Effect of Storage of Platelet Concentrate and Products of Myeloperoxidase Reaction on Initial Platelet Aggregation

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An original nephelometric method allows one to measure initial platelet aggregation after a long-term storage of platelet concentrate. This attests to a high level of platelet activity despite the fact that final platelet aggregation cannot be detected by usual turbidimetric method. N-chloroalanine more slowly inhibits initial platelet aggregation in comparison with sodium hypochlorite, which implies different mechanisms of their membrane-modifying effects.

Key Words: *aggregation; storage; platelet; hypochlorite; chloramine*

Enhanced platelet activity is a possible cause of thrombosis, therefore the search for new antiplatelet drugs is an important problem [4,9]. Transfusion of platelet concentrate is currently used in clinical practice to stop internal hemorrhage [12]. In all cases the ability to form aggregates in response to addition of an inductor is used as a measure of platelet activity. Platelet aggregation (PA) is usually assessed *in vitro* by Born's turbidimetric method, which detects the formation of large platelet aggregates [5,10]. However, direct microscopy showed that addition of an inductor first leads to the formation of small aggregates and this can be the only manifestation of platelet activity. This initial PA (iPA) cannot be detected by turbidimetric aggregometers [6]. Recently, we proposed a new nephelometric analysis of iPA; this method was approved for ADP-induced platelet activation [10]. The primary objective of the present study was to explore the possibility of nephelometric recording of iPA induced by thrombin and calcium ions in the presence of plasma (these conditions induce rapid coagulation of the plasma) and, second, to use this method for evaluation of platelet activity during a long-term storage of platelet concentrate.

Sometimes management of patients with platelet-mediated thrombosis requires general inactivation of platelets, i.e., suppression of their activity regardless the type of inductor and the mechanism of cell signaling [4]. This can be achieved by chemical modification of the plasma membrane [1,2], for instance with hypochlorite anion, a product of myeloperoxidase reaction, and chloramine amino acid derivatives, which can be considered as the secondary products of this reaction [13].

The second objective was to study the dynamics of the effect of hypochlorite anion and a chloramine alanine derivative on the initial aggregation of isolated platelets. The studied effects were compared with the effect of acetylsalicylic acid (aspirin), a standard antithrombotic drug.

MATERIALS AND METHODS

Experiments were carried out on rabbit platelets in the presence of plasma (platelet-rich plasma, PRP) and on pure human platelets (diluted platelet concentrate). Rabbit platelets were isolated as described previously [1,2]. Human platelet concentrate (from

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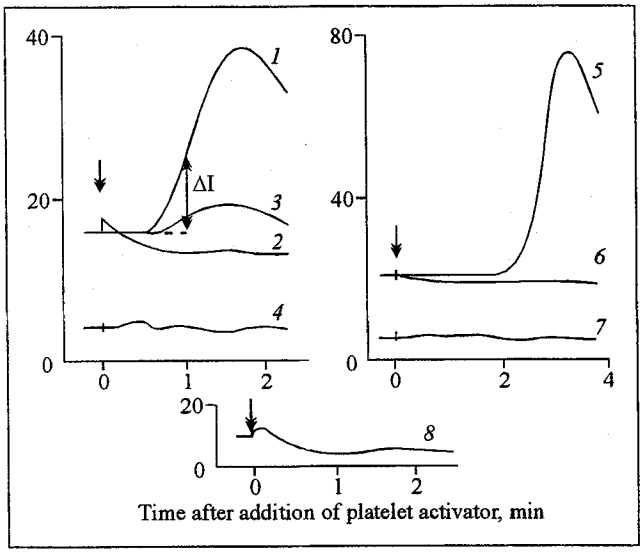

Fig. 1. Typical kinetic curves of light scattering intensity and light transmission in rabbit platelet-rich (PRP) and platelet-free plasma after addition of platelet activator. Ordinate: *1-7*) intensity of scattered light, arb. units; *8*) light transmission, %. Addition of thrombin (0.4 U/ml) and $CaCl_2$ (9 mM) is indicated by arrows. *1-3*) PRP (activation with thrombin) under stirring, without stirring, and in the presence of 1 mM EDTA under stirring, respectively; *5* and *6*) PRP (activation with $CaCl_2$) with and without stirring, respectively; *4* and *7*) plasma after addition of thrombin and $CaCl_2$, respectively, under stirring; *8*) PRP (activation with thrombin), light transmission measured in a Chrono-log aggregometer.

blood transfusion station) represented a suspension of a volume of 40 ml prepared from 400 ml blood. The concentrate was kept in a thermostat (22°C) in a plastic bag (Green Cross Medical Corp.) under constant shaking (1 rock per sec). Before measurements the concentrate was 5-fold diluted with a buffer containing (in mM): 10 HEPES, 134 NaCl, 5 KCl, 1 $MgSO_4$, 0.5 Na_2HPO_4, and 0.5 glucose, pH 7.4. Isolated rabbit platelets were suspended in the same buffer.

