JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1984, p. 534–537
0095-1137/84/040534-04$02.00/0

Vol. 19, No. 4

Streptococcus pyogenes Streptolysin O as a Cause of False-Positive CAMP Reactions

JOHN W. TAPSALL* AND EDNA A. PHILLIPS

Department of Microbiology, The Prince of Wales Hospital, Randwick, New South Wales 2031, Australia

Received 13 July 1983/Accepted 5 January 1984

The synergistic hemolysis of sheep erythrocytes in the CAMP reaction by the sequential action of staphylococcal beta-lysin and the CAMP factor of group B streptococci is the only known function of this extracellular product of group B streptococci. The reaction forms the basis of the CAMP test used to identify group B streptococci because the CAMP factor is believed to be restricted to this group of organisms. However, on occasion other streptococci, notably group A streptococci, may produce a similar synergistic lysis of sheep erythrocytes. The nature of the synergistic lytic factor of group A streptococci responsible for this sequential hemolysis was investigated in a tube CAMP reaction system. The properties of this synergistic lytic factor were found to correspond to those of streptolysin O of group A streptococci. The synergistic lytic factor, like streptolysin O, was produced during the logarithmic phase of growth; the activity was increased by reducing agents and greatly decreased or abolished by heat, trypsin, cholesterol, and anti-streptolysin O, and it was immunogenic in rabbits. This would suggest that the synergistic hemolysis seen in the CAMP reaction system with group A streptococci is due to the action of those small amounts of streptolysin O which remain unoxidized and thus have a capacity to lyse the fragile beta-lysin-treated sheep erythrocytes.

The lysis of sheep erythrocytes (SRBC) by the combined action of staphylococcal beta-lysin and an extracellular product of Lancefield group B streptococci (GBS) was first described by Christie et al. in 1944 (3). Subsequently, the acronym CAMP has been applied not only to this extracellular product of GBS but also to the lytic reaction of the SRBC. It has been shown that the staphylococcal beta-lysin is a sphingomyelinase (9) which depletes the erythrocyte membrane of lipids, making the erythrocytes susceptible to subsequent lysis by a variety of physical, chemical, or biological agents. These agents include mechanical damage, temperature or pH changes, and EDTA as well as the CAMP factor of GBS (14, 16). The extracellular CAMP factor is now known to be a thermostable protein with a molecular weight of 23,500 and the ability to lyse beta-lysin-sensitized, but not intact, SRBC (1).

Among the streptococci, the CAMP factor is believed to be restricted to GBS, and based on this a positive CAMP reaction is regarded as a presumptive test for the identification of these organisms. Esseveld et al. (5) and Darling (4) have modified the original plate CAMP test, and in their reports they refer to false-positive CAMP tests which were caused by group A streptococci. Darling noted also that the phenomenon of false-positive CAMP tests due to group A streptococci was more pronounced in an anaerobic atmosphere. Facklam et al. (6), in a subsequent investigation with the CAMP test of Darling, although noting that care in interpretation of intermediate results was required, recommended use of the CAMP test in conjunction with other tests.

Subsequently, a tube CAMP test (RTCT) was described in which a suspension of SRBC previously exposed to the action of staphylococcal beta-lysin (β-SRBC) was mixed with streptococcal culture supernatants (13). These supernatants were obtained under conditions which allowed most GBS to multiply and produce the CAMP factor. Under the same conditions it was found that the more fastidious group A streptococci failed to grow optimally, thereby circumventing the problem of false-positive CAMP test results due to these organisms. In experiments during the developmental stages of the RTCT, it was noted that variation of the culture conditions used would permit group A organisms to produce a false-positive CAMP test result (E. A. Phillips, M. App. Sc. thesis, New South Wales Institute of Technology, Sydney, Australia, 1981). These variations included use of different media or a prolonged incubation time.

In this study the RTCT system was used to investigate the phenomena of CAMP-like reactions of group A streptococci. The nature of the synergistic lytic factor (SLF) of group A streptococci responsible for false-positive CAMP reactions was examined, and in particular, the relationship between this hypothetical SLF and streptolysin O (SO), the thiol-activated hemolysin of *Streptococcus pyogenes*, was investigated.

MATERIALS AND METHODS

Staphylococcal beta-lysin was prepared, using *Staphylococcus aureus* S32A (2), by the semisolid-agar technique of Haque and Baldwin (10), and beta-lysin (hot-cold) titers were determined as previously described (13). The lytic titer at 4°C (hot-cold titer) was taken as the last tube showing more than 50% hemolysis as determined visually (12) and recorded as the reciprocal of the dilution of staphylococcal beta-lysin causing this lysis. A 1:8 dilution of the staphylococcal beta-lysin preparation diluted in phosphate-buffered saline (PBS) and containing 1 mM SO_4 was used in experiments in this study.

