Platelet aggregation studies: autologous platelet-poor plasma inhibits platelet aggregation when added to platelet-rich plasma to normalize platelet count

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Manuscript received November 3, 2006.
Manuscript accepted March 9, 2007.

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ABSTRACT

Adjusting platelet count (PC) in platelet-rich plasma (PRP) using platelet-poor plasma (PPP) is recommended for platelet aggregation (PA) studies, but it could also affect PA independently of the decrease in PC. Analysis of aggregation tracings from healthy controls showed that PC correlated with PA in 47 diluted-PRPs, but not in 104 undiluted-PRPs. Dilution of 9 PRPs with PPP progressively decreased PA, while dilution of washed platelets with buffer hardly affected PA. Apyrase partially prevented the inhibitory effect of PPP. Therefore, the practice of diluting PRP with PPP to adjust platelet count should be avoided because it artefactually inhibits PA.

Key words: platelet aggregation, platelet-rich plasma, platelet count.

Haematologica 2007; 92:694-697

Light transmission aggregometry is the most widely used laboratory method to screen patients with suspected abnormalities of primary hemostasis due to inherited or acquired defects of platelet function.[1,2] It measures the increase in light transmission through platelet-rich plasma (PRP) that occurs when platelets are aggregated by an agonist. There are many pre-analytical and analytical variables that affect the results of platelet aggregation.[1-3] Even when all variables are accounted for the accuracy of the technique and its reproducibility are very poor.[4] For this reason, results obtained with PRP of the index patient should be compared to those of control PRP, run in parallel.[1] Because platelet count is considered a major determinant of *in vitro* platelet aggregation,[1,2] platelet counts in the two PRP samples (from the index patient and the normal control) should be adjusted to the same value, using autologous platelet-poor plasma (PPP) for correct dilution. However, this practice could also affect platelet function independently of the induced change in platelet count, as PPP may contain substances affecting platelet function that are released by platelets or other blood cells during high-speed centrifugation of blood samples necessary to obtain PPP.[5] This study examines whether or not dilution of PRP with autologous PPP also affects the results of platelet aggregation studies independently of the induced decrease in platelet count.

Design and Methods

Adenosine diphosphate (ADP), platelet-activating factor (PAF), collagen and apyrase were from Sigma (St. Louis, MO, USA). All other products were at least reagent grade. Human fibrinogen was purified from citrated plasma according to the method described by Kazal *et al.*[6] Studies of platelet aggregation in patients and healthy controls from 1995 through 2002 were carried out according to a specific protocol, established at our Centre in 1975. This carefully standardizes pre-analytic and analytical variables of the test. According to this protocol, individuals who had been taking drugs known to interfere with platelet function for the 15 days previous to blood sampling were not included in the study. Blood samples were collected from healthy volunteers in 12.9 mM sodium citrate and centrifuged at 150 g for 15 mins to obtain PRP. After separation of PRP, tubes were centrifuged again at 1,200 g for 15 mins to obtain PPP.

Washed platelets were washed using the method described by Mustard *et al.*,[7] and resuspended in Tyrode's solution containing

$CaCl_2$ 2 mM, $MgCl_2$ 1 mM, 0.1% dextrose, 0.35% bovine serum albumin, 0.05 U/mL apyrase, pH 7.35. Platelet aggregation was studied using a light transmission aggregometer (Dual Aggregometer, Chrono-Log Corporation, Havertown, PA, USA) and recorded for 3 min after stimulation of platelets with the indicated platelet agonists as described.[8] Fibrinogen (0.4 mg/mL) was added to washed platelet suspensions before stimulation with the platelet agonists. The statistical methods used to analyze the results of the study are detailed in figure legends.

