Similarities between Complement-mediated and Streptolysin S-mediated Hemolysis*

The oxygen-stable hemolysin streptolysin S (SLS) of Streptococcus pyogenes is encoded in part by the pel/sagA gene product. Antibodies to a synthetic peptide from the C terminus of the Pel/SagA open reading frame inhibited hemolysis mediated by both culture supernatants from multiple M serotypes of S. pyogenes isolates or a commercially available SLS preparation. Analysis of the SLS-mediated hemolytic reaction demonstrated that it was temperature- and concentration-dependent. Like complement-mediated hemolysis it conforms to the prediction of a one-hit mechanism of hemolysis. A number of intermediates in the SLS-mediated hemolysis of sheep erythrocytes could be distinguished. SLS could bind to erythrocytes below 17 °C; however, lysis could only occur at temperatures >23 °C. Following binding of SLS and washing, a papain-sensitive intermediate could be distinguished prior to insertion of the SLS complex into the erythrocyte membrane, which resulted in formation of a transmembrane pore and led to irreversible osmotic lysis of the cell. These intermediates were similar to those described previously during complement-mediated hemolysis.

Despite β-hemolysis being one of the most widely recognized phenotypes of streptococci, the molecular nature and mode of action of the oxygen-stable bacterial hemolysin streptolysin S (SLS) is not understood (1, 2). SLS has been difficult to characterize because it consists of a peptide that is stabilized by a second molecular species that may be RNA, albumin, or, in model systems, a detergent like Tween (3–6).

Recent genetic studies have identified the gene pel/sagA that is required for β-hemolysis in Streptococcus pyogenes (7–9). This gene is present in group A, C, and G but not group B streptococci (10), in which a distinct gene has been shown to encode the hemolysin (11, 12). The pel/sagA gene encodes a 53-amino acid polypeptide. Based on genetic evidence, this polypeptide is predicted to contain a 17-amino acid leader sequence that would be processed to yield a 36-amino acid polypeptide (7, 8). Studies of mutants and complementation experiments using nonhemolytic Lactococcus isolates support the conclusion that the polypeptide encoded by the pel/sagA gene is indeed a key constituent of streptococcal SLS (9).

In this study we have generated monospecific polyclonal antibodies to two peptide sequences present in the Pel/SagA primary sequence and have found that the antibody to a C-terminal peptide neutralizes hemolysis of sheep erythrocytes mediated by SLS isolated from opacity factor-positive and -negative S. pyogenes isolates of various M serotypes. The availability of this antibody now enables the study of a defined streptococcal hemolysin and analysis of its mode of action in mediating red cell hemolysis.

EXPERIMENTAL PROCEDURES

Bacteria and Culture Conditions—S. pyogenes strains were grown in Todd-Hewitt broth yeast broth supplemented with 0.5% yeast extract at 37 °C with 10% CO₂.

Buffers and Solutions—Isotonic veronal-buffered saline (VBS), pH 7.4, containing 0.1% gelatin (Mann Research Labs, New York) (VBS gel) was prepared as described (13).

Chemicals and Enzymes—Glucose-monohydrate was obtained from Baker Chemical Co. (Philadelphia, PA), sucrose (ultracentrifuge grade) was obtained from Schwarz-Mann (Orangeburg, NY), and raffinose pentahydrate was obtained from (Vega Chemicals, Tucson, AZ). All sugar solutions were 0.3 M in water. Trypan blue (Matheson, Coleman, and Bell, Norwood, OH) was prepared as a stock solution in distilled water at 1.6 mM. Papain was obtained from Sigma.

SLS—A commercial source of SLS (Sigma) was used for certain experiments. This preparation was derived from S. pyogenes and contains the SLS peptide with an RNA core.

SLS-mediated Hemolysis Assay—Sheep red blood cells (Pel Freez Biologicals, Rodgers, AR) were collected and washed as described in Ref. 13. Red cells were standardized to a concentration of 1.5 × 10⁸/ml based on their hemoglobin content as described previously (13). An aliquot of 100 µl of culture supernatant or a dilution of a commercial SLS preparation was added to a test tube containing a fixed amount of red blood cells (1.5 × 10⁷ cells) in a final volume of 1.5 ml VBS gel. The samples were incubated at the indicated temperature. The extent of hemolysis was determined by measuring the hemoglobin released into the supernatants at A₅₄₁ following pelleting of unlysed erythrocytes by centrifugation at 3000 × g for 5 min.

This basic hemolytic assay was modified to include binding of SLS at different temperatures and washing to remove unbound SLS to generate a SLS-bound erythrocyte intermediate. For incubation studies using trypan blue, the hemolysin release in the supernatant could not be quantified because of interference from the dye. Consequently, the number of unlysed cells was determined following washing and lysis of the cell pellet with 1.5 ml of H₂O and determining the concentration of hemoglobin at A₅₄₁.

Analysis of the effect of papain treatment of erythrocytes to which SLS was then bound on subsequent hemolytic events was performed as described in the text. In related experiments erythrocytes with SLS bound were incubated in isotonic solutions of different sugars to determine the effect of molecules of varying Stokes radius on SLS-mediated...
hemolysis. This experimental approach has previously been used to identify differences in the pore size of complement channels present in erythrocyte membranes as a function of C8:C9 ratios (14).

Generation of Antibodies to Synthetic Pel Peptides and Hemolysis Inhibition Experiments—A synthetic C-terminal peptide (N-TGS-GNSSQGSGSYTPGK-C) that corresponded to amino acids 37–53 of the predicted pel ORF from S. pyogenes was synthesized by Eurogentec (Herstal, Belgium). It was coupled to keyhole limpet hemocyanin and used to immunize rabbits following standard immunization protocols. Enzyme-linked immunosorbent assays were run to monitor the generation of specific antibodies to the C-terminal synthetic peptide. IgG was purified from the final bleed and further affinity-purified on a peptide-Sepharose affinity column.

The second peptide (N-CCCCCTTCFFSIA-C) corresponded to amino acids 24–36 of the predicted pel ORF from S. pyogenes and was synthesized by Bachem (Heidelberg, Germany). Antibodies to the N-terminal peptide were produced as described for the C-terminal peptide (Eurogentec, Herstal, Belgium). For inhibition experiments culture supernatants or a commercial SLS preparation was preincubated with the indicated concentration of antibody for 10 min at 37 °C, before the mixture was added to erythrocytes and the hemolytic assay was performed as described above.

SELDI-TOF Analysis of SLS Preparations—SELDI-TOF mass spectrometry analysis was carried out using the Ciphergen protein chip system (Ciphergen, Palo Alto, CA) as described in Ref. 15. For all of the studies described, SELDI analysis was performed using a hydrophobic H4 protein chip CiphergenTM. The H4 chip contains a long chain aliphatic surface that binds proteins by reverse phase interaction.

Three to five microliters of sample diluted in distilled water were applied to a spot on an H4 chip and allowed to air dry and then washed with 3 µl of H2O and allowed to dry. To the dry spot, 0.5 µl of an energy absorbing molecule (EAM) was added. The EAM consisted of a saturated solution of 3.5-dimethoxy-4-hydroxycinnamic or sinapinic acid (Sigma), 50% acetonitrile, and 0.5% trifluoroacetic acid. EAM was added and allowed to air dry. Once dry, a second application of EAM solution was added and allowed to cristalize. The sample was transferred to the Ciphergen SELDI reader and analyzed following desorption of bound proteins by short intense probes from an N2 320-nm UV laser. The profile of bound proteins was determined by time of flight in a mass spectrometer.

RESULTS

Properties of SLS Preparation—As noted in the introduction, the precise chemical nature of SLS is unknown. In this study we have used a commercially available, functionally active form of the hemolysin derived from S. pyogenes. This preparation (lot 0274 4112) contained 2.9% protein by weight, and the balance of the material was core RNA and salt. The majority of the sample was insoluble in water at 4 °C; however, the soluble fraction post-centrifugation (13,000 × g for 10 min) contained >90% of the hemolytic activity (data not shown).

