Synergistic Hemolysis Phenomenon Shown by an Alpha-Toxin-Producing *Clostridium perfringens* and Streptococcal CAMP Factor in Presumptive Streptococcal Grouping

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A new phenomenon of synergistic hemolysis by *Clostridium perfringens* alpha-toxin and the streptococcal CAMP factor on human and guinea pig erythrocytes is described. A possible mode of action of the CAMP factor is suggested. On human blood agar all of the tested isolates of group B streptococci gave an arrowhead-shaped zone of hemolysis; 74% of group A gave a crescent-shaped lytic zone, whereas all isolates of groups C and G and the remaining 26% of group A streptococci gave a bullet-shaped lytic zone. By comparison, in the CAMP test incubated aerobically and anaerobically, 70 and 91%, respectively, of streptococci other than group B gave positive, arrowhead-shaped lytic zones. If all intermediate positive reactions in the CAMP tests were read as negative after aerobic incubation, only 89% of group B streptococci would be properly identified. The synergistic hemolysis phenomenon, using an alpha-toxin-producing *C. perfringens* and human blood agar, provided a reliable test for presumptive identification of group B streptococci, with promising potential to differentiate in the same test group A streptococci from other groups.

One of the presumptive tests for differentiating group B streptococci from other groups is the CAMP test developed by Christie et al. in 1944 (3). This test depends on synergistic hemolysis between *Staphylococcus aureus* beta-toxin and a streptococcal factor, later frequently called the CAMP factor, on sheep blood agar (SBA) or ox blood agar. Human blood agar (HBA) cannot be used for this purpose. If staphylococci are inoculated as a streak across the plate and the streptococci are inoculated at right angles, but not touching, the lytic zone acquires an arrowhead shape, with its tip pointing to the staphylococci (17).

Originally the CAMP test was considered as highly specific for group B streptococci, all of which, whether hemolytic or nonhemolytic on first isolation, were thought to give positive reactions (3, 16, 17). In later studies, depending on cultural conditions and assessment of the lytic zones, between 20 and 100% of group A streptococci, and a number of strains of groups C and G, gave positive reactions, although many of these were weaker than those of group B. At the same time, up to 5% of group B streptococcal cultures gave negative results (6, 9-12, 20).

Recently, two attempts were made to standardize the CAMP test and obviate false positive reactions. Darling (6) found that they did not occur under aerobic and candle jar atmospheres.

Wilkinson (23) has noticed, using disks soaked with staphylococcal beta-toxin, that an excess CO₂ atmosphere was not superior to anaerobic conditions and that false positive reactions could be best avoided by using aerobic incubation and neglecting weaker synergistic hemolytic reactions. In another recent study (15) it was stated that synergistic hemolysis in the CAMP test did not occur when the plates were incubated anaerobically.

The initial purpose of this study was to evaluate the use of the CAMP test in the presumptive identification of group B streptococci. Subsequently, a newly observed phenomenon of synergistic hemolysis between alpha-toxin-producing *Clostridium perfringens* and group B streptococci was explored. This synergistic hemolysis was correlated with the results of the CAMP test and with Lancefield serological grouping. Also, an attempt was made to explain the synergistic mode of action between the CAMP factor and *C. perfringens* alpha-toxin or *S. aureus* beta-toxin.

MATERIALS AND METHODS

Bacterial strains. One hundred and eleven isolates of beta-hemolytic streptococci were obtained from routine clinical specimens from different patients over a period of several months. The sources of the specimens were throat swabs, sputa, wounds, ulcers, urines,

female genital tract, and stool. Isolates were stored at 4°C on sealed blood agar plates until tested.

An isolate of alpha-toxin-producing *C. perfringens* was taken from a routine clinical specimen. Storage of that strain was similar to that of streptococci.

Eight strains of phospholipase C-producing *Bacillus cereus* were tested. Strains 9E₂ and 4E₁ were obtained from P. Chadwick, strain 58262 is a quality control strain from our laboratory, and the rest were isolated as laboratory contaminants.

