.....	Yes	No
Activity destroyed by heating 100 C 10 minutes.....	No	Yes

Vaughan (1950) that the hemolytic activity of the delta-lysin is inhibited by various animal sera, and both of the albumin and globulin fractions of a normal horse serum were capable of neutralizing this hemolysin. The hemolysin inhibiting substance may be similar to the protein fractions in the animal sera which neutralize the delta-lysin.

DISCUSSION

Observation of the above mentioned phenomenon has never been described, but it is not particularly surprising because the Staphylococcus and the Micrococcus are closely related. Productions of bacterial exotoxins may be due to the excretion of an incomplete product which is a part of a nontoxic respiratory enzyme. In an unfavorable condition, the organism may not be able to complete the process of enzyme synthesis and the half products; either the prosthetic group or the protein carriers of the enzyme are excreted into the surrounding medium. The best example is the mechanism for toxin production of *Corynebacterium diphtheriae* (Pappenheimer and Hendee, 1947). In the absence of iron which is necessary for the synthesis of cytochrome b, the protein carrier of this enzyme is excreted into the surrounding medium. It was postulated by these workers that the toxicity of the protein carrier (the toxin) is due to its affinity to some respiratory enzyme in the tissue because the combination of this substance may interfere with some normal enzyme synthesizing process.

In a pair of closely related organisms such as *Staphylococcus* and *Micrococcus*, some of their enzymes may be similar in structure, and when their half products are mixed, it is possible that they will combine readily. The combination thus results in the inhibition of the hemolytic activity.

It is also possible that the receptors for the

delta-lysin on human red cells and the substance in normal animal sera which inhibits the hemolytic activity of the delta-lysin are similar in structure with the hemolysin inhibiting substance produced by the micrococcus. The data presented above, although limited, seemed to indicate that the hemolysin inhibiting substance is a protein.

A search was made to find this hemolysin inhibiting phenomenon among closely related organisms. The combinations of organisms employed included groups A, C, and G hemolytic streptococci with a nonhemolytic *Streptococcus*, hemolytic varieties of fecal streptococci with nonhemolytic type, hemolytic varieties of *Escherichia coli* with a nonhemolytic type. None of these combinations exhibited a phenomenon similar to that described above on blood agar plate. However, only a few strains were used for each of these combinations, and it is possible that the same phenomenon may be found with other combinations of organisms.

SUMMARY

The delta-hemolysin of staphylococci was found to be inhibited by a soluble substance produced by a nonhemolytic yellow pigment forming *Micrococcus* species. The production of this hemolysin inhibiting substance seemed to be quite common because all of the five strains of yellow pigment forming *Micrococcus* species isolated from clinical materials were found to produce this substance.

The hemolysin inhibiting substance has no effect on the human red cells, and its activity is exhibited by combining directly with the delta-hemolysin. This substance seems to be a protein in nature, and a possible mode of its production and activity is discussed.

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