The following chemicals were used: salts of the pure for analysis and chemically pure grades, bovine α-thrombin (Medio), and commercial preparations of acetylsalicylic acid (aspirin, Sigma), ADP (Chrono-log) and α-alanine (Reanal). N-Chloroalanine was synthesized by mixing the amino acid with sodium hypochlorite [1].

Platelet aggregation was analyzed by two optical methods: Born's turbidimetry [5] and nephelometry. Usual turbidimetric analysis was carried out in an aggregometer constructed on the basis of a KFK-2MP photoelectrocolorimeter and equipped with a magnetic stir with a magnet positioned on the side of the measuring cell. The cuvette was 1-cm thick. Light transmission at 670 nm was recorded with an automatic recorder. In some cases turbidimetric analysis of PA was performed in a Chrono-log aggregometer.

We developed a new nephelometric method based on the fact that the formation of small platelet aggregates is accompanied by an increase in the intensity of light scattered at sharp angles [10]. The measurements were performed in a nephelometric aggregometer. The intensity of light scattered within 0.7-1.5° from the incident beam emitted by a helium-neon laser (632.2 nm) was measured.

RESULTS

Addition of thrombin (final concentration 0.4 U/ml) to rabbit PRP under constant stirring induced an increase in the intensity of scattered light, which was noted after a certain latency and attained the maximum 1.5-2 min after addition of the inductor (Fig. 1, *1*). Similar changes but with a longer latency were observed after addition of high concentrations (3-10 mM) of calcium ions (Fig. 1, *5*). Both thrombin and Ca^{2+} induced coagulation of PRP, this process is initiated 2.5-4 min after addition of the inductor, i.e., later than light scattering peak.

The observed increase in light scattering was not due to aggregation of plasma proteins or other changes in the plasma, since the addition of thrombin and calcium to platelet-free plasma did not induce changes in light scattering (Fig. 1, *4, 7*). Two types of experiments showed that intensification of light scattering reflects PA. First, the effect markedly decreased in the presence of 1 mM EDTA (Fig. 1, *3*), an inhibitor of PA [3]. Second, the intensity of light scattering did not increase if stirring was stopped immediately after addition of thrombin and calcium (Fig. 1, *2, 6*). It is known that PA proceeds only with stirring, which makes effective cell collisions possible [8]. It should be noted that when aggregation was measured in a Chrono-log aggregometer, no thrombin-induced rise of light scattering was noted (Fig. 1, *8*). This implies that large aggregates are not formed within the studied time range (final PA). Our findings suggest that nephelometry detects the formation of small aggregates, i.e., iPA. The increment of light scattering intensity ΔI (degree of aggregation) certain time after the addition of a PA inductor can serve as a quantitative measure of iPA (Fig. 1). It should be emphasized that this method allows one to measure PA in the presence of plasma before the beginning of its coagulation.

In the concentrate stored for 1 day, thrombin-induced initial aggregation of human platelets was clearly pronounced (Fig. 2, *a, 1*) as well as their secondary turbidimetry-detectable aggregation (Fig. 2, *b, 1*). After a 4-day storage, the secondary aggregation was not detected (Fig. 2, *b, 2*), but platelets were still active. Indeed, nephelometry revealed