A beta-toxin-producing strain of *S. aureus* was supplied by the Public Health Microbiology Laboratory, Kingston, Ontario. On SBA the strain gave a very narrow zone of complete hemolysis around the colonies but a fairly broad (4 to 5 mm) outer zone of blackened, partially hemolyzed erythrocytes, indicating a good beta-toxin production with a minimal amount of alpha- and delta-toxins.

Blood agar plates. Approximately 15 ml of 5% citrated sheep blood (Woodland Laboratory Ltd., Guelph, Ontario, Canada), outdated human blood bank blood, or citrated guinea pig blood in a tryptose blood agar base (Oxoid, London, England) was poured into disposable petri dishes (15 by 85 mm). The depth of the blood agar was 3 mm. Washed erythrocytes were not used, but every new batch of SBA was tested for the presence of inhibitory factors to *S. aureus* beta-toxin or the CAMP factor. If the beta-toxin-producing strain of *S. aureus* developed a broad darkened zone around the colonies, and if the CAMP test was obtainable with the control group B streptococcus strain after incubation at 37°C overnight, the blood was considered suitable for use in the test. Similar tests were done with SBA and HBA when *C. perfringens* was used.

Cell-free supernatant of *C. perfringens*. A strain of *C. perfringens* was grown in egg yolk-coagulated broth (Difco Laboratories, Detroit, Mich.) under aerobic conditions for 6 h at 37°C in a water bath. The growth was sedimented by centrifugation, and the supernatant was separated and stored at 4°C. Filter paper strips (Whatman no. 1) were sterilized by UV light, soaked with the supernatant, and applied wet, or dried, to blood agar plates. The supernatant originally gave a positive Nagler reaction, but after 1 week of storage at 4°C this property was lost.

Lancefield grouping. Lancefield grouping was performed by using a capillary pipette precipitation test (22), with the streptococcal antigen extracted by the autoclave-Pronase B method of Edwards and Larson (8), and commercially available grouping antisera (Baltimore Biological Laboratory, Cockeysville, Md.).

Synergistic mode of action between the CAMP factor and type C phosphohydrolases. For this purpose, three type C phosphohydrolase-producing bacterial species were chosen: (i) *S. aureus*, which produces a narrow-spectrum sphingomyelinase C (7); (ii) *C. perfringens*, whose alpha-toxin contains sphingomyelinase C fraction similar to that of *S. aureus* (19) and a broader-spectrum phospholipase C (lecithinase), hydrolyzing phosphatidylcholine (lecithin), sphingomyelin, and phosphatidylethanolamine (cephalin) (4, 14; J. de Gier, G. H. de Haas, and L. L. M. van Deenen, Biochem. J. 81:33p-34p, 1961); and (iii) *B. cereus*, whose phospholipase C attacks phos-

phatidylcholine, phosphatidylethanolamine, and phosphatidylserine, but not sphingomyelin, causing a nonlytic degradation of phospholipids in different mammalian erythrocytes (5, 13; de Gier et al., Biochem. J. 81:33p-34p, 1961).

In addition, three types of erythrocytes were used as substrates for the above bacterial enzymes: (i) sheep, whose outer leaflet of the membrane phospholipid bilayer consists almost entirely of sphingomyelin; (ii) human, where the major phospholipid in the outer leaflet is phosphatidylcholine, but with a substantial amount of sphingomyelin; and (iii) guinea pig, with a phosphatidylcholine as a predominant phospholipid in the outer leaflet of the membrane and a small amount of sphingomyelin. The inner leaflet of the lipid bilayer of all three types of the tested erythrocytes is similar, consisting mostly of phosphatidylethanolamine and phosphatidylserine (1, 18, 26).

