Evaluation of the Anticoagulants EDTA and Citrate, Theophylline, Adenosine, and Dipyridamole (CTAD) for Assessing Platelet Activation on the ADVIA 120 Hematology System

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Background: Monitoring of platelet activation by the ADVIA® 120 Hematology System requires an anticoagulant and protocol that ensures that platelets are sphered and their activation status is not altered artificially in vitro.

Methods: Blood from healthy controls was collected into tripotassium EDTA; citrate, theophylline, adenosine, and dipyridamole (CTAD); or a combination of both (E/C) and stored at ambient temperature or at 4 °C (E/C only) and then analyzed between 0 and 180 min later on the ADVIA 120. In addition, immunofluorescent flow cytometry was used to identify activated platelets and platelet-leukocyte aggregates.

Results: In blood stored with all three anticoagulants, the platelet count changed little, but the mean platelet volume (MPV) at first decreased and then increased, whereas the mean platelet component (MPC; an indicator of activation) changed in a reciprocal manner. The changes in MPV and MPC, which reflect platelet spherating and swelling, were greatest between 30 and 60 min in blood stored at ambient temperature, irrespective of which anticoagulant was used, and between 60 and 180 min when blood anticoagulated with E/C was stored at 4 °C. In all anticoagulants, the percentages of platelets expressing CD62P and of leukocytes in platelet-leukocyte aggregates increased significantly (P < 0.01) over 180 min at ambient temperature. Only minimal (<2%) increases occurred when blood with E/C was stored at 4 °C.

Conclusions: When determining platelet activation ex vivo on the ADVIA 120, blood should be collected into E/C, stored at 4 °C, and analyzed between 60 and 180 min later; these conditions ensure maximum platelet sphering without concurrent artifactual platelet activation.

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It has been suggested by multiple studies [for original references see Refs. (1, 2)] that the measurement of indicators of platelet activation during routine hematologic investigations might offer advantages in the clinical evaluation and management of patients at risk from thrombotic and other diseases. The ADVIA® 120 Hematology System, recently introduced by the Bayer Corporation, is an automated analyzer that in addition to measuring the conventional hematologic indices, also provides some activation-related information about platelets. It measures the intensity of light scattered by platelets at two different cone angles (2–3° and 5–15°) and from the paired values computes platelet volume (PV) and platelet component (PC) concentration on a cell by cell basis. The latter values are then averaged to provide the mean platelet component (MPC) concentration (expressed in g/dL), a measure of platelet density that is correlated with the platelet activation state (1). Mean platelet mass (in pg) is com-

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4 Nonstandard abbreviations: PV, platelet volume; PC, platelet component; MPC, mean PC; MPV, mean PV; ACD, acid-citrate-dextrose; CTAD, citrate, theophylline, adenosine, and dipyridamole; E/C, tripotassium EDTA plus CTAD; TS, Tyrode’s salt solution; FITC, fluorescein isothiocyanate; and PE, phycoerythrin.
puted from the mean PV (MPV) and MPC. Data for individual platelets are presented in cytograms, whereas the mean value for each index is tabulated (3). Several studies in which the ADVIA 120 has been used to monitor platelet activation have already been published, but little information is available at present concerning the effects of preanalytical variables and, in particular, the correct choice of anticoagulant and optimum conditions for sample storage. For reasons of simplicity and economy, the ideal anticoagulant would be one that enabled information on platelet activation to be obtained as part of the full blood profile, but this might not be practicable.

EDTA is the anticoagulant recommended for full blood cell counts and white blood cell differential analysis by the NCCLS (4), principally for its cell preservation properties. However, an attempt to select and standardize on a particular salt of EDTA (5) has been abandoned and replaced by the H1-A standard. The International Council for Standardization in Hematology currently recommends the dipotassium salt of EDTA as the anticoagulant for full blood counts (6). In Europe and Japan, it is the preferred anticoagulant for this purpose, whereas in the US and the United Kingdom the tripotassium salt of EDTA is more commonly used (8). Under optimal conditions (appropriate anticoagulant concentration and analysis within 1–4 h after phlebotomy), the choice of dipotassium EDTA or tripotassium EDTA makes little difference to the results of full blood cell counts and white blood cell differential analyses (9). Heparin is not used because it is considered too expensive, it activates platelets (10), and it affects the staining properties of cells. Citrate is used as an anticoagulant primarily for coagulation studies. However, a pilot study using the CELL-DYN 4000 hematology system (Abbott) indicated that it can be used instead of EDTA for routine full blood cell counts, provided that corrections are made to take account of the different dilution factor. Furthermore, peripheral blood smears stained with Wright’s Giemsa prepared from citrated blood are indistinguishable from those prepared from EDTA-anticoagulated blood, thus allowing blood collected into just one tube to be used for both hematologic and coagulation analyses (11).

