Human Hyperimmune Globulin Protects against the Cytotoxic Action of Staphylococcal Alpha-Toxin In Vitro and In Vivo

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Alpha-toxin, the major cytolytic of Staphylococcus aureus, preferentially attacks human platelets and cultured monocytes, thereby promoting coagulation and the release of interleukin-1 and tumor necrosis factor. Titers of naturally occurring antibodies in human blood are not high enough to substantially inhibit these pathological reactions. In the present study, F(ab')2 fragment preparations from hyperimmune globulin obtained from immunized volunteers were tested for their capacity to inhibit the cytotoxic action of alpha-toxin in vitro and in vivo. These antibody preparations exhibited neutralizing anti-alpha-toxin titers of 80 to 120 IU/ml, whereas titers in commercial immunoglobulin preparations were 1 to 4 IU/ml. In vitro, the presence of 2 to 4 mg of hyperimmune globulin per ml protected human platelets against the action of 1 to 2 μg of alpha-toxin per ml. Similarly, these antibodies fully protected human monocytes against the ATP-depleting and cytokine-liberating effects of 0.1 to 1 μg of alpha-toxin per ml. Intravenous application of 0.5 mg (85 to 120 μg/kg of body weight) of alpha-toxin in cynomolgus monkeys elicited acute pathophysiological reactions which were heralded by a selective drop in blood platelet counts. Toxin doses of 1 to 2 mg (170 to 425 μg/kg) had a rapid lethal effect, the animals presenting with signs of cardiovascular collapse and pulmonary edema. Prior intravenous application of 4 ml of hyperimmune globulins per kg inhibited the systemic toxic and lethal effects of 1 mg (200 μg/kg) of alpha-toxin. In contrast, normal human immunoglobulins exhibited no substantial protective efficacy in vitro and only marginal effects in vivo. It is concluded that high-titered anti-alpha-toxin antibodies effectively protect against the cytotoxic actions of alpha-toxin.

Many strains of Staphylococcus aureus produce alpha-toxin (3, 11, 12, 14, 18, 20), a 34,000- Mr, proteinaceous cytolytin that damages cells by forming hydrophilic pores across the plasma membrane (1, 5, 7, 10, 15, 21, 22). There is consensus regarding the pathogenetic relevance of this toxin in animal models (16), but its significance in humans has been debated because human cells have been thought to be relatively resistant towards toxin action (14). Furthermore, if preincubated with alpha-toxin, naturally occurring antibodies as well as lipoproteins effectively neutralize toxin activity in vitro (4). For these reasons, many investigators deem it improbable that alpha-toxin plays a relevant role as a pathogenicity factor in the human host.

The possible relevance of alpha-toxin must, however, today be reconsidered in the light of two recent findings. First was the discovery that human platelets represent highly susceptible targets for toxin action (6). When applied in nonhemolytic concentrations of 0.5 to 2 μg/ml, alpha-toxin was found to bind to and stimulate platelets in human blood, this process in turn promoting coagulation. Second, human monocytes were identified as another highly vulnerable cell type, and toxin attack on cultured cells was found to evoke rapid release of interleukin-1 (IL-1) and low to moderate levels of tumor necrosis factor (TNF) (S. Bhakdi et al., submitted for publication). From these findings, it has become apparent that alpha-toxin is endowed with the capacity to provoke cellular reactions that may augment the severity of disease.

A significant recognition emerging from the above studies is related to the unexpected inability of naturally occurring antibodies and lipoproteins to effectively inhibit the action of alpha-toxin on the highly sensitive cells. The fact that certain cytolsins may successfully attack susceptible cells in the presence of plasma inactivators presumably derives from more rapid binding to these cells, as opposed to their interaction with antibodies when the latter are present at low physiological concentrations. In the light of these recognitions, attempts were launched to produce an effective antitoxic agent. In this study, we report the preparation of hyperimmune human antibodies against alpha-toxin. These antibodies, but none of the tested, commercially available immunoglobulin preparations, effectively protected human platelets and monocytes from attack by alpha-toxin. In addition, application of therapeutic doses of the hyperimmune globulin protected monkeys against the toxic and lethal action of alpha-toxin.

