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Characteristics of Streptolysin S Hemolysis

JAMES L. DUNCAN* AND LESLIE MASON

Department of Microbiology-Immunology, Northwestern University Medical and Dental Schools, Chicago, Illinois 60611

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The characteristics of hemolysis produced by streptolysin S (SLS) were investigated in rabbit erythrocytes. Treatment of erythrocytes with SLS at various temperatures prior to incubation at 37 C revealed an initial temperature-dependent interaction between toxin and the cells. No subsequent hemolysis occurred when erythrocytes were exposed to SLS at 0 to 10 C; exposure to toxin at temperatures above 10 C gradually increased the amount of hemolysis that occurred at 37 C. Very little binding of toxin to erythrocytes or their ghosts, as detected by a decrease of hemolytic activity from toxin preparations, could be demonstrated at any temperature. The release of hemoglobin after the temperature-dependent process occur at virtually the same rate at 0, 22, or 37 C. The loss of intracellular rubidium-86 (Rb) and hemoglobin from SLS-treated erythrocytes was studied. Rb^+ release significantly preceded the escape of hemoglobin, suggesting that colloid-osmotic processes play a role in SLS hemolysis.

Streptolysin S (SLS) is a cytolytic toxin produced by group A streptococci which is responsible for zones of beta-hemolysis surrounding colonies of the organism on blood agar plates. SLS is a non-immunogenic, oxygen-stable toxin; these characteristics help distinguish it from the other important hemolysin produced by these streptococci, streptolysin O (SLO). SLS can apparently exist either as a cell-bound hemolysin or as a cell-free complex with one of several carrier molecules. Cell-free SLS presumably consists of a hemolytic moiety bound to ribonucleic acid (RNA), serum albumin, Tween, or Triton. The hemolytic moiety is thought to be a polypeptide of 11 to 17 amino acids, which can be inactivated with certain proteolytic enzymes. The unrelated molecules to which the hemolytic moiety is complexed remove the active peptide from the surface of the cells and serve as carrier molecules for it. The hemolytic portion can be transferred from one carrier to another, and destruction of either the hemolytic moiety or the carrier molecule inactivates the toxin (9, 12).

In addition to its hemolytic effects on erythrocytes, SLS has been shown to be cytotoxic for a number of nucleated eukaryotic cells in culture; it is lytic for platelets and intracellular organelles, as well as for bacterial protoplasts and L-forms (3, 13). Liposome vesicles consisting of lecithin and dicetylphosphate are also readily disrupted by SLS (2, 18). Although the toxin is thought to act on the plasma membrane of susceptible cells, the mechanism of this process and the membrane alterations that are produced are not understood. Unlike SLO, SLS produces no visible alterations in the cell membrane (7, 10). The lytic activity of SLS can be inhibited by the addition of certain phospholipids, an observation that, together with the liposome studies, suggests that SLS interacts with phospholipids in the cell membrane. The nature of this interaction is not known, but SLS does not appear to be a phospholipase (11, 16).

In a recent review of streptococcal hemolysins, Bernheimer (5) suggested that SLS might alter the membrane lipids such that the cell could no longer prevent the free passage of ions across the membrane. As a result of osmotic processes, the cell would swell and the membrane eventually rupture. We have examined this possibility by studying the kinetics of the release of hemoglobin (Hb) and intracellular rubidium (Rb) from SLS-treated erythrocytes. Our findings support the idea that cell lysis by SLS is the result of an osmotic process.

MATERIALS AND METHODS

SLS preparation. SLS was prepared from strain S23g of *Streptococcus pyogenes*, which had grown for 14 h in 1 liter of Todd-Hewitt broth. After centrifugation, the cells were washed twice in phosphate-buffered saline (PBS) (NaCl, 0.137 M; K_2HPO_4, 0.01 M; K_2HPO_4, 0.003 M; pH 7.2) and incubated in 140 ml of an RNA-maltose medium (RNA core, 54.4 mg; maltose, 0.014 M; KH_2PO_4, 0.021 M; $MgSO_4$, 0.002 M) for 4 h at 37 C, essentially as described by Bernheimer (3). After centrifugation, the SLS-containing supernatant was stored at −20 C.