Two main requirements must be met when monitoring platelet activation ex vivo: (a) a venipuncture procedure must be used that minimizes spontaneous platelet activation and (b) blood must be collected into a medium that will not only prevent coagulation, but will also preserve the activation status of platelets until the samples can be analyzed (12–15). None of the above-mentioned commonly used anticoagulants is able to prevent platelet activation, and the extent to which preanalytical activation occurs is markedly dependent on the anticoagulant into which blood is collected (10). If blood is collected into EDTA, platelets quickly change shape from discs with a 2–4 μm diameter and a thickness of 0.5 μm to spheres covered by long thin filopodia (8). The spherical of platelets in EDTA is initially isovolumetric (16,17), but almost immediately, their apparent size changes over ~1–2 h until a state of semiequilibrium is reached (8, 17, 18). During this time, there may be apparent increases (>20%) or decreases (~10%) in the MPV, depending on whether measurements are made by impedance or light scattering, respectively [see Refs. (19, 20) for original references]. This has led to the suggestion that EDTA is not a suitable anticoagulant for PV analysis (21–25).

Furthermore, if blood from certain individuals is anticoagulated with EDTA, the platelets aggregate, causing an apparent thrombocytopenia to be recorded (8, 26) that in some, but not all, cases may result from the presence of agglutinating anti-platelet antibodies (24). EDTA was used as an anticoagulant when a prototype of the ADVIA 120 was being evaluated (3) and has also been used more recently in several studies of platelet status (1, 27, 28). Provided that the effects of EDTA on platelets are understood, the data generated could still be clinically useful (20).

For many years, citrate was the anticoagulant preferred by most investigators undertaking platelet studies [see Ref. (2) for original references], mainly because sodium citrate causes less spontaneous activation of platelets in vitro than does EDTA (10). When blood is collected into citrate, there is initially little or no change in platelet shape and volume. However, in citrate, platelets slowly adopt a spherical shape (1) and, as in EDTA, swell progressively over a period of 1–2 h [3–10% increase in volume by impedance procedures, depending on the concentration of the sodium citrate used (17, 18, 20, 29)]. For these reasons, citrate was originally considered unreliable for the measurement of platelet volume (29), but acid-citrate-dextrose (ACD) (25) and a combination of ACD and sodium EDTA (29) have been recommended instead because the MPV values obtained are stable over time. Citrate-based anticoagulants have been used for the determination of platelet indices in the ADVIA 120 (1,3), suggesting that platelet sphering may not be essential for the analysis. However, the standard deviation of the MPC (recorded as platelet component distribution width) is initially greater in citrate than in EDTA because the light scattering characteristics of disc-shaped platelets, unlike those of spheres, are dependent on their orientation (3).

Specific inhibitors of platelet function have been added to anticoagulants in attempts to minimize preanalytical activation in vitro. An example of this strategy, which is available commercially in Diatube-H Vacutainers (BD Biosciences), comprises citrate, theophylline, adenosine, and dipyridamole (commonly known as CTAD) (30). Theophylline and dipyridamole inhibit cAMP phosphodiesterase activity (31), and adenosine stimulates membrane adenylyl cyclase (32). The consequent increase in platelet cAMP and the inhibition of Ca²⁺-mediated responses lead to a reduction in platelet activation (33,34). Dipyridamole has the disadvantage of being light sensitive, and CTAD anticoagulant tubes should be stored appropriately. Flow cytometric studies of platelet surface
antigens have shown that whole-blood samples anticoagulated with CTAD are stable at 20 °C under standard laboratory conditions for up to 4 h after venesection (2, 35).

Although platelets are neither perfectly spherical nor homogeneous in EDTA, the platelet sphering that occurs in EDTA is considered advantageous for obtaining accurate PV and PC (and hence MPV and MPC) values in the ADVIA 120 because the computations are based on the assumption that scattering is by spherical particles (3). However, the platelet-activating property of EDTA makes its use as an anticoagulant for measuring patient platelet activation status questionable. CTAD was recently used with the ADVIA 120 system in a study (36) in which it was shown that patients with chest pain had lower MPC values than controls, but being citrate-based, CTAD is unlikely to sphere platelets. We therefore investigated, in a comparative manner, the consequences of using CTAD, EDTA, and the two anticoagulants together (E/C) for assessing platelet activation ex vivo. Samples were analyzed in the ADVIA 120 and by immunofluorescent flow cytometry for up to 180 min postvenesection to assess the relative stability of the platelets in each anticoagulant. The first aim of the study was to determine which anticoagulant would be optimal in minimizing platelet activation, whereas the second was to assess whether the optimal anticoagulant for platelet studies would also be suitable for making full blood cell counts and white blood cell differentials. We present data showing that the two anticoagulants together (E/C; UK patent application number 0027309.4) ensure platelet sphering with minimal activation and that blood in the combined anticoagulant is even more stable over time when stored at 4 °C rather than at ambient temperature.