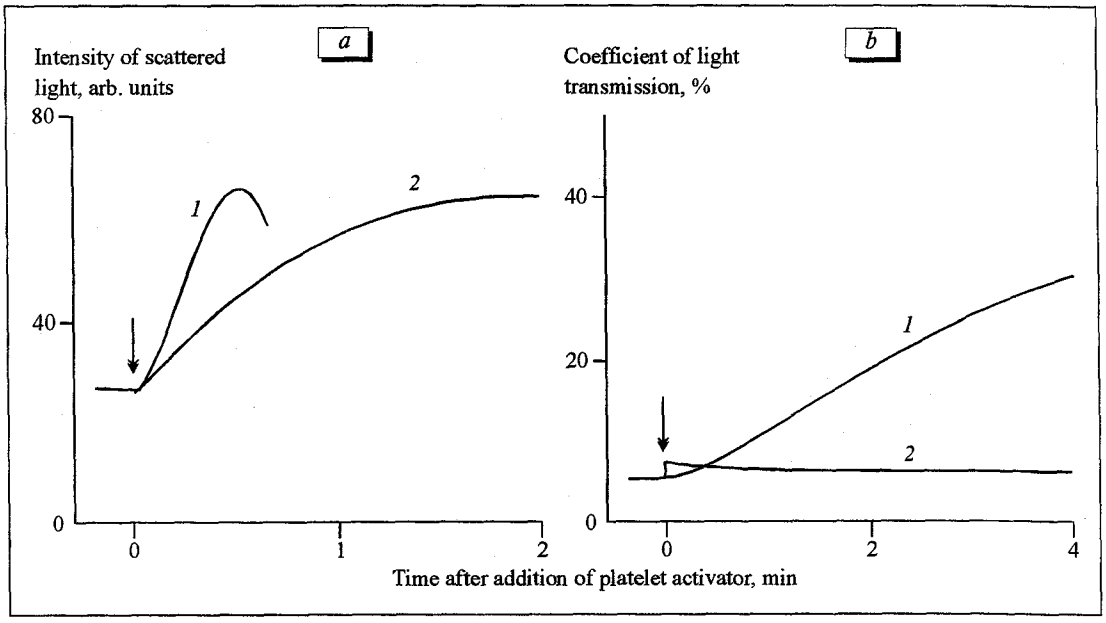

Fig. 2. Kinetic curves of initial (*a*) and final (*b*) aggregation of conserved human platelets. *1*) 1-day storing, *2*) 4-day storing. Arrow indicates the addition of thrombin (0.05 U/ml).

potent, although slower iPA (Fig. 2, *a*, *2*). Thus, nephelometry provides a more adequate information on platelet functional activity in the concentrate for transfusion.

Using rapid nephelometric analysis of iPA we studied the dynamics of the effect of sodium hypochlorite, N-chloroalanine, and aspirin on isolated rabbit platelets. Platelets were incubated in the presence of these agents for different times and then their thrombin-induced aggregation activity was assessed. Aggregation was measured 1 min after addition of thrombin; the data were presented as the ratio of $\Delta I/\Delta I_c$, where ΔI_c and ΔI are the change in the intensity of scattered light in the control and treated platelets, respectively. The inhibiting effect of sodium hypochlorite attained the maximum after 0.5-1 min of incubation (Fig. 3, *1*), whereas N-chloroalanine acted more slowly, and its effect peaked on the 5th minute of incubation (Fig. 3, *2*). This difference implies that although the activity of both agents is due to the presence of active chlorine atom, the mechanism of their effect is different. It is obvious that a highly reactive compound is inconvenient for the use as a platelet inhibitor in the organism, since special care should be taken for its equal distribution in the circulation. Chloramine amino acid derivatives are more preferable inhibitors of platelet activity in the blood.

Acetylsalicylic acid (aspirin) is currently used for prevention and treatment of platelet-mediated thrombosis [4,14]. Its inhibiting effect is due to irreversible modification (acetylation) of cyclooxygenase [1] and probably other proteins. Preincubation with aspirin (1 min) markedly suppressed the thrombin-induced iPA: 60% of the control level (p=0.01, Student's t test, Fig. 3, *3*). Interestingly, the inhibiting effect of aspirin inversely depended on the time of platelet preincubation with aspirin. For instance, the degree of PA after a 20-min incubation with aspirin constituted 80% of the control (p=0.05 in comparison with 1-min incubation, nonparametric Wilcoxon T

