INFECTION AND IMMUNITY, Dec. 1971, p. 683-687

Vol. 4, No. 6
Printed in U.S.A.

Characteristics of Streptolysin O Action

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Received for publication 11 August 1971

A study of the lysis of rabbit erythrocytes by streptolysin O (SO) revealed at least two steps in the hemolytic process. The initial interaction between SO and erythrocytes is the adsorption of the toxin molecule to the cell surface. Adsorption occurred at 4 C and was independent of ionic strength and *p*H; these results suggest that hydrophobic interactions between SO and the cell may be important in this process. Cholesterol was shown to prevent the adsorption of toxin to the cell, and it is proposed that cholesterol in the red cell membrane may be the site of toxin adsorption. The concept of a lipid attachment site is supported by the findings that proteolytic enzymes and sulfhydryl inhibitors known to affect external erythrocyte proteins did not affect SO hemolysis. Although the number of toxin molecules that will adsorb to a cell is limited, more than one toxin molecule was required for hemolysis. The step(s) following adsorption was dependent on temperature, ionic strength, and *p*H. Thus, it is evident that this step(s) is readily separable from adsorption, suggesting that an ionic interaction occurs between toxin and an erythrocyte membrane molecule. The step(s) following adsorption was also inhibited by divalent cations. Since *N*-ethyl maleimide will also inhibit lysis after toxin adsorption, it is possible that divalent cations may prevent SO hemolysis by reacting with free sulfhydryl groups on the toxin molecule.

Streptolysin O (SO) is a toxic protein secreted into the culture medium during growth of certain group A streptococci. The toxin is lethal for laboratory animals (12), and in vitro destroys tissue culture cells (9) and lyses erythrocytes (15), leukocytes (11), macrophages (7), and blood platelets (5). SO is one of a group of bacterial cytolytic toxins thought to act on the cell membrane to bring about lysis (4). Hemolysis of erythrocytes by SO occurs in a matter of minutes, suggesting that lysis is the result of a direct effect of toxin on the cell membrane rather than a secondary effect of toxin acting on cellular metabolism. In addition, isolated erythrocyte membranes (6) as well as cholesterol (10), an important component of the cell membrane, inhibit SO activity. Although the cell membrane has been implicated in the action of SO, the process of toxin-induced lysis is not understood. The following report presents information on the characteristics of hemolysis of erythrocytes (RBC) by SO.

MATERIALS AND METHODS

Purification of SO. SO was prepared from a 16-hr culture of *Streptococcus pyogenes* type 3, Richards strain, grown in Todd-Hewitt broth (Difco). The bacteria were removed from the culture by centrifugation at 10,400 × *g*, and the supernatant fluid was concentrated to 1/25 the original volume by freeze-thawing the supernatant fluid twice and collecting the first fraction to thaw. The concentrate was precipitated with 60% saturated ammonium sulfate, and the precipitate was dissolved in phosphate-buffered saline (NaCl, 0.083 M; Na_2HPO_4, 0.047 M; KH_2PO_4, 0.020 M; *p*H 7.1). A 6-ml amount of this solution was then applied to a 500-ml Sephadex G-100 column which had been equilibrated with phosphate-buffered saline at 4 C (16). Fractions were collected and assayed for hemolytic activity. Active material was found in a single elution peak, and the fractions in this peak were pooled. The hemolytic activity in the pooled fractions could be increased by treatment with various reducing agents and was completely inactivated by cholesterol, antistreptolysin O, and trypsin. These properties are characteristic of SO.

Assay of SO. Blood was collected from normal female New Zealand White rabbits and centrifuged at 1,100 × *g*. The plasma and buffy coat were aspirated, and the cells were washed three times in phosphate-buffered saline (PBS: NaCl, 0.126 M; KH_2PO_4, 0.023 M; Na_2HPO_4, 0.013 M; *p*H 6.5) and then suspended in PBS to a final concentration of 0.35%. The suspensions were used within 48 hr of preparation.

