Yersinia pestis Toxin is Able to Reduce the Functional Response of Human Platelets to Hormonal Stimulation

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Clinical observations, as well as numerous experimental data obtained for inoculating animals with both *Y. pestis* bacteria and autolysed *Yersinia* microbe ("murine" toxin), indicate changes in the number and functional state of the platelets, which is probably a major factor in the development of the syndrome of disseminated intravascular coagulation (DIC) and of shock resulting in the animal's death [1,2,4,12]. We have previously shown that *Yersinia* toxin (YT) (fraction II after Baker) intraperitoneally injected in rats causes a decrease in thrombin-induced platelet aggregation due to alterations in the concentration of cyclic nucleotides and prostaglandins in the cells [3]. *In vivo*, the functional changes in the state of cells may be associated both with a direct effect of *Yersinia* toxin on the platelets and with the development of an intoxication-induced complex of hemodynamic and metabolic disturbances in the organism. In the present study we explored the effect of *Yersinia* toxin on the receptor-dependent regulation of calcium ions in human platelets, which governs their functional activity.

MATERIALS AND METHODS

Toxin from EB-76 *Yersinia pestis*, strain 1290, line NIIEG, prepared after Baker *et al.* [1] (fraction II), was obtained at the Rostov-on-Don Anti-Plague Research Institute of Epidemiology. The lethal dose (LD_{50}) for mice weighing 16-18 g was 15-17 μg. Purified "murine" toxin (MYT) was obtained by the method of ion-exchange chromatography on a column (2.0×20 cm) with DEAE-Trisacryl equilibrated with 50 mM NaCl, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride (PMSF), and 25 mM sodium phosphate buffer, pH 7.4. The initial freeze-dried preparation was dissolved in the buffer, dialyzed for 24 h at 4°C, loaded on a column, and washed with buffer (60 ml). The protein was eluted with a 0.05-0.5 M linear gradient of NaCl in the same buffer. The toxic fraction was eluted with 0.15-0.2 M NaCl. The preparation obtained was concentrated and loaded on a column with Sephacryl-S-200 (1.6×100 cm) preliminarily equilibrated with a 25 mM sodium phosphate buffer (pH 7.4, 150 mM NaCl and 0.1 mM PMSF). Toxin was eluted with this buffer. All the procedures of toxin isolation were performed at 4°C. LD_{50} of purified MYT was 0.7-1 μg. The protein recovery was 6.9%. Electrophoresis in 10% polyacrylamide gel with 0.1% SDS showed that 90% of the total protein in the final toxin preparation was represented by antigens with a molecular weight of 24 and 12 kD. Platelet-enriched plasma (PEP) was obtained by centrifugation (200 g, 15 min) of sodium citrate-stabilized (1:6) donor blood. Aggregation of PEP was induced by adenosine triphosphate (ATP) (5×10^{-7} M) and assessed by a method described elsewhere [7]. For obtaining cells

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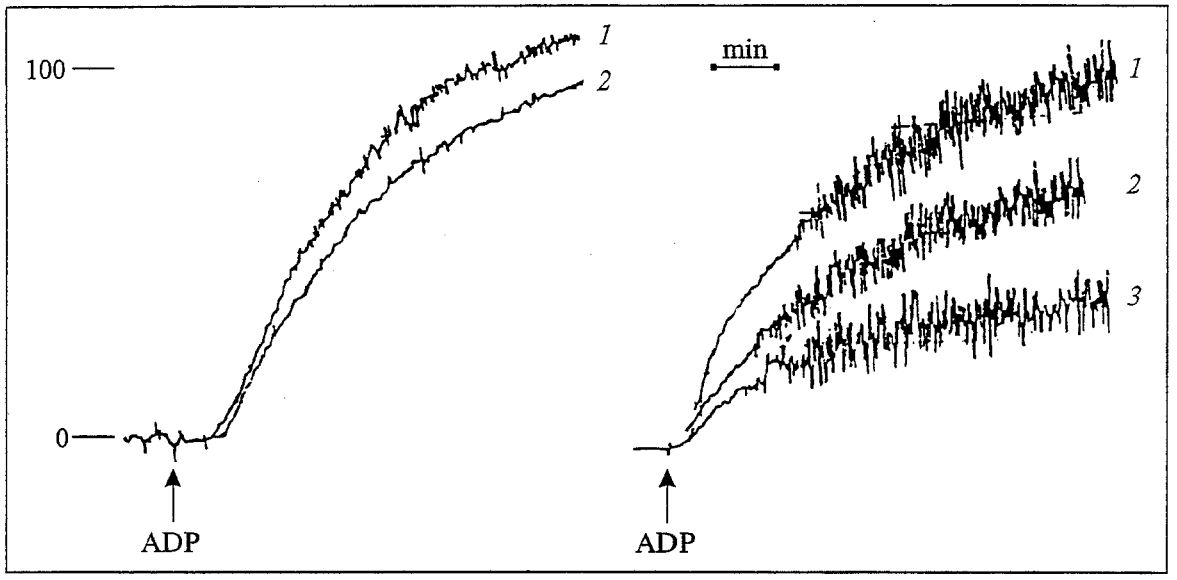

