

Evidence that *Clostridium perfringens* Theta-Toxin Induces Colloid-Osmotic Lysis of Erythrocytes

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***Clostridium perfringens* theta-toxin was shown to lyse target erythrocytes by a colloid-osmotic mechanism. Analysis showed the onset of lysis of erythrocytes by theta-toxin could be temporarily stabilized with 0.3 M sucrose. Flow cytometry analysis of the size distribution of theta-toxin-treated erythrocytes showed swelling of the erythrocytes prior to lysis.**

Clostridium perfringens theta-toxin (perfringolysin O or PFO) is a secreted hemolysin/cytolysin which is similar in activity and primary structure (12) to toxins from *Streptococcus* (8, 14), *Bacillus* (6), and *Listeria* (9) species. The mechanism of membrane damage produced by this group of toxins remains unresolved, although it has been concluded that lysis of erythrocytes by these toxins does not proceed by a colloid-osmotic mechanism. Early evidence of this came from Bernheimer (1), who reported that erythrocytes did not swell in volume prior to lysis with streptolysin O (SLO). In addition, Duncan (5) found that the efflux of hemoglobin approximated the rate of ⁸⁶Rb release in SLO-treated erythrocytes, suggesting that ion equilibration does not precede hemolysis. Similar results were observed by Blumenthal and Habig (3) for tetanolysin. In the present report, we provide evidence that a colloid-osmotic mechanism may be responsible for *Clostridium perfringens* theta-toxin-dependent erythrocyte lysis.

Two hallmarks of colloid-osmotic lysis are that (i) compounds such as sucrose or polyethylene glycol can delay the onset of erythrocyte lysis by a temporary osmotic stabilization of the cells and (ii) cell swelling occurs with the influx of water into the cell, presumably via toxin-induced membrane pores. Initially, osmotic stabilization of PFO-treated erythrocytes was examined at various PFO concentrations. Measurement of hemolytic activity was accomplished by using changes in right-angle scatter of erythrocytes as previously described (7). Briefly, the hemolysis of washed human erythrocytes (buffy coat removed) was determined by right-angle scatter in a 1-cm-pathlength cuvette containing a 2-ml stirred erythrocyte suspension with an SLM 8000 spectrofluorimeter (SLM Instruments, Urbana, Ill.). Various amounts of PFO were injected into PBS (10 mM sodium

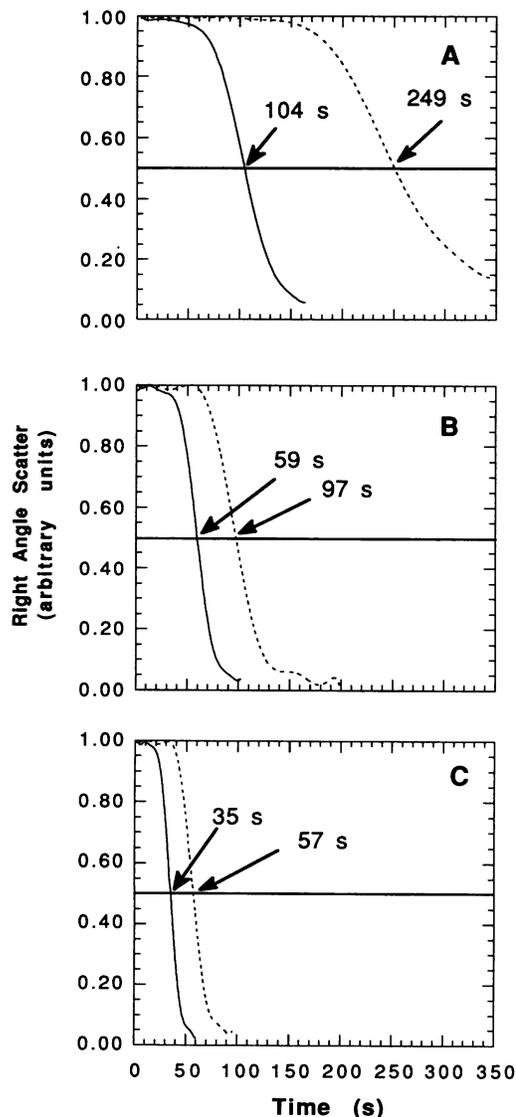


FIG. 1. Effects of sucrose on PFO-induced lysis of erythrocytes. Right-angle scatter measurements were performed to determine the effect of a hypertonic medium on PFO-induced lysis of erythrocytes. (A) 10 ng of PFO was injected into a solution of erythrocytes in PBS (—) or PBS + 0.3 M sucrose (- -); (B) same as in panel A except that 50 ng of PFO was injected; (C) same as in panel A except that 100 ng of PFO was injected. The arrows show the time required to reach 50% hemolysis in each experiment.