SO was activated by the addition of 0.2 M sodium thioglycolate and diluted in PBS to the desired concentration. In the following experiments one hemolytic unit (HU) is arbitrarily defined as the amount of SO which produces 50% lysis of a 0.35% RBC suspension after 30 min at 37 C. Lysis was measured by determining hemoglobin release colorimetrically with a

Spectronic 20 spectrophotometer at 540 nm. All assays were performed in a final volume of 4.2 ml. A complete hemolysis standard was prepared by adding one drop of 1% saponin to the same volume of RBC suspension.

Cholesterol preparation. Cholesterol (0.001 M) was prepared by suspending 0.0406 g of cholesterol in 5 ml of methanol and adding this mixture to 100 ml of boiling water. The suspension was then filtered to remove insoluble material.

Reagents. Cholesterol, papain, and Pronase were obtained from Sigma Chemical Co., St. Louis, Mo. Trypsin and antistreptolysin O were preparations of Difco, Detroit, Mich.

RESULTS

Adsorption of SO. Alouf and Raynaud (1) observed that no hemolysis occurred when RBC were exposed to SO at 4 C; when the cells were subsequently incubated at 37 C, however, they lysed completely. We used this procedure to separate adsorption from subsequent events leading to hemolysis in order to study the characteristics of SO adsorption. Toxin and RBC were incubated together at 4 C; no lysis occurred during the 60-min incubation period. However, when the cells were subsequently centrifuged and resuspended in fresh PBS at 37 C, complete lysis occurred within 30 min, indicating that SO had adsorbed to the cells at 4 C. The addition of antistreptolysin O to the cells just prior to resuspension at 37 C prevented hemolysis. Thus, SO adsorbs to RBC at 4 C and remains susceptible to antitoxin neutralization, but hemolysis does not occur at this temperature.

The amount of SO that will adsorb to the standard cell suspension was studied by adding increasing amounts of toxin to RBC suspensions and incubating the mixtures at 4 C. After 60 min, the mixtures were centrifuged and the clear supernatant fluid was removed. SO activity in the supernatant fluid and pellet was assayed as described above. The results (Fig. 1) showed that between 1 and 2 HU of toxin could be completely adsorbed to the cells; at higher concentrations, hemolytic activity began to appear in the supernatant fluid.

Effect of ionic strength and pH on SO adsorption. The effect of ionic strength and pH on SO adsorption was studied by exposing RBC to 1.2 HU of toxin at various ionic strengths or *p*H's for 60 min at 4 C. If adsorption takes place under these experimental conditions, approximately 60% lysis should occur when the cells are resuspended in PBS at 37 C. The results of these experiments showed that maximum hemolysis occurred at all ionic strengths (Fig. 2) and all *p*H's tested (Fig. 3), indicating that the adsorption of SO to the cell is independent of ionic strength and *p*H.

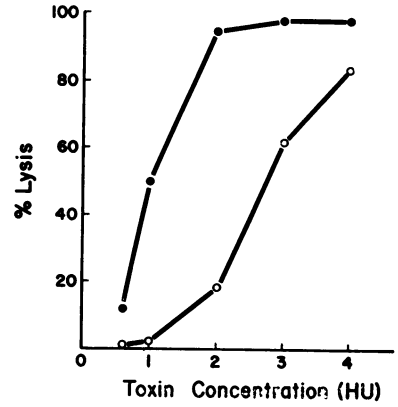

FIG. 1. *Adsorption of streptolysin O. Toxin and erythrocytes were incubated at 4 C for 60 min and the amount of toxin activity on the cells* (●) *and the supernatant fluid* (○) *was determined.*

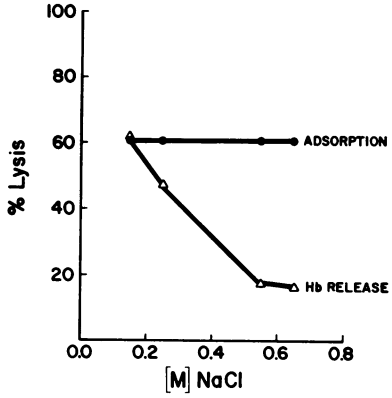

FIG. 2. *Effect of ionic strength on streptolysin O adsorption and hemoglobin release.*