The yeast RNA core was prepared by digestion of RNA with bovine pancreatic ribonuclease. Yeast

RNA (100 mg/ml) was incubated with 0.1 mg of bovine pancreatic ribonuclease per ml (Schwarz/Mann) at 37 C for 7 h. The mixture was periodically adjusted to pH 7 with NaOH, and at the end of the incubation period it was dialyzed against cold running water for 1 h and then against distilled water for 18 h at 4 C. After centrifugation and filtration to remove insolube material, the RNA was applied to a diethylaminoethyl-Sephadex column (1.5 by 20 cm) equilibrated with PBS at 4 C. RNA was eluted with a 0.3 to 1 M KCl gradient, and the fractions were tested for their ability to "induce" SLS in RNA-maltose medium described above. The RNA fractions active in SLS production were pooled and subsequently used in SLS preparation.

The SLS-containing supernatants (see above) were filtered and concentrated by methanol precipitation as described by Ginsburg et al. (14). The precipitate was resuspended in PBS, dialyzed against PBS at 4 C, and applied to a Sephadex G-50 column (2.5 by 45 cm) equilibrated with PBS at 4 C. Fractions containing the peak of hemolytic activity were pooled and applied to a diethylaminoethyl-Sephadex column (1.5 by 20 cm) equilibrated in the same buffer. The toxin was eluted with a 0.3 to 1 M KCl gradient, and the fractions containing peak hemolytic activity were pooled and stored at −20 C.

Assay of SLS. Rabbit erythrocytes were obtained and washed as previously described (8). Toxin was diluted in PBS and added to tubes containing 2 ml of a 0.6% (vol/vol) rabbit erythrocyte suspension; the total volume of the mixture was always 4 ml. The tubes were incubated at 37 C for 30 min, and lysis was measured by determining Hb release colorimetrically at 540 nm in a Spectronic 20 or Coleman model 44 spectrophotometer. Complete hemolysis (absorbance = 0.800) was determined by including 0.1 ml of 1% saponin in the suspension. One hemolytic unit (HU) is defined as that amount of toxin that produces 50% hemolysis in 30 min at 37 C.

Erythocyte membranes. Rabbit erythrocytes were washed four times in PBS, and the cells from a 25-ml portion of the 0.6% suspension were hypotonically lysed in 0.01 M tris(hydroxymethyl)aminomethane hydrochloride, pH 7.2, or 0.005 M Na_2HPO_4, pH 7.4. The membranes were recovered by centrifugation at 35,000 × *g*, washed once in the same buffer, and resuspended in 25 ml of PBS.

Measurement of ^{86}Rb efflux. Rabbit erythrocytes were washed in potassium-free PBS (PBS-K) and resuspended in PBS-K containing 0.5% glucose as previously described (8). A 30-ml portion of the cell suspension was incubated with 0.1 ml (100 μCi) of $^{86}Rb^+$ (New England Nuclear Corp.) at 37 C for 90 min. After being washed, the cells were resuspended to the same volume in PBS-K, and 2-ml portions were distributed to a series of tubes in a 37 C water bath. Two-milliliter samples of SLS or control solution were added, and, after incubation for various times, the tubes were removed and centrifuged for 60 s at 2,000 × *g*. A 0.1-ml sample of the supernatant was quickly removed and placed in scintillation vials. The tubes were read at 540 nm to determine Hb release. Rubidium release into the supernatant was determined by counting the beta-emitting energy of the $^{86}Rb^+$ in a Packard Tri-Carb liquid scintillation spectrometer. The total intracellular amount of Hb and $^{86}Rb^+$ was determined in cell samples lysed by saponin. Less than 1% of the intracellular Rb leaked out of untreated cells during the 10 to 15 min required to carry out each experiment.