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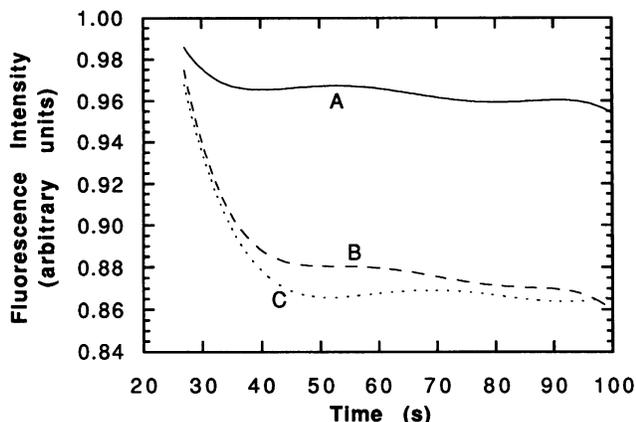


FIG. 2. PFO aggregation kinetics on erythrocyte ghost membranes in the absence and presence of sucrose. Fluorescence energy transfer was used to measure the kinetics of polymerization of PFO on erythrocyte membranes. No energy transfer is observed when the acceptor (PFO_A) is replaced with unlabeled PFO (line A), whereas maximum energy transfer is observed when PFO_A is used as the acceptor (lines B and C). (A) 150 ng of PFO_D and 750 ng of unlabeled PFO; (B) same as for A except the unlabeled PFO was replaced with PFO_A; (C) same as for B except the buffer contained 0.3 M sucrose. Fluorescence intensity at 520 nm (emission maximum for fluorescein) was monitored over time.

phosphate [pH 7.0], 145 mM NaCl, 5 mM KCl, 100 μg of gelatin per ml) containing 5×10^7 erythrocytes per ml 25 s after the start of the scan. The right-angle scatter was measured at 1-s intervals. The emission and excitation

monochromators were set at 590 nm, with a slit width of 2 nm. After addition of PFO (11), the intensity of erythrocyte right-angle scatter decreases rapidly after the onset of hemolysis (Fig. 1). We have shown that the curve exhibited by the right-angle scatter is related to the percentage of lysis on the basis of hemoglobin release (7), with 0% hemolysis corresponding with a right-angle scatter value of ≈ 1 in this case. At all concentrations of PFO, the $t_{1/2}$ (time required to achieve 50% hemolysis) approximately doubled for the ex-