Nature of toxin-cell binding. The possibility that a receptor site on the erythrocyte membrane is involved in SO adsorption was examined. Since adsorption of SO occurs only if the toxin molecule is in the reduced form (1), toxin may adsorb by forming disulfide bridges with an erythrocyte membrane protein. To determine whether sulfhydryl groups on the erythrocyte membrane are necessary for SO action, RBC were treated for 30 min at 37 C with 0.002 M *N*-ethyl maleimide (NEM). This reagent irreversibly inactivates sulfhydryl groups and has been shown to affect sulfhydryl groups on intact erythrocytes (13). The cells were washed and exposed to 1 HU of toxin. Cells pretreated with NEM in this manner remained completely susceptible to SO action; similar results were obtained with 0.0005 M

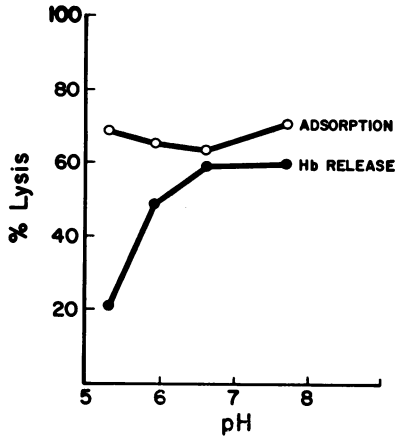

FIG. 3. *Effect of pH on streptolysin O adsorption and hemoglobin release.*

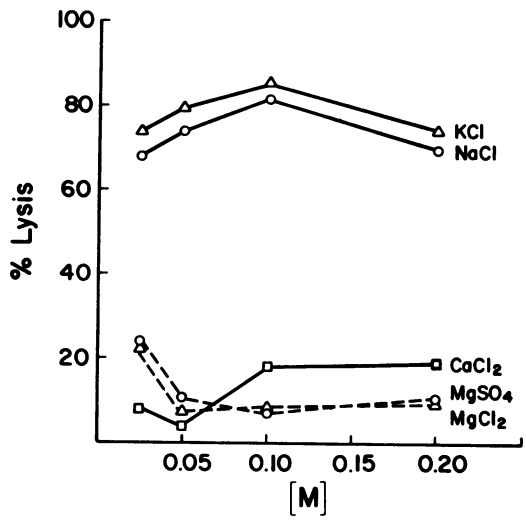

FIG. 4. *Effect of divalent cations on streptolysin O hemolysis.*

p-chloromercuribenzoate, suggesting that disulfide bridges between toxin and an external erythrocyte protein do not play a role in SO adsorption. In an additional experiment, the possibility that membrane proteins are involved in adsorption of SO was examined by treating the cells with the proteolytic enzymes trypsin, papain, or Pronase at a concentration of 1 mg/ml for 30 min at 37 C before the addition of toxin. Although exposure of cells to trypsin (14) and Pronase (3) under similar conditions has been shown to completely hydrolyze surface membrane proteins, the cells in this experiment remained completely susceptible to SO.

Low concentrations of cholesterol prevent SO hemolysis (10), and Bernheimer (4) has suggested that this compound may serve as a receptor or substrate for SO. The possibility that cholesterol affects adsorption of SO was tested indirectly by incubating 2 HU of SO with 0.6 μmoles of cholesterol for 10 min at room temperature. No lysis occurred when the toxin-cholesterol mixture was incubated with RBC for 30 min at 37 C. If toxin adsorbs to the cells under these conditions but is inhibited by the presence of cholesterol in the suspension, lysis should occur when cholesterol is removed. The cells which had been incubated in toxin-cholesterol were washed two times with PBS and resuspended in fresh PBS at 37 C; again, no lysis occurred. Finally, the same cells were shown to have remained susceptible to toxin by the fact that complete lysis occurred when they were subsequently incubated with SO. Since the amount of SO which will adsorb to the standard cell suspension is limited (Fig. 1), the possibility that cholesterol-inhibited toxin had adsorbed to the cells appears unlikely. These results suggest that cholesterol prevents the adsorption of toxin to the cell.