Sources of SO and SLF. SO and SLF were obtained from culture supernatants of *Streptococcus pyogenes* ATCC 38601 (kindly donated by M. Wilson, University of New South Wales), and *Streptococcus pyogenes* A_3, originally a clinical isolate in this department and a control strain for the RTCT (13). Fifty-milliliter volumes of Todd-Hewitt broth (Oxoid Ltd.) were heavily inoculated with the group A

* Corresponding author.

streptococcal strains grown overnight on horse blood agar. The inoculated broths were incubated for 4 h at 37°C and then centrifuged. The supernatants were collected, and the pH was adjusted to 7.2. The culture supernatants were deemed to contain SO if, when activated by thiol, they caused lysis of SRBC and if this lysis was inhibited by anti-SO (ASO). SLF was deemed to be contained in the same supernatants if, in the absence of thiol activation, β-SRBC were lysed at 37°C. Commercially obtained SO (Behring), reconstituted and used according to the directions of the manufacturer, was used as a control throughout the series of experiments.

SO and SLF activity on SRBC and β-SRBC. SO titers were determined by adding 0.75 ml of an SRBC suspension to 0.25-ml volumes of culture supernatants serially diluted in PBS with 0.024 M sodium thioglycolate. The SRBC suspension comprised 0.25 ml of a 2% suspension of triple-washed SRBC and 0.5 ml of PBS. The SRBC-supernatant mixture was incubated for 30 min at 37°C, and the titer was recorded as the reciprocal of the last dilution showing more than 50% hemolysis.

SLF titers were determined by adding 0.75 ml of a β-SRBC suspension to 0.25-ml volumes of culture supernatants serially diluted in PBS without sodium thioglycolate. The β-SRBC suspension comprised 0.5 ml of the staphylococcal beta-lysin preparation mixed with 0.25 ml of a 2% suspension of triple-washed SRBC and incubated for 30 min at 37°C. The β-SRBC–supernatant mixture was incubated for a further 30 min at 37°C, at which time endpoints were determined as for SO titers. The activity of thiol-activated supernatants on β-SRBC and of unactivated supernatants on SRBC were determined by similar procedures.

Comparison of the characteristics of SO and SLF. (i) Effect of temperature. The effect of temperature on SO and SLF titers was determined by heating equal volumes of culture supernatants at 60 and 100°C for 60 min and, after cooling, comparing the titers present with those demonstrated in unheated portions of the same supernatants.

(ii) Effect of SO inhibitors. The effect of SO inhibitors on SO and SLF titers was determined in the presence of cholesterol, trypsin, and ASO. To test for the effects of cholesterol, 0.025 ml of a 1 mM preparation was incubated with 4.2 ml of culture supernatant for 10 min before being mixed with SRBC or β-SRBC. For trypsin, preincubation of supernatants and their dilutions for 15 min at 37°C in the presence of 250 μg of the enzyme per ml was employed. ASO (Hyland) was reconstituted according to the directions of the manufacturer and added to serial dilutions of culture supernatants. The ASO-supernatant mixtures were incubated at 37°C for 30 min, at which time either SRBC or β-SRBC was added and titers were determined. As a control for staphylococcal beta-lysin activity, the above experiments were repeated with β-SRBC in the presence of cholesterol, trypsin, and ASO but with PBS substituted for culture supernatants.

(iii) Kinetics of SO and SLF production. The kinetics of SO and SLF production by strain A_3 were studied by inoculating 2×10^7 CFU of the organism per ml into a number of tubes each containing 3 ml of Todd-Hewitt broth (Oxoid Ltd.). The inoculated broths were incubated at 37°C in a shaking water bath, and the optical density of the cultures was determined at 2-h intervals for 8 h of incubation and also after 24 h of incubation with a Bausch & Lomb Spectronic 20 spectrophotometer. SO and SLF titers in culture supernatants were determined after 2, 4, 6, 8, and 24 h of incubation.

Production of immune rabbit sera and determination of immune titers. Culture supernatants from strains A_3 and ATCC 38601 were prepared as previously described and filter sterilized. The culture filtrates were mixed with equal volumes of Freund incomplete adjuvant (Difco Laboratories), and 6 ml of each filtrate-adjuvant mixture was injected intramuscularly into a separate pair of New Zealand white rabbits. Unused filtrate was stored at −4°C, and further intramuscular injections of 6 ml of filtrate only were given at 9 and 12 weeks after the initial injection. Sera were obtained from each animal and from an uninoculated control rabbit before the first injection and at 14 weeks after the immunization procedure.

Anti-SO and anti-SLF titers in rabbit sera were determined by a microtiter technique by using serum from the uninoculated rabbit as a negative control and commercial SO, used with and without thiol activation, as a further control. The SO preparations used were derived from culture supernatants of strains A_3 and ATCC 38601 and from the commercial SO preparation, all diluted with PBS to obtain an SO titer of 2 when thiol activated. These SO preparations were added as 0.025-ml volumes to 0.025-ml volumes of serially diluted (with PBS) rabbit sera. The SO-rabbit serum mixtures were incubated at 37°C for 30 min, at which time 0.05 ml of a 1% suspension of SRBC was added, and the mixture was incubated for a further 45 min at 37°C. The ASO titer was recorded as the reciprocal of the last dilution of rabbit serum which inhibited lysis of 50% or more of the SRBC. The SLF preparations were similarly derived and prepared to obtain an SLF titer of 2, but were not thiol activated; for anti-SLF titers β-SRBC were substituted for SRBC. The final incubation period after addition of β-SRBC was reduced to 15 min. Procedures for anti-SLF determinations were otherwise similar to ASO titer estimation.

RESULTS

SO and SLF activity in culture supernatants of group A streptococci. Supernatants of 4-h broth cultures of strains A_3 and ATCC 38601 contained a thiol-activated hemolysin (SO) whose activity on SRBC was neutralized by ASO. The same culture supernatants, in the absence of thiol, had a capacity to lyse β-SRBC, i.e., SLF activity. The titers of SO and SLF obtained are shown in Table 1, which represents the results of typical experiments.

In addition, it was noted that thiol-activated supernatants produced higher lytic titers for both SRBC and β-SRBC than did unactivated supernatants (Table 1). Also, lytic titers for

TABLE 1. Lysis of SRBC and β-SRBC by culture supernatants of group A streptococci and a commercial SO preparation[a]

Source of lysins[b]	Lytic titers using:	
	SRBC	β-SRBC
Strain A_3		
Unactivated	0	2
Activated	4	8
Strain ATCC 38601		
Unactivated	1	8
Activated	8	16
Commercial SO		
Unactivated	2	8
Activated	4	16

[a] Titers obtained with and without thiol activation.
[b] Activation was by 0.024 M sodium thioglycolate.

β-SRBC induced by activated and unactivated preparations were higher than those obtained with the same preparation acting on SRBC. The commercial SO preparation gave a pattern of results similar to those obtained with the streptococcal culture supernatants.

Comparison of properties of SO and SLF. (i) Effect of temperature and SO inhibitors. The effect of temperature and SO inhibitors on SO and SLF titers are shown in Table 2. These titers represent the results of typical experiments. Heating at 60 or 100°C for 60 min completely abolished all SO and SLF activity in the culture supernatants and the commercial SO preparation. Similarly, the presence of cholesterol, trypsin, or ASO either abolished or greatly decreased both SO and SLF activity simultaneously. (The activity of staphylococcal beta-lysin as revealed by hot-cold titers is necessary for the demonstration of SLF titers. This activity was not affected when heated culture supernatants, cholesterol, or trypsin were employed. Addition of ASO to the test system decreased this hot-cold titer from 16 to 8 [see above].)

(ii) Kinetics of production of SO and SLF. Both SO and SLF were produced during the exponential phase of growth. The maximum titer of 4 obtained for SO and the maximum SLF titer of 8 were recorded after 4 h of incubation. These levels of activity were maintained in samples titrated over the next 4 h, at which time both titers then declined. SLF was not detected in 24-h cultures, and SO was present in a titer of 2 after 24 h of incubation.

(iii) Neutralizing (anti-SO and anti-SLF) titers obtained in immunized rabbits. Rabbits immunized with sterile culture supernatants of strains A_3 and ATCC 38601 developed inhibitory titers to SO and SLF. This was demonstrated by the presence of rising immune titers in sera obtained 14 weeks after immunization and the absence of any such rise in serum from the control animal. The rising antibody titers to SO and SLF demonstrated in sera from each immunized rabbit were directed against the SO and SLF contained in culture supernatants of strains A_3 and ATCC 38601. These sera also inhibited the lytic activity of the commercial SO preparation on SRBC and β-SRBC. The immune titers demonstrated by using the commercial SO preparation as the lytic antigen are shown in Table 3. These titers represent the results of typical experiments. (Immune rabbit sera and serum from the control rabbit decreased the staphylococcal hot-cold titers from 16 to 8, an effect coinciding with that observed with the commercial ASO control. This effect was observed in a large number of repeat tests and probably represents stabilization of the depleted erythrocyte membrane by serum factors.)

