Effect of Toxic Substances of Diphtheria Corynebacteria on Aggregation of Human Platelets

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The effect of the toxic substances of diphtheria corynebacteria (diphtheria toxin, diphtheria anatoxin, and codivac) on the aggregation of human platelets *in vitro* was demonstrated using platelet-rich plasma prepared from citrated blood and the standard platelet activator ADP (2×10^{-5} M). These substances induce platelet aggregation in a dose-dependent manner. Incubation of diphtheria toxin and anatoxin with platelets reduces ADP-induced and total platelet aggregation, the effect being dependent on the inducer dose and incubation time. By contrast, codivac stimulates ADP-induced and total platelet aggregation in all experimental series.

Key Words: *platelet aggregation; diphtheria corynebacteria; diphtheria toxin; diphtheria anatoxin; codivac*

The role of platelets in the pathogenesis of thrombohemorrhagic complications in diphtheria has scarcely been studied. Meanwhile, it is known that platelets are target cells for bacterial toxins, which induce alterations in their shape, aggregation, and secretory activity, leading to serious disorders in the blood coagulation system [1,2].

Studies of the blood coagulation system in diphtheria reveal clear manifestations of the thrombohemorrhagic syndrome, the intensity of which depends on the form and severity of the disease.

In order to elucidate the role of diphtheria corynebacteria (DCB) in the impairment of the platelet element of the blood coagulation system in diphtheria we studied the effects of DCB metabolites on platelet aggregation *in vitro*.

MATERIALS AND METHODS

The experiments were performed on platelet-rich donor plasma with the standard number of cells in a sample ($250\text{-}300\times10^3$ cells/μl). Platelet aggregation was measured in an aggregometer designed in the experimental workshops of the Russian Academy of Medical Sciences. Adenosine diphosphate (ADP, 2×10^{-5} M, Sigma), diphtheria toxin ($2.0\times\times10^{-5}$ - 2.0 Lf/ml), diphtheria anatoxin (2.0×10^{-5} - 2.0 Lf/ml), and codivac (an antidiphtheria toxin based on the surface glycoprotein of DCB) (0.11-2.0 mg/ml) were used as inducers of platelet aggregation. Diphtheria toxin was obtained at the N. F. Gamaleya Institute of Epidemiology and Microbiology, diphtheria anatoxin was from the I. I. Mechnikov Institute of Vaccines and Sera, and codivac was produced at the G. N. Gabrichevskii Institute of Epidemiology. The inducers were added to platelet-rich plasma with continuous monitoring of platelet aggregation, which allowed us to assess the activity of each substance. For a study of platelet aggregation as a function of the time of toxic substance action, platelet-rich plasma was incubated with diphtheria toxin, diphtheria anatoxin, and codivac for various times (from 30 sec to 30 min). In control experiments the DCB preparations were replaced with normal saline.

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RESULTS

The addition of any of the DCB preparations to platelet-rich plasma prepared from the blood of healthy individuals induced a statistically significant increase in platelet aggregation compared with the control. Using different concentrations of the toxins, we found that these substances affect platelet aggregation in a dose-dependent manner. For example, a high dose of diphtheria toxin (0.2-2.0 Lf/ml) induced the most pronounced aggregation (29.7±3.0 *vs.* 11.5±1.1 mm, $p<0.05$), while low toxin concentrations (2×10^{-2} - 2×10^{-5} Lf/ml) reduced aggregation to 21.6±1.7 and 23±1.6 mm, respectively ($p<0.05$ compared with the control).

The same concentrations of diphtheria anatoxin added to platelet-rich plasma induced lesser aggregation: 21.7±1.7 mm in response to high concentrations, 20.4±0.8 mm to medium concentrations (2×10^{-2} - 2×10^{-3} Lf/ml) and 17.8±1.9 mm to low concentrations (2×10^{-4} - 2×10^{-5} Lf/ml) ($p<0.05$ compared with the control). The addition of high (2.0-1.0 mg/ml), medium (0.4 mg/ml), and low (0.2-0.1 mg/ml) doses increased platelet aggregation to 22.0±1.0, 19.2±1.4, and 16.7±1.4 mm, respectively ($p<0.05$ compared with the control).

These results indicate that DCB preparations induce platelet aggregation in a dose-dependent manner.

In reality, patients' blood contains different biologically active substances, including inducers of platelet aggregation (ADP, platelet-activating factor, etc.). We studied the effect of ADP, a standard inducer of platelet activation, on donor platelets in the presence of DCB toxic substances.

Successive addition of diphtheria toxin and ADP to platelet-rich plasma at 5-7-min intervals had no effect on the ADP-induced aggregation, which was equal to 69.4±3.5 mm, while a 30-min incubation with diphtheria toxin reduced it markedly: 28.7±8.0 mm ($p<0.01$ compared with the control). After control incubation of platelets in normal saline for 5-7 and 30 min, the ADP-induced aggregation was the same: 67.0±4.7 mm.

Successive addition of diphtheria anatoxin and ADP to platelet-rich plasma at 5-7-min intervals as well as a 30-min incubation with diphtheria anatoxin led to a statistically significant inhibition of ADP-induced aggregation: to 49.7±1.6, $p<0.01$, and 54.6±1.4 mm, $p<0.05$, respectively.