To explore the range of synergistic hemolytic activity, separate plates with SBA, HBA, and guinea pig blood agar (GPBA) were inoculated with beta-toxin-producing *S. aureus*, alpha-toxin-producing *C. perfringens*, and phospholipase C-producing *B. cereus*. Alternatively, filter paper strips soaked with the supernatant of *C. perfringens* were placed on plates of SBA and HBA. Isolates of group A, B, C, and G streptococci were then inoculated similarly to the method of Munch-Petersen et al. (17). Incubation was under aerobic and/or anaerobic conditions overnight at 37°C.

Application of the synergistic hemolysis tests. In the CAMP test, SBA plates were inoculated and incubated as above. Reactions were recorded as "large-positive," "intermediate," and "negative" (20). In an intermediate reading, the size of the hemolytic zone was at least one-third smaller than that given by the control group B strain, which was selected at the beginning of the experiment.

When *C. perfringens* was used for the presumptive identification of group B streptococci, circular inoculation of *C. perfringens* was done on HBA plates, using a turntable. The circle was 3.5 cm in diameter. Streptococci were streaked radially outside the clostridial circle, starting at a distance of 1 to 2 mm from the circle. This technique allowed 12 isolates to be tested on the same plate and permitted more uniformity in the shape of the hemolysis. The plates were incubated anaerobically overnight at 37°C in GasPak jars.

Inhibition of the synergistic hemolysis of *C. perfringens*. *C. welchii* type A antiserum (Wellcome Reagents Ltd., Beckenham, England) and group A, B, C, and G streptococcus antisera (Baltimore Biological Laboratory) were used. Plates were dried, smeared over their whole surface with a swab soaked in the appropriate antiserum, redried, and inoculated as described above.

RESULTS

Lancefield grouping showed that 39 strains were group A streptococci; 44, group B; 8, group C; 13, group G; and 3, group D. Four beta-hemolytic strains did not belong to any of the above groups.

When beta-toxin-producing *S. aureus* was

used, synergistic hemolysis with different groups of streptococci was elicited on SBA but not on HBA or GPBA. The results of the CAMP test were as follows. All 44 isolates of group B streptococci gave positive CAMP test reactions under both aerobic and anaerobic conditions. However, except for 20 (30%) isolates of streptococci other than group B, incubated aerobically, and 6 (9%) isolates, incubated anaerobically, all other tested isolates gave positive CAMP test reactions in both atmospheres. All three isolates of group D were negative (Table 1). The majority of the CAMP-positive, non-group B streptococci gave an intermediate-sized lytic zone. One isolate of group A produced a large zone under anaerobic conditions, and five isolates of group B gave an intermediate zone under aerobic conditions. Some isolates of group A or B produced a different zone size if tested repeatedly. Group C and G streptococci usually produced the smallest zone sizes of all tested groups if incubated aerobically. The lytic zones had an arrowhead shape pointing towards the staphylococci, except in a very weak or small-sized reaction, and the extent and intensity of lysis depended on the size of the streptococcal inoculum. If the inoculum was too light, so that growth was less than confluent, the reaction tended to be very weak, and such strains were retested with heavier inocula before the result was recorded. If test plates were kept at room temperature beyond the usual incubation time, reading of the test was difficult due to "hot-cold" lysis of the sheep erythrocytes.

The use of the alpha-toxin-producing *C. perfringens* isolate to condition erythrocytes enabled the elicitation of synergistic hemolysis with different groups of streptococci on SBA, HBA, and GPBA in an anaerobic atmosphere. This synergistic hemolysis could be completely inhibited by the *C. perfringens* type A antiserum but not by the streptococcal antisera. The area of synergistic hemolysis on SBA was in the shape

of an arrowhead with group B streptococci but was bullet shaped with groups A, C, and G (Fig. 1).

Synergistic hemolysis was most intense on SBA, followed by HBA and GPBA in decreasing order. But differentiation between streptococcal groups was clearest when human blood was used in the medium (Fig. 2). Group B streptococci continued to give arrowhead shapes, with more hemolysis at the periphery of the *C. perfringens* alpha-toxin-induced partial hemolytic zone. Many group A streptococci gave crescent-shaped synergistic hemolysis at the edge of the zone of partial hemolysis, with a convex side towards the clostridial circle. Group C, G, and the remainder of group A streptococci gave only a somewhat enlarged hemolytic zone with a bullet-shaped, hazy margin around the streptococcal streak, inside the area of clostridial lytic action.