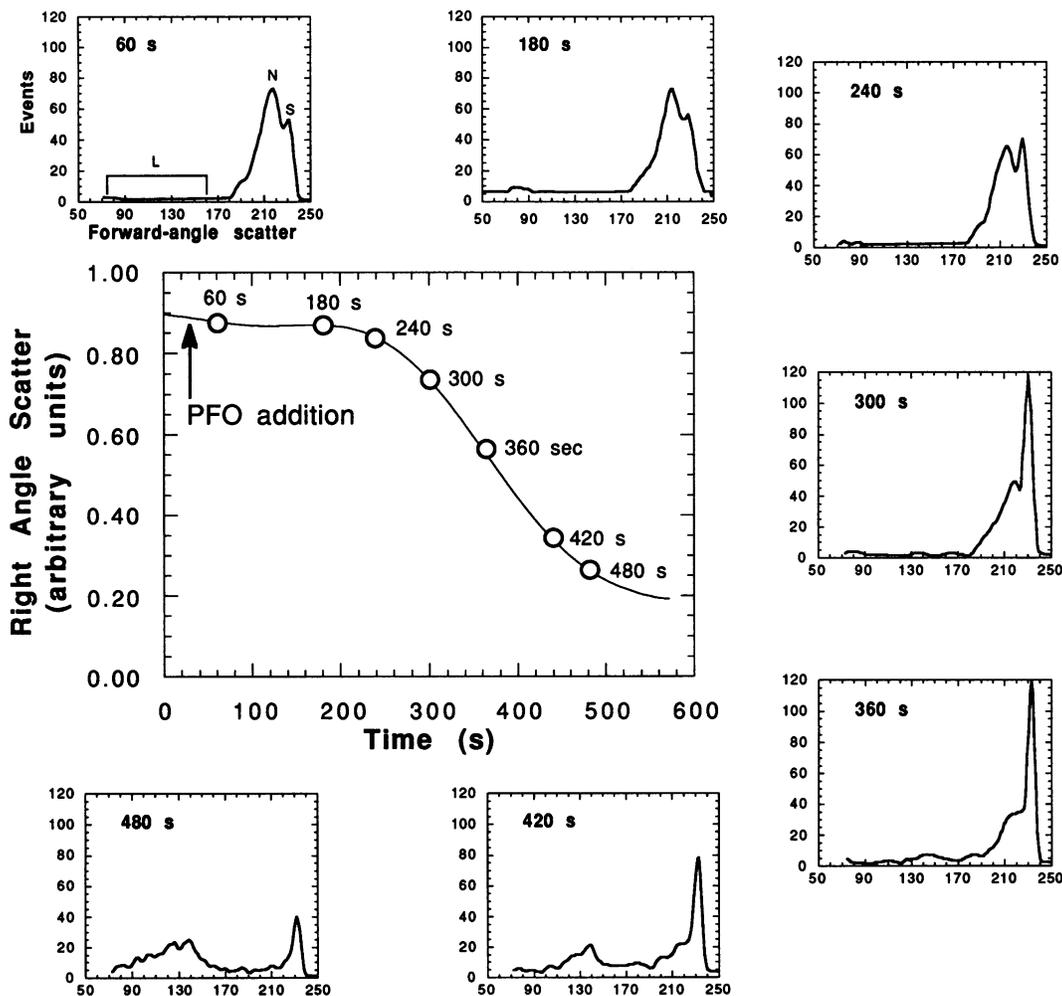


FIG. 3. Erythrocyte size analysis by flow cytometry during PFO-induced hemolysis. A hemolytic plot based on the change in right-angle scatter of erythrocytes is shown as the central graph. The size distributions of erythrocytes represented by forward-angle scatter histograms were obtained at 60 s, 180 s, 240 s, 300 s, 360 s, 420 s, and 480 s and are represented by the seven outlying graphs. In the 60 s histogram, the locations of the various erythrocyte derivatives are denoted. N, normal erythrocytes; S, swollen erythrocytes; L, lysed erythrocytes.

periments carried out in the presence of sucrose, which indicated that sucrose had a stabilizing effect on the PFO-treated erythrocytes. These data resemble observations reported for *Staphylococcus aureus* alpha-toxin-induced hemolysis in isotonic and hypertonic environments (4). *S. aureus* alpha-toxin lyses erythrocytes by means of colloid-osmotic swelling (2).

A fluorescence-energy-transfer (FET)-based aggregation assay for PFO (7) was used to show that the increased lag period and decreased hemolytic rate in the presence of sucrose were not due to decreased PFO binding or aggregation rates on the membrane. It has been shown (7) that FET occurs between PFO-conjugated fluorescein isothiocyanate (donor or PFO_D) and PFO-conjugated tetramethylrhodamine isothiocyanate (acceptor or PFO_A) in the presence of erythrocyte membranes. If the addition of sucrose affected PFO membrane binding or membrane aggregation of the PFO, then it would have been reflected in the aggregation rate of PFO. Fluorescence intensity measurements were made with a spectrofluorimeter (SLM 8000) with the excitation monochromator set at 470 nm and a slit width of 16 nm. Erythrocyte ghost membranes (1×10^8) were injected into a 2-ml stirred cuvette with a 1-cm pathlength containing PBS (± 0.3 M sucrose) with PFO_D and PFO_A or unlabeled PFO. Shown in Fig. 2 is the aggregation kinetics of PFO in the presence and absence of sucrose, as determined by FET. Little change was observed in the aggregation rates in the presence of sucrose; in fact, in the presence of sucrose, the aggregation rate was slightly accelerated. This clearly showed that neither binding nor aggregation was significantly affected in the presence of the sucrose. The effect of the sucrose must therefore occur after these stages of PFO assembly on the membrane.

As indicated above, colloid-osmotic lysis implies a prelytic period of volume expansion (10). Forward-angle scatter as determined by flow cytometry has proven to be useful in differentiating cell volumes of hemopoietic cells (13) and was employed to observe changes in the size of erythrocytes during PFO-induced lysis of erythrocytes. Hemolysis was initiated by the injection of 100 μ g of PFO into a stirred cuvette containing 1×10^6 erythrocytes per ml of PBS. Hemolysis was monitored by right-angle scatter as described above. In a duplicate hemolytic assay samples from the various times represented along the hemolytic curve were analyzed by flow cytometry using forward-angle scatter. Histograms were generated on a FACScan system (Becton-Dickinson, Mountain View, Calif.) by the aspiration of samples from the PFO-treated erythrocytes at the selected time intervals. Total acquisition time was approximately 10 to 20 s per sample, and a total of 5,000 events were recorded per sampling period. Data were collected in the log collection mode and displayed from 50 to 250 units of forward-angle scatter on the ordinate. Shown in Fig. 3 is a composite of a right-angle scatter plot showing lysis of erythrocytes by PFO and the distribution of erythrocyte sizes determined by forward-angle scatter histograms at selected times during hemolysis. Little change in the erythrocyte size distribution was observed for the first 180 s, which was consistent with our previous observation that the lag period largely resulted from the time required for PFO aggregation (7). At the onset of hemolysis (≈ 240 s), a shift occurred in the size distribution such that the swollen erythrocytes increased in number. As hemolysis progressed, the swollen erythrocytes predominated in the erythrocyte population, which was followed by the appearance of lysed cells.

The data presented here conflict with the current hypoth-

esis in which PFO and related cytolysins supposedly do not lyse cells by a colloid-osmotic mechanism. This hypothesis was largely based on previous investigations which did not detect a prelytic stage of ion release in SLO-treated (5) or tetanolysin-treated (3) erythrocytes. However, the apparent absence of prelytic ion release may reflect the rapidity of the ion equilibration and water influx in toxin-treated cells and the inability of such methods to distinguish this process from lysis. It is clear from the present results that PFO-treated erythrocytes can be temporarily stabilized with an osmotic stabilizer and that the cells apparently undergo a prelytic volume expansion. Both features are hallmarks of a colloid-osmotic mechanism of lysis.

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