Results and Discussion

First, historical platelet aggregation tracings obtained in 151 healthy controls who had been studied in parallel with index patients from 1995 to 2002 were re-analyzed. Forty-seven PRPs had been diluted to a mean platelet count of 306×10^9/L (range, 120-437) to match the platelet count in the PRP of index patients, while 104 had been used undiluted at a mean platelet count of 382×10^9/L (range, 177-569) ($p<0.001$). The mean maximal and final extents of PA induced by platelet-activating factor (PAF) 0.2 μM or adenosine diphosphate (ADP) 2 μM were significantly lower in diluted-PRPs than in undiluted-PRPs (maximal extent: 28.6 ± 24.3 vs 54.4±26.3, $p<0.001$ for PAF; 40.8±20.8 *vs* 54.5±22.2, $p=0.001$, for ADP; final extent, 21.2±28.8 *vs* 49.9±32.5, $p<0.001$ for PAF; 36.4±25.4 vs 52±26, $p=0.001$ for ADP). Those induced by collagen 2 μg/mL were not significantly different (maximal and final extent were identical, because platelet deaggregation did not occur: 68.9±17.3 *vs* 71.8±14.7 p=0.422). There was no correlation between platelet count in undiluted-PRPs and either the maximal (*not shown*) or final extent (Figure 1) of platelet aggregation. By contrast, there was a statistically significant correlation between platelet count and both maximal (not shown) and final (Fig. 1) extent of platelet aggregation induced by PAF (0.2 μM) or ADP (2 μM) in diluted-PRPs. The correlation did not reach statistical significance when higher concentrations of the agonists were used. This analysis suggested that platelet count in PRP is not a major determinant of platelet aggregation, at least in the range of about 200 and 600×10^9/L, and that the decrease in platelet aggregation observed after dilution of PRP is due to inhibitory effects of PPP. Therefore, the effects of dilution of control PRP samples with PPP (n=9) were compared with those of dilution of washed platelet suspensions with suspending buffer (n=5). In both cases, platelet aggregation was studied in undiluted samples and in samples that had been diluted to 300, 225 and 150×10^9/L. Both maximal (Figure 2) and final (*not shown*) extent of platelet aggregation in PRPs decreased as a function of the decrease in platelet count. The extent of platelet aggregation in all diluted PRPs stimulated with ADP or PAF

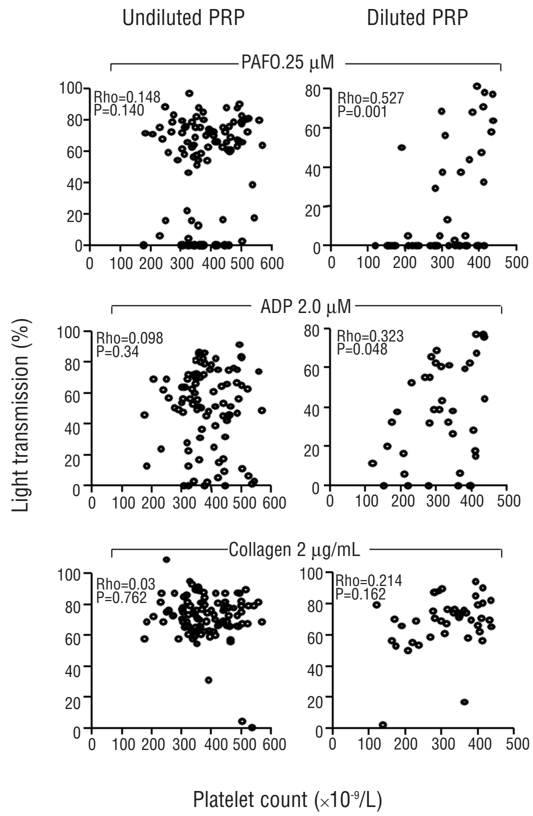

Figure 1. Correlations between platelet count and final extent of platelet aggregation in citrated PRP from healthy subjects studied in parallel with patients with suspected abnormalities of platelet function. Left graphs refer to PRP samples that were not diluted with autologous PPP (n=104). Right graphs refer to PRP samples that had been diluted with autologous PPP to match the platelet count in index patients (n=47). Platelet aggregation was recorded for 3 mins after stimulation with the aggregating agent. Spearman's rank-order correlation.

was significantly lower than that observed in undiluted PRPs, while the effect of dilution on collagen-induced platelet aggregation was statistically significant only at the lowest platelet count tested (150×10^9/L). By contrast, in washed platelet suspensions, a very slight effect of sample dilution was observed. This reached statistical significance only at the lowest platelet count tested (150×10^9/L) when PAF or ADP were the aggregating agents. The results obtained in these experiments with PRP agree with those of previous studies with a similar design. These are usually thought to confirm that platelet count is a major factor influencing results of platelet aggregation studies with light transmission aggregometry.[3] However, the combined analysis of our results obtained with PRP and washed platelet suspensions suggests that at least in the range of about 200 and 600×10^9/L it is not the decrease in platelet count that affects platelet aggregation, but rather the use of PPP to dilute PRP samples.

These results could be explained by the suggestion that substances released from blood cells during the

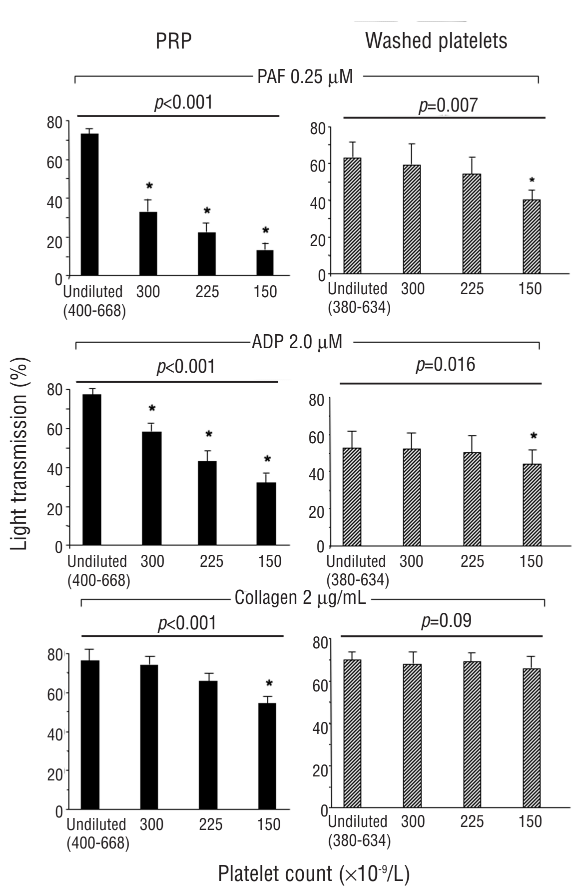

Figure 2. Effects of dilution of PRP samples with autologous PPP (left graphs) or washed platelet suspensions with suspending buffer (right graphs) on the maximal extent of platelet aggregation. Platelet aggregation was induced by PAF (0.2 μM), ADP (2 μM) or collagen 2 μg/mL. The suspending buffer used for studies with washed platelet suspensions was Tyrode's solution containing CaCl2 2 mM, MgCl2 1 mM, 0.1% dextrose, 0.35% bovine serum albumin, 0.05 U/mL apyrase, pH 7.35. Means ± SD of 9 experiments with PRP and 5 experiments with washed platelet suspensions. Fibrinogen (0.4 mg/mL) was added to platelet suspensions before stimulation. *p* values: ANOVA for repeated measures. **p*<0.05, *post-hoc* Bonferroni test.

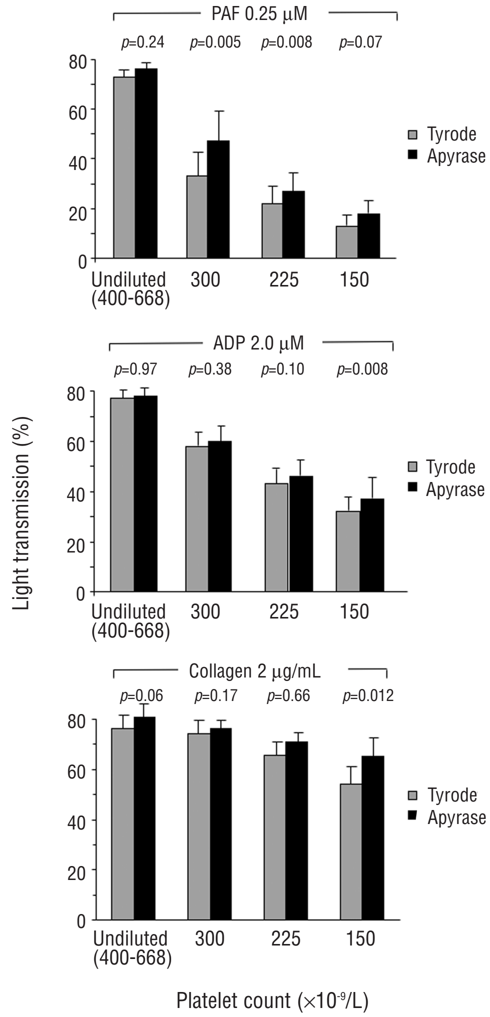

Figure 3. Effects of dilution of PRP samples with autologous PPP in the presence or absence of apyrase (0.5 U/mL) on the maximal extent of platelet aggregation. Apyrase or equal volumes of Tyrode buffer was added to PRP and PPP samples. Dilutions of apyrase-PRP were made with apyrase-PPP, and those of Tyrode-PRP with Tyrode-PPP. Platelet aggregation was induced by PAF (0.2 μM), ADP (2 μM) or collagen 2 μg/mL. Means ± SD of 9 experiments. P values: t-test for paired samples.

high-speed centrifugation of blood samples necessary to obtain PPP may be responsible for the observed inhibitory effect on platelet aggregation. One such substance could be ADP, normally contained in red blood cells and platelets. This could induce desensitisation of its receptors in PRP[9-12] thus impairing platelet response to both exogenous and endogenous ADP.[13] The experiments of PRP dilution with PPP were therefore repeated in the presence or absence of 0.5 U/mL apyrase. This prevents ADP receptors desensitization by degrading adenine nucleotides.[14] Apyrase only partially prevented the inhibitory effect of PPP on the aggregation of PRP, (Figure 3) suggesting that other substances, besides ADP, may also be responsible for the observed inhibitory effect of PPP.

In conclusion, this study challenges the common belief that platelet count (at least in the range of about 200-600×10^{9}/L) is a major determinant of platelet aggregation studies with light transmission aggregometer.[1,3] The observed reduction in the extent of platelet aggregation after dilution of PRP with PPP is due to the inhibitory effect of substances contained in PPP. One of these is ADP, which probably desensitizes its receptors in PRP. It is suggested, therefore, that, when comparing two or more PRP samples, platelet counts should not be adjusted with PPP, as is common practice, because this generates an artifact that inhibits platelet aggregation. These observations could be particularly relevant when comparing patients with very high platelet counts to healthy controls since the extent to which platelet aggregation is inhibited by PPP is a function of the dilution factor. For instance, some of the abnormalities of platelet aggregation described in patients with essential thrombocythemia (ET)[15] could be caused by the extensive dilution of their PRP samples with PPP. This is, however, unlikely to be responsible for abnormalities that are intrinsic to the ET platelet, such as defects of α adrenergic receptors and δ granules.[16-18] This issue should be addressed in appropriately designed studies; it is of interest, however, to note that a study in which platelet aggregation of ET patients was studied with both light transmission and whole blood aggregometry, platelet aggregation was normal or defective in most diluted PRP samples, while it even increased in most undiluted, whole blood samples.[1] In addition, preliminary experiments in 5 patients with ET performed in our laboratory, showed that platelet aggregation was significantly lower after dilution of patients' PRP (range of baseline platelet counts: 714-1,660×10^9/L; range of platelet counts after dilution: 423-600×10^9/L) with autologous PPP than with Tyrode buffer.

Authors' Contributions

MC: conception and design; drafting the article; final approval of the version to be published; AL: conception and design, acquisition of data; revising the manuscript; final approval of the version to be published; MLZ: acquisition of data; critically reviewing the article; final approval of the version to be published; FL: analysis and interpretation of data; revising the article critically; final approval of the version to be published.

Conflict of Interest

The authors reported no potential conflicts of interest.

References

1. Dacie JV, Lewis SM, Pitney WR, Brozovic M. Quantitative assay of coagulation factors. In: Dacie JV, Lewis SM, eds. Practical Haematology. Edinburgh: Churchill Livingstone; 1984:248-58.
2. Cattaneo M. Inherited platelet-based bleeding disorders. J Thromb Haemost 2003;1:1628-36.
3. Philp RB. In vitro tests of platelet function and anti-platelet inhibiting drugs. In: Philp RB. Methods of Testing Proposed Antithrombotic Drugs. Boca Raton, FL: CRC Press; 1981. p. 129-69.
4. Cattaneo M. Aspirin and clopidogrel: efficacy, safety, and the issue of drug resistance. Arterioscler Thromb Vasc Biol 2004;24:1980-7.
5. Mani H, Luxembourg B, Klaeffling C, Erbe M, Lindhoff-Last. Use of native or platelet count adjusted platelet rich plasma for platelet aggregation measurements. J Clin Pathol 2005;58:74750
6. Kazal LA, Amsel S, Miller OP, Tocantins LM. The preparation and some properties of fibrinogen precipitated from human plasma by glycine. Proc Soc Exp Biol Med 1963; 113:989-94.
7. Mustard JF, Perry DW, Ardlie NG, Packham MA. Preparation of suspensions of washed platelets from humans. Br J Haematol 1972;22:193-204.
8. Cattaneo M, Canciani MT, Mannucci PM. Human platelet aggregation and release reaction induced by platelet activating factor (PAF-acether)-effects of acetylsalicylic acid and external ionized calcium. Thromb Haemost 1985;53:221-4.
9. Packham MA, Mustard JF. Platelet aggregation and adenosine diphosphate/adenosine triphosphate receptors: a historical perspective. Sem Thromb Hemost 2005;31:129-38
10. Baurand A, Eckly A, Hechler B, Kauffenstein G, Galzi JL, Cazenave JP, et al. Differential regulation and relocalization of the platelet P2Y receptors after activation: a way to avoid loss of hemostatic properties? Mol Pharmacol 2005;67:721-33.
11. Hardy AR, Conley PB, Luo J, Benovic JL, Poole AW, Mundell SJ. P2Y1 and P2Y12 receptors for ADP desensitize by distinct kinase-dependent mechanisms. Blood 2005;105:3552-60.
12. Enjyoji K, Sevigny J, Lin Y, Frenette PS, Christie PD, Esch JS 2nd, et al. Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. Nat Med 1999;5:1010-17.
13. Cattaneo M, Gachet C. ADP receptors and clinical bleeding disorders. Arterioscler Thromb Vasc Biol 1999; 19:2281-5.
14. Robson SC, Wu Y, Sun X, Knosalla C, Dwyer K, Enjyoji K. Ectonucleotidases of CD39 family modulate vascular inflammation and thrombosis in transplantation. Semin Thromb Hemost 2005;31:217-33.
15. Tefferi A. Thrombocytosis and essential thrombocythemia. In: Michelson AD, ed. Platelets. Orlando, FL: Academic Press; 2002. p. 667-79.
16. Kaywin P, McDonough M, Insel PA, Shattil SJ. Platelet function in essential thrombocythemia. Decreased epinephrine responsiveness associated with a deficiency of platelet alpha-adrenergic receptors. N Engl J Med 1978;299:505-9
17. Pareti FI, Gugliotta L, Mannucci L, Guarini A, Mannucci PM. Biochemical and metabolic aspects of platelet dysfunction in chronic myeloproliferative disorders. Thromb Haemost 1982;47:84-9.
18. Malpass TW, Savage B, Hanson SR, Slichter SJ, Harker LA. Correlation between prolonged bleeding time and depletion of platelet dense ranule ADP in patients with myelodysplastic and myeloproliferative disorders. J Lab Clin Med 1984;103:894-904.
19. Balduini CL, Bertolino G, Noris P, Piletta GC. Platelet aggregation in platelet-rich plasma and whole blood in 120 patients with myeloproliferative disorders. Am J Clin Pathol 1991;95:82-6.