Effect of ionic strength and pH on hemoglobin release. Although ionic strength and *p*H do not affect adsorption of SO, the possibility that subsequent events in the hemolytic process are affected was examined. 1.2 HU of SO was added to RBC in solutions of varying NaCl concentration, and hemoglobin release was measured after 30 min at 37 C. The results in Fig. 2 show that, as the concentration of NaCl increased above 0.14 M, the extent of cell lysis decreased. Similar results were obtained with KCl. The effect of *p*H on hemolysis was studied by incubating 1.2 HU of SO with RBC suspensions at different *p*H's. Maximum hemolysis occurred at *p*H 6.6 and higher, but at *p*H 5.3 significant inhibition of SO action was observed (Fig. 3). Non-toxin-treated control cells did not lyse under these conditions of ionic strength or *p*H.

Effect of divalent cations on SO hemolysis. The effect of divalent cations on SO hemolysis was studied by exposing RBC suspended in isotonic 0.3 M sucrose to 2 HU of toxin in the presence of appropriate cation concentrations. The cells were suspended in isotonic sucrose in this experiment in order to examine the effect of divalent cations in the absence of ions present in PBS. The cells were almost completely protected by divalent cations, even at low concentrations where monovalent cations had little effect (Fig. 4). The divalent cations did not prevent SO adsorption, and the possibility that they inhibit a subsequent step leading to hemoglobin release was tested by allowing 2 HU of SO to adsorb to the cells as

previously described. When the cells were suspended in 0.05 M divalent cation in isotonic sucrose, almost complete protection was observed.

Mechanism of cation inhibition. The inhibition of SO activity by either monovalent or divalent cations could indicate that SO action involves the disruption of cationic gradients; thus, lysis would be prevented because the RBC are stabilized by external cations. If this were true, increasing SO concentration should have little effect on cation inhibition. RBC suspended in varying NaCl or $MgCl_2$ concentrations were incubated with increasing amounts of toxin for 30 min at 37 C. In both cases increasing SO concentration overcame the cation inhibition. In an additional experiment, 1 HU of toxin was allowed to adsorb to RBC at 4 C, and the cells were suspended in 0.2 M $MgCl_2$ or 0.6 M KCl. As expected, very little hemolysis occurred during the subsequent incubation at 37 C. The cells in half of the tubes treated in this manner were suspended in PBS containing 500 Todd units of antistreptolysin O. Cells from duplicate samples were suspended in PBS alone. The cells suspended in PBS showed maximal lysis, whereas the suspensions containing antibody were protected, indicating that the protective effect of cations is not due simply to osmotic stabilization.

Effect of NEM on SO hemolysis. To test whether free sulfhydryl groups are necessary for hemolysis even after toxin adsorption has occurred, SO and RBC were incubated together for 60 min at 4 C. The cells were suspended in PBS containing 0.001 M NEM and held at room temperature for 30 min. No lysis occurred when these cells were subsequently incubated at 37 C for 30 min, whereas the cells in control tubes which had been suspended in PBS alone lysed completely.

Effect of RBC concentration on SO activity. The number of toxin molecules necessary to lyse a single erythrocyte is unknown. If one molecule of SO is sufficient for lysis, then the amount of hemoglobin released by a constant amount of toxin should be proportional to RBC concentration until a plateau is reached; if more than one molecule of SO is required for lysis, then the amount of hemoglobin released should increase to a peak and then decrease. The effect of RBC concentration on SO activity was studied by keeping the amount of SO constant and varying the proportion of cells in the assay mixture. After 30 min at 37 C, the mixtures were centrifuged and the supernatant fluid was assayed for hemoglobin. The results (Fig. 5) are consistent with the hypothesis that more than one toxin molecule is required to lyse one erythrocyte.

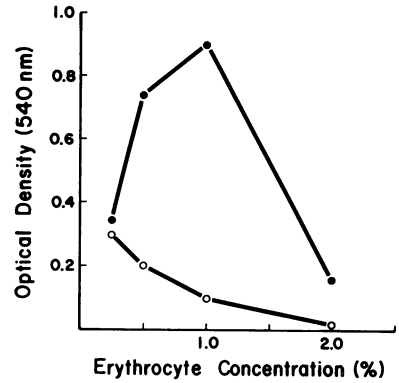

FIG. 5. *Effect of erythrocyte concentration on streptolysin O activity. Symbols:* ●, *2 HU;* ○, *1 HU.*

DISCUSSION

Based on the experiments described, it is possible to define at least two steps in the lysis of erythrocytes by SO. The initial interaction between SO and erythrocytes appears to be the adsorption of the toxin molecule to the cell surface. The adsorption process occurs at 4 C and is independent of ionic strength and *p*H; in addition, the protection observed when antistreptolysin O is added to cells preincubated with SO at 4 C shows that the toxin molecule remains completely accessible to antitoxin neutralization during this step. These results demonstrate the unique nature of the adsorption process and distinguish it from subsequent events leading to hemolysis.