TABLE 2. Effect of temperature and SO inhibitors on SO[a] and SLF[b] titers

Source of lysins	Titers obtained with supernatants			
	Untreated	Exposed to:[c]		
		Cholesterol	Trypsin	ASO
SO				
Strain A_3	4	0	0	0
Strain ATCC 38601	8	0	1	1
Commercial SO	4	1	0	1
SLF				
Strain A_3	2	0	0	0
Strain ATCC 38601	8	1	1	2
Commercial SO	8	1	0	1

[a] SO action of thiol-activated supernatants on SRBC.
[b] SLF action of unactivated supernatants on β-SRBC.
[c] All titers were 0 for lysins exposed to heat.

TABLE 3. SO and SLF neutralizing titers obtained in rabbits 14 weeks after immunization with group A streptococcal culture supernatants

Source of immunizing culture[a]	Rabbit	Neutralization titer		
		Anti-SO		Anti-SLF[b]
		0 wk	14 wk	14 wk
Strain A_3	1	4	64	16
Strain A_3	2	2	128	32
Strain ATCC 38601	3	8	256	64
Strain ATCC 38601	4	4	64	32
No immunization	5	2	2	2

[a] Commercial SO was used as the lytic antigen in this series of neutralization experiments.
[b] Titers were 1 for all rabbits at 0 week.

DISCUSSION

Under appropriate conditions, group A streptococci will produce, with staphylococcal beta-lysin (sphingomyelinase), a synergistic lysis of SRBC similar in appearance to the CAMP reaction of GBS (5). It would appear from the results of this study that the synergistic lytic effect observed with group A streptococci in the CAMP test system is due to the action of streptolysin O on sphingomyelinase-depleted SRBC.

The hypothetical SLF, postulated for the purposes of this study as being responsible for the CAMP-like reactions of group A streptococci, would appear to have properties identical to those of SO. Both SO and SLF were produced during the logarithmic phase of growth, and the activity of each lysin was increased in the presence of thiol but abolished or decreased by cholesterol, trypsin, ASO, and heating. Further, SLF was antigenic when injected into rabbits, with the rise in anti-SLF titers paralleling the rise in ASO titers in the same animals. SLF also behaved in a similar fashion to commercially prepared SO, and anti-SLF was indistinguishable in its action from commercial ASO. It is possible that SLF and SO are separate extracellular products of group A streptococci and that commercial SO and ASO contain contaminating SLF and anti-SLF, respectively. However, it is highly unlikely that two separate lysins with characteristics which appear to be identical exist when the properties of SO can account for the phenomenon observed.

Streptolysin O is a cytolysin capable of altering membranes of erythrocytes from a large number of species and also of affecting the permeability of membranes associated with subcellular organelles (15). This activity occurs in the reduced state, but SO is readily oxidized (11), and for maximal SO activity, activation by thiol or other reducing agents is employed (15). However, SO produced in broth cultures is never entirely oxidized, and some activity is present even in the absence of reducing agents (11). Sphingomyelinase-depleted SRBC (β-SRBC) are highly susceptible to lysis by a wide variety of physical, chemical, and biological agents (14) and are also more liable to lysis by SO than SRBC not exposed to staphylococcal sphingomyelinase (Table 1). It would seem that the SO produced by group A streptococci in the CAMP test system has sufficient activity to lyse the fragile β-SRBC used.

It is difficult to implicate streptolysin S, the other major

extracellular hemolysin of *Streptococcus pyogenes*, as the agent involved in the reactions demonstrated. The activity of streptolysin S is expressed in cell-free culture filtrates only when a carrier molecule, e.g., Tween or albumen, is available to stabilize the hemolytic portion (8). In addition, streptolysin S is oxygen stable and non-immunogenic (8).

The properties of SO of group A streptococci are also distinct from those of the CAMP protein of GBS. The CAMP factor causes a nonenzymatic disruption of sphingomyelin-depleted bovine erythrocytes or SRBC only, having no effect on the erythrocytes of other species or on intact erythrocytes (1). Further differences between SO and CAMP factor include the relative heat stability of the CAMP factor (1) and the failure to increase CAMP factor activity with thiol (unpublished data).