**Materials and Methods**

**MATERIALS**

Tyrode’s salt solution (TS; 0.265 g/L CaCl$_2$·2 H$_2$O, 0.214 g/L MgCl$_2$·6 H$_2$O, 0.2 g/L KCl, 1.0 g/L NaHCO$_3$, 8.0 g/L NaCl, 0.05 g/L Na$_2$HPO$_4$, 1.0 g/L glucose) and human thrombin (10 units) were from Sigma. Tripotassium EDTA and CTAD in Vacutainers were from BD Biosciences; the latter were stored in light-protective boxes and removed just before use.

**ANTISERA**

Fluorescein isothiocyanate (FITC)-conjugated mouse IgG1, FITC-CD62P, and phycoerythrin (PE)-conjugated CD45 were from Immunotech. Mouse FITC-IgG2a and FITC-CD42a were from BD Biosciences.

**BLOOD SAMPLES**

Blood was collected from the antecubital vein of seven healthy individuals (median age, 35 years) who had not taken any medication, including aspirin or aspirin-containing products, in the previous 48 h and who had previously given informed consent. All procedures were in accordance with the current (October 2000) revision of the Helsinki Declaration.

**ASSESSMENT OF PLATELET ACTIVATION ON THE ADVIA 120**

Whole-blood samples were collected into Vacutainers that contained tripotassium EDTA, CTAD, or E/C. For the last, blood was collected first into tripotassium EDTA and then immediately transferred to a Vacutainer containing CTAD. Samples were stored at ambient temperature and analyzed immediately and at 30, 60, 120, and 180 min after venesection. The platelet count, MPV, and MPC were determined using the ADVIA 120 hematology system (Bayer Corporation). Platelet counts made in blood anticoagulated with CTAD and E/C were corrected for dilution (dilution factors were 1.11 and 1.125, respectively). The system was calibrated and standardized before use with ADVIA SETpoint Hematology Control and ADVIA OPTIpoint, respectively (Bayer). In one series of experiments (n = 4), these analyses were performed on blood samples anticoagulated with E/C but stored at 4 °C to investigate the effect of cooling on platelet activation.

**MEASUREMENT OF PERCENTAGES OF PLATELETS EXPRESSING CD62P AND OF LEUKOCYTES WITH PLATELETS ATTACHED (PLATELET-LEUKOCYTE AGGREGATES)**

Anticoagulated blood (5 μL) was labeled at ambient temperature with FITC-isotype control (5 μL); FITC-CD62P (5 μL); PE-CD45 (5 μL) and FITC-isotype control (5 μL); or PE-CD45 (5 μL) and FITC-CD42a (5 μL) in 90 μL of TS for 5 min. Previous studies have shown that antibody binding is complete within this time (37). Samples were diluted to 1 mL with TS and analyzed immediately by flow cytometry.

**FLOW CYTOMETRY**

Blood cells were analyzed on a FACScan (BD Biosciences) equipped with CellQuest® software. The flow cytometer was calibrated and standardized before use with fluoro-chrome-labeled beads (Fluospheres; Dako).

For the analysis of CD62P expression, data were acquired in real time with a primary gate set on a dual-parameter histogram of forward light scatter logarithmic scale (abscissa) and side light scatter logarithmic scale (ordinate). This facilitated identification of the platelets within the blood and was confirmed by the analysis of CD42a expression. Background fluorescence was assessed with platelets labeled with the FITC-conjugated isotype control antibody. Cursors were set in a single-parameter dot plot of frequency (ordinate) and green fluorescence intensity (abscissa) so that <1% of the platelets stained positively with the control antibody. Changes in CD62P expression (green fluorescence logarithmic scale), together with those of forward and side light scatter, were then recorded on the gated platelets.

