

## STAPHYLOCOCCAL $\alpha$ TOXIN PROMOTES BLOOD COAGULATION VIA ATTACK ON HUMAN PLATELETS

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*Staphylococcus aureus* plays a major role as a bacterial pathogen in human medicine, causing diseases that range from superficial skin and wound to systemic nosocomial infections. The majority of *S. aureus* strains produces  $\alpha$  toxin, a proteinaceous exotoxin whose hemolytic, dermonecrotic, and lethal properties have long been known (1–6). The toxin is secreted as a single-chained, nonglycosylated polypeptide with a  $M_r$  of  $3.4 \times 10^4$  (7, 8). The protein spontaneously binds to lipid monolayers and bilayers (9–14), producing functional transmembrane pores that have been sized to 1.5–2.0-nm diameters (15–18). The majority of pores formed at high toxin concentrations (20  $\mu\text{g/ml}$ ) is visible in the electron microscope as circularized rings with central pores of  $\sim 2$  nm in diameter. The rings have been isolated, and molecular weight determinations indicate that they represent hexamers of the native toxin (7). We have proposed that transmembrane leakiness is due to embedment of these ring structures in the bilayer, with molecular flux occurring through the central channels (15, 19). Pore formation is dissectable into two steps (20, 21). Toxin monomers first bind to the bilayer without invoking bilayer leakiness. Membrane-bound monomers then laterally diffuse and associate to form non-covalently bonded oligomers that generate the pores. When toxin pores form in membranes of nucleated cells, they may elicit detrimental secondary effects by serving as nonphysiologic calcium channels, influx of this cation triggering diverse reactions, including release of potent lipid mediators originating from the arachidonate cascade (22–24).

That  $\alpha$  toxin represents an important factor of staphylococcal pathogenicity has been clearly established in several models of animal infections through the use of genetically engineered bacterial strains deleted of an active  $\alpha$  toxin gene (25–27). Whether the toxin is pathogenetically relevant in human disease, however, is a matter of continuing debate. Doubts surrounding this issue originate from two main findings. First, whereas 60% hemolysis of washed rabbit erythrocytes is effected by  $\sim 75$  ng/ml  $\alpha$  toxin,  $\sim 100$ -fold concentrations are required to effect similar lysis of human cells (4–6, 13). The general consensus is that human cells display a natural resistance towards toxin attack. The reason for the wide inter-species variations in susceptibility towards  $\alpha$  toxin is unknown but does not seem to be due to the presence or

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This study was supported by the Deutsche Forschungsgemeinschaft and the Verband der Chemischen Industrie. Address correspondence to S. Bhakdi, Institut für Medizinische Mikrobiologie, Klinikum der Justus-Liebig-Universität Giessen, D-6300 Giessen, Federal Republic of Germany.

absence of high-affinity binding sites on the respective target cells (20, 21). Second, low-density lipoprotein (28) and neutralizing antibodies present in plasma of all healthy human individuals inactivate a substantial fraction of  $\alpha$  toxin in vitro. These inactivating mechanisms presumably further raise the concentration threshold required for effective toxin attack, and it is most unlikely that such high toxin levels will ever be encountered during infections in the human organism.

The foregoing arguments rest on the validity of two general assumptions. First, the noted natural resistance of human erythrocytes to  $\alpha$  toxin must be exhibited by other human cells. Second, toxin neutralization by plasma components, usually tested and quantified after their preincubation with toxin in vitro, must be similarly effective under natural conditions, and protection afforded by these components must not be restricted to specific cell species.

In 1964, Siegel and Cohen (29) presented suggestive evidence that human platelets may be more susceptible towards attack by  $\alpha$  toxin than erythrocytes. These authors described shape changes undergone by isolated platelets upon incubation with a crude toxin preparation. They also noted that such platelets apparently released (a) procoagulatory factor(s) parallel to leaking  $K^+$  and nicotinamide adenine dinucleotide in the absence of overt cell lysis. At the same time, Bernheimer and Schwartz (30) reported that  $\alpha$  toxin induced a dramatic decrease in turbidity of rabbit platelet suspensions that, however, appeared to be due to frank platelet lysis. Since the time of these interesting observations, no further detailed studies have been conducted on the interaction of  $\alpha$  toxin with human platelets.

In the present communication, we report that human platelets indeed differ from human erythrocytes in being as sensitive towards  $\alpha$  toxin attack as rabbit erythrocytes. It will be demonstrated that neutralizing antibodies to  $\alpha$  toxin, although fully effective when assayed by standard procedures, fail to protect platelets against even low levels of  $\alpha$  toxin in human blood.  $\alpha$  toxin becomes the first bacterial cytolysin recognized to activate human platelets and promote blood coagulation in subcytolytic concentrations, processes that bear high potential relevance in staphylococcal infections.

### Materials and Methods

Monomeric  $\alpha$  toxin was recovered from a Sephacryl S-300 column after chromatography of a partially purified lyophilized toxin preparation (kindly donated by Behringwerke, Marburg, FRG), as described (15). Additionally, the toxin was purified in our laboratory from culture supernatants of *S. aureus* Wood 46 following a protocol that is to be reported elsewhere. These toxin preparations contained only very few residual contaminants that were approximated to represent <1-2% of total protein according to densitometric analyses of SDS-polyacrylamide gel stained with Coomassie Blue (Fig. 1). The toxin solutions with a protein content of 0.5-0.8 mg/ml were stored in aliquots at  $-20^\circ\text{C}$ .

**Hemolysis Assays.** Whole citrated human blood from healthy adults, or washed erythrocytes suspended to 50% hematocrit in PBS, were treated with 1 vol of  $\alpha$  toxin doubly diluted in PBS. Hemolysis was quantified by measuring hemoglobin absorbance at 412 nm in the supernatant after 60 min at  $37^\circ\text{C}$ .

**Whole Blood Samples.** Blood was drawn in citrate, heparin (3 U/ml final concentration), or EDTA (10 mM, final concentration) and held at room temperature until used.