RESULTS

SLO is known to bind to erythrocytes at 0 C, but no hemolysis occurs at that temperature (1, 15). This observation has made it possible to separate the SLO hemolytic process into a temperature-independent adsorption step and a subsequent temperature-dependent step(s) that results in cell lysis; the separable adsorption and hemolysis steps have been useful in defining the characteristics of these processes. The possibility that similar temperature effects are found in SLS hemolysis was examined. Rabbit erythrocytes were incubated with 2 HU of SLS for different times at 0, 14, 22, or 37 C. No hemolysis occurred during this initial incubation period. The cells were then washed, resuspended in PBS, and incubated at 37 C for 30 min. The amount of hemolysis that occurred during the 37 C incubation period was determined colorimetrically. The results in Fig. 1 show that no 37 C hemolysis was observed after an initial exposure to SLS at 0 C; the amount for 37 C hemolysis that occurred after exposure at higher temperatures increased as the time and temperature of the initial incubation period increased. The possibility that the initial interaction between SLS and erythrocytes might show one or more sharp temperature transitions was examined by incu-

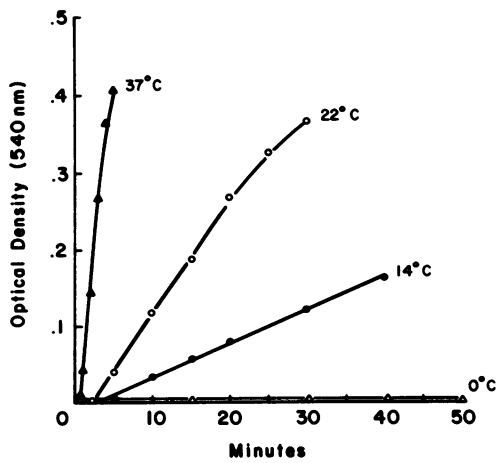

FIG. 1. *Effect of temperature on SLS action. Erythrocytes were exposed to 2 HU of SLS at each temperature for various times, centrifuged, and then incubated in fresh PBS at 37 C for 30 min.*

bating the erythrocytes with SLS for 15 min at temperatures between 10 and 28 C. After the incubation period, the cells were pelleted and resuspended in PBS, and the amount of hemolysis that occurred at 37 C after 30 min was determined. The results in Fig. 2 depict a gradual increase in hemolysis with increasing temperature (above 10 C), and no abrupt temperature break is obvious.

As part of both these experiments, the amount of SLS remaining in the supernatant (unadsorbed toxin) after the initial incubation period was determined by incubating the supernatant with fresh erythrocytes at 37 C for 30 min. Very little SLS activity was lost from the supernatants during the initial exposure to toxin, even at 22 and 37 C, where considerable cell lysis occurred during the subsequent 37 C incubation. Figure 3 shows the extent to which the treated cells lysed at 37 C and the hemolytic activity remaining in the supernatant after an initial 22 C incubation. The finding that most of the SLS activity was not removed by the cells from toxin suspensions was examined in a second type of experiment, shown in Table 1. Erythrocytes were treated with between 1 and 2 HU of SLS for 3 min at 37 C. The cells were removed by centrifugation, and the toxin activity remaining in the supernatant was determined by resuspending a fresh erythrocyte pellet in it and incubating the mixture for 30 min at 37 C (first column of double figures in Table 1). Other toxin solutions were exposed for 3 min to fresh erythrocyte preparations two or three different times before the amount of SLS remaining in the supernatants

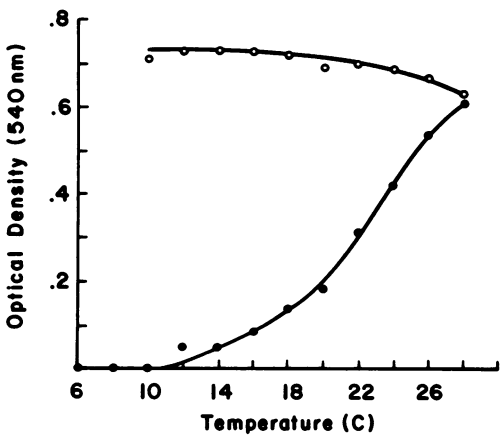

FIG. 2. *Effect of temperature on SLS action. Erythrocytes were exposed to 2 HU of SLS at each temperature for 15 min, centrifuged, and then incubated in fresh PBS at 37 C for 30 min. Symbols: Cell lysis (●); hemolytic activity remaining in the supernatant (○).*