For the analysis of platelet-leukocyte aggregates, cells...
were analyzed first in histograms (Fig. 1, A and D) of side light scatter (logarithmic scale; ordinate) and orange fluorescence (logarithmic scale; abscissa). Leukocytes identified by their positive staining with PE CD45 were gated (R1) to dot plots (Fig. 1, B and E) of green fluorescence (logarithmic scale; abscissa) and orange fluorescence (logarithmic scale; ordinate). Events that were both green and orange (R2) were considered platelet-leukocyte aggregates and recorded as a percentage of a total of 10,000 gated leukocytes. Platelet-leukocyte aggregates could then be gated to histograms (Fig. 1, C and F) of side light scatter (logarithmic scale; ordinate) and orange fluorescence (logarithmic scale; abscissa) to identify, by their characteristic side light scatter, which leukocytes were forming platelet-leukocyte aggregates.

**INVESTIGATION OF INHIBITORY EFFECT OF CTAD ON THROMBIN-ACTIVATED PLATELETS**

In one series of experiments (n = 3), the effect of CTAD on platelet activation was investigated in blood anticoagulated with tripotassium EDTA that had been incubated with a suboptimal concentration (determined previously by titration) of human thrombin. A concentration of thrombin was chosen that would stimulate low-level platelet activation as determined by CD62P expression. Thrombin (15 μL) was added to blood (210 μL) that had been anticoagulated with tripotassium EDTA to give a final concentration of 1.2 units/L and incubated at ambient temperature with FITC-CD62P (25 μL). After 10 min, a sample (40 μL) was removed, added to CTAD (5 μL), and incubated for an additional 20 min. Control blood samples anticoagulated with tripotassium EDTA or CTAD to which no thrombin had been added were also incubated with FITC-CD62P for 30 min. Aliquots (5 μL) of blood were removed from each reaction tube at 0, 10, 20, 40, and 60 min; diluted with TS (995 μL); and analyzed immediately by flow cytometry.

**STATISTICAL ANALYSIS**

Results from the flow cytometer and the ADVIA 120 Hematology System were compared using the paired t-test to test for significant differences between the same sample analyzed at different times. To take into account multiple comparisons, P < 0.01 was considered significan-

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**Fig. 1. Analysis of platelet-leukocyte aggregates in whole blood.**

Blood was stained with PE-conjugated CD45 and FITC-conjugated CD42a. Leukocytes were identified (region R1) by their positive staining with PE-CD45 in a plot of side scatter (logarithmic scale; ordinate) vs orange fluorescence (logarithmic scale; abscissa; dot plots A and D) and were displayed in a plot of green fluorescence (logarithmic scale; abscissa) and orange fluorescence (logarithmic scale; ordinate; dot plots B and E). Events that were both green (CD42a) and orange (CD45; region R2) were considered platelet-leukocyte aggregates. Back-gating of these events to a plot of side scatter (logarithmic scale; ordinate) vs orange fluorescence (logarithmic scale; abscissa; dot plots C and F) showed that the majority of aggregates had the side light scatter characteristics of granulocytes and monocytes. An example of the analysis performed on blood from a healthy control is illustrated in plots A–C and that from a patient with inflammatory bowel disease in plots D–F.
Results

Platelet count

Platelet counts in all three anticoagulants immediately after venesection did not differ significantly and were within the reference interval. No significant change in platelet count occurred over 180 min in blood stored with the different anticoagulants at ambient temperature (Table 1) or when blood anticoagulated with E/C was stored at 4 °C.

MPV

MPV values decreased initially in all anticoagulants and then increased again. When blood was stored at ambient temperature, the nadir was at 30 min in all anticoagulants, but when blood anticoagulated with E/C was stored at 4 °C, MPV values remained low between 60 and 180 min. When blood was stored at ambient temperature, the MPV values obtained at all times were significantly lower (P < 0.04) when it had been anticoagulated with tripotassium EDTA than when it had been anticoagulated with CTAD or E/C. Moreover, in blood anticoagulated with E/C, the MPV values after storage at 4 °C and at ambient temperature differed significantly (P < 0.01) at all times between 30 and 180 min inclusive (Fig. 2).

MPC

In direct contrast to the results for MPV values (above), the MPC values increased initially and then remained approximately constant or decreased. Maximal values were reached at 30 min in CTAD and E/C and between 30 and 60 min in tripotassium EDTA when blood was stored at ambient temperature and at 60 to 180 min when it was stored in E/C at 4 °C. MPC values were significantly higher at all times in blood stored at ambient temperature with tripotassium EDTA than with CTAD (P < 0.04) or E/C (P < 0.02). In blood stored with CTAD or tripotassium EDTA at ambient temperature, the MPC values at 30 and 180 min differed significantly (P < 0.01). In blood anticoagulated with E/C and stored at 4 °C or at ambient temperature, the values differed significantly (P < 0.01) at all times between 30 and 180 min inclusive (Fig. 3).