SDS-polyacrylamide gel electrophoresis analysis and silver staining of the hemolytically active soluble SLS failed to identify any polypeptide band. (Note that a 1:10,000 dilution of this preparation could completely lyse 1.5 × 106 sheep erythrocytes within 30 min at 37 °C.) An aliquot of the soluble hemolysin preparation was subjected to analysis by SELDI-TOF (Ciphergen) using an H4 protein chip. The H4 chip contains a long chain aliphatic surface that binds proteins by reverse phase interactions.

Analysis of soluble fraction containing the hemolysin revealed the presence of a predominant 4,702 ± 20-Da peak (Fig. 1, top panel), which was removed after incubation with 0.5 ml of packed sheep erythrocytes on ice (Fig. 1, bottom panel). The erythrocyte preparation used for affinity purification was completely washed and further affinity-purified at 37 °C for 60 min.

In addition to its hemolytic potential, a second well described property of SLS is the ability of the vital dye trypan blue to inhibit hemolysis (1, 2). To evaluate the effects of trypan blue on the SLS preparation, red cells were incubated in the presence of 25% of differing concentrations of trypan blue and a SLS dose that resulted in ~50% end point hemolysis in the absence of any inhibitor. Following a 1-h incubation at 37 °C, unlysed cells were pelleted and washed to remove any released hemoglobin and trypan blue. The remaining intact cells were lysed by addition of water and the number of intact erythrocytes determined. By comparing the quantity of hemoglobin present in lysates of untreated cells, cells treated with SLS alone or cells treated with SLS and trypan blue, the effect of the dye on hemolysis was determined. In agreement with previous reports (1, 2), trypan blue acted as a dose-dependent inhibitor of SLS-mediated hemolysis (data not shown).

The SLS preparation was further characterized using an immunological approach. Antibodies generated to synthetic peptides from the predicted processed form of the pel ORF were generated as described under “Experimental Procedures.” Two antibody preparations were evaluated. One antibody was generated to an N-terminal synthetic peptide of the predicted processed pel gene product, and the second was generated to a predicted C-terminal synthetic peptide. Dilutions of the affinity-purified N-terminal and C-terminal antibodies were tested for their ability to inhibit SLS-mediated hemolysis of sheep red blood cells.

The C-terminal antibody was able to inhibit SLS-mediated hemolysis in a dose-dependent manner (Fig. 2). Neither the N-terminal antibody nor normal rabbit serum control was able to neutralize the hemolytic effects of SLS. The failure of the N-terminal peptide antibody to inhibit SLS-mediated hemolysis might relate to the failure of the peptide immunogen to adopt the three-dimensional configuration of the native active SLS molecule or may show that this region of the protein is not present in the active molecule.

The C-terminal antibody was also found to be capable of neutralizing the hemolysin present in culture supernatants of different M serotypes of S. pyogenes (Fig. 3). These studies further support the conclusion that the oxygen-stable hemolysin of streptococci involves the product of the pel/baga gene.

Characteristics of SLS-mediated Hemolysis—The next series of experiments were designed to analyze the mechanism of hemolysis mediated by SLS. In the initial experiments, differing concentrations of SLS were added to a fixed number of red cells (1.5 × 107), and the kinetics of lysis were analyzed. After a short lag period hemolysis occurred and reached an end point within 30–45 min at 37 °C (Fig. 4A). At high SLS concentrations, 100% of the erythrocytes could be lysed.