Fig. 1. Effect of initial preparation of YT on ADP-induced aggregation of human platelets. Abscissa: time, min; ordinate: light transmission. *a*) platelet-enriched plasma; *b*) platelets suspended in modified TIRODE solution. *1*) cells not treated with toxin; *2* and *3*) cells treated for 2 min with toxin in a dose of 40 and 20 μg/ml, respectively. ADP concentration is 5×10^{-7}.

washed out of the plasma, PEP was centrifuged (100 g, 10 min), and the pellet was resuspended in buffer A (150 mM NaCl, 1 mM $MgCl_2$, 2.7 mM KCl, 0.37 mM NaH_2PO_4, 5 mM glucose, 10 mM HEPES-NaOH, pH 7.40, 0.1 mg/ml apyrase, and 0.1 mg/ml bovine serum albumin) (medium A). Directly prior to the measurement of ATP-induced aggregation, 1 mM $CaCl_2$ and 0.5 mg/ml fibrinogen were added to the suspension of platelets washed out of the plasma.

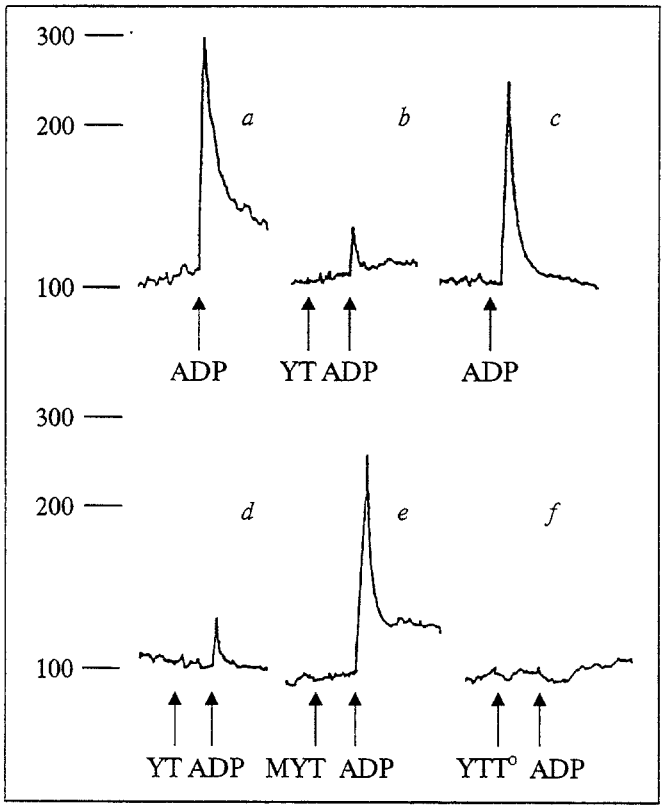

Fig. 2. ADP-induced $[Ca^{2+}]$ changes in platelets loaded with Fura-2. Abscissa: time, min; ordinate: Ca^{2+} concentration, nm. Cells were suspended in modified TIRODE medium containing 1 mM $CaCl_2$ (*a*, *b*, *e*, *f*) and 1 mM EGTA (*c*, *d*) in the absence of toxin (*a*, *c*) and after a 2-min treatment with initial (*b*, *d*) and heating-inactivated (*f*) preparation of toxin (40 μg/ml), and with purified "murine" lethal toxin (*e*). ADP concentration is 5×10^{-7}.

