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Discovery and Correction of Spurious Low Platelet Counts due to EDTA-Dependent Pseudothrombocytopenia

Jianhua Lin,[1] Yaoling Luo,[1] Shaoyu Yao,[2] Miansheng Yan,[2] Jianpei Li,[1] Wenting Ouyang,[2] and Miaohuan Kuang[1]*

[1]*Department of Clinical laboratory, State Key Laboratory of Oncology in South China/Cancer Center Sun Yat-sen University, Guangzhou, P.R. China*

[2]*Department of Clinical laboratory, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, P.R. China*

Background: Ethylene diamine tetraacetic acid dependent pseudothrombocytopenia (EDTA-PTCP) is a laboratory artifact that may lead to unnecessary evaluation and treatment of patients. The purpose of this article is to discuss how to identify EDTA-PTCP and correct spurious low platelet counts in clinical laboratories. *Methods:* We use two criteria to screen for platelet aggregation: (1) an abnormal platelet count in EDTA-treated blood from a patient lacking clinical signs of a platelet disorder, and (2) an instrument flag for platelet clumps. EDTA-PTCP was confirmed by microscopic examination for platelet agglutination and by platelet counts that corrected with citrate sample. In addition, the time course of EDTA-PTCP was investigated in samples from 26 patients anticoagulated with EDTA-K_2 and sodium citrate. Amikacin (5 mg/ml) was added to tubes with EDTA-K_2 or sodium citrate from seven additional cases in order to confirm its dissociative effect on platelet aggregation. *Results:* In our laboratory, the overall incidence of EDTA-PTCP was approximately 0.09%; and the duration was between 2 weeks and 6 months. EDTA-PTCP was time-dependent and occurred as early as 10 min after sample collection. Weaker agglutination could also occur in most corresponding citrate-treated samples. The dissociative effect of amikacin on platelet agglutination was case-specific and not concentration-dependent. *Conclusions:* The method of screening for platelet clumping with the help of XE5000 images is convenient. The decline in the platelet count is related to the length of time and the intensity of chelation. Amikacin supplement is not always effective for correcting platelet counts in vitro. J. Clin. Lab. Anal. 29:419–426, 2015.

Key words: citrate; chelation; ethylene diamine tetraacetic acid (EDTA); manual microscopy; pseudothrombocytopenia

Abbreviations

CPT	=	trisodium citrate pyridoxal 5′ phosphate and tris
DIFF	=	leukocyte differential
EDTA-PTCP	=	EDTA-dependent pseudothrombocytopenia
ICSH	=	International Council for Standardization in Hematology
IMI	=	immature myeloid information
LIS	=	laboratory information system
NRBC	=	nucleated red blood cell
PDW	=	platelet distribution width
P-LCR	=	platelet-to-large-cell ratio
PLT	=	platelet
PTCP	=	pseudothrombocytopenia

INTRODUCTION

Pseudothrombocytopenia (PTCP) is an in vitro phenomenon with multiple etiologies, including anticoagulant-related platelet (PLT) agglutination, PLT satellitism, cold agglutination, large PLTs, tiny clots,

*Correspondence to: Miaohuan Kuang, Department of Clinical laboratory, Cancer Center of Sun Yat-sen University, 651 Dongfeng East Road, Guangzhou 510060, P.R. China. E-mail: kuangmiaohuan@163.com

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and improper blood sampling technique (1–3). Among these reasons, ethylene diamine tetraacetic acid dependent PTCP (EDTA-PTCP) is the most frequent cause of PTCP (2,4), which is usually caused by EDTA-dependent antiplatelet antibodies that recognize the cytoadhesive receptors gpIIb-IIIa (5–7). This interaction stimulates the expression of activation antigens, triggers activation of tyrosine kinases, platelet agglutination, and clumping in vitro, which finally leads to a spurious reduction in platelet counts by an automated hematology analyzer (7,8).