**Platelet Preparations.** Platelet-rich plasma (PRP)<sup>1</sup> was obtained from five healthy adults

<sup>1</sup> Abbreviations used in this paper: PF-4, platelet factor 4; PPP, platelet-poor plasma; PRP, platelet-rich plasma.

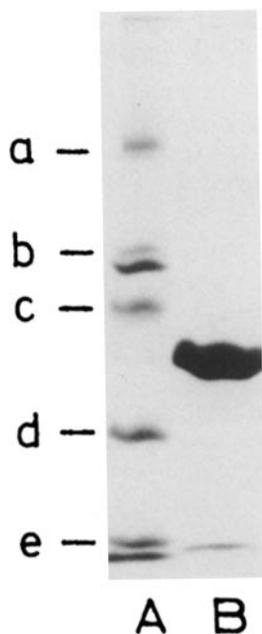


FIGURE 1. SDS-PAGE of purified  $\alpha$  toxin (B) and calibration proteins (A). 20  $\mu$ g of protein were applied to gel B. The calibration proteins (Serva, Heidelberg, Federal Republic of Germany) and their molecular weights were: (a) phosphorylase b ( $9.4 \times 10^4$ ); (b) BSA ( $6.7 \times 10^4$ ); (c) OVA ( $4.3 \times 10^4$ ); (d) carbonic anhydrase ( $3 \times 10^4$ ); (e) lactalbumin (20,100). Gels were stained with Coomassie Brilliant Blue.

through centrifugation of citrated blood samples (20–30 ml) at 200 *g* for 20 min (Minifuge, Heraeus Christ, Osterode, FRG) at 21°C. The PRP was carefully aspirated, the pH adjusted to 7.3 with 0.2 M maleic acid, and preparations were gently agitated at 12°C in a water bath. Platelet-poor plasma (PPP) was obtained by recentrifuging PRP at 5,600 *g* for 10 min, followed by four 2-min centrifugations of the supernatants at 8,000 *g* in a table top Eppendorf centrifuge. Platelet counts in PRP ranged between  $2.3$  and  $7.5 \times 10^5/\mu\text{l}$ ; PPP contained  $<100$  platelets/ $\mu\text{l}$ .

Platelets were isolated from PRP according to Mustard et al. (31). The final platelet suspensions contained no white blood cells. The washed platelets were suspended in 135 mM NaCl, 2.7 mM KCl, 12 mM NaHCO<sub>3</sub>, 0.3 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.35% (wt/vol) human serum albumin, 0.1% (wt/vol) glucose, and were held at room temperature until used.

**Measurements of Platelet Aggregation and ATP Release.** These measurements were conducted simultaneously in a Lumi-Aggro-Meter (model 400, Chrono-Log Corp., Coulter Electronics, Krefeld, FRG; reference 32). The aggregometer was equipped with a double-channel Omni-Scribe<sup>®</sup>II recorder (Coulter Electronics) for continuous recordings of optical measurements. ATP was assayed by the firefly method using luciferin/luciferase reagent (50  $\mu\text{l}$  reagent per assay; Chrono-Lume #395 from Chrono-Log Corp.; reference 33). Experiments were conducted with PRP and with washed platelets. PRP samples (0.4 ml) were preincubated at 37°C for 3 min before aggregation assays. Washed platelet suspensions were supplemented with 2 mM Ca<sup>2+</sup>, 1 mM Mg<sup>2+</sup>, and 30  $\mu\text{g}/\text{ml}$  fibrinogen (Kabi Vitrum, München, FRG) before addition of the stimuli.

**Measurements of Platelet Factor 4 (PF-4) Release.** These measurements were conducted using a commercially available ELISA (Enzygnost PF-4, Behringwerke). Experiments were conducted with whole blood samples and isolated platelets. In the latter, platelet suspensions were supplemented with 5 mM Ca<sup>2+</sup> before addition of  $\alpha$  toxin. After an incubation for 20 min at 37°C, samples were centrifuged at 2,000 *g* (60 min, 4°C) to sediment the platelets, and PF-4 was determined in the supernatants.

**Determination of Lactate Dehydrogenase (LDH).** LDH was determined with the use of a commercially available test (aca-LDH-Testpack, DuPont Lab., Bad Nauheim, FRG).

**Measurements of Clot Time.** These were performed with the use of a Mecrolab Clottimer 202 A (Heller Laboratories, Santa Rosa, CA). Experiments were conducted at 37°C with

citrated whole blood, citrated PRP, PPP, and mixtures of PRP and PPP containing varying platelet concentrations. Clot reactions were initiated through addition of 12 mM Ca plus or minus  $\alpha$  toxin to the samples.

**Assessment of Platelet-bound Toxin.** Washed platelets were suspended to  $7-9 \times 10^5$  platelets/ $\mu$ l and treated with  $\alpha$  toxin in the absence of Ca and fibrinogen for 20 min, 37°C. The toxin-treated platelets were washed thrice in PBS by centrifugation at 8,800 g for 3 min in a table top Heraeus Christ Biofuge A centrifuge. The platelets were solubilized either through addition of 50 mM Triton X-100 for quantification of bound monomers, or by boiling for 30 s in 70 mM SDS for determination of total bound toxin. The specificity and performance of the sandwich ELISA used has previously been described in detail (20, 21).

**Other Reagents.** ADP was obtained from Boehringer (Mannheim, FRG). Fibrinogen was from Kabi Vitrum (Münich, FRG). mAb  $\alpha$ 4C1 against  $\alpha$  toxin has been described (20). Another mAb, 4G3, was produced that does not inhibit toxin binding but inhibits lateral aggregation and oligomer formation in the cell membrane (Hugo, F., B. Eberspächer, and S. Bhakdi, unpublished data). Indomethacin was purchased from MSD Sharp and Dome (Münich, FRG) and the thromboxane receptor antagonist BM 13 177 was a gift from Boehringer (Mannheim, FRG). Commercial human Ig preparations were from Sandoz (Sandoglobin<sup>R</sup>; Basel, Switzerland) and Behringwerke (Beriglobin<sup>R</sup>; Marburg, FRG).