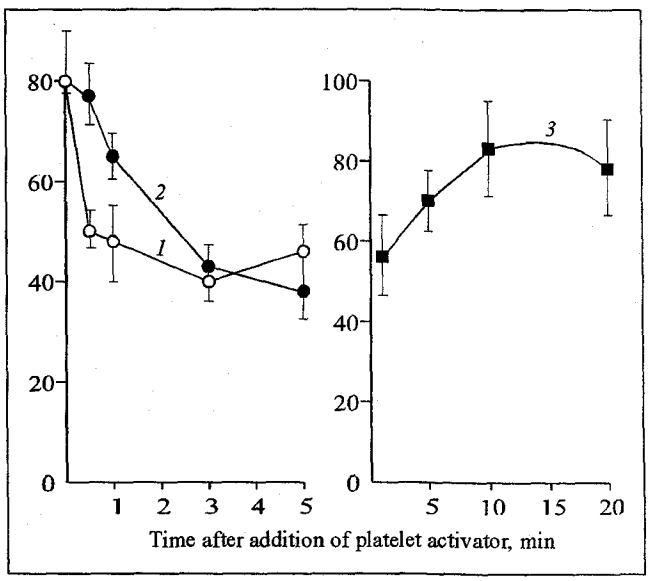

Fig. 3. Suppression of initial aggregation of rabbit platelets with various agents. Ordinate: degree of aggregation, % of control. *1*) sodium hypochlorite (0.02-0.04 mM)+0.05-0.06 U/ml thrombin for platelet activation; 2) N-chloroalanine (0.05-0.12 mM)+0.04-0.07 U/ml thrombin for platelet activation; 3) acetylsalicylic acid (0.25-0.5 mM)+0.05-0.07 U/ml thrombin for platelet activation.

test). It has been previously found that aspirin suppresses thrombin activity via its acetylation [7]. In light of this our findings can be explained as follows. Platelet suspension incubated with aspirin for a short time contains considerable amounts of acetylsalicylic acid which partially inactivates added thrombin, thus weakening PA. Under these conditions, no direct effect of acetylsalicylic acid on platelets is detected. During longer incubation, the bulk of acetylsalicylic acid disappears in the reaction with platelets, thrombin inactivation is reduced, while PA is enhanced. Thus, our findings confirm the concept that thrombin inactivation is a component of antithrombotic effect of aspirin [7].

Thus, nephelometry allows one to analyze iPA under conditions of plasma coagulation. The method also demonstrates that platelets in the concentrate for transfusion retain high aggregation ability over a 4-day storing, which cannot be detected by usual turbidimetric method. Nephelometric iPA assay proposed by us can be recommended for a more adequate control of the quality of a platelet concentrate. Judging from the dynamics of the effects of hypochlorite anion and N-chloroalanine, their mechanisms are different.

REFERENCES

1. M. A. Murina, D. I. Roshchupkin, N. N. Trunilina, *et al.*, *Byull. Eksp. Biol. Med.*, **119**, No. 5, 488-490 (1995).
2. D. I. Roshchupkin, M. A. Murina, N. N. Trunilina, and V. I. Sergienko, *Biofizika*, **40**, 569-575 (1995).
3. H. Affolter and A. Plaescher, *Thromb. Haemost.*, **48**, 204-207 (1982).
4. R. C. Becker, *Am. J. Cardiol.*, **69**, 39A-51A (1992).
5. G. V. R. Born, *Nature*, **194**, 927-929 (1962).
6. M. M. Frojmovik, J. G. Milton, and A. Duchastel, *J. Lab. Clin. Med.*, **101**, 964-976 (1983).
7. P. Han and N. G. Ardlie, *Br. J. Haematol.*, **26**, 357-372 (1974).
8. H. Holmsen, L. Salganicoff, and M. M. Fucami, in: *Haemostasis. Biochemistry, Physiology, and Pathology*, D. Ogston and B. Bennet (Eds.), New York (1977), pp. 241-319.
9. A. J. Marcus, *Blood*, **76**, 1903-1907 (1990).
10. D. I. Roshchupkin, A. Yu. Sokolov, and V. V. Berzhitskaya, *Phys. Chem. Biol. Med.*, **3**, No. 2, 17-22 (1996).
11. G. J. Roth, N. Stanford, and P. W. Majerus, *Proc. Natl. Acad. Sci. USA*, **72**, 3073-3076 (1975).
12. E. L. Snyder, *Transfusion*, **32**, 500-502 (1992).
13. E. L. Thomas, M. B. Grisham, and M. M. Jefferson, *J. Clin. Invest.*, **72**, 441-454 (1983).
14. J. Valles, M. T. Santos, J. Aznar, *et al.*, *Blood*, **78**, 154-162 (1991).