EDTA-PTCP was first reported by Gowland et al. in 1969 (9), but its pathophysiology still remains unclear. EDTA-dependent, antiplatelet autoantibodies, which have not been associated with any disease pathology in vivo, are mostly of the IgG, IgM, or IgA subclass (5, 10–13). According to previous reports (5–7, 14–17), the calcium-chelating effect of EDTA results in aggregation of platelets in vitro. EDTA causes dissociation of the Ca^{2+}-dependent gpIIb-IIIa (15, 18–20) heterodimer. This may allow autoantibodies to bind certain cryptoantigens that are only revealed in the dissociated form of gpIIb, which results in platelet aggregation in vitro (21–23).

According to the recommendation of the International Council for Standardization in Hematology (ICSH), EDTA is widely used as an anticoagulant for routine hematologic testing in laboratory medicine. The incidence of EDTA-PTCP has been reported to be as low as 0.07–0.20% (2, 10, 24, 25). However, this rare phenomenon, which has no clinical significance, often leads to misdiagnosis or mismanagement, such as bone marrow biopsy, initiation of corticosteroid therapy, platelet transfusion, or even splenectomy (1, 26–28). Thus, it is important to identify and reliably correct spurious low platelet counts in a timely manner, in order to avoid the unnecessary treatment of healthy individuals or select patient populations, such as cancer patients whose hematopoiesis is often affected by chemo- or radiotherapy. The purpose of this article is to introduce the methods used in our hospital for identifying EDTA-PTCP, and to suggest a better approach for correcting spurious low platelet counts.

MATERIALS AND METHODS

Patient Samples

We analyzed approximately 55,000 EDTA-K3 anticoagulated whole blood samples that were sent to the clinical laboratory of Sun Yat-sen University Cancer Center for routine hematological analysis between June 2010 and May 2012, using the Sysmex XE-5000 Hematology Analyser (Sysmex Co., Japan). Excluding samples with cold agglutination or clotting, we identified 49 cases (0.09%) of EDTA-PTCP. Of these, 33 cases involved hospitalized in-patients who were followed-up and included for further observation study. The primary diagnoses of these patients included hepatocellular carcinoma ($n = 13$), lung carcinoma ($n = 4$), nasopharyngeal carcinoma ($n = 4$), malignant lymphoma ($n = 3$), esophageal carcinoma ($n = 2$), ovarian carcinoma ($n = 2$), thyroid carcinoma ($n = 2$), colon rectal carcinoma ($n = 1$), pancreatic carcinoma ($n = 1$), and gastric carcinoma ($n = 1$). Study participants included 19 males and 14 females. The age of participants ranged from 19 to 84 years old, with a mean age of 57 years old. The study was approved by the Clinical Research Ethics Committee of Sun Yat-sen University Cancer Center.

Detection and Confirmation of EDTA-PTCP

Before reporting results of routine hematological analysis, we used the following rules to screen for platelet aggregation:

(1) The platelet count reported by a hematologic analyzer was $<100 \times 10^9$/l, which was the first reported low platelet count, or which was significantly lower than historical platelet counts in the same patient within the last 3 days in our laboratory information system (LIS). In addition, the patient had no symptoms of a platelet disorder.
(2) Presence of a "platelet aggregation" alert flag from the software of the XE5000 hematologic analyzer.

When one or both of the above rules was fulfilled, we performed the following procedures, in sequence, to confirm the diagnosis of EDTA-PTCP:

(1) Check the sample to exclude the presence of a clot or cold agglutination of red blood cells.
(2) Repeat the complete blood count to eliminate the possibility of random error or improper operation of the hematologic analyzer.
(3) Confirm the presence of platelet aggregates or clumps by microscopic examination of blood smears stained with Wright's & Giemsa stains (Baso Diagnostics, Inc., China).
(4) Consider repeating phlebotomy using tubes containing EDTA-K2 or other anticoagulants, such as sodium citrate or heparin sodium. EDTA-PTCP can be confirmed if the platelet count obtained in the presence of other anticoagulants is obviously higher than that measured in the presence of EDTA.