Tests for synergistic hemolysis between *C. perfringens* and 111 isolates of streptococci on HBA gave the following results (Table 2). All 44 isolates (100%) of group B streptococci gave an arrowhead-shaped lytic zone, even when repeatedly tested. Twenty-nine of 39 isolates of group A streptococci (74%) gave characteristic crescent-shaped synergistic lysis, and the remainder (26%) gave bullet-shaped zones. It must be noted that crescent production was inconstant with some isolates inoculated at different times on different batches of blood agar. All 8 isolates of group C, and 13 of group G, streptococci (100%) consistently gave bullet-shaped synergistic hemolysis, as did 2 isolates of streptococci not belonging to these groups. Three isolates of group D streptococci and two nongrouped isolates did not show any distinct change in their usual hemolysis around the streak. Hot-cold lysis did not interfere with the readings, but after a day or so hemolytic zones obtained a double-image appearance, due to diffusion of the clostridial toxin. A comparison of the results

TABLE 1. Results of the CAMP test under aerobic and anaerobic incubation and relative size of arrowhead-shaped lytic zone

Streptococcus Lancefield group	No. of strains	Relative size of lytic zone					
		Aerobic incubation			Anaerobic incubation		
		Large	Intermediate	No lytic zone	Large	Intermediate	No lytic zone
A	39	0	35	4	1	38	0
B	44	39	5	0	44	0	0
C	8	0	4	4	0	6	2
G	13	0	6	7	0	13	0
D	3	0	0	3	0	0	3
Not grouped	4	0	2	2	0	3	1

FIG. 1. SBA plate demonstrating an arrowhead-shaped zone of synergistic hemolysis of group B streptococci (B) and bullet-shaped zones of group A, C, and G streptococci (A, C, G). *C. perfringens* is circularly inoculated. This plate shows the beginning of the hot-cold lysis due to some delay in photographing.

obtained by the CAMP test and the *C. perfringens* synergistic hemolysis test is given in Table 3.

When phospholipase C-producing strains of *B. cereus* were used, no synergistic hemolysis with streptococci could be noticed on SBA, HBA, or GPBA.

The supernatant of *C. perfringens* growth in coagulated egg yolk broth, which was devoid of phospholipase C activity (Nagler plate negative), showed a bullet-shaped synergistic hemolysis with streptococcal groups A, C, and G but no reaction with group B streptococci on HBA (Fig. 3). The same supernatant gave a typical CAMP-like arrowhead reaction with group B streptococci as well as bullet-shaped lytic zones with other groups when tested on SBA (Fig. 4). Before storage, the supernatant gave a characteristic synergistic hemolysis with all tested groups on both types of agar plates.

While the above experiments were being done, an interesting observation was made on a post-mortem culture of a lung tissue specimen that revealed a heavy growth of group B streptococci mixed with a light growth of *C. perfringens*, showing synergistic hemolysis on an HBA plate. Figure 5 represents a subculture of the mixed

growth, in which the hemolytic pattern may be seen more clearly. Three *C. perfringens* colonies mixed in the dense growth of group B streptococci along the streak extending away from the original inoculum formed an ellipsoidal zone of partial hemolysis. The synergism appeared only at the poles of the partly lysed area. This observation may prove to be of value in presumptive identification of both group B streptococci and *C. perfringens* in mixed cultures on primary plates and was helpful in the attempt to explain the mode of action of the CAMP factor.