## Results

**Hemolysis of Human Erythrocytes by  $\alpha$  Toxin.** As known from the early literature (4), washed human erythrocytes are lysed only by high concentrations of  $\alpha$  toxin, 60% lysis of a 50% cell suspension occurring at  $\sim 12-15$   $\mu$ g/ml  $\alpha$  toxin. In the presence of plasma proteins, onset of hemolysis was markedly retarded and comparable hemolysis of cells in whole citrated blood occurred at toxin concentrations of  $\sim 50$   $\mu$ g/ml (Fig. 2). Hemolysis in whole blood was always nil at 5  $\mu$ g/ml and 0 to  $<2\%$  at 10  $\mu$ g/ml of  $\alpha$  toxin. These results reiterate the intrinsic high resistance of human erythrocytes towards toxin attack and show that plasma factors, presumably antibodies and low-density lipoprotein, further effectively protect red cells against  $\alpha$  toxin in whole blood.

**Release of PF-4 Invoked by  $\alpha$  Toxin in Whole Blood.** In sharp contrast to the resistance of erythrocytes towards lytic toxin action, human platelets present in citrated or heparinized whole blood responded to nonhemolytic levels of  $\alpha$  toxin by release of granular constituents. Fig. 3 depicts the release of PF-4 in blood of one donor anticoagulated with citrate, heparin, or EDTA. Essentially, the same patterns were reproduced with two other donors. The background levels of PF-4 measured in controls not treated with the toxin were somewhat high (200-600 ng/ml) due to the

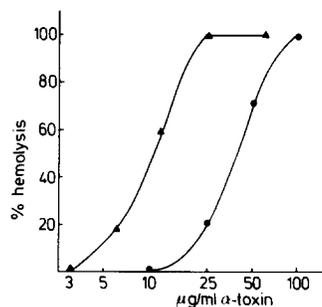


FIGURE 2. Lysis of human erythrocytes by staphylococcal  $\alpha$  toxin. (▲) 50% suspension of washed cells in PBS; (●) whole citrated blood.

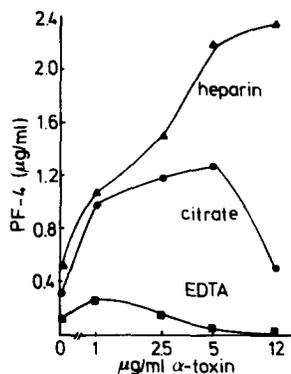


FIGURE 3. Release of PF-4 from platelets in (10 mM) whole human blood anticoagulated with heparin (3 U/ml), citrate (10 mM), or EDTA (10 mM) upon treatment with subhemolytic doses of staphylococcal  $\alpha$  toxin. PF-4 was quantified in the supernatants after a 20-min incubation with the toxin.

incubation of samples at 37°C. PF-4 release was noted at toxin levels of  $\sim 1$   $\mu\text{g/ml}$ , and plateaued at 2.5–5.0  $\mu\text{g/ml}$  toxin in heparin and citrated blood. Unexpectedly, less PF-4 was measurable in supernatants of platelets treated with 12.5  $\mu\text{g/ml}$  toxin in the presence of citrate. The presence of 10 mM EDTA abolished the effects evoked by 2.5–12.5  $\mu\text{g/ml}$  toxin, whereby PF-4 measured at 12.5  $\mu\text{g/ml}$  again presented the lowest values. At levels of  $\sim 1$   $\mu\text{g/ml}$ ,  $\alpha$  toxin appeared to induce release of very small amounts of PF-4, even in the presence of EDTA.

*$\alpha$  Toxin Induces Aggregation of Platelets and ATP Release in PRP.* In classical aggregation tests,  $\alpha$  toxin in the same concentration range of 1–2.5  $\mu\text{g/ml}$  induced platelet aggregation and ATP release in PRP from five healthy individuals. Fig. 4 A depicts the classical platelet response to an ADP stimulus (34); shape change is followed first, by primary aggregation without ATP release, and then by secondary, irreversible aggregation that is paralleled by ATP liberation (35). Fig. 4 B depicts the platelet response in one donor evoked by 2.5  $\mu\text{g/ml}$   $\alpha$  toxin. A virtually identical aggregation pattern was noted after a short lagphase of  $\sim 30$  s. However, ATP release occurred earlier than with ADP, coinciding with the commencement of the aggregation response. ATP release was also always enhanced compared with the ADP response. At 1  $\mu\text{g/ml}$  toxin, the lag-phase was prolonged to 60–80 s, and the aggregation rate was slower. The simultaneous commencement of aggregation and ATP release is well recognizable at this threshold toxin concentration (Fig. 4 C). Aggregation always appeared irreversible even at low toxin levels. Toxin concentrations of  $< 1$   $\mu\text{g/ml}$  usually did not elicit aggregation or ATP release (maximum time of observation: 5 min). With one donor, however, a platelet response similar to that shown in Fig. 4 C was observed with 0.5  $\mu\text{g/ml}$  toxin. With another donor, the platelet response commenced at 2.5  $\mu\text{g/ml}$  rather than at 1  $\mu\text{g/ml}$  toxin. These differences are presently attributed to the varying levels of antitoxin antibodies and low density lipoprotein in the individual plasma samples.

*Release of PF-4 and ATP Is not Due to Platelet Lysis.* Aliquots of PRP containing  $3 \times 10^5$  platelets/ $\mu\text{l}$  were incubated with 0–12.5  $\mu\text{g/ml}$   $\alpha$  toxin for 15 min at 37°C. After removal of platelets by centrifugation, LDH was determined in the supernatants. A sonicated PRP sample served as positive control. Whereas a concentration of 500 U/ml LDH was measured in the latter, all  $\alpha$  toxin-treated samples presented

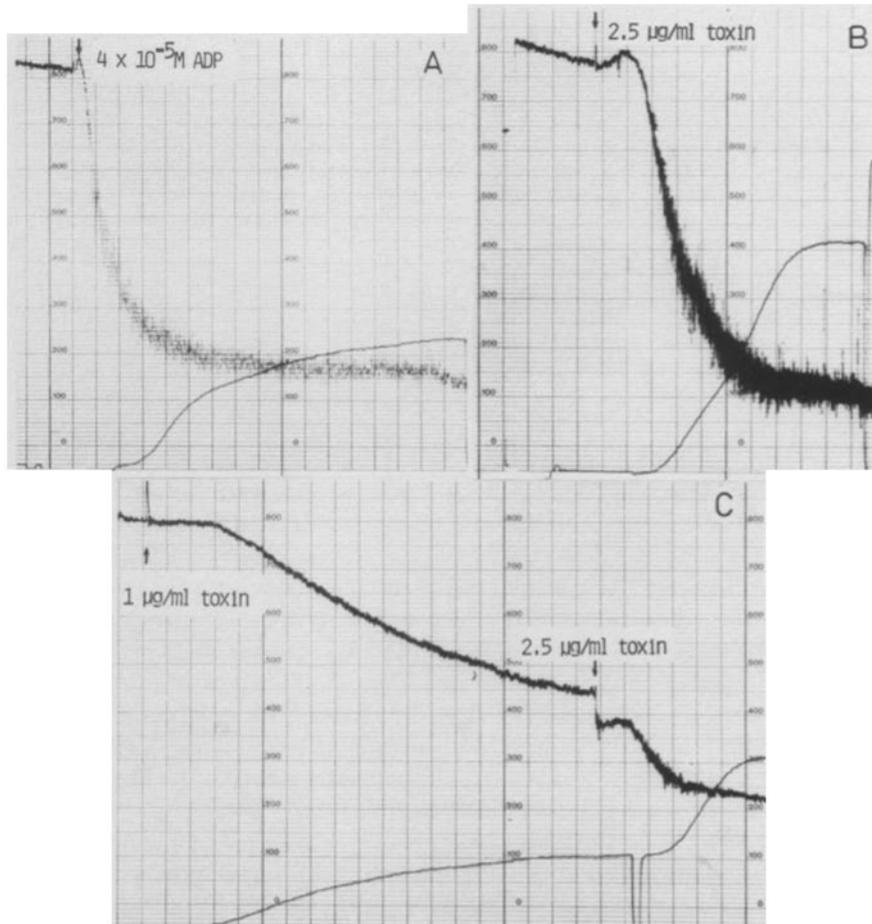


FIGURE 4. Recordings of OD changes due to platelet aggregation (*upper traces*) and of ATP release (*lower traces*) in platelet-rich plasma induced by ADP (*A*) and staphylococcal  $\alpha$  toxin (*B* and *C*).  $2 \mu\text{M}$  ATP was added as a calibration to the sample at the end of the experiment in *B*. Chart speed: two and a half columns correspond to 1 min. The traces representing ATP precede OD traces by one and a quarter columns.

concentrations of 110 U/ml, identical to the LDH level of the saline control. Hence,  $\alpha$  toxin applied in the given doses does not lyse human platelets in PRP.

*Neutralizing mAbs Impart only Partial Protection of Platelets against Toxin Action.* Two mAbs that were capable of neutralizing hemolytic toxin effects were used. The mAb  $\alpha 4\text{C}1$  binds to native toxin monomers and prevents their binding to target cells. mAb 4G3 does not inhibit toxin binding, but blocks oligomerisation of membrane-bound toxin monomers. Both antibodies suppressed release of PF-4 from platelets in heparinized whole blood when preincubated with  $\alpha$  toxin before its administration (Fig. 5 *A*). As controls, six purified IgG murine mAbs directed against streptolysin-O or terminal C5b-9 complement complexes (for review, see reference 19) were used at similar concentrations. These irrelevant antibodies did not suppress the action

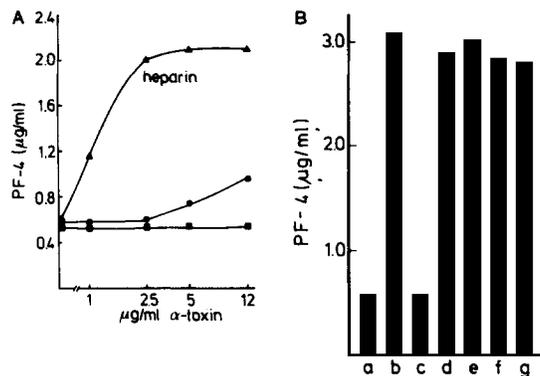


FIGURE 5. (A) Inhibition of toxin-induced platelet activation by mAbs. Heparinized whole blood was treated with a toxin ( $\blacktriangle$ ), toxin preincubated (3 min) with mAb  $\alpha$ 4C1 ( $\blacksquare$ ), or mAb 4G3 ( $\bullet$ ), and the concentrations of PF-4 were subsequently measured in the cell-free supernatants. The molar ratios of toxin to antibody were  $\sim$ 1:2 in all cases. (B) Specificity of toxin neutralization by mAbs. a toxin was preincubated with saline (b); with neutralizing mAb  $\alpha$ 4C1 (c; molar ratio of toxin to antibody, 1:3); or with one of four irrelevant mAbs (d-g) for 2 min, 22°C, and then added to heparinized blood. The final toxin concentration was 2.5  $\mu\text{g/ml}$ . After 20 min at 37°C, PF-4 was determined in the cell-free supernatant. (a) Control without  $\alpha$  toxin. Similar results were obtained in another experiment using two further irrelevant mAbs (not shown).

of  $\alpha$  toxin on platelets (Fig. 5 B). The neutralizing capacity of both neutralizing mAbs was, however, overrun if the antibodies were not preincubated with toxin. In the aggregation experiment of Fig. 6, mAb  $\alpha$ 4C1 was used at a concentration of 10  $\mu\text{g/ml}$ . If preincubated with 2.5  $\mu\text{g/ml}$  toxin for 2 min at 22°C, the antibody effected total neutralization, and no platelet aggregation was noted (molar ratio of toxin:antibody,  $\sim$ 1:1). Upon posttreatment with an additional 2.5  $\mu\text{g/ml}$  toxin, aggregation ensued (Fig. 6 A). If antibodies were applied simultaneously with the toxin, protracted aggregation occurred (Fig. 6 B) after a slightly prolonged lag-phase. If given 30 s after toxin application, the mAb was totally incapable of preventing platelet aggregation (Fig. 6 C). These results demonstrate that  $\alpha$  toxin binds rapidly to platelets, and neutralizing antibodies are rather ineffective in protecting platelets against toxin action. At the same time, these results show that the noted platelet responses are due to binding and oligomerisation of  $\alpha$  toxin, and not to a contaminant possibly contained in the toxin preparation. Preincubation of  $\alpha$  toxin with any of the six irrelevant mAbs (molar ratio of toxin to antibody,  $\sim$ 1:3) failed to affect toxin-induced aggregation.

**Response of Washed Platelets to Toxin Attack.** Isolated platelets exhibited a yet higher susceptibility towards  $\alpha$  toxin, aggregation, and ATP release already commencing at toxin levels of 50–100 ng/ml and always maximal at 0.5–1.0  $\mu\text{g/ml}$ . Release of PF-4 was also noted at these low toxin concentrations. If platelet suspensions were reconstituted with preparations of pooled human Igs (final IgG concentration: 10 mg/ml), the toxin levels required to elicit aggregation and ATP release returned to the region of  $\sim$ 1–2  $\mu\text{g/ml}$ . These results indicate that human IgG antibodies impart partial protection against the platelet activation effects of  $\alpha$  toxin. However, as noted in whole blood and PRP, this protection ends at a relatively low threshold concentration in the range of 1–2  $\mu\text{g/ml}$   $\alpha$  toxin.

**Toxin-dependent Platelet Stimulation Bypasses Cyclooxygenase Pathway.** The presence of 50  $\mu\text{M}$  indomethacin (cyclooxygenase inhibitor) or 5  $\mu\text{M}$  BM 13177 (thromboxane receptor blocker) abrogated ATP release and secondary platelet aggregation induced

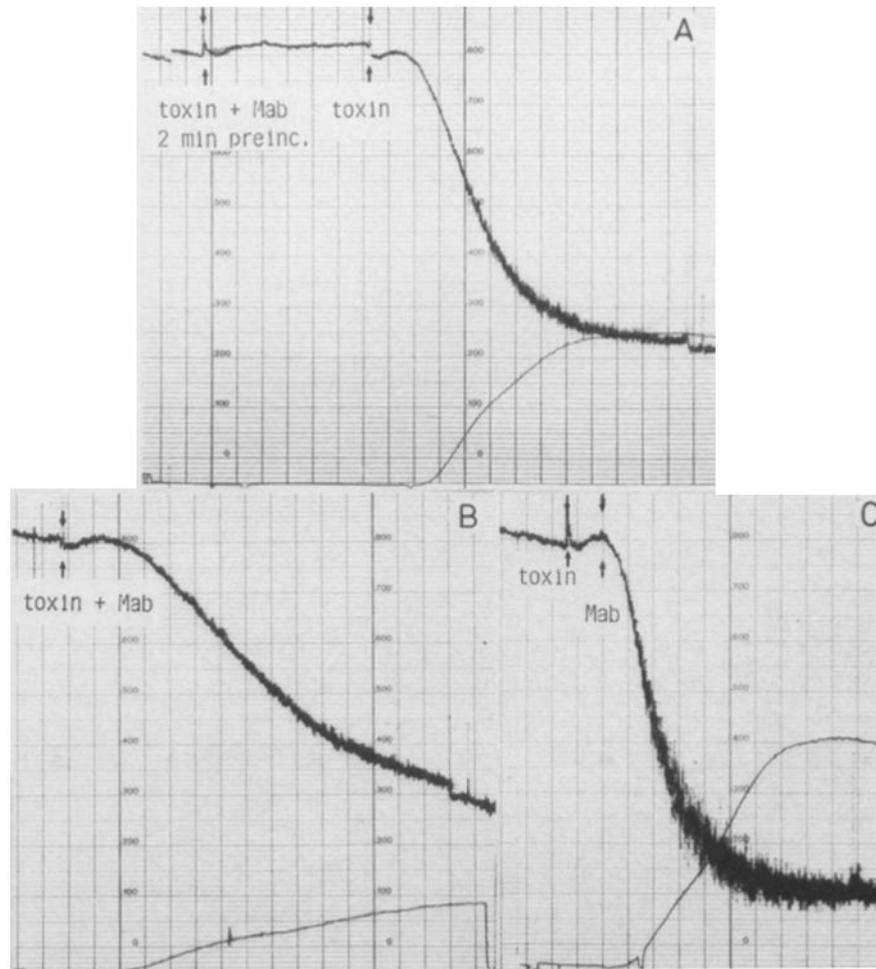


FIGURE 6. Inhibition of platelet aggregation and ATP release by mAbs. (A)  $\alpha$  toxin was preincubated with mAb  $\alpha$ 4C1 at a molar ratio of 1:1 (toxin/mAb) for 2 min at 22°C, and then added to PRP (final toxin concentration in PRP: 2.5  $\mu$ g/ml); no toxin effects were discerned. Addition of another 2.5  $\mu$ g/ml toxin to the sample resulted in platelet aggregation and ATP release. (B) Toxin and mAb were applied in the same dose as in A simultaneously. In this case, the mAb could not prevent platelet activation and aggregation. (C) toxin (2.5  $\mu$ g/ml) was applied 30 s before the mAb; no protective effect of the antibody was observed.

by ADP (Fig. 7, A and B). However, neither inhibitor was able to influence the platelet response to  $\alpha$  toxin (Fig. 7, C and D). Hence, toxin-induced platelet activation bypasses the cyclooxygenase pathway (36–38) and is thromboxane independent.

**Quantitation of Toxin Binding to Platelets.** Washed platelets were suspended in buffer without  $\text{Ca}^{2+}$  and fibrinogen, treated with  $\alpha$  toxin, and bound toxin was subsequently quantified by ELISA. As shown in Fig. 8, measurable binding of  $\alpha$  toxin to washed platelets commenced at levels  $\sim$ 100 ng/ml and increased with the amount of toxin offered. The binding exhibited no recognizable saturation and displayed

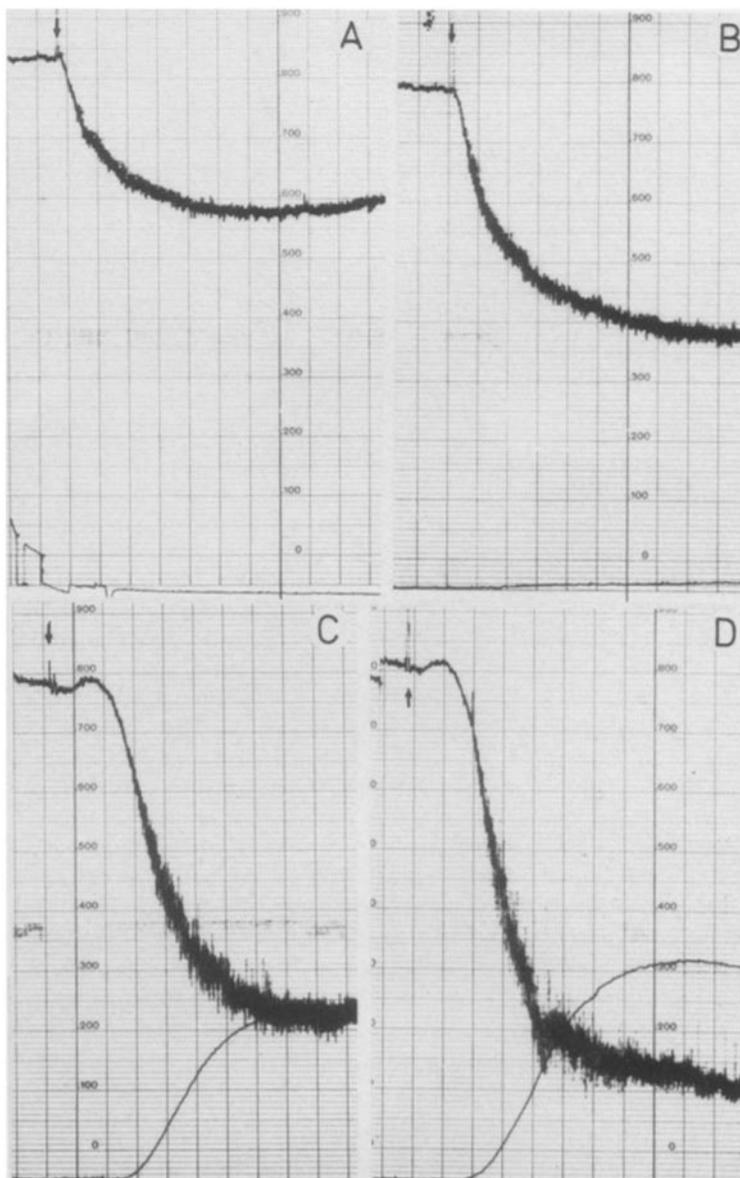


FIGURE 7. Toxin activation of platelets bypasses cyclooxygenase pathway. PRP samples were given 50  $\mu\text{M}$  indomethacin (*A* and *C*) or 5  $\mu\text{M}$  thromboxane receptor blocker BM 13 177 (*B* and *D*) and treated with  $2 \times 10^{-5}$  M ADP (*A* and *B*) or 2.5  $\mu\text{g/ml}$   $\alpha$  toxin (*C* and *D*). ADP-dependent secondary aggregation and ATP release were blocked by both agents, whereas toxin-induced effects remained unchanged.

no characteristics of a receptor-ligand interaction; the total net binding was calculated to be  $\sim 10\%$  at all toxin concentrations between 0.5 and 12.5  $\mu\text{g/ml}$ . A similar binding behavior was previously noted with rabbit erythrocytes (21). The ELISA permitted quantitative differentiation between toxin monomers and oligomers. At

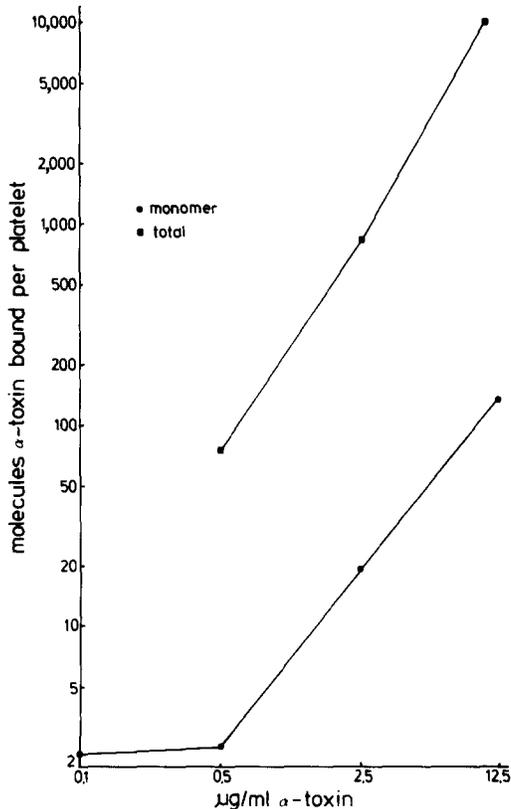


FIGURE 8. Quantitation of the binding of  $\alpha$  toxin to isolated human platelets. A suspension of platelets ( $9 \times 10^5/\mu\text{l}$ ) was given  $\alpha$  toxin at the depicted final concentrations, and platelet-bound toxin was quantified by ELISA, which differentiated between monomeric and total toxin. Net binding was  $\sim 10\%$  of total toxin offered in each sample, and no saturability of binding was observed in the measured range.

all toxin doses applied, monomers constituted  $\sim 2\%$  of total toxin. The number of oligomeric toxin molecules bound at lowest toxin concentrations (100 ng/ml) was below the detection limit of the ELISA. Assuming that the major population of cell-bound oligomers represented hexamers, we estimate that ATP release and platelet aggregation commences upon average binding of  $<10$  toxin hexamers, and is maximal upon binding of  $<100$  hexamers per platelet (Fig. 8). These results emphasize the high efficiency of  $\alpha$  toxin in activating human platelets.

*$\alpha$  Toxin Accelerates Blood Coagulation.* These experiments were conducted with citrated blood, PRP, and PPP. Clotting was initiated by recalcification in the absence or presence of  $\alpha$  toxin. As shown in Fig. 9, the presence of  $\alpha$  toxin dose-dependently enhanced the rate of clot formation, significant effects already being noted at 1  $\mu\text{g/ml}$  toxin concentration. Maximal reduction of clot times of  $\sim 70\%$  occurred when the toxin was applied to PRP at levels of 2.5–5  $\mu\text{g/ml}$ . PPP exhibited no response to  $\alpha$  toxin; hence acceleration of clot formation was dependent on the presence of platelets. Reductions in clot times similar to those observed in normal PRP (containing  $2.3 \times 10^5$  platelets/ $\mu\text{l}$ ) were already observed in plasma containing  $2.3 \times 10^4$  platelets/ $\mu\text{l}$ , and a clot-enhancing effect of  $\alpha$  toxin was noted even at platelet counts of  $1-2 \times 10^3/\mu\text{l}$ . Similar acceleration in clot formation was observed upon addition of  $\alpha$  toxin to whole citrated blood. The reductions in clot times were

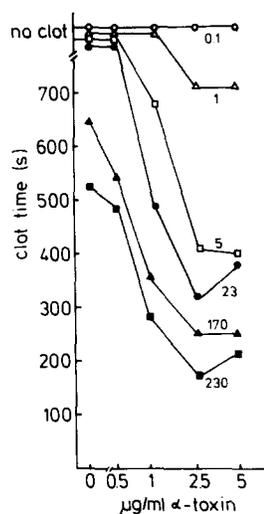


FIGURE 9 Reduction in clot times of recalcified human plasma by  $\alpha$  toxin. Citrated plasma samples containing the depicted numbers of platelets (per nanoliter) were given 12 mM  $\text{Ca}^{2+}$  and  $\alpha$  toxin at the given final concentrations. Marked reductions clot times were noted at toxin concentrations of 1–2.5  $\mu\text{g}/\text{ml}$ , dependent on the presence of platelets in the plasma sample. *No clot* indicates lack of clot formation within 900 s.

totally abrogated if  $\alpha$  toxin was preincubated with mAb  $\alpha 4\text{C}1$  (two molecules antibody per molecule toxin, 2 min, 22°C).

### Discussion

Unexpected aspects regarding the action and potential pathophysiological relevance of a major bacterial cytolyisin in the human organism arise from the present study. The dogma that human cells are generally insensitive towards staphylococcal  $\alpha$  toxin attack must be rectified. In fact, human platelets exhibit similar susceptibility towards the toxin as rabbit erythrocytes, responding to toxin levels as low as 50 ng/ml. The platelet reaction comprises a classical irreversible aggregation upon stirring of PRP at 37°C in an aggregometer, paralleled by release of  $\alpha$  granule constituents (39–44), documented in the present study through measurements of liberated PF-4. Toxin-treated platelets also released ATP, whereby differentiation between ATP released from the cytosol as opposed to release from dense bodies was not yet undertaken. Binding of  $\alpha$  toxin to platelets is rapid, ensuing within 30–90 s at 37°C. Elicitation of the platelet reaction requires not only binding of monomers, but also formation of membrane-bound oligomers, since it can be prevented by preincubation of toxin with two mAbs, one acting to inhibit toxin binding, the other blocking toxin oligomerisation. The rapidity and high efficiency of toxin action on platelets could partially account for the second unexpected finding that neutralizing antibodies fail to effectively protect platelets against  $\alpha$  toxin. In PRP and whole blood, the threshold concentration required for successful toxin attack is raised in the presence of human antibodies to only  $\sim 1\text{--}2 \mu\text{g}/\text{ml}$ . In contrast, red cell lysis in whole blood is usually nil even at toxin concentrations of 10  $\mu\text{g}/\text{ml}$ . We have found that human white blood cells display a similar resistance towards  $\alpha$  toxin as erythrocytes (unpublished data). Our results thus confirm the suspicion of Siegel and Cohen (29) that they had “identified a human cell (or cell fragment) highly susceptible to this agent,” and identify platelets as the primary cell targets for  $\alpha$  toxin attack in human blood. The cause for the high susceptibility of platelets compared with other blood

cells is unknown. The unsolved enigma regarding the widely differing susceptibility of various cell targets to toxin attack has recently been discussed in detail (21, 45), and we have no new data to justify any further speculation at present. The binding data obtained in this study are very similar to those found for toxin binding to rabbit erythrocytes and speak against the presence of specific, saturable receptors on the platelets. As discussed previously (21, 45), we believe that surface properties of the respective cell target, such as density and orientation of charged groups, are important in determining the concentration threshold at which successful toxin attack will occur. Even when this takes place, overall net toxin binding will be rather ineffective, and toxin molecules will not be quantitatively taken up by the cell targets. In the present study (Fig. 8), we estimate that only  $\sim 10\%$  of toxin offered in solution becomes platelet bound, the bulk of toxin thus remaining available for attack on other cells. The binding inefficiency of  $\alpha$  toxin to rabbit platelets was indeed already noted by Bernheimer and Schwartz in 1965 (30). These authors showed that continued loss of turbidity occurred when several aliquots of rabbit platelets suspensions were consecutively added to toxin solution.