The preparation of platelets and incorporation of the fluorescent calcium probe Fura-2/AM in them were performed as described previously [9]. The donor blood was stabilized with anticoagulant (2.5% sodium citrate, dihydrate; 1.4% citrate, monohydrate; 2% D-glucose). PEP was centrifuged (1000 g, 15 min) and the pellet was resuspended in medium A (pH 6.55) containing 2.5 mM Fura-2/AM. After cell incubation for 1 h at 30°C, the cells were centrifuged (1000 g, 10 min) and resuspended in medium A (pH 7.4). The fluorescence of the cell-incorporated calcium probe was measured on a Hitachi 3000 spectrometer. The calcium concentration $[Ca^{2+}]$ was determined using Conrad and Rink's formula [9]. The basal level of free intracellular calcium was 87-23 nm (n=10). Protein was determined after Peterson [14].

The results were statistically processed after Student.

RESULTS

As is seen from Fig. 1, *a*, ADP in a final concentration of 5×10^{-7} M, added to PEP incubated for 2 min in the presence of 40 μg/ml YT, caused a markedly lower cell aggregation as compared with

the control (blood plasma not treated with toxin). Under the same conditions the aggregation of the cells washed out of the plasma (Fig. 1, *b*) and treated with toxin was reduced vs. the control. Toxin produced a more marked effect on the cells washed out of the plasma, this evidently being due to the binding of some of the toxin molecules by the plasma proteins or blood cell structures. It should be mentioned that YT incubated with platelets for 20-30 min did not cause their spontaneous aggregation. The inhibiting effect of the toxin depended on the dose (Fig. 1, *b*).

Aggregation, changes of platelet shape, and hormone-induced secretion of intracellular granules are known to be mediated by an increase of the cytoplasmic calcium concentration. Such substances as thrombin, vasopressin, ADP, platelet activation factor (PAF), etc. exert a similar effect upon cells. The effect of these substances is produced by specific receptors on the outer surface of the plasma membrane, which are capable of high-affinity binding of the corresponding hormones [15].

As is seen from Fig. 2, *a*, the treatment of Fura-2-loaded platelets with ADP (5×10^{-7} M) causes a sharp rise of the concentration of free intracellular calcium, which peaks within a few seconds. Incubation of cells in the presence of 40 μg/ml YT markedly reduces the ADP-induced activation (Fig. 2, *b*). Incubation of platelets for 5-10 min with YT is not attended by a change in the basal level of intracellular calcium. The blocking effect depends on the dose of toxin (Fig. 3) and virtually does not depend on the type of agonist used. A 50% blocking of $[Ca^{2+}]$ increase is observed at a YT concentration of 20 μg/ml for the use of such aggregation inducers as ADP, PAF, and thrombin. As is seen from Fig. 4, the blocking effect develops as soon as 1-1.5 min after the beginning of cell preincubation with toxin.

The majority of hormones which activate platelets are known to raise the $[Ca^{2+}]$ not only due to calcium release from the membranes of the endoplasmic reticulum, but also due to stepped-up entry of these ions from the extracellular space [8,15]. The presence of receptor-activated calcium entry in platelets has been confirmed by a number of scientists [5,10]. It was of interest to explore which of the pathways of $[Ca^{2+}]$ increase is blocked by *Yersinia* toxin. As is seen from Fig. 2, *c*, removal of calcium ions from the extracellular medium by using EGTA, a known ion chelator, leads to a drop of the ADP-induced calcium response. The component of the calcium signal which is preserved in a calcium-free medium correlates with the Ca^{2+} release from the reticulum,