FIG. 1. SELDI-TOF analysis of soluble SLS preparation following preincubation with or without sheep erythrocytes. 100 µl of SLS diluted 1:10 was incubated with buffer alone (top panel) or 107 packed sheep erythrocytes (bottom panel) for 15 min on ice. The erythrocytes were removed by centrifugation, and 3 µl of the absorbed or unabsorbed sample was spotted on an H4 hydrophobic chip (Ciphergen) and reacted with an EAM before analysis in the SELDI-Ciphergen protein chip reader. Note the disappearance of the major 4,702-Da peak following absorption with sheep erythrocytes.
mediated hemolysis was time-dependent. In addition, evidence for with previous studies using rabbit erythrocytes (19), SLS-kinetics of hemolysis were measured (Fig. 5). In agreement determined. Differing concentrations of SLS were incubated the effect of temperature on SLS-mediated hemolysis was temperature-dependent mechanism (16). Bacterial toxins have been shown to act through a multi-step, monitored. The average number of lytic sites/cell (Fig. 4) was calculated using the Poisson transformation:

\[ Y = \frac{C}{Z} \]

where

\[ C \] is the number of lytic sites/cell and \( Z \) is the SLS concentration. To control for nonspecific effects, dilutions of normal rabbit serum (filled circles) from 1:10 to 1:800 were included. Only the C-terminal specific antibody had any affect on SLS-mediated hemolysis.

To determine the dose-dependent characteristics of SLS-mediated hemolysis, a fixed number of erythrocytes (1.5 \( \times 10^8 \)) were incubated with differing concentrations of SLS, and the extent of hemolysis was determined following 60 min of incubation at 37 °C (Fig. 4B). The dose response was concave to the abscissa, consistent with a one-hit mechanism of SLS-mediated hemolysis, i.e. one functional SLS molecule being necessary and sufficient to lyse one erythrocyte.

To confirm the one-hit nature of SLS-mediated hemolysis, the effect of varying the number of target erythrocytes on the extent of hemolysis mediated by a fixed concentration of SLS was determined. The average number of lytic sites/cell (Z) was calculated using the Poisson transformation: Z = −ln (1 - Y), where Y is the fraction of cells lysed. The average number of lytic sites/cell was directly proportional to the SLS concentration for each cell concentration tested. Furthermore, the average number of lytic sites/cell varied proportionally with cell number (Fig. 4C).

Taken together, these results demonstrate that SLS-mediated hemolysis follows the predictions for a one-hit mechanism of hemolysis, i.e. one molecule of the SLS complex is necessary and sufficient to lyse one red blood cell. This enables a sensitive assay for functional SLS activity in any sample to be performed.

Effect of Temperature on SLS-mediated Hemolysis—Several bacterial toxins have been shown to act through a multi-step, temperature-dependent mechanism (16–20). Consequently, the effect of temperature on SLS-mediated hemolysis was determined. Differing concentrations of SLS were incubated with sheep erythrocytes at 17, 20, 24, and 37 °C, and the kinetics of hemolysis were measured (Fig. 5). In agreement with previous studies using rabbit erythrocytes (19), SLS-mediated hemolysis was time-dependent. In addition, evidence for a critical temperature-dependent step was apparent (Fig. 5).

At 17 °C no hemolysis was seen (Fig. 5A); however, as the temperature was increased (Fig. 5, B–D), hemolysis was observed over time. For each SLS concentration a similar end point was reached at each temperature above 23 °C. Below 23 °C no lysis occurred for any SLS concentration (data not shown). The time required for maximal lysis was dependent on the temperature of the reaction. The lag time before any hemolysis was detected correlated with temperature. At lower temperatures, the lag period was prolonged (compare the 1:800 dilutions of SLS in Fig. 5, B and D).

A similar pattern of temperature dependence has previously been described for complement-mediated hemolysis (21). In that system, a critical temperature was also identified that was required to enable the membrane attack complex to insert into erythrocyte membrane as well as a subsequent temperature-dependent reaction that influenced the rate of hemolysis (21). Previous studies of complement-mediated hemolysis have identified a series of intermediate steps in the hemolytic reaction mediated by C9 (22). These included binding of the final complement component (C9), which can occur on ice, a temperature-dependent event that was related to C9 insertion into the erythrocyte membrane followed by rearrangement or activation to yield a transmembrane pore that allowed lysis to occur in a temperature-independent colloid osmotic step (22).