Thus, diphtheria toxin and anatoxin reduce ADP-induced platelet aggregation. The anatoxin, being a more highly purified preparation, lowers platelet aggregation after a short-term incubation, while diphtheria anatoxin elicits its effect only after a long-term incubation (30 min).

The opposite effect was observed upon incubation of platelets with codivac, which stimulated their aggregation depending on the period of incubation. After a short-term incubation, the ADP-induced aggregation increased 14% and after long-term incubation it increased 22.3% compared with the norm ($p<0.05$), being 76.6±2.7 and 82.0±4.0 mm, respectively. These data indicate that the effect of DCB toxic substances on platelets depends not only on the dose of preparation but also on the incubation time.

The total aggregation under the action of diphtheria toxin and ADP, diphtheria anatoxin and ADP, and codivac and ADP also depended on the incubation time. For example, a 30-sec incubation of platelet-rich plasma with diphtheria toxin significantly increased the total aggregation to 116.5% of the control, whereas a 30-min incubation reduced aggregation to 61% ($p<0.01$).

Analysis of the total aggregation under the influence of diphteria anatoxin and ADP showed that for just a 30-sec incubation of platelets with the anatoxin the degree of aggregation dropped to 86.7% of the control level and remained lowered after 5-7 and 30 min of incubation (85.6 and 94%, respectively). Incubation of codivac with platelet-rich plasma for 30 sec raised total aggregation 14% vis-a-vis the control ($p<0.05$), and after 30 min of incubation the level rose by 22.3% ($p<0.05$). Total platelet aggregation also depended on the dose of the toxin. For example, in experiments with diphteria toxin in most cases the degree to which aggregation activity dropped increased as the toxin dose was increased ($2\text{-}2\times10^{-3}$ Lf/ml).

Analysis of the frequency of alterations in the total aggregation depending on the dose of DCB toxic substance revealed different effects of these preparations on the platelets. Diphtheria toxin inhibited platelet aggregation in 52-76.2% of cases, while diphtheria anatoxin inhibited it in 81.3-88% of experiments, which confirms our hypothesis on its higher activity.

In contrast to diphtheria toxin and anatoxin, codivac stimulated the total platelet aggregation in a dose-dependent manner. High doses stimulated platelet aggregation in 92.9, medium doses in 82.3, and low doses in 72% of observations. Aggregation increased with prolongation of the incubation period: 114.3% after 30 sec, 120.3% after 5-7 min, and 125% after 30 min of incubation. These findings indicate that the total platelet aggregation depends on the incubation time. The stimulatory effect of codivac may be due to the fact that its

components increase the sensitivity of platelets to ADP by actively interacting with the cell membrane. This assumption does not contradict the findings of other researchers who demonstrated a stimulatory effect of peptoglycans of nontoxigenic DCB on cultured immune cells *in vitro*.

Thus, this study has shown that diphtheria toxin, diphtheria anatoxin, and codivac are agents that directly affect the functional activity of human platelets. In addition, it was found that diphtheria toxin and anatoxin reduce ADP-induced and total platelet aggregation, the decrease being dependent on preparation dose and incubation time. However, codivac, a glycopeptide from the cell wall of nontoxigenic DCB, stimulates platelet aggregation in all experimental series.

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Blast Transformation of Lymphocytes and the Activity of Natural Killer Cells in the Presence of γ-Globulin *In Vitro*

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The cytotoxic activity of natural killer cells against ^{3}H-uridine-labeled target cells (human erythromyeloleukosis cells K-562) and the intensity of spontaneous blast transformation are studied *in vitro* in the presence of human serum γ-globulin. It is shown that spontaneous blast transformation is 49-51% due to the presence of aggregated γ-globulin, while the aggregate-free γ-globulin fraction does not induce this reaction. The cytotoxic activity of natural killer cells *in vitro* declines in the presence of native γ-globulin, which is related to the influence of aggregated γ-globulin, the intensity of whose formation may increase upon a manyfold decrease in the γ-globulin content of the preparation.

Key Words: *blast transformation; natural killer cells; γ-globulin*

The receptors for the Fc portion of IgG (FcγR) or the CD16 antigens [1], the expression of which has been reported on the lymphocyte surface [6,9,16,17], are regarded, along with others, as potential receptors (structural elements) participating in the interaction between natural killer cells (NK) and target cells. It is believed that the FcγR-III expressed by NK [5], which is similar to the neutrophil FcγR but different from the B-cell FcγR [17], is the sole type of receptor providing for the antibody-dependent lysis of target cells [16]. The FcγR of NK (molecular weight 50-70 kD [9]) mediates the cell-to-cell contact upon lysis of antibody-covered target cells [17] and triggers the formation of NK cytotoxic factor [3].

Like most FcγR$^+$ cells, activated NK generate 40-50-kD soluble forms of FcγR-III [4,13] that can become involved in the natural cytotoxicity reaction which is presumed to occur at the early stages via the contact and transfer of receptor-

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