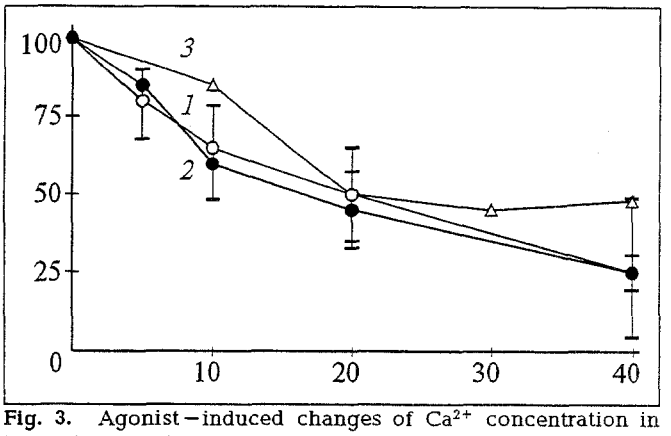

Fig. 3. Agonist–induced changes of Ca^{2+} concentration in Fura–2–loaded platelets as a function of toxin concentration. Abscissa: μg protein/ml; ordinate: increase of $[Ca^{2+}]$, % of control. Time of preincubation with toxin is 2 min. Here and in Fig 4.: *1*) ADP (5×10^{-7}); *2*) PAF (10^{-9} M); *3*) thrombin (0.01 IU/ml).

whereas the component which disappears after EGTA addition probably reflects Ca^{2+} entry from the external medium. As is seen from Fig. 2, *b*, *d*, platelet preincubation with toxin results in $[Ca^{2+}]$ inhibition, irrespective of whether calcium is present or absent in the incubation medium. Thus, we may conclude that toxin blocks not only the release of calcium ions from the endoplasmic reticulum membranes, which is induced by inositol-1,4,5-triphosphate produced during hormonal activation of phospholipase C, but also the calcium entry into the cytoplasm of platelets from the extracellular medium. We have shown the fluorescent probe signal arising in Quin-2-loaded platelets in response to the agonist (PAF, ADP, and thrombin) to be markedly reduced as compared to the signal in the control cells for replacement of extracellular Ca^{2+} with Ba^{2+} (results not presented). Evidently, these data may be regarded as additional

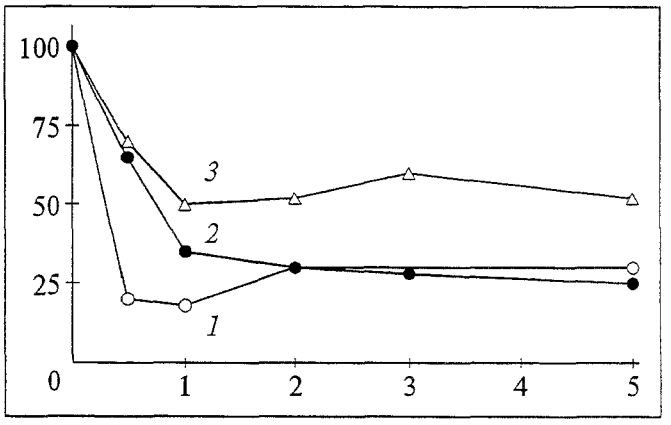

Fig. 4. Agonist–induced changes of Ca^{2+} concentration in Fura–2–loaded platelets as a function of time of preincubation with unpurified toxin preparation (40 μg/ml). Abscissa: time of preincubation with YT, min; ordinate: $[Ca^{2+}]$ increase, % of control.

testimony of the YT-induced blocking of receptor-controlled calcium channels, since Ba^{2+} entry is known to be activated by the agonists via this mechanism [5].

The toxin used in our experiments (fraction II after Baker) is a protein-lipopolysaccharide complex containing some ten antigens [11,13]. MYT found in this fraction is known to kill mice and rats in the case of intravenous or intraperitoneal administration and consists of two thermostable proteins with a molecular weight (MW) of 240 and 120 kD, which are composed by subunits with a MW of 24-12 kD (as was shown by SDS-electrophoresis) [11,13]. The purification of MYT from Baker's fraction II led to a 20-fold decrease in the LD_{50} in mice, but, at the same time, the preparation lost the ability to block Ca^{2+} in human platelets in response to the agonist used in a concentration of 40 μg/ml (Fig. 2, *e*). On the other hand, boiling of YT (15-20 min on a boiling water bath) did not result in the loss of calcium-blocking activity (Fig. 2), although this procedure wiped out the ability of this preparation to cause mouse death after intraperitoneal administration in a dose of 15-100 μg per animal.

Thus, these data provide evidence that the antiaggregating and calcium-blocking activity is not associated with lethal MYT but is apparently due to a previously unknown thermostable factor that is a constituent of Baker's fraction II, obtained from *Y. pestis*.

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