Comparison Between Different Methods for Correcting Platelet Counts

Testing a substitute sample anticoagulated with sodium citrate is the most common method for correcting platelet counts. But, over the past few years we have found that platelet aggregates may also occur in citrate-anticoagulated blood. To determine the optimal period for diagnosing and correcting PTCP after phlebotomy, we studied the time course of platelet clumping in 26 patients with EDTA-PTCP. Whole blood samples were collected simultaneously in EDTA-K2 and sodium citrate anticoagulated tubes. Platelet counts were then determined by an automated hematology analyzer at room temperature in 10, 60, 120, and 240 min after phlebotomy. Blood smears were also made at each time point to assess the degree of platelet clumping. Manual platelet counts were performed using the classical method (23–25) for every specimen within 10 min after phlebotomy, as a gold standard, to evaluate the extent of platelet clumping.

Others have reported that platelet aggregation due to EDTA-PTCP can be prevented and reversed by supplementing whole blood with kanamycin or amikacin (29–31). In this study, whole blood from additional seven patients with EDTA-PTCP was collected in either EDTA-K2 or sodium citrate anticoagulated tubes and immediately mixed with amikacin at the final concentration of 5 mg/ml, as previously reported (29). Platelet counts were also determined in 10, 60, 120, and 240 min after phlebotomy. At each time point, blood smears were also made to examine the degree of platelet clumping.

Statistical Analysis

Statistical analyses were performed using the SPSS software package (ver. 17.00; SPSS, Inc., Chicago, IL). Comparisons between means of matched samples were performed by a Wilcoxon signed-rank test. Results were expressed as a mean ± standard deviation (SD). A P-value < 0.05 was considered significant.

RESULTS

Clinical Features of EDTA-PTCP Patients

The overall prevalence of EDTA-PTCP was approximately 0.09% in our laboratory. We did not find any correlation with age, sex, specific disease, or drug treatment in our follow-up of cases with EDTA-PTCP. But, we found that it may occur within a short period of treatment in some patients, and can continue to occur for 2 weeks to 6 months. Interestingly, the phenomenon appeared intermittently in some patients. However, we did not identify any related clinical manifestations resulting from EDTA-PTCP in our patient cohort.

Laboratory Characteristics and Identification of EDTA-PTCP

Using our criteria, we were able to identify most patients with EDTA-PTCP, including those with normal platelet counts (100–300 $\times$ 10^9/l). We may have missed those patients who simultaneously had real thrombocytopenia resulting from cancer chemo- or radiotherapy.

Sufficient quantities of platelet aggregates can be identified by the Sysmex XE 5000 hematology analyzer and displayed in specific areas of images from different channels on the analyzer, including the impedance counting channel, the leukocyte differential (DIFF) channel, the immature myeloid information (IMI) channel, and the nucleated red blood cell (NRBC) channel, as show in Figure 1. Platelet aggregates produced a zigzag tail on the platelet histogram (Fig. 1A3), which resulted in a spurious wide platelet distribution width (PDW) and a high platelet-to-large-cell ratio (P-LCR). Because of their specific shape and rate of fluorescence absorption after treatment with different XE5000 reagents, platelet aggregates can be separated into a special area in DIFF, IMI, or NRBC scatter diagrams (Fig. 1B to D).

All suspicious samples should undergo microscopic examination to confirm platelet aggregation (Fig. 2), and to rule out platelet satellitism or the presence of giant platelets. Simultaneous phlebotomy into EDTA- and citrate-containing tubes can help to control for complicating factors, such as faulty phlebotomy techniques, unexplained or occasional platelet activation in vitro, presence of microthrombi, and equipment failure. EDTA-PCTP can be identified by recurrent platelet aggregation in blood collected containing EDTA, but not in blood collecting tubes containing citrate as the anticoagulant.