Table 1. PCs in blood stored with different anticoagulants.

<table>
<thead>
<tr>
<th>Time, min</th>
<th>K3EDTA*</th>
<th>CTAD</th>
<th>E/C</th>
<th>E/C at 4 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>235 ± 13</td>
<td>229 ± 14</td>
<td>230 ± 14</td>
<td>231 ± 14</td>
</tr>
<tr>
<td>30</td>
<td>246 ± 14</td>
<td>229 ± 14</td>
<td>238 ± 14</td>
<td>233 ± 19</td>
</tr>
<tr>
<td>180</td>
<td>246 ± 5</td>
<td>224 ± 7</td>
<td>225 ± 10</td>
<td>237 ± 18</td>
</tr>
</tbody>
</table>

*Mean (± SE) of seven experiments at ambient temperature except where otherwise indicated.

**Tripotassium EDTA.

Expression of CD62P on platelets

Only low percentages of platelets expressed CD62P shortly after venesection (mean ± SE, 1.10% ± 0.61%, 1.22% ± 0.62%, and 1.28% ± 0.85% in tripotassium EDTA, CTAD, and E/C, respectively), but the percentages increased when blood was stored at ambient temperature. Values at 180 min were significantly higher in blood stored with tripotassium EDTA (P < 0.04) or E/C (P < 0.02). In blood stored with CTAD or tripotassium EDTA at ambient temperature, the MPC values at 30 and 180 min differed significantly (P < 0.01), whereas in blood anticoagulated with E/C and stored at 4 °C or at ambient temperature, the values differed significantly (P < 0.01) at all times between 30 and 180 min inclusive. Symbols represent the mean values of four (E/C at 4 °C) or seven (CTAD, E/C, and tripotassium EDTA) experiments. Bars, SE.
anticoagulated with tripotassium EDTA (23.05% ± 1.54%) than with E/C (8.45% ± 0.79%; \( P < 0.01 \)), and were lowest in blood anticoagulated with CTAD (4.14% ± 0.79%; \( P < 0.01 \)). When blood samples anticoagulated with E/C were stored at 4 °C, there were only minimal increases in the number of CD62P-positive platelets, from 0.53% ± 0.12% at 0 min to 1.07% ± 0.57% at 180 min (Fig. 4).

**PLATELET-LEUKOCYTE AGGREGATE FORMATION**
Immediately after venesection, a small percentage of leukocytes that were associated with platelets could be found in blood from all donors irrespective of the anticoagulant into which it had been collected (3.50% ± 0.71%, 3.95% ± 0.97% and 2.82% ± 1.03% in tripotassium EDTA, CTAD, and E/C, respectively). In all anticoagulants, the percentage of platelet-leukocyte aggregates increased markedly when blood was stored at ambient temperature. The increases at 180 min were greater in blood anticoagulated with CTAD (18.88% ± 2.06%) than with tripotassium EDTA (13.50% ± 1.74%) and were lowest in blood that had been anticoagulated with E/C (7.81% ± 1.43%). However, when blood samples anticoagulated with E/C were incubated at 4 °C, there were only minimal increases in the percentage of platelet-leukocyte aggregates over 180 min (Fig. 5).

**EFFECT OF CTAD ON THROMBIN-ACTIVATED PLATELETS**
To ascertain whether CTAD could effectively inhibit further responses by platelets that had already encountered an agonist, blood that had been collected into tripotassium EDTA was incubated alone or with a suboptimal concentration of thrombin. After 10 min, an aliquot of the thrombin-stimulated blood was added to CTAD. Platelet activation, as monitored by CD62P expression, was completely inhibited by the addition of CTAD, whereas progressive activation occurred in blood that had been anticoagulated only with tripotassium EDTA and, as expected, was greater in these samples when thrombin had been added than when it had been omitted (Fig. 6).

**STABILITY OF EDTA AND CTAD WHEN MIXED BEFORE USE**
Because the above-mentioned results suggested that E/C might be a better anticoagulant for platelet studies than either tripotassium EDTA or CTAD alone, the effect of
premixing the two components was investigated. Blood was collected into mixtures of tripotassium EDTA and CTAD that had been prepared either 14 days or immediately before use and into tripotassium EDTA that was then mixed with CTAD (as had been done previously throughout the study). All samples were subsequently stored at 4 °C. Values for routine hