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Inhibiting Effect of *Yersinia Pestis* Protein Factor on the Hormone-Stimulated Response of Human Platelets

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In a previous study we have showed that a short-term incubation of *Yersinia* toxin (fraction II after Baker) with human platelets reduces the hormone-stimulated increase of the calcium concentration in the cytoplasm ($[Ca^{2+}]$) and causes a decrease of the aggregative properties of these cells. The purified preparation of lethal "murine" toxin does not exhibit such an effect.

In the present research we obtained a factor blocking the hormone-dependent increase of the cytoplasmic calcium concentration in the platelets and determined a number of its properties and the mechanism of action.

MATERIALS AND METHODS

Toxin (YT) 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 activity of the preparation was assessed by the ability to block the ADP-induced (5×10^{-7} M) increase of $[Ca^{2+}]$ in Fura-2-loaded human platelets [2]. The time of platelet preincubation with toxin was 2 min. The initial preparation in a dose of 20 μg/ml (EC_{50}) caused a 50% inhibition of ADP-dependent increase of $[Ca^{2+}]$ in the platelets. The method of ion-exchange chromatography was used for purification of the factor which blocks hormone-stimulated increase of $[Ca^{2+}]$ (FYT) in the platelets. Ammonium-sulfate extract (fraction II) of the *Y. pestis* cells was dissolved in buffer A, containing 20 mM HEPES-NaOH, pH 7.4 (20°C), 1 mM $MgCl_2$, 1 mM EDTA, and 200 μM phenylmethylsulfonylfluoride at room temperature, dialysed against buffer A, heated on a water bath for 20 min, centrifuged at 30,000 g for 20 min, and loaded on a column with DEAE-Toyopearl (1.6× ×10 cm) preliminarily equilibrated with buffer A. The column was washed with 2 volumes of buffer A, then with a 0-0.5 M linear gradient of NaCl in the buffer, and then with 1 M NaCl (20 ml).

TABLE 1. Properties of Factor Inhibiting ADP – Stimulated Increase of $[Ca^{2+}]$ in Platelets

Experimental conditions	Reduction of calcium-blocking activity, % of control
Control (without admixtures)	100
Dialysis (24 h in 10 mM phosphate buffer, pH 7.6 at 4°C)	100
Heating (20 min, 100°C)	90±9
Ammonium sulfate (80% saturation)	85±10
Papain (1:50, 2 h, 37°C)	16.3±1.13
4 M urea	22.4±1.21
2 M urea	79±8

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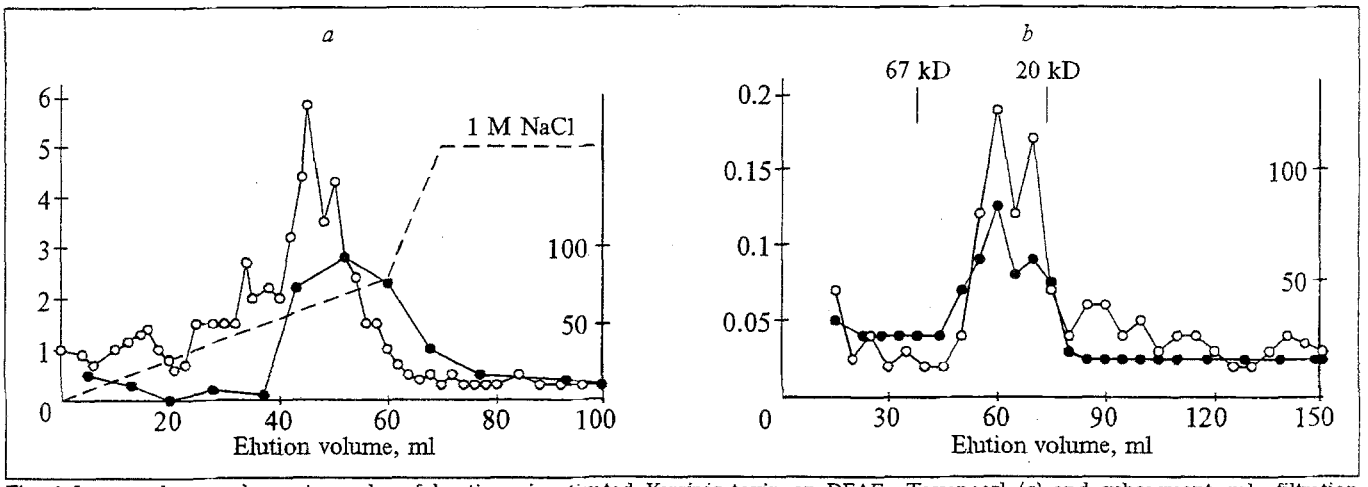

Fig. 1. Ion-exchange chromatography of heating-inactivated *Yersinia* toxin on DEAE-Toyopearl (*a*) and subsequent gel-filtration on Sephadex G-75 (*b*). Ordinate: right – inhibition of ADP-stimulated [Ca^{2+}] in platelets; left – protein concentration, mg.

The samples selected according to their biological activity were pooled and transferred by dialysis (24 h, 4°C) to buffer B (20 mM sodium phosphate buffer, pH 7.4 (20°C), 150 mM NaCl), frozen in liquid nitrogen, and freeze-dried. The freeze-dried preparation was dissolved in 2 ml of buffer B, centrifuged at 30,000 g for 20 min, and loaded on a column with Sephadex G-75 (1.6×100 cm) preliminarily equilibrated with buffer B. Elution was performed using buffer B. The protein recovery constituted 4.2%; the EC_{50} was 3.5 μg/ml. The EC_{50} of the initial YT and purified FYT was assessed using a platelet preparation derived from the blood of one and the same donor.

The content of cAMP and cGMP was measured using Amersham radioimmunological kits (UK). A platelet suspension (5×10^8 cells) obtained after Conrad [2] was incubated with toxin in medium C: 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 7.4, 0.1 mg/ml bovine serum albumin, and 0.1 mM 3-isobutyl-1-methylxanthine (IBMX), at 30°C; the reaction was stopped by the addition of 3 volumes of 96% ethanol.

The activity of adenylate cyclase (AC) in the toxin preparation and in the platelets was determined after Gentile [6], and that of guanylate cyclase (GC) by a modified method [12]. For GC determination in the platelet suspension in buffer C, the cells were disrupted by thrice-repeated freeze-thawing in liquid nitrogen. The activity of GC was measured in medium containing 50 mM Tris-HCl (pH 7.6 at 30°C), 5 mM $MgCl_2$, 1 mM GTP [2-3×10 cpm] (α-^{32}P-GTP), 2 mM theophylline IBMX, 0.1% (w/v) bovine serum albumin, 15 mM creatine phosphate, 135 IU/ml creatine phosphokinase, and 5 mM cGMP. The final volume was 50 μl, and the toxin concentration was 50-100 μg protein. The samples were incubated for 15 min, the reaction was stopped with 200 μl 0.5 N HCl, boiled for 9 min, cooled, and neutralized with 200 μl imidazole. The entire contents of the test-tubes was loaded on columns with 1 g aluminium oxide (neutral, according to Brockman), previously equilibrated with 5 ml of 10 mM imidazole (pH 7.4), and washed with 10 ml of 10 mM imidazole. The cGMP recovery was 90-100%. The protein content was determined after Peterson [11].

TABLE 2. Activation of GC in Human Platelets by Different YT preparations

Toxin preparation	GC, pmol/mg protein/min
Platelets (without toxin)	1.6±0.01
Platelets + initial YT (40 μg/ml)	2.1±0.05
Platelets + heating-inactivated YT (40 μg/ml)	2.4±0.07
Platelets + YT obtained by gel-filtration on Sephadex G-75 (10 μg/ml)	5.2±0.5

RESULTS

As is seen from Fig. 1, *a*, FYT is eluted at a salt concentration of 0.5 M, the same as for the majority of the proteins of *Y. pestis*. Subsequent filtration on Sephadex G-75 (Fig. 1, *b*) showed that FYT had a molecular weight of about 20 kD. Similar results were obtained by gel-filtration of the initial preparation of YT, not denatured by heating, on columns with Sephacryl S-200 and AcA 44 Ultragel (data are not presented).

On the basis of these data, as well as the results presented in Table 1, it can be postulated that FYT is a protein with a molecular weight of 20 kD.

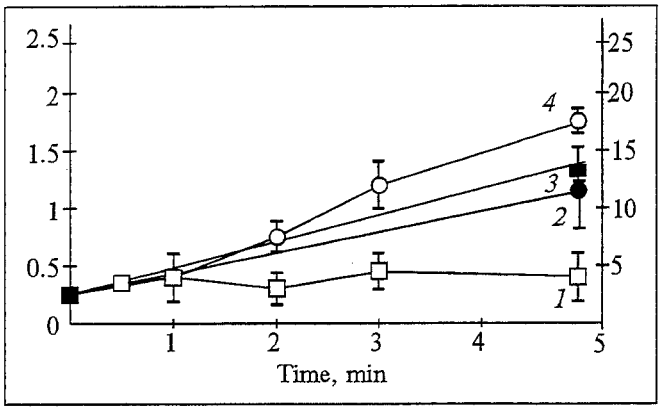

Fig. 2. Effect of YT on cAMP (*1*, *3*) and cGMP (*2*, *4*) content in platelets. Ordinate: right – cAMP content, pmol/10^8 cells; left – cGMP content, pmol/10^8 cells. *1* and *4*) cAMP and cGMP content in the presence of toxin (40 μg/ml); *2*) cGMP content in the presence of sodium nitroprusside (10^{-4} M); *3*) cAMP content in the presence of forskolin (10^{-6} M).