MATERIALS AND METHODS

The preparation of alpha-toxin has been described previously (6). Toxin preparations yielded a single polypeptide band of Mr 34,000 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (6). All cytotoxic effects described in this paper could be abrogated by preincubation of alpha-toxin with equimolar amounts of neutralizing monoclonal antibody clone α4C1 (13). We therefore assume that alpha-toxin was the sole active agent in the preparations. The following methods were performed as described previously (see reference 6; Bhakdi et al., submitted): clot tests with whole blood and platelet-rich plasma (PRP), measurements of platelet aggregation, quantitation of ATP by bioluminiscence, and quantitation of IL-1β and TNF-α by radioassays (both from IRE Diagnostic, Düsseldorf, Federal Republic of Germany). Additionally, platelet prothrombinase activity was measured by following a conventional procedure (8). Briefly, platelets
were isolated from PRP by a passage over a Sepharose 2B column (Pharmacia, Uppsala, Sweden; 2 by 18 cm) equilibrated in Hanks balanced salt solution containing 1 mM EDTA and 0.5% bovine serum albumin. The gel-filtered platelets were suspended to a density of approximately 10^6 particles per ml, and prothrombinase activity was assessed in microtiter plates (Nunc, Wiesbaden, Federal Republic of Germany) after the addition of stimulus, 3.3 mM Ca\(^{2+}\), functionally pure factor Xa (Thame, Oxford, England), and prothrombin (a gift of K. Preissner, Giessen) by using the chromogenic thrombin substrate S-2238 (2) from Kabi Vitrum (Stockholm, Sweden). Absorbsances were read at 405 nm in an EAR 400 reader (SLT Lab Instruments, Overath, Federal Republic of Germany). Assays were calibrated with thrombin which was prepared as previously described (17). The thrombin preparation had a fibrinogen clotting activity of 2,200 to 2,500 NIH units per mg, as determined by the method of Fenton and Fasco (9). As to be detailed elsewhere, 100 ng of alpha-toxin per ml provoked factor Va-dependent prothrombinase activity in this system.

Production of hyperimmune human immunoglobulins against alpha-toxin. Volunteers were immunized by subcutaneous application of two doses of the developmental vaccine from Behringwerke AG at a 4-week interval. The vaccine consisted of 0.1 mg of Formol-activated alpha-toxin (0.5 ml of solution, applied without adjuvant). Hyperimmune serum specimens were drawn several weeks after immunization, and the immunoglobulin G (IgG) fraction was isolated according to conventional procedures. The IgG fraction was treated with pepsin at a slightly acid pH, and the resulting F(ab')\(_2\) fragments were dialyzed, stabilized with glycine, and lyophilized. Five-percent solutions of these gamma globulin preparations exhibited antitoxin titers of 80 to 120 IU/ml as defined by the World Health Organization "List of Standards," 1982.

Experiments with cynomolgus monkeys. In vivo toxicity of alpha-toxin was tested in a total of six adult cynomolgus monkeys (Macaca fascicularis). The animals were from a breeding colony of the Behringwerke. They were housed in quarantine for a minimum of 3 days prior to experiments in an air-conditioned room maintained in an environment of 25 ± 1°C, 55 ± 10% relative humidity, and 12 h of light-12 h of darkness cycles. Food was withheld for 18 h before each trial. The body weights of the animals ranged from 4.1 to 5.2 kg.

Animals were anesthetized by intravenous application of ketamin HCl (Ketaneest; Parke, Davis & Co., Detroit, Mich.; 0.2 ml/kg) and pentobarbital sodium (Nembutal; Compagnie Rousset, Paris; 20 mg/kg of body weight). Catheters for injection and blood collection and devices for recording of physiological body functions were placed under narcosis. Test and control reagents were administered by intravenous injection into the jugular vein. Intravenous application was chosen because this is the route of administration of immunoglobulins in humans. Each toxin dose was applied in a total volume of 2 to 5 ml of saline over a period of 30 s. The following parameters were measured: systolic blood pressure; heart rate; partial O\(_2\) pressure (pO\(_2\); transcutaneous); electrocardiogram (ECG) (1st lead); numbers of blood leukocytes, erythrocytes, and platelets; hemoglobin; hematocrit; platelet aggregation capacity; quick time; partial prothrombin time; plasma fibrinogen; and plasma antithrombin III.