Time Course of PTCP Resulting From Platelet Aggregation

We examined the time course of EDTA-PTCT in 26 patients who had blood drawn simultaneously in tubes containing either EDTA or citrate as the anticoagulant. According to the results shown in Table 1 and Figures 2 and 3, EDTA-PTCP can occur as early as 10 min after sample collection. Platelet counts then decline sharply over the next 2 h and appear to plateau after 2 h (Fig. 3). Citrate anticoagulation corrected platelet counts in samples with evidence of EDTA-PTCP, as shown in Figure 3B. However, platelet aggregation also occurred in 17 cases (65.4%) of citrate anticoagulated sample an hour later (Table 1 and Fig. 2), but anticoagulation with citrate caused less

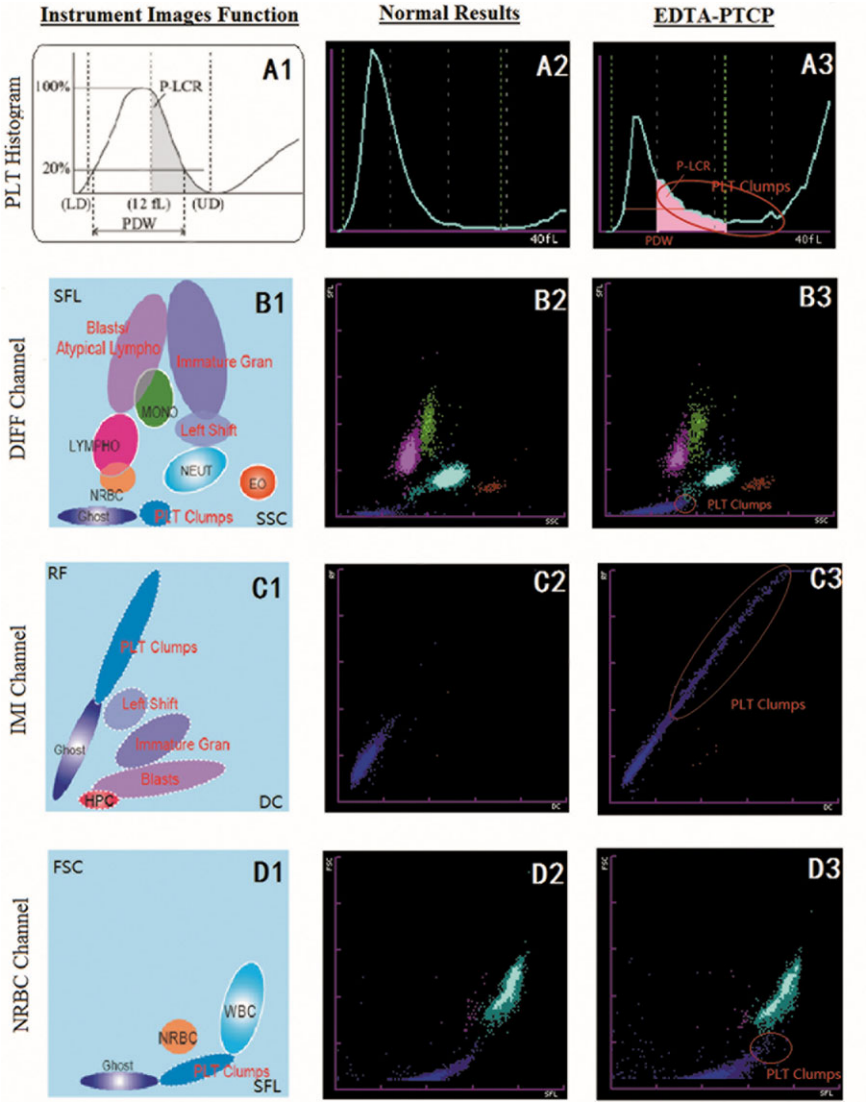

Fig. 1. Images captured from different channels of the XE-5000 showing how to distinguish platelet clumps. (A) PLT histogram by electrical impedance technology for platelet counts. P-LCR, platelet-to-large-cell ratio; PDW, platelet distribution width. (B) DIFF channel scattergram for WBC classification. SFL, sideward fluorescence; SSC, sideward scatter. (C) IMI channel scattergram showing immature myeloid information. RF, radio frequency; DC, direct current. (D) NRBC channel scattergram for nucleated red blood cells counts. FSC, forward scatter; SFL, sideward fluorescence.

dramatic effects on platelet counts compared to matched samples anticoagulated with EDTA (Fig. 3).