DISCUSSION

In this study the CAMP test showed unacceptably high numbers of false positive reactions under both aerobic and anaerobic conditions. This was due to the large proportion of intermediate positive reactions; if, however, these reactions were read as negative, 11% of the group B streptococci would not be recognized by this test. Although difficult to compare, results of this experiment are roughly in agreement with those of Esseveld et al. (9) but somewhat less favorable than those of others, notably in aerobic tests (6, 10, 12, 20, 23), probably due to the use of different criteria for intermediate zone sizes,

FIG. 2. HBA plate showing an arrowhead-shaped zone of synergistic hemolysis of group B streptococci (B), a crescent-shaped zone of group A streptococci (A), and bullet-shaped zones of groups C and G (C, G). *C. perfringens* is circularly inoculated.

TABLE 2. Results of the synergistic hemolysis test by *C. perfringens* and streptococci of different Lancefield groups on HBA and the corresponding shape of the lytic zone

Streptococcus Lancefield group	No. of strains	No. with synergistic lytic zone shape:		
		Arrow- head	Crescent	Bullet
A	39	0	29	10
B	44	44	0	0
C	8	0	0	8
G	13	0	0	13
D	3	0	0	0
Not grouped	4	0	0	2

whose assessment may be very subjective. In addition, the existence of many factors that can influence the test results (2, 6, 16, 23) makes standardization of the CAMP test very difficult.

The newly detected synergistic hemolysis phenomenon between *C. perfringens* alpha-toxin and the CAMP factor on HBA showed more clear-cut results than those in the CAMP test. Also, the hot-cold effect was not pronounced, so that delayed reading was easier. The arrowhead shape of the hemolytic zone separated all group B streptococci from those of

other groups, and the crescent-shaped lytic zone produced by group A streptococci appeared to be fairly specific. It seemed that no special standardization was necessary, and difficulties were encountered only when the percentage of erythrocytes in the agar was very small, when blood was not evenly mixed with agar, and when the streptococcal streak did not show a confluent growth. No differences in the performance of the media were noticed with different batches of HBA. Variations in the production of the crescent-shaped lysis by the same isolate of group A streptococci at different times and on different batches of HBA might be due to variations in the production of the responsible factor with time, or to other as yet unexplained influences.

Inhibition of the synergistic lysis with the specific type A antiserum on SBA, HBA, and GPBA showed that *C. perfringens* alpha-toxin was involved in this phenomenon. When phospholipase C activity of the alpha-toxin was lost, its sphingomyelinase C fraction was still preserved, as indicated by the synergism obtainable only on SBA, and resembled that of *S. aureus* beta-toxin (Fig. 3 and 4). However, a phospholipase C-producing *B. cereus* isolate did not show lytic synergism with the CAMP factor on any of the three types of erythrocytes. Finally, as illus-

TABLE 3. Comparative incidence of arrowhead-shaped lytic zones in CAMP test and *C. perfringens* synergistic hemolysis test on HBA

Streptococcus Lancefield group	No. of strains	CAMP test ^a				<i>C. perfringens</i> synergistic hemolysis test (on HBA)	
		Positive ^b		Negative		Positive	Negative
		O ₂	An O ₂	O ₂	An O ₂		
A	39	35	39	4	0	0	39
B	44	44	44	0	0	44	0
C	8	4	6	4	2	0	8
G	13	6	13	7	0	0	13
D	3	0	0	3	3	0	3
Not grouped	4	2	3	2	1	0	4

^a Regardless of the relative size of the lytic zone.^b O₂, Incubation under aerobic conditions; An O₂, incubation under anaerobic conditions. FIG. 3. HBA plate laid with a filter paper strip (CP) soaked with the supernatant of *C. perfringens* growth in coagulated egg broth which has lost its phospholipase C activity. Note bullet-shaped zones of synergistic hemolysis of group A, C, and G streptococci (A, C, G). Group B streptococci do not show any synergistic hemolytic activity (B).