To determine whether SLS-mediated hemolysis followed a sequence of events similar to that of the complement-mediated hemolytic reaction, a parallel experimental strategy was employed. Initially the ability of SLS to bind to red cells at 17 °C, a temperature at which no hemolysis occurs (Fig. 5A), was tested. A concentration of SLS that resulted in ~50% hemolysis, if incubated with the same number of red cells for 1 h at 37 °C, was used. Parallel samples were incubated for 5, 10, or 15 min at 17 °C, the cells were centrifuged at 3000 × g for 5 min and washed with VBS gel to remove any unbound SLS. The washed cell pellet was resuspended in VBS gel buffer, and duplicate samples were incubated at either 17 or 37 °C for 1 h. At the end of this second incubation period hemolysis was determined by pelleting intact erythrocytes and measuring released hemoglobin. The binding of SLS to the red cells was found to be complete within 15 min at 17 °C, and no erythrocytes were lysed unless subsequently incubated at 37 °C (data not shown).

The kinetics of lysis of red blood cells to which SLS was bound for 15 min at 17 °C was determined over time at either 17 or 37 °C. The kinetics of hemolysis for SLS bound at 17 °C and subsequently incubated at 37 °C was similar to that observed when SLS was present at 37 °C throughout the reaction (compare Figs. 4B and 6). No lysis occurred if the cells were incubated at 17 °C throughout (Fig. 6). These results were consistent with some temperature-dependent rearrangement of the bound SLS molecule being required before hemolysis occurred.

In an attempt to determine whether the hemolytic potential of bound SLS could be reversed by protease treatment, SLS was bound to sheep erythrocytes at 17 °C for 15 min. The cells were washed to remove unbound SLS, and aliquots were treated with different concentrations of a number of different proteases before washing and incubation at 37 °C to determine the extent of hemolysis. The hemolytic potential of bound SLS was most efficiently reversed by treatment with papain (data not shown).

Having established conditions of papain treatment at 17 °C that could reverse the potential hemolysis of SLS bound to red cells, the next experiment was designed to determine whether, at some point prior to the cells lysing, an intermediate could be identified that was resistant to papain treatment, i.e. papain was no longer able to prevent SLS bound to red cells from

![Fig. 2. Effect of a polyclonal antibody specific for either a C- or N-terminal peptide of Pel on SLS-mediated hemolysis. Affinity-purified antibodies raised against either an N-terminal or C-terminal synthetic peptide, corresponding to the predicted sequence of the Pel polypeptide, were tested for their ability to inhibit SLS-mediated hemolysis. Antibodies to both the N-terminal (filled diamonds) and C-terminal (filled circles) peptides were diluted 2-fold starting at a 100 μg/ml. Aliquots of different concentrations of each antibody (100 μl) were added to a dilution of SLS (Sigma) that would cause ~50% lysis at end point when added to sheep erythrocytes in a standard hemolytic assay. To control for nonspecific effects, dilutions of normal rabbit serum (filled squares) from 1:10 to 1:800 were included. Only the C-terminal specific antibody had any affect on SLS-mediated hemolysis.](http://www.jbc.org/)
mediating lysis. For these studies, SLS-bound red cells were prepared by incubating red cells with SLS for 15 min at 17 °C and washing. Aliquots of cells to which SLS was bound were incubated for varying periods at 23 °C before being subjected to a papain treatment at 17 °C. The effects of papain treatment were then evaluated by incubating the washed enzyme-treated cell pellet at 37 °C for 60 min.

A time-dependent conversion of SLS-coated erythrocytes from a papain-sensitive state to a papain-resistant, nonlysed, intermediate was observed (Fig. 7, black bars). Incubation at 23 °C, in the absence of papain, did not affect the subsequent ability of SLS to lyse the erythrocytes (Fig. 7, hatched bars). These results are consistent with SLS becoming an integral membrane protein as a result of inserting into the erythrocyte membrane in a time- and temperature-dependent reaction.

Based on previous studies of complement-mediated hemolysis (24, 25), it should be possible to distinguish between the insertion of SLS into the membrane and the osmotic lysis of the cells, provided SLS mediates its hemolytic potential in a similar way to complement by formation of a transmembrane pore. To test this prediction SLS was bound to red cells at 17 °C, washed, and then suspended in osmolar solutions of NaCl (Stoke’s radius, 0.14 nm) glucose (0.36 nm), sucrose (0.46 nm), or raffinose (0.56 nm). The cells were incubated for 1 h at 37 °C, and the extent of hemolysis was determined. In the presence of isotonic solution of either glucose, sucrose, or raffinose hemolysis of SLS-coated erythrocytes was inhibited as compared with aliquots of cells incubated in an isotonic NaCl solution (Fig. 8, black bars).

This inhibition was attributable to the osmotic blocking by the sugar solutions because reincubation of any cell pellets in NaCl results in the expected level of hemolysis when incubated at 4 °C for 60 min (Fig. 8, hatched bars). The results of these studies indicate that SLS-mediated hemolysis involves disruption of the semi-permeable properties of a red cell by formation of a defined size pore in the membrane. Based on the properties of the blocking sugars tested, the predicted pore size is between 0.14 and 0.36 nm.

DISCUSSION

The oxygen-stable hemolysin SLS of group A (S. pyogenes), C, and G streptococci has never been fully characterized. Despite the functional property of hemolysis being easy to quantify the predicted protein composition of SLS from different isolates has varied widely (4–6). Recent genetic studies have identified a locus in S. pyogenes that has been convincingly associated with encoding this oxygen-stable hemolysin (7–9). The predicted ORF encoding the hemolysin, pel/sagA, is present in group A, C, and G streptococci but not group B (10). These findings are consistent with earlier studies that have indicated the oxygen-stable β hemolysin associated with group
B isolates is functionally distinct from that of other \( \beta \) hemolytic streptococci (11, 12).

By using antibodies generated to synthetic peptides based on the predicted primary sequence of the pel/sagA gene ORF, we have demonstrated that an antibody specific to a C-terminal peptide of Pel totally inhibits SLS-mediated hemolysis by several different serotypes of \( S. \) pyogenes as well as a commercial preparation of SLS. This is the first report of a neutralizing antibody specific for SLS. Biochemically purified SLS is not immunogenic, and no anti-SLS antibodies are generated in the human host during infection (1).

FIG. 5. Effects of temperature on SLS-mediated hemolysis. Two concentrations of SLS (1:100, triangles; 1:800, circles) were incubated with \( 1.5 \times 10^7 \) sheep erythrocytes in a fixed volume of 1.5 ml of VBS gel at 17 °C (A), 20 °C (B), 24 °C (C), or 37 °C (D), and the kinetics of hemolysis were measured as described under “Experimental Procedures.”

FIG. 6. Effect of temperature on SLS binding and SLS-mediated hemolysis. SLS was preincubated with red blood cells at 17 °C for 15 min with a SLS concentration that resulted in ∼50% hemolysis if incubated with the same number of red cells for 1 h at 37 °C. The cells were washed with ice-cold VBS gel and then resuspended in 1.5 ml of VBS gel and incubated at either 37 or 17 °C for varying times. Each sample was resuspended. At varying times, hemolysis was determined by pelleting intact erythrocytes and measuring the release of hemoglobin as described under “Experimental Procedures.”

FIG. 7. Effect of papain treatment on SLS-mediated hemolysis. Red cells to which SLS had been bound by incubation at 17 °C, followed by a washing step, were treated with papain for 15 min at 17 °C to determine whether bound SLS had the properties of an extrinsic or intrinsic membrane protein. This procedure was repeated with a pretreatment step at 23 °C for varying times prior to papain treatment. Following a washing step, the cell pellet was incubated for 1 h at 37 °C, and the extent of hemolysis was determined (solid bars). A control sample of erythrocytes with SLS bound but treated with buffer alone instead of papain was included as a control (hatched bars). Under these conditions, no hemoglobin was released from erythrocytes prior to the final incubation step at 37 °C (data not shown).
neutralizing antibody to a defined synthetic peptide could have potential implications in diagnostics and therapy of S. pyogenes.

The antibody generated to an N-terminal peptide sequence of Pel failed to neutralize SLS activity. This region of the hemolysin is rich in cysteine residues (7 of 15 residues are cysteines), and thus the synthetic peptide may not have had the appropriate three-dimensional configuration present in the native SLS molecule to form neutralizing antibodies. The inhibitory effects of the C-terminal antibody, however, indicate that the major oxygen-stable hemolysins secreted by S. pyogenes are related to the pel/sagA gene product (Fig. 2).

Based on the efficiency of the SLS preparation to mediate hemolysis at dilutions below which any protein could be detected, we cannot conclude unequivocally that the peaks observed in the SELDI-TOF analysis I (Fig. 1A) represent the functional form(s) of SLS. The finding that one SLS complex is necessary and significant to lyse an erythrocyte allows the functional assay to detect active SLS at concentrations in the subattomolar range. This coupled with the small size of the SLS peptide makes it difficult to unequivocally characterize the SLS peptide by conventional physiochemical methods.

Analysis of the hemolytic activity of a partially purified SLS preparation derived from a S. pyogenes isolate indicated that the mechanism of hemolysis was similar to that mediated by complement. Both hemolytic systems are time- and temperature-dependent as well as conforming to the predictions of a one-hit mechanism (14, 25), i.e. one complex of SLS peptide(s) and carrier or one molecule of C9 is necessary and sufficient to lyse one red blood cell.

The overall mechanism of hemolysis was also similar, involving a series of defined steps (Fig. 9). The SLS-bound intermediates were analogous to the intermediate generated by binding of C9 to EAC1–8 in the complement-dependent hemolytic reaction (22). In the complement model (Fig. 9) it was possible to distinguish two forms of cell-associated C9: a bound form that could be removed by treatment with a protease and an inserted form that could not be removed by enzymatic treatment (23).

In this study we have demonstrated equivalent intermediates in the SLS-mediated hemolytic reaction. Initially SLS bound at 17 °C could be removed or inactivated by treatment with papain; however, following a brief incubation at 23 °C, an intermediate state could be identified in which treatment of papain failed to reverse the hemolytic potential of the bound SLS molecule.

This step is associated with a change in SLS from having the properties of an extrinsic protein, which can be removed by treatment with papain to a papain-resistant form having the properties of an intrinsic membrane protein. This reaction is temperature-dependent and occurs prior to the red cell undergoing irreversible hemolysis. Analysis of the effect of antibody and trypan blue indicated that they exerted their inhibitory effect prior to binding of SLS to the erythrocyte. Neither reagent had any effect on hemolysis once SLS was bound (data not shown).

The SLS-mediated hemolysis also appears to result in pore formation. Addition of osmotic blockers of different Stokes’ radii (Fig. 8) was used to inhibit SLS-mediated hemolysis. The ability of isotonic solutions of molecules with varying Stokes’ radii to inhibit SLS-mediated hemolysis was tested. SLS was bound to the red cells at 17 °C and washed to yield a population of cells that would result in ~50% lysis when subsequently incubated in NaCl at 37 °C for 1 h. Aliquots of this cell preparation were incubated in osmolar solutions of NaCl (Stokes’ radius, 0.14 nm), glucose (Stokes’ radius, 0.36 nm), sucrose (Stokes’ radius, 0.44 nm), or raffinose (Stokes’ radius, 0.56 nm). The extent of lysis was determined following a 1-h incubation at 37 °C (solid bars) as described under Experimental Procedures. At the end of the incubation period at 37 °C unlysed cells were resuspended in 1.5 ml of NaCl, and the amount of hemolysis following a second incubation step at 4 °C for 30 min was determined (hatched bars).