Although SO must be in its reduced form for adsorption to occur, the possibility that disulfide bridges between the toxin molecule and an erythrocyte membrane protein are involved seems unlikely; cells treated with sulfhydryl inhibitors remained susceptible to toxin. A similar observation was reported by van Epps and Andersen (16), who showed that alkylation of membrane sulfhydryl groups with iodoacetamide did not prevent SO hemolysis. In addition, the finding that cells treated with various proteolytic enzymes were lysed by SO argues against a role for external membrane proteins in the adsorption process.

The fact that toxin adsorption is independent of ionic strength and *p*H suggests that hydrophobic interactions between the toxin molecule and the cell may be important in this step. Furthermore, cholesterol may be the membrane component directly involved in adsorption; cholesterol has long been known to inhibit hemolysis by SO, and only those cells which contain cholesterol or related sterols in their membranes are

susceptible to SO action (4). Our results indicate that in the presence of cholesterol SO does not adsorb to erythrocytes; the simplest explanation for the inhibitory effect of this compound is that exogenous cholesterol competes with cholesterol in the membrane for a "binding" site on the toxin molecule. Preliminary studies with ^{14}C-cholesterol indicate that cholesterol does in fact bind to streptolysin O (*unpublished data*).

The step(s) following adsorption which results in lysis of the cell is inhibited at low temperatures and is sensitive to high ionic strength and low *p*H. In addition, low concentrations of divalent cations gave almost complete protection against SO hemolysis even when added after adsorption had taken place. Cells protected against hemolysis by cations were found to lyse when suspended in PBS; however, if they were suspended in PBS containing antitoxin, no lysis occurred, indicating that the protective effect of cations is not simply due to an osmotic stabilization of SO-damaged erythrocytes, and further showing that the toxin molecule remains susceptible to antitoxin throughout the period of protection. The effect of *p*H and high ionic strength could indicate that, after adsorption, an ionic interaction occurs between toxin and an erythrocyte membrane molecule. Another mechanism must be proposed to explain the inhibition of SO activity by divalent cations. Van Epps and Andersen (16) observed a similar effect by using even lower concentrations of divalent cations and proposed that they may prevent hemolysis by reacting with free sulfhydryl groups on the toxin molecule. This interpretation is compatible with the protective effect observed when NEM is given after toxin has adsorbed to the cell and suggests that the maintenance of free sulfhydryl groups on the toxin molecule is necessary for a subsequent event in the hemolytic process. The fact that toxin adsorption occurs in the presence of divalent cations does not necessarily argue against such an explanation for the cation effect; adsorption requires that the toxin molecule be in its reduced form, and an interaction between sulfhydryl groups and divalent cations may not preclude a molecular configuration necessary for adsorption.

It is interesting to speculate whether our results may be applied to other bacterial toxins. Arbuthnott (2) suggested that staphylococcal alpha toxin may become adsorbed to the cell surface by a hydrophobic interaction and, once specifically oriented, may become enzymatically active. In addition, Gill and Pappenheimer (8) recently suggested that diphtheria toxin is able to penetrate the cell membrane, because the toxin molecule contains a segment which is hydrophobic in nature. Most cytolytic bacterial toxins are inhibited by various lipid membrane components (4), and an initial hydrophobic interaction between the toxin molecule and the target cell may be characteristic of a number of bacterial toxins.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant AI 08793 from The National Institute of Allergy and Infectious Diseases. T. D. O. was a predoctoral trainee supported by microbiology training grant 5 TO 1 GM 00724-10 from The National Institute of General Medical Sciences.

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