Dissociation Effect of Amikacin Supplementation on Platelet Counts

To validate the dissociation effect of amikacin, amikacin at a final concentration of 5 mg/ml was added to both EDTA- and sodium citrate anticoagulated blood samples of seven additional cases with EDTA-PTCP. As shown in Figure 4, we observed obvious dissociation of platelet clumping over time just in three cases, both in EDTA-anticoagulated (Fig. 4A) and sodium citrate (Fig. 4B) anticoagulated samples. In four other cases, we observed slight dissociation of platelet clumping in sodium citrate anticoagulated samples (Fig. 4B), but not in EDTA-anticoagulated samples (Fig. 4A). When we increased the concentration of amikacin to 20 mg/ml in those four unaffected cases, no dissociation was observed after 60 min of incubation.

DISCUSSION

It is now generally acknowledged that EDTA, which exists in three salt formulations, EDTA-Na_2, EDTA-K_2, and EDTA-K_3 (8, 32), is the most widely used blood

TABLE 1. Spurious Low Platelet Counts Resulting From Platelet Aggregation

	Manual ($N = 26$)	EDTA ($N = 26$)			Citrate ($N = 26$)		
Counts	Mean ± SD ($\times 10_9$/l)	Mean ± SD ($\times 10_9$/l)	EDTA/manual (%)	Aggregation cases	Mean ± SD ($\times 10_9$/l)	Citrate/manual (%)	Aggregation cases
10 min	146.4 ± 54.1	109.4 ± 39.6	75	20	139.2 ± 57.1	95	3
1 h		63.2 ± 28.8	43	25	102.8 ± 55.9	71	16
2 h		53.9 ± 23.4	37	26	98.1 ± 55.5	67	17
4 h		50.5 ± 19.1	35	26	91.2 ± 55.1	62	17

anticoagulant in vitro for routine hematologic examination. Unfortunately, platelet clumping due to EDTA is the most common cause of PTCP. Although EDTA-PTCP has been reported to occur at a low frequency of approximately 0.09–0.11% in the general population (10, 33, 34), identification and prevention of this artifact is very important for clinical laboratory staff to avoid misdiagnosis or unnecessary treatment. In this article, we introduced our experience on how to discover EDTA-PTCP, and investigated solutions to correcting platelet counts.

The criteria for EDTA-PTCP that we set in our laboratory is based on the finding of an abnormal platelet count

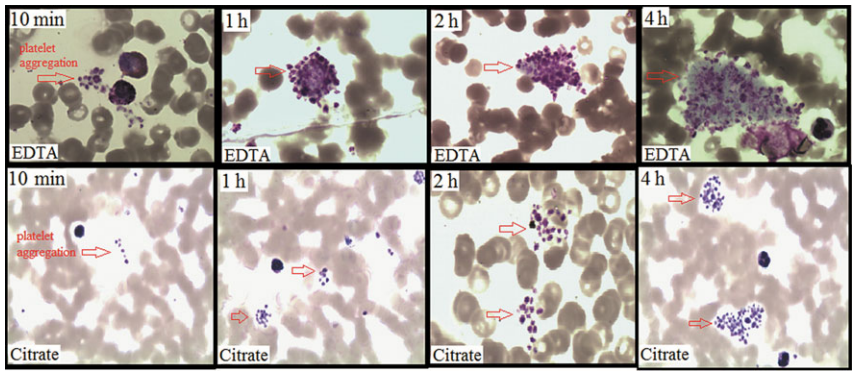

Fig. 2. Exemplary pictures (Wright Stain, 100×) of platelet aggregation (red arrow) in blood smears from EDTA anticoagulated samples and citrate anticoagulated samples at different time points.