**FIG. 8.** Effect of molecules with varying Stokes’ radii on SLS-mediated hemolysis. The ability of isotonic solutions of molecules with varying Stokes’ radii to inhibit SLS-mediated hemolysis was tested. SLS was bound to the red cells at 17 °C and washed to yield a population of cells that would result in ~50% lysis when subsequently incubated in NaCl at 37 °C for 1 h. Aliquots of this cell preparation were incubated in osmolar solutions of NaCl (Stokes’ radius, 0.14 nm), glucose (Stokes’ radius, 0.36 nm), sucrose (Stokes’ radius, 0.44 nm), or raffinose (Stokes’ radius, 0.56 nm). The extent of lysis was determined following a 1-h incubation at 37 °C (solid bars) as described under Experimental Procedures. At the end of the incubation period at 37 °C unlysed cells were resuspended in 1.5 ml of NaCl, and the amount of hemolysis following a second incubation step at 4 °C for 30 min was determined (hatched bars).

**FIG. 9.** Schematic representation comparing the mechanism of SLS-mediated and complement-mediated hemolysis. The mechanism of SLS-mediated lysis has been derived from the data in this study. The profile for complement-mediated lysis was summarized from published studies (22).
radii suggested formation of a pore whose size was between 0.14 and 0.36 nm. There was no evidence in these studies that these pores can aggregate or combine to create larger transmembrane channels. In earlier studies of complex-mediated hemolysis, differences in the pore size could be identified based on differences in the ratio of C8 to C9 (26, 27) or the polymerization of C5b-9 complexes (28–31) in a given complex, but the maximum size lesion was less than the Stokes’ radius of albumin, 2.5 nm.

It is of interest that earlier electron microscopy studies of SLS-mediated hemolysis failed to identify complement-like pores in red cell membranes (32). In similar studies of complement-mediated red cell lysis under conditions of limiting C9, we also failed to demonstrate the classical pore structures when examined by electron microscopy. These EM structures are only observed under conditions of excess complement that facilitate polymerization of C5b-9 complexes (33, 34). Bhakdi and Tranum-Jensen (30) have demonstrated 30-nm pore-like structures formed in erythrocyte membranes following lysis with the oxygen sensitive streptococcal hemolysin streptolysin, which has recently been shown to be part of a system mimicking type III secretion in Gram-positive bacteria (34).

The studies presented in this paper confirm the importance of the pel/sagA gene in encoding the polypeptide backbone of SLS; the precise chemical nature of the effective molecule has not yet been elucidated. The importance of the core structure is apparent because pel/sagA expressed in Escherichia coli fails to produce an active hemolysin (data not shown). The ability of group A, C, and G streptococci to secrete SLS in a soluble functionally active form that can bind to and insert into cell membranes forming a transmembrane channel is intriguing. This complex binds to an erythrocyte membrane under aqueous conditions and then undergoes a temperature-dependent rearrangement to create a stable pore in a biomolecular lipid membrane. This represents a highly evolved mechanism for a single toxin molecule.

In the case of the membrane attack complex formation of a complement pore involves five soluble plasma proteins that interact to produce a hydrophobic core that inserts and forms a pore in a red cell membrane (35–37). This hydrophilic-hydrophobic transition is achieved by structural rearrangements that occur as the five terminal complement components interact with each other and form intermediate complexes that expose hydrophobic regions that align to form the transmembrane pore (34–36). In this case, SLS, a similar pore-forming complex can be generated from a single soluble molecular complex secreted by the bacteria that on binding to a cell membrane can insert and form a transmembrane pore. At this time it is not clear whether the polypeptide component of the SLS complex is involved with the hydrophilic activity of maintaining solubility of this complex or the pore forming potential or both. A detailed analysis of the precise composition and structure of SLS will help to elucidate why this streptococcal complex is such an effective bacterial toxin.

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