Arterial blood pressure was measured in the carotid artery with a stethoscope transducer and an electromanometer (Hellige Lab, Freiburg, Federal Republic of Germany). ECGs (1st lead) were recorded with subcutaneous needle electrodes and an ECG recording device. Blood cell counts were performed with a Coulter Counter T 660 (Coulter Electronics, Krefeld, Federal Republic of Germany). Plasma antithrombin III was determined with the chromogen substrate method (Berichrom AT III kit; Behringwerke). Transcutaneous pO\(_2\) measurements were performed with an oxymonitor (Hellige Lab).

Other reagents. Polyclonal immunoglobulin preparations from human plasma for intravenous (IVIG) or intramuscular application were obtained from Behringwerke (Gamma-Veinin, Venimun, and Beriglobin), Sandoz (Basel, Switzerland; Sandoglobin), Biotest (Frankfurt, Federal Republic of Germany; Intraglobin F, Pentaglobin), Armour Pharma (Eschwege, Federal Republic of Germany; Purimum), Kabi Vitrum (Munich, Federal Republic of Germany; Kabi-globin), Troponwerke (Cologne, Federal Republic of Germany; Polyglobin N), and Immuno (Heidelberg, Federal Republic of Germany; Endoglobin). Human serum albumin (5% solution) was obtained from Behringwerke.

Determination of antibody titers against alpha-toxin. These assays were performed according to a standard procedure with the neutralization assay (Antistaphylolysin Reaction) from Behringwerke. It was found that commercial IVIG preparations displayed antitoxin titers ranging from 1 to 4 IU/ml. The antibody titers in plasma from healthy adults are uniformly in the range of 0.5 to 0.8 IU/ml. In contrast, preparations of hyperimmune globulin exhibited antitoxin titers ranging from 80 to 120 IU/ml. The mean antitoxic titer in serum from five adult monkeys that were used in this study was 2.4 IU/ml, i.e., considerably higher than the neutralization titer found in normal human plasma.

RESULTS

Hyperimmune immunoglobulin protects human platelets against attack by alpha-toxin. As shown in Fig. 1A, the addition of 2 µg of alpha-toxin per ml to human PRP rapidly induced irreversible platelet aggregation and release of ATP. These effects of alpha-toxin have been described in detail (6). Figure 1B depicts an experiment conducted with the same PRP supplemented with a preparation of commercially available IgG. It is apparent that the presence of these pooled human antibodies did not substantially inhibit the action of alpha-toxin on platelets. Essentially the same negative results were obtained with all of the tested commercial immunoglobulin preparations. In contrast, the presence of 0.9 mg of hyperimmune immunoglobulins per ml almost entirely inhibited toxin-dependent platelet responses, and full protection was observed with immunoglobulin concentrations of 1.8 mg/ml. These plasma levels are readily attainable by intravenous administration of immunoglobulins in humans.

The next set of experiments examined the effect of antibodies on the development of prothrombinase activity in toxin-treated platelets. As to be reported in detail elsewhere, isolated human platelets attacked by alpha-toxin develop FXa-dependent prothrombinase activity due to the enhanced expression of FVa on the cell surface. This process forms the basis for the previously demonstrated procoagulatory activity of alpha-toxin. Figure 2 depicts the results of an experiment wherein the platelets were treated with 0.1 µg of alpha-toxin per ml and the ensuing prothrombinase activity was measured through quantification of thrombin generation. Whereas non-toxin-treated controls exhibited virtually no prothrombinase activity (open circles), thrombin generation
was rapidly noted in the presence of alpha-toxin (triangles). This stimulation of FVa-associated prothrombinase activity was fully prevented in the presence of 1 mg of hyperimmune globulins per ml (closed circles). In contrast, a commercial immunoglobulin preparation applied at the same concentration entirely failed to suppress development of prothrombinase activity.

Figure 3 depicts the results of an experiment wherein coagulation times were determined in samples of whole citrated blood after recalcification in the presence of increasing amounts of alpha-toxin. The results are mean values of data obtained in six independent experiments with different donors. As reported previously, alpha-toxin dose-dependently caused a reduction in coagulation times, here expressed as percentage of those registered for control non-toxin-treated samples. The dose-response behavior varied from donor to donor, but the overall procoagulatory effect was always noted. Whereas the addition of commercial IgG preparations (3.6 mg/ml, final concentration) led to only slight reduction of this effect, presence of hyperimmune globulin almost totally abrogated the procoagulatory action of 0.1 to 0.5 μg of alpha-toxin per ml, as tested in this system.

**Hyperimmune globulin inhibits induction of monokine release by alpha-toxin.** Next, the capacity of hyperimmune globulins to inhibit the cytotoxic action of alpha-toxin on human monocytes was investigated. Monocytes in short-term culture were treated with increasing doses of alpha-toxin in the presence of 50% autologous serum for 6 h (37°C). Thereafter, cellular ATP content was determined. With this donor (Fig. 4), ATP depletion commenced at approximately 1 μg of alpha-toxin per ml and was essentially complete at 5 μg/ml (Fig. 4A). As previously reported, cytotoxic, ATP-depleting toxin effects were accompanied by the appearance of high levels of IL-1β in the cell supernatant, whereas lower toxin doses stimulated the production of TNF-α (Fig. 4B and C). Again, supplementation of 50% normal human serum with 2 mg of hyperimmune globulin per ml almost totally abrogated both the ATP-depleting and cytokine-liberating effects of alpha-toxin in the concentration range of 0.1 to 1.25 μg/ml. At the high dose of 5 μg of alpha-toxin per ml,
VOL. 57, with a toxin were taken as respective IVIG let-rich plasma. 

Assays supernatants were IVIG per ml. 

FIG. 3. Determination of coagulation times in recalcified platelet-rich plasma containing no additional IgG (□), 4 mg of commercial IVIG per ml (▲), and 4 mg of hyperimmune globulin per ml (●). The respective original clotting times determined in the absence of added toxin were taken as 100%. Data represent mean values obtained with a total of six donors.

FIG. 5. Protection of monkey platelets against attack by 6 μg of alpha-toxin per ml by hyperimmune globulin. A, Control, hyperimmune globulin applied (arrow, 1.8 mg/ml final concentration) without alpha-toxin; B, hyperimmune globulin applied (first arrow) and this procedure followed by 6 μg of alpha-toxin per ml (second arrow); C, commercial IVIG (Gamma-Venin) applied prior to alpha-toxin.

ATP depletion and liberation of cytokines were still markedly depressed compared with controls. In contrast, the addition of IVIG to cell cultures entirely failed to prevent the action of alpha-toxin on monocytes in this system. Essentially the same pattern of results was reproduced with monocytes from a second individual.

Action of alpha-toxin on platelets of cynomolgus monkeys. PRP was prepared from citrated blood of monkeys. Considerably higher doses of alpha-toxin (≥3 μg/ml) were required to induce platelet aggregation and ATP release compared with the situation with human PRP samples. We attribute this finding to the markedly higher titer of naturally occurring toxin inhibitors (presumably, antibodies and lipoproteins) in monkeys. As shown in Fig. 5, hyperimmune globulin (1.8 mg/ml) substantially protected the platelets from attack by 6 μg of alpha-toxin per ml (Fig. 5A and B). In contrast, IVIG did not display this protective efficacy (Fig. 5C).

Hyperimmune globulins protect monkeys against the toxic and lethal action of alpha-toxin. Bolus injection of up to 0.1 mg of alpha-toxin elicited no discernable, acute toxic effects in monkeys. First toxic manifestations were noted in two animals after application of 0.5 mg of alpha-toxin (85 and 100 μg/kg, respectively). The animals presented with a slight but significant drop in arterial blood pressure, a selective fall in blood platelet counts, and ECG alterations. The latter included prolongation of the QRS complex, depression of the ST segment, extra systoles, and arrhythmias. There was also a slight but significant reduction in plasma antithrombin III in one animal. These toxin reactions were all noted within 5 min after toxin application, whereas other measured blood parameters remained essentially unchanged. Subsequent infusion of 1 mg of alpha-toxin (170 and 200 μg/kg, respectively) was rapidly fatal in one animal and produced highly toxic reactions in a second one (Fig. 6), where a sharp drop in P0₂, heart rate, and systolic blood pressure, marked ECG alterations, and further lowering of blood platelet counts were registered (Fig. 6). In addition, a pronounced reduction in platelet aggregation capacity and a slight reduction of plasma antithrombin III were noted. Other measured param-
FIG. 6. Systolic blood pressure, heart rate (beats per minute), arterial pO₂, and blood platelet counts monitored in a cynomolgus monkey that received bolus infusions of alpha-toxin at the depicted times.

Parameters remained within the normal range. To ensure that the noted in vivo effects were due to alpha-toxin, we preincubated 1 mg of alpha-toxin with 4 mg of monoclonal antibody α4C1 for 15 min, 22°C. Application of this solution failed to evoke any pathological effects in a control animal. Four animals were used in the next set of experiments. The control monkey received physiological saline; the second and third were given 2 and 4 ml of hyperimmune globulin per kg, respectively, five minutes prior to toxin application; and the fourth animal received 5 ml of conventional IVIG per kg. Application of 0.5 mg (120 μg/kg) of alpha-toxin in the control animal again induced a rapid fall in pO₂ and systolic blood pressure, a 50% reduction in blood platelet counts 5 min after administration, and alterations in the ECG. In contrast, prior application of 2 ml of hyperimmune globulin per kg almost totally prevented the manifestation of these acute intoxication symptoms, and no toxic effects whatsoever were observed in the animal that had received 4 ml of these antibodies per kg. A slight protective effect was noted in the animal that received the high dose of IVIG at this toxin concentration (0.5 mg). Figure 7 summarizes the results of blood platelet determinations in the different experiments, and Fig. 8 shows the course of a prolonged experiment conducted with the monkey that had received 4 ml of hyperimmune globulin per kg. This animal showed no signs of toxic responses 30 min after injection of another 1 mg of alpha-toxin (210 μg/kg). In contrast, the animal that had received 5 ml of normal IVIG presented with highly toxic cardiovascular reactions and a 75% reduction in platelet counts upon treatment with the same toxin doses (Fig. 7).

Upon postmortem examination, all animals that had succumbed to the acute lethal action of alpha-toxin were found to display pulmonary edema. No consistent pathological alterations were observed in other organs.

FIG. 7. Summary of data on blood platelet counts in five cynomolgus monkeys determined 5 min after application of alpha-toxin at the depicted concentrations. The original counts determined prior to toxin application were taken as the respective 100% values. Animals received the following agents 5 min prior to application of toxin: 1, 4 ml of hyperimmune globulin per kg; 2, 2 ml of hyperimmune globulin per kg; 3, 5 ml of human serum albumin per kg (5% solution); 4, 5 ml of saline per kg; 5, 5 ml of commercial IVIG per kg.

DISCUSSION

The major impetus for this work stemmed from recent recognitions that attack by subhemolytic concentrations of alpha-toxin on platelets promotes coagulation and that cultured monocytes damaged by the toxin release large amounts of IL-1β. Although naturally occurring antibodies and lipoproteins do not efficiently inhibit these processes, we report that hyperimmune antisera can be raised in humans and can be successfully used to protect cells against toxin action.

In vitro models were first used to test the efficacy of protection attainable with our antibody preparations. Whereas no substantial protection was noted with any of the tested, commercially available immunoglobulins, hyperimmune antitoxin antibodies prevented aggregation and ATP release from platelets in PRP, prevented manifestation of factor Va activity in isolated platelets, almost suppressed the procoagulatory effects of moderate toxin doses, and abrogated the effects of alpha-toxin on monocytes. The levels of immunoglobulins required to attain this protective efficacy were well within the conventional therapeutic range of 2 to 4 mg/ml.

We deemed it essential to test the in vivo effects of the antibodies in primates because of the well-known difference in susceptibility of cells from various animal species. For example, low doses (2 μg/ml) of alpha-toxin provoke hemolysis when applied to rabbit blood (14; unpublished data). Under the same conditions, no hemolysis was observed in human or monkey blood. Intravascular hemolysis would obviously trigger an array of pathological systemic reactions that are, however, irrelevant to the situation in humans. For
this reason, a limited number of experiments were conducted with cynomolgus monkeys. Because monkey plasma contains much higher levels of toxin inhibitors (2 to 3 IU/ml as opposed to 0.5 to 0.8 IU/ml in humans), two- to threefold higher concentrations of alpha-toxin were required to provoke aggregation of platelets and ATP release from these cells in PRP. In vivo, the threshold of acute toxicity lay in the range of 500 μg of alpha-toxin per animal (100 μg/kg). If we assume that the blood volume corresponds to approximately 8% of body weight, i.e., 320 to 400 ml, we find that the level of alpha-toxin eliciting first acute toxic effects is in the order of 1 to 2 μg/ml of whole blood or 2 to 4 μg/ml of plasma. (Since the toxin cannot enter the intact red blood cells, the bulk of applied protein will initially distribute in the plasma volume only.) The latter concentration corresponds excellently with in vitro findings, where first effects of alpha-toxin on platelets were noted at approximately 3 μg/ml. By extrapolation, one might predict that the threshold of acute toxicity in humans would be in the toxin concentration range of 0.1 to 0.25 μg/ml of whole blood, which corresponds to the levels at which first accelerations of clotting times are noted. In the absence of plasma proteins, the toxicity threshold will probably be considerably lower; such situations may arise at local sites of infections, e.g., in wounds and abscesses. Intravenous application of 2 to 4 ml of hyperimmune globulins per kg (7 to 14 g, total dosage), which represents a realistic dosage in conventional immunoglobulin therapy, would generate plasma levels of 3 to 5 mg of these antibodies per ml. According to our present data, such levels should suppress the major effects of circulating alpha-toxin. In monkeys, it was indeed found that similar immunoglobulin doses effectively prevented the development of acute reactions elicited by otherwise highly toxic or rapidly fatal toxin doses. It is noteworthy that the first and most sensitive parameter heralding the advent of acute toxic reactions was a fall in blood platelet counts. In contrast, significant alterations in red or white blood cell counts were not noted within the periods of observation. White blood cells were not differentiated, and a selective fall in monocytes cannot be excluded. Due to the constraints imposed on in vivo experimentation, we did not attempt to detect monokines in monkey plasma. These and other temporally longer-range effects of alpha-toxin were not addressed, but we assume that such effects will also be significantly inhibited by the hyperimmune globulin. As to be expected in acute and rapidly fatal intoxications, postmortem pathological alterations were minimal, pulmonary edema representing the most consistent finding. Whether the edema resulted from the concerted effects of alpha-toxin on platelets and monocytes and the ensuing disturbances in the microcirculation and cardiac function or whether it was due to toxin effects on endothelial cells cannot be decided at present. Pulmonary edema has been shown to develop as a direct consequence of toxin perfusion through isolated, blood-free rabbit lungs, probably due to an attack on endothelial cells (19). It is not yet known whether primate endothelial cells represent targets for toxin attack under physiological conditions.

The exceptionally high antibody titers against alpha-toxin exhibited by hyperimmune globulin preparations are never found in pooled IVIG from normal donors, accounting for the fact that the latter fail to display a similar protective capacity. Since normally occurring antibody titers against alpha-toxin are uniformly low in healthy individuals, it has not been possible to correlate any differences in severity of disease with preexisting antibody titers in the past. The protective effects described in the present study were all registered when toxin was applied in the presence of hyperimmune globulin. Experiments wherein antibody administration follows at different times after toxin application are called for, but these will probably have to be conducted in nonprimates.

Studies correlating toxin production by clinical isolates with defined parameters of human disease are also wanting. Toxic shock syndrome is caused by strains that produce little or no alpha-toxin, yet shock occurs frequently. Clearly, we do not suggest that alpha-toxin is the major staphylococcal factor responsible for coagulopathy or septic shock. However, attention is drawn to the possibility that this cytolsin could contribute to the overall severity of disease. Whether or not early administration of hyperimmune globulin might be useful as a supplement to the therapy of acute, life-threatening S. aureus infections warrants further investigation.

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LITERATURE CITED