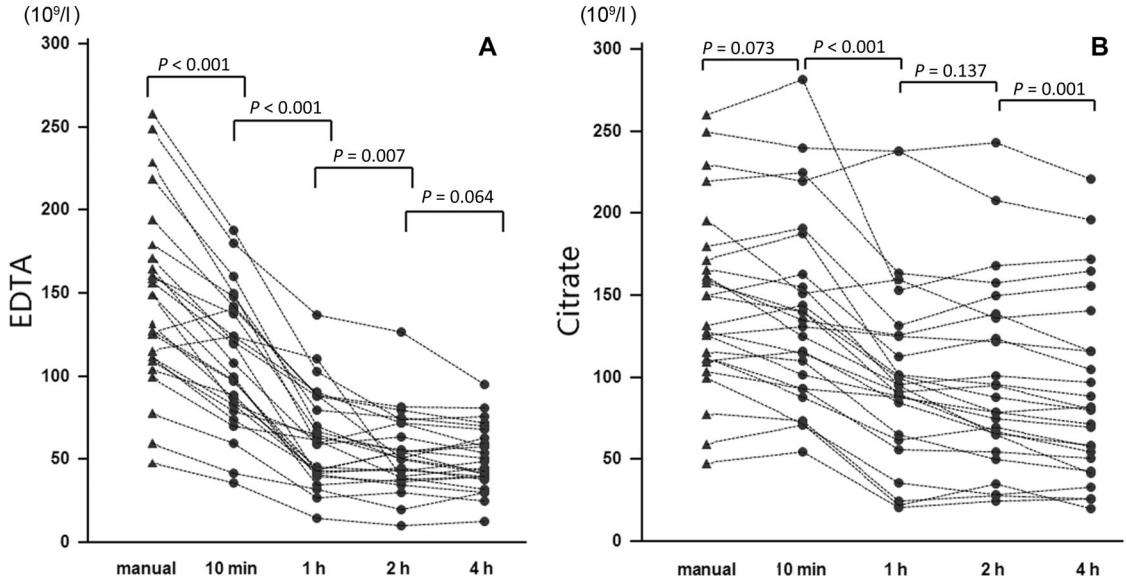

Fig. 3. The time courses of platelet counts of EDTA anticoagulated samples (A) and citrate anticoagulated samples (B) from 26 ADP patients.

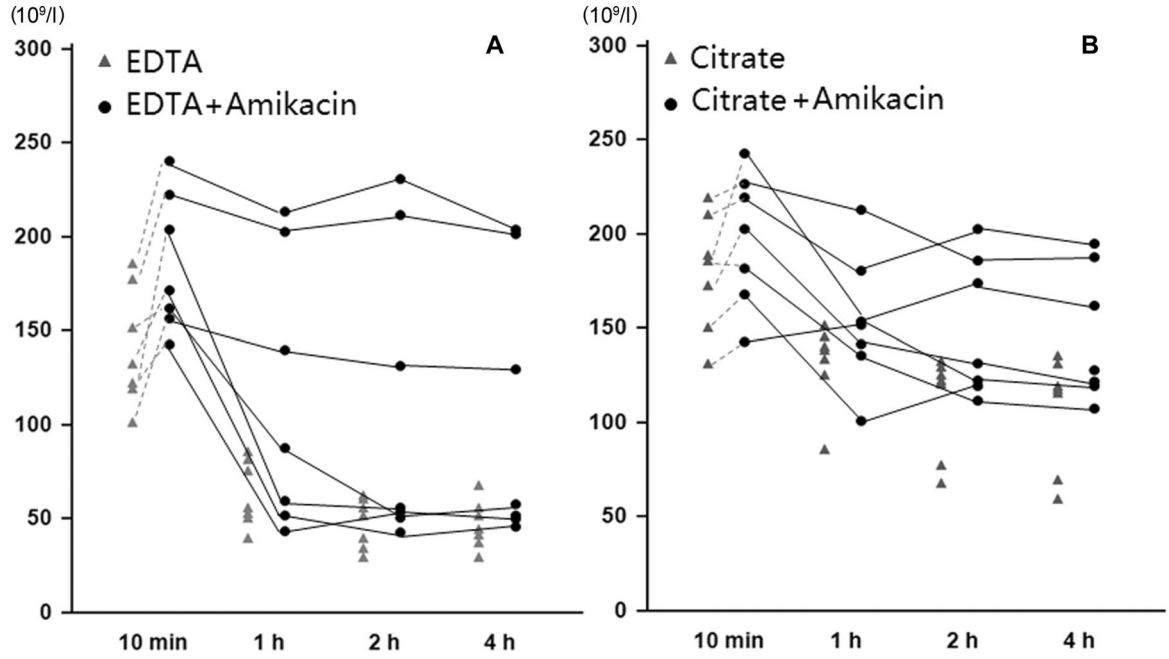

Fig. 4. The effects of amikacin supplementation on the time course of platelet counts of EDTA anticoagulated samples (A) and citrate anticoagulated samples (B) from additional seven ADP patients.