trated in Fig. 5, no synergistic lysis appeared in the vicinity of *C. perfringens* colonies. The above observations and what is known about the CAMP factor (2, 3, 9, 16), action of bacterial type C phosphohydrolases (4, 5, 7, 13, 14, 19, 25; de Gier et al., Biochem. J. 81:33p-34p, 1961), and architecture of different erythrocyte membranes (1, 18, 26) suggest an explanation of the mode of action of the CAMP factor as follows. It appears that the CAMP factor manifests spe-

cific synergism with sphingomyelinase C and is most effective when present before or nearly simultaneously with the enzymatic hydrolysis of sphingomyelin. This action depends on a relatively high concentration of the factor and the slower rate of the degradation of the substrate, e.g., due to the dilution of sphingomyelinase C (Fig. 5). Under such conditions the CAMP factor might interfere with the precipitation of ceramide (*N*-acyl-sphingosine) in situ. This water-in-

FIG. 4. SBA plate laid with a filter paper strip (CP) soaked with the superantant of *C. perfringens* growth in coagulated egg broth which has lost its phospholipase C activity. Note an arrowhead-shaped zone of group B streptococci (B), indicating that the sphingomyelinase activity of the supernatant was preserved, and bullet-shaped synergistic lytic zones of groups A, C, and G (A, C, G).

soluble end product of the degradation of sphingomyelin is thought to contribute to the relative stability of the erythrocyte membrane after action of *S. aureus* beta-toxin at 37°C (5). Removal of ceramide would leave wide open areas in the outer layer of the sheep erythrocyte membrane, rendering the inner one susceptible to the pressure from inside the cell and to a possible enzymatic attack, or enough open areas in the outer layer of human and guinea pig erythrocytes to expose the inner one to the action of the broader-spectrum phospholipase C of *C. perfringens*, culminating in the hydrolysis of phosphatidylethanolamine and consequent complete hemolysis. The possible significance of the degradation of phosphatidylcholine by *C. perfringens* alpha-toxin in the synergistic hemolysis on HBA and GPBA could not be clarified by experiments in this study.

The difference in the intensity of the synergistic hemolytic zones on SBA, HBA, and GPBA is probably due to the different amounts of sphingomyelin, difference in ionic contents of different erythrocytes, and the less effective hydrolysis of phosphatidylethanolamine in the inner layer of the erythrocyte membrane by *C. perfringens* alpha-toxin (4, 18, 21).

The CAMP factor/enzyme ratio necessary to produce complete hemolysis might be in part responsible for the arrowhead shape of the synergistic lytic zone, whereas different shapes may be caused by changed conditions of inoculation. The size of the zone depends on the amount of the bacterial product and its diffusibility through the medium.

In addition, crescent-shaped synergistic hemolysis of some group A streptococci suggests a possible production of a small amount of the CAMP factor by these isolates. Contrary to that, the phenomena shown in Fig. 3 and 4 indicate that the bullet-shaped lytic zones of streptococcal groups A, C, and G are not due to the production of the CAMP factor or to the action of *C. perfringens* phospholipase C or sphingomyelinase C. The role of these factors in the false positive CAMP tests must await further investigations.

In conclusion, this is, as far as is known, the first time that synergistic hemolysis between *C. perfringens* alpha-toxin and the CAMP factor has been demonstrated on erythrocytes from species other than sheep. Williams and Harper in 1947 (24) indicated in Fig. 1V of their article that synergistic hemolysis was obtainable be-

FIG. 5. Subculture on an HBA plate of an autopsy specimen of the left lung tissue. A streak, extending away from the original inoculum (long arrow), shows a heavy growth of group B streptococci mixed with a few colonies of *C. perfringens* (short arrows). Synergistic hemolysis (SH) occurred only at the poles of the ellipsoidal zone of partial hemolysis by alpha-toxin.

tween *C. perfringens* and group B streptococci on SBA but did not explore this further in the paper. The test using HBA showed apparent superiority over the CAMP test, with promising potential to differentiate both group B and group A streptococci from other groups. Paper strips, soaked with stabilized *C. perfringens* alpha-toxin, would obviate the need for anaerobic incubation.

Experiments for the application of the test in the identification of phospholipase C-producing clostridia are being done in this laboratory.

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