The activators of AC and GC, as well as the inhibitors of phosphodiesterase (PDE), are known to block platelet aggregation and to reduce the effect of hormones and agonists on the level of intracellular calcium by raising the level of cAMP or cGMP [9,10]. All these effects of cyclic nucleotides are realized via the corresponding protein kinases and correlate with phosphorylation of a number of proteins [13]. Microbial cells possess a cAMP metabolism apparatus of their own, and in some cases the bacterial AC are capable of penetrating inside the eucaryotic cell and of sharply raising the cAMP level in it [3,6]. In our study of the effect of YT on the content of cAMP and cGMP in human platelets, it was shown that the toxin slightly affected the level of cAMP, but sharply raised the cGMP concentration (Fig. 2). The effect of YT on the cGMP content in the cells was even more marked than the effect of sodium nitroprusside, a known activator of GC in the cytosol. It should be stressed that the toxin preparations used in our study (YT, FYT) did not exhibit either adenylate- or guanylate-cyclase activity.

The YT-induced increase of the cGMP concentration in the platelets discovered by us attests to the presence of either a GC activator or a PDE inhibitor in the YT preparation. As is seen from Table 2, YT and different fractions of it possessing the ability to block the ADP-dependent increase of intracellular calcium cause GC activation in the platelet suspension. The degree of enzyme activation directly depends on the purity of the preparation used.

Thus, the protein of *Y. pestis* discovered and isolated by us is a GC activator and is capable of penetrating inside human platelets, increasing the cGMP content in them.

It is worthy of note that activators of membrane GC have been found within the group of thermostable enterotoxins of many Gram-negative bacteria [5,8]. However, in contrast to them, the *Y. pestis* protein has a molecular weight at least 10 times as high as that known for enterotoxins represented by 18-19 amino acid-long peptides [5,8]. In addition, we have not come across any evidence of the presence of receptors to thermostable enterotoxins on human and animal platelets. Receptors for these toxins have only been shown to exist in the enterocytes and endothelial cells of the kidneys [4,14].

Thus, the bacteria *Y. pestis* EB 76, strain 1290 contain a protein factor which is an activator of GC and is able to interact with human platelets. In turn, activation of the processes of cGMP-dependent phosphorylation in the platelets results in the blocking of the hormone-induced increase of the cytoplasmic calcium level and cell aggregation, which evidently plays an important role in the pathogenesis of plague intoxication.

REFERENCES

1. E. E. Baker, H. Sommer, L. E. Foster, and K. F. Meyer, *J. Immunol.*, **68**, 131-138 (1952).
2. G. W. Conrad and T. J. Rink, *J. Cell Biol.*, **103**, 439-450 (1986).
3. H. G. Donovan and D. R. Storm, *J. Cell Physiol.*, **145**, 444-449 (1990).
4. K. Ivens, H. Gazzano, P. Hanley, and S. A. Waldman, *Infect. Immunol.*, **58**, 1817-1820 (1990).
5. H. Gazzano, H. I. Wu, and S. A. Waldman, *Ibid.*, **59**, 1552-1557 (1991).
6. F. Gentile, L. G. Khipling, D. L. Sackett, *et al.*, *J. Biol. Chem.*, **265**, № 18, 10686-10692 (1990).
7. V. Gordon, W. Young, S. Lechler, *et al.*, *J. Biol. Chem.*, **264**, 14792-14796 (1989).
8. A. Guazino, R. Gianella, and M. R. Thompson, *Infect. Immunol.*, **57**, 649-652 (1989).
9. S. Nakashima, T. Tohmatsu, H. Hattori, *et al.*, *Biochem. Biophys. Res. Commun.*, **135**, 1099-1104 (1986).
10. M. A. Packham, *Thromb. Haemostas.*, **50**, 610-619 (1983).
11. G. L. Petersen, *Anal. Biochem.*, **83**, 346-356 (1947).
12. S. Schulz, S. Singh, R. A. Bellet, *et al.*, *Cell*, **58**, 1155-1162 (1989).
13. R. Waldmann, S. Bauer, C. Gobel, *et al.*, *Eur. J. Biochem.*, **158**, 203-210 (1986).
14. A. A. White, W. T. Krauze, J. T. Turner, and L. R. Forte, *Biochem. Biophys. Res. Commun.*, **159**, 363-367 (1989).