($<100 \times 10^9$/l), as reported by an automated hematology analyzer. However, it also requires historical information about a patient's platelet count and medical history. Duration of EDTA-PTCP was approximately 2 weeks to 6 months. Consistent with previous reports, none of the patients with EDTA-PTCP had clinical signs or symptoms of a platelet disorder, with either thrombotic or hemorrhagic diatheses (3,10,33). But, also consistent with previous reports, EDTA-PTCP may be associated with certain treatment courses (35).

In addition to the platelet count, criteria for EDTA-PTCP can also come from an instrument flag for PLT clumps or from an abnormal PLT histogram from different channels (Fig. 1). In this case, the PLT count may not always be below 100×10^9/l. If PLT clumps reach the size of a white blood cell (WBC), hematology analyzers, based on impedance technology, may falsely elevate the WBC count or identify these clumps as a definite population of WBCs (1,33,34). But that situation likely would not occur in our laboratory analyzer (Sysmex XE5000) that identifies PLT clumps in three channels based on parameters other than impedance, which include optical parameters, cell staining, and radio-frequency parameters (Fig. 1).

Microscopic examination is necessary to confirm platelet aggregation indicated from our criteria for EDTA-PTCP. In this study, we found that platelet aggregation due to EDTA-PTCP is time-dependent (Fig. 2), and is associated with a time-dependent fall in the platelet count (Fig. 3). However, we also observed the same phenomenon in most samples anticoagulated with citrate, as incubation time extended, as shown in Figures 2 and 3. This suggests that a calcium chelation effect, rather than an EDTA-dependent or citrate-dependent platelet aggregation, is perhaps the real trigger mechanism. Because citrate exhibits a weaker chelation effect, compared to EDTA, the fall in the platelet count is slower (Fig. 3) and its effect on the intensity of platelet aggregation is weaker (Fig. 2). That may be why citrate can correct platelet counts due to EDTA-PTCP.

Other than the anticoagulant citrate, other substances have been reported to prevent this artifact, including heparin, ammonium oxalate (10), magnesium salt (36, 37), calcium chloride (15, 37), sodium fluoride (38, 39), CPT (trisodium citrate, pyridoxal 5′ phosphate, and tris; (40, 41)), amikacin, and other aminoglycosides (29, 30). We investigated effects of amikacin on platelet clumping using an additional seven cases of EDTA-PTCP. We found that its effects were case-specific and did not appear to be concentration-dependent. Thus, the mechanism by which aminoglycosides promote dissociation of platelets remains unknown. One report (30) hypothesized that a cross-reacting antibody arising from aminoglycosides treatment is also the antiplatelet antibody. Supplementation of aminoglycoside to blood samples may neutralize the reaction of antiplatelet antibodies, resulting in the prevention of or dissociation of platelet clumping. We

doubt this hypothesis because few of our patients received aminoglycoside antibiotics and none of them received amikacin, but we could also observe obvious amikacin dissociation of platelet clumping over time in three cases.

In conclusion, EDTA-PTCP is an inherent problem of using EDTA as an anticoagulant for counting platelets. Although the prevalence of EDTA-PTCP is as low as 0.1% in the general population, this artifact may lead to unnecessary patient evaluations and treatment. In the clinical laboratory, microscopic review for platelet aggregation due to EDTA-PTCP is triggered by two criteria: (1) an abnormal platelet count ($<100 \times 10^9$/l) in EDTA-treated blood from a patient lacking clinical signs of a platelet disorder, and (2) instrument flag for platelet clumping. The decline of the platelet count is related to the length of time of chelation and the intensity of the chelation effect. Therefore, it still may be most convenient and practical to correct the platelet count using a citrate-anticoagulated sample as soon as possible (10 min) after phlebotomy.

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CONFLICT OF INTEREST

None declared.

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