The precise nature of the toxin-induced reactions of human platelets has not yet been delineated, but it appears probable that the toxin forms transmembrane pores, and activation results from influx of calcium ions. The release of  $\alpha$  granule constituents and ATP elicited by  $\alpha$  toxin is not due to simple lysis of the platelets, since no release of cytoplasmic LDH was noted even at 12.5  $\mu\text{g/ml}$   $\alpha$  toxin. This finding fully confirms the early report of Siegel and Cohen (29), who registered rapid  $\text{K}^+$  and NAD-efflux from toxin-treated platelets in the absence of protein release. Electron microscopic studies are presently underway in this laboratory, and preliminary results also indicate that platelet lysis does not occur at the given toxin concentrations. Analogous, calcium-dependent cell activation after binding of pore-forming cytolysins to membranes has been demonstrated in several recent studies. Examples include the stimulation of arachidonate metabolism invoked by  $\alpha$  toxin in endothelial cells (23) and leukocytes (24), and by complement C5b-9 in various cell targets (e.g. references 46-49). With special regard to platelets, it is notable that earlier studies by Polley and Nachman (50, 51), more recently followed up by Hänsch et al. (52) and Wiedmer and Sims (53, 54), have similarly demonstrated platelet activation by C5b-9. In our present study, toxin-induced activation unsurprisingly exhibited a requirement for  $\text{Ca}^{2+}$ , and release of PF-4 by 2.5-12  $\mu\text{g/ml}$   $\alpha$  toxin was totally abrogated in the presence of EDTA. Why PF-4 release is apparently diminished when platelets in citrated and EDTA-anticoagulated PRP are given high doses of toxin (12.5  $\mu\text{g/ml}$ ) is unclear. We currently speculate that this may be due to rapid codiffusion of citrate and EDTA across the toxin pores into the cells. The cause of minimal PF-4 release observed in EDTA at marginal toxin levels (1  $\mu\text{g/ml}$ ) also remains unknown.

The failure of indomethacin and the thromboxane receptor blocker to suppress platelet response to  $\alpha$  toxin contrasts with the effects of these inhibitors on ADP-dependent stimulation and indicates that the platelet response to  $\alpha$  toxin bypasses the cyclooxygenase/thromboxane pathway (36-38). These preliminary results are presented because the realization that the described processes are refractory towards inhibition by related pharmacotherapeutic agents may be of practical importance. From a positive viewpoint,  $\alpha$  toxin could become valuable as a membrane-perme-

abilizing agent for probing the minimal requirements for granule exocytosis in platelets. Several recent studies have already begun to exploit the use of this toxin in the study of exocytic processes (e.g., reference 55). The toxin could also be used as a tool to probe the importance of thromboxane in the induction of platelet aggregation (56, 57).

A priori, it may not be surprising that a cytolysin that generates transmembrane pores stimulates platelets. The unexpected findings made in the present study relate to the extreme susceptibility of human platelets and the capacity of  $\alpha$  toxin to evoke procoagulatory responses in these cells in whole blood at low concentrations such as may be expected to be present in tissues during severe infections with *S. aureus* and systemic disease. In vitro,  $\alpha$  toxin can reduce clotting times by up to 70%, an effect that is dependent on the presence of platelets. In vivo,  $\alpha$  toxin may thus act synergistically with staphylocoagulase to cause local thrombus formation. In severe deep infections and septicemia, systemic platelet responses to  $\alpha$  toxin might even contribute to the pathogenesis of disseminated intravascular coagulation. The present study is a long overdue continuation of the pioneering work by Siegel and Cohen (29) and Bernheimer and Schwartz (30). It is the first demonstration that low, non-hemolytic levels of a bacterial cytolysin can promote coagulation by selectively activating platelets in human blood.

Staphylococcal  $\alpha$  toxin in the nonhemolytic concentration range of  $\sim 1$   $\mu\text{g/ml}$  binds to and stimulates platelets in human blood. After addition of the toxin to stirred platelet-rich plasma, a short lag-phase (30–70 s) is followed by platelet shape change and irreversible aggregation. The stimulation of platelets in whole citrated or heparinized blood has also been demonstrated by measurements of platelet factor 4 release. Aggregation and release of granule constituents are not inhibitable by indomethacin or by the thromboxane receptor blocker BM 13177. Washed human platelets are sensitive to even lower concentrations of 0.05–0.10  $\mu\text{g/ml}$   $\alpha$  toxin. In the presence of human IgG antibodies, the threshold for effective toxin attack returns to levels of  $\sim 1$   $\mu\text{g/ml}$ . An mAb that inhibits toxin binding to cells totally suppresses platelet activation if preincubated, but not if applied simultaneously with the toxin. Activation of washed platelets correlates with binding of toxin oligomers to the cells, maximal activation occurring upon binding of an average of  $<100$  hexamers per platelet. When added to recalcified citrated blood or to platelet-rich plasma,  $\alpha$  toxin reduces clotting times by up to 70%; this effect is dependent on the presence of platelets. The collective data identify platelets as primary targets for  $\alpha$  toxin attack in human blood, and demask its potential to invoke imbalance of hemostasis that may be of pathophysiological relevance during severe local and systemic staphylococcal infections in the human host.

We thank Drs. J. Trantum-Jensen, O. Behnke, K. Preissner, W. Seeger, S. Santoso, J. Bux, and Prof. A. Akatov for advice and valuable discussions.

*Received for publication 1 February 1988 and in revised form 28 March 1988.*

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