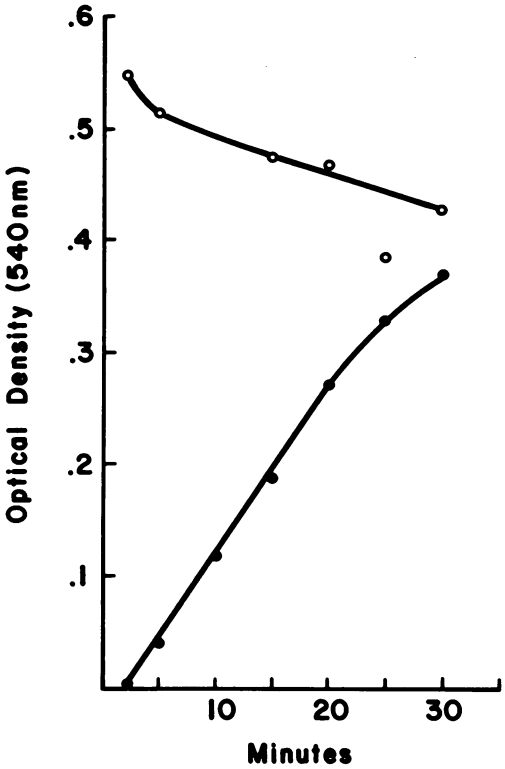

FIG. 3. *SLS adsorption and hemolysis. Erythrocytes were exposed to 1 to 2 HU of SLS at 22 C for various times. The extent of subsequent cell lysis at 37 C (●) and the amount of unadsorbed toxin remaining in the supernatant (○) were determined.*

TABLE 1. *Adsorption of SLS hemolytic activity*[a]

No. of incubations with erythrocytes	Supernatant hemolytic activity[b] (OD_{540})		Cell-associated hemolytic activity[c] (OD_{540})
	+ Cells	− Cells	
1	(0.544) (0.685)	(0.542) (0.695)	(0.450) (0.590)
2	(0.530) (0.535)	(0.546) −	(0.288) (0.440)
3	(0.328) (0.438)	(0.508) (0.470)	(0.070) (0.144)

[a] Toxin preparations (1 to 2 HU) were exposed one to three times to fresh erythrocytes for 3 min at 37 C. The results of two separate experiments are given.

[b] Assayed by incubating fresh erythrocyte pellets with the supernatants for 30 min at 37 C. OD_{540}, Optical density at 540 nm. −, Not determined.

[c] Amount of lysis observed in test cells exposed to SLS for 3 min, after resuspension in fresh PBS and incubation at 37 C for 30 min.

was determined. The amount of toxin that adsorbed to the cells during the 3-min exposures was also determined by resuspending those cells in fresh PBS and incubating the mixtures at 37 C for 30 min (third column of double figures in Table 1). Controls included a toxin solution that was carried through the incubations and centrifugations in the absence of erythro-

cytes (second column of double figures). The results of the two separate experiments shown in Table 1 again demonstrate that much of the hemolytic activity remains in the supernatant even after exposing the toxin solutions to two or three fresh erythrocyte preparations. Although the supernatants retained much of their original hemolytic activity after multiple exposures (as measured by fresh cell lysis after incubation at 37 C for 30 min), the amount of hemolysis observed in the test cells exposed for the 3-min time period was greatly reduced after the third incubation.

Comparable results were obtained when SLS was incubated with erythrocyte ghosts prepared in either tris(hydroxymethyl)aminomethane or phosphate hypotonic buffer and resuspended in PBS. After a 5-min incubation period at 37 C, the ghosts were removed by centrifugation at 30,000 × *g*, and the hemolytic activity remaining in the supernatant was determined. The results of several experiments showed that less than 20% of the hemolytic activity was removed by incubation with ghosts, and in most cases only 3 to 4% of the hemolytic activity was removed.

The results described above suggest that an early step in the toxin-cell interaction is temperature dependent. To determine whether the lytic step itself was affected by temperature, erythrocytes were incubated with SLS for 4 min at 37 C, during which time no hemolysis occurred. The toxin-treated cells were removed by centrifugation and incubated in PBS at 0, 22, or 37 C. The rate of hemolysis at each temperature was determined. The results in Fig. 4 demonstrate that the kinetics of the lytic process were almost identical at the three temperatures.