The cause of the false-positive reactions by group A streptococci in the plate CAMP test was not investigated here. It is interesting to note, however, that when anaerobic conditions, which enhance SO activity, were employed for the performance of the plate CAMP test, the lytic effect of group A streptococci on β-SRBC was more pronounced (4). In this context it is pertinent to recall that false-positive CAMP tests have also been recorded with strains of *Listeria monocytogenes* (7). These organisms produce an extracellular lysin, listeriolysin, with properties closely related to those of SO (15).

This knowledge of the probable mechanism of false-positive CAMP reactions of group A streptococci not only accounts for this previously unexplained phenomenon but also raises the possibility of eliminating these reactions. In theory this may be achieved by employing SO inhibitors which do not simultaneously affect staphylococcal beta-lysin or CAMP factor of GBS, so that increased specificity, without loss of sensitivity, may be obtained in CAMP tests. Preliminary results indicate that with the RTCT system at least, this aim may be realized through use of heated culture supernatants. This inactivates the heat-labile SO of group A streptococci but not the heat-stable CAMP factor of GBS.

LITERATURE CITED

1. **Bernheimer, A. W., R. Linder, and L. S. Avigad.** 1979. Nature and mechanism of action of the CAMP protein of group B streptococci. Infect. Immun. **23**:838–844.
2. **Bryce, L. M., and P. M. Rountree.** 1936. The production of beta-toxin by staphylococci. J. Pathol. Bacteriol. **43**:173–189.
3. **Christie, R., N. E. Atkins, and E. Munch-Petersen.** 1944. A note on a lytic phenomenon shown by group B streptococci. Aust. J. Exp. Biol. Med. Sci. **22**:197–200.
4. **Darling, C. L.** 1975. Standardization and evaluation of the CAMP reaction for the prompt, presumptive identification of *Streptococcus agalactiae* (Lancefield group B) in clinical material. J. Clin. Microbiol. **1**:171–174.
5. **Esseveld, H., M. S. M. Daniels-Bosman, and B. Leijnse.** 1958. Some observations about the CAMP reaction and its application to human beta-haemolytic streptococci. Antonie van Leeuwenhoek J. Microbiol. Serol. **24**:145–156.
6. **Facklam, R. R., J. F. Padula, E. C. Wortham, R. C. Cooksey, and H. A. Rountree.** 1979. Presumptive identification of group A, B, and D streptococci on agar plate media. J. Clin. Microbiol. **9**:665–672.
7. **Fraser, G.** 1964. The effect on animal erythrocytes of combinations of diffusible substances produced by bacteria. J. Pathol. Bacteriol. **88**:43–53.
8. **Ginsberg, I.** 1970. Streptolysin S, p. 99–171. *In* T. C. Montie, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. 3. Bacterial protein toxins. Academic Press., Inc., New York.
9. **Gow, J. A., and J. Robinson.** 1969. Properties of purified staphylococcal β-hemolysin. J. Bacteriol. **97**:1026–1032.
10. **Haque, R., and J. N. Baldwin.** 1964. Purification and properties of staphylococcal beta-hemolysin. I. Production of beta-hemolysin. J. Bacteriol. **88**:1304–1309.
11. **Herbert, D., and E. W. Todd.** 1941. Purification and properties of a haemolysin produced by group A haemolytic streptococci (streptolysin O). Biochem. J. **35**:1124–1139.
12. **Liao, S. J.** 1951. A modification of the anti-streptolysin test. J. Lab. Clin. Med. **38**:648–659.
13. **Phillips, E. A., J. W. Tapsall, and D. D. Smith.** 1980. Rapid tube CAMP test for identification of *Streptococcus agalactiae* (Lancefield group B). J. Clin. Microbiol. **12**:135–137.
14. **Pulsford, M. F.** 1954. Factors affecting the lysis of erythrocytes treated with staphylococcal beta-lysin. Aust. J. Exp. Biol. Med. Sci. **32**:347–352.
15. **Smyth, C. J., and J. L. Duncan.** 1978. Thiol-activated (oxygen-labile) cytolysins, p. 129–183. *In* J. Jeljaszewicz and T. Wadstrom (ed.), Bacterial toxins and cell membranes. Academic Press, Inc., New York.
16. **Smyth, C. J., R. Möllby, and T. Wadström.** 1975. Phenomenon of hot-cold hemolysis: chelator-induced lysis of sphingomyelinase-treated erythrocytes. Infect. Immun. **12**:1104–1111.