The possibility that a colloid-osmotic process is responsible for SLS-induced hemolysis was examined by comparing the rates of Hb and ^{86}Rb release from toxin-treated cells. Rubidium is transported and maintained in erythrocytes in a manner similar to potassium (17). Rabbit erythrocytes were labeled with $^{86}Rb^+$, as described in Materials and Methods, and exposed to 2 HU of SLS at 37 C. At various times, samples were removed, and, after centrifugation, the amount of Rb^+ and Hb present in the supernatant was determined. The results of a typical experiment are shown in Fig. 5, where Y denotes the proportion of zero-time intracellular material released from the cells at the time of sampling. It is apparent that the loss of intracellular $^{86}Rb^+$ precedes the escape of Hb by several minutes, so that, at a time when less than 4% of the Hb had been released, 50% of the intracellular Rb^+ had escaped. These results

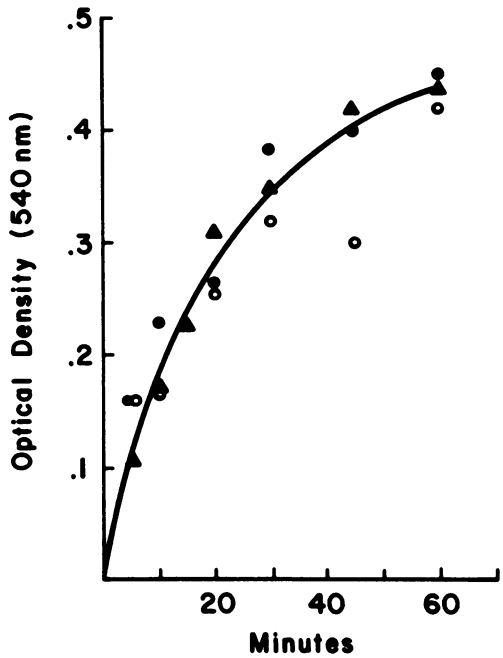

FIG. 4. *Effect of temperature on Hb release from toxin-treated cells. Erythrocytes were exposed to 1 HU of SLS for 4 min at 37 C. After resuspension of the cells in fresh PBS, the extent of hemolysis at 0 C (○), 22 C (●), or 37 C (▲) was determined.*

demonstrate that osmotic processes are responsible for the lysis of SLS-treated erythrocytes.

DISCUSSION

The results of the experiments reported here make it possible to describe some of the events in SLS-induced hemolysis. The initial interaction of toxin with the cell appears to be a temperature-dependent phenomenon, as shown by the fact that no lysis occurred (even at 37 C) when erythrocytes were initially treated with SLS at 0 to 10 C. Exposure of the cells to toxin at increasing temperatures gradually increased the amount of hemolysis that subsequently occurred.

In a second group of experiments, it was demonstrated that, even with toxin concentrations that lyse 60 to 70% of the standard erythrocyte preparation, most of the hemolytic activity is not irreversibly bound to the cells and can be detected in the supernatants after centrifugation of toxin-cell mixtures. Similar results were obtained when toxin preparations were incubated with erythrocyte ghost preparations. Although the hemolytic activity of the toxin preparations (assayed by treating fresh cells for 30 min at 37 C) is somewhat decreased after a third 3-min exposure to erythrocytes, the subse-

quent lysis of those same cells (exposed for 3 min) is greatly reduced. This result is difficult to understand. One possible explanation is that SLS consists of a heterogeneous population of molecules having different affinities for the cells. Accordingly, those molecules that readily interact with the cells would be removed by one or two 3-min exposures, and subsequent short exposures would produce little lysis of test cells. If the population of toxin molecules having a high affinity for the cells is relatively small, the loss (by adsorption) of those molecules may therefore not be readily detectable when fresh cells are exposed to the toxin preparation for 30 min at 37 C.

An additional observation can be made from the experiment reported in Table 1. A toxin solution capable of lysing 50 to 60% of a standard erythrocyte suspension (about 1 HU) retains most of its hemolytic activity after one short exposure to erythrocytes. Yet, 40 to 60% of those test cells (exposed for 3 min) lyse upon further incubation. Thus, 1 HU of SLS appears to be able to produce about 50% hemolysis in two (or three) different erythrocyte suspensions, but in none of the suspensions is 100% of the cells lysed. This finding probably reflects heterogeneity of the erythrocyte population in response to the toxin. Our observations on toxin adsorption tend to support those of Cinader and Pillemer (6), who also found that a large proportion of the hemolysin remained in the supernatant even though the toxin had already acted on the original cells. Elias et al. (11), on the other hand, found that 75% of the hemolytic activity of SLS had disappeared after a 10-min incubation with erythrocyte ghosts; the reasons for the differences in the results of these investigators, and our own, are not known.

The unusual temperature dependence of the initial step in SLS hemolysis, and the finding that little of the total toxin activity is irreversibly adsorbed to the cell, suggest that SLS may not bind to peripheral membrane components, as has been proposed for several bacterial toxins. An alternative explanation might be that a small number of SLS molecules (perhaps just the hemolytic moieties) become inserted into the bimolecular lipid structure of the membrane during this first step. Thus, the temperature effects seen might be related to thermal transitions in the membrane that allow the toxin molecules to penetrate into the lipid bilayer.

As a consequence of the temperature-dependent process, the cell appears to lose its ability to prevent the free exchange of ions across the membrane; the cell then swells and bursts due to a colloid-osmotic process. The evidence for

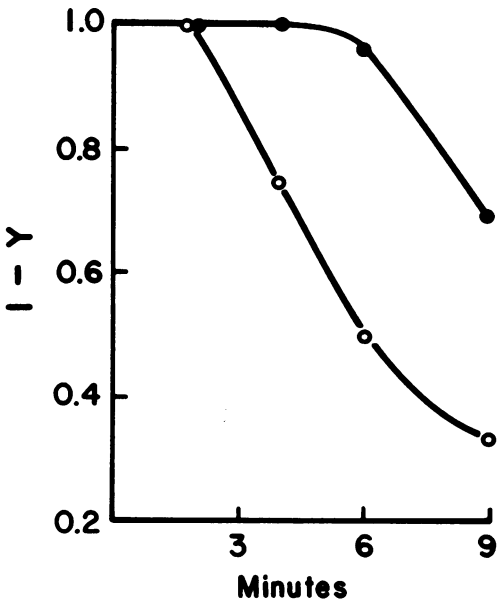

FIG. 5. *Loss of $^{86}Rb^+$ (○) and Hb (●) from SLS-treated erythrocytes.*

this comes from the finding that rubidium ions escape from toxin-treated erythrocytes prior to the release of Hb; almost half of the intracellular concentration of Rb^+ is released before any Hb escape can be detected (Fig. 5). Once the functional membrane lesions have been produced, the ion flux, cell swelling, and eventual membrane rupture would be expected to be only slightly affected by temperature. This statement is supported by the experimental results shown in Fig. 4, which demonstrate that Hb release occurs at about the same rate at 4, 22, and 37 C in cells briefly exposed to SLS at 37 C. Furthermore, if toxin-treated cells are resuspended in sucrose, the subsequent release of Hb after 30 min at 37 C is decreased by about 75% (data not shown). Sucrose in the extracellular medium presumably stabilizes the damaged cells by preventing their swelling.

The observations described here for SLS are completely different from the results of similar experiments with SLO, the other cytolytic toxin produced by group A streptococci. Up to 2 HU of SLO can be completely adsorbed to erythrocytes, even at 0 C; the subsequent steps that result in Hb release, however, are temperature dependent (15). Studies with $^{86}Rb^+$ showed that ion release does not precede Hb escape from SLO-treated erythrocytes, indicating that colloid-osmotic processes are not involved in hemolysis by that toxin (8). Cholesterol is thought to be the membrane component involved in SLO action, and an electron microscopic study

of SLO-treated erythrocytes, liposomes, and lipid dispersions revealed the presence of 38-nm rings and C-like structures, presumably due to the interaction of SLO molecules with cholesterol (10). No membrane lesions have been seen in SLS-treated eryrocytes. At the present time, the precise interaction of these toxins with their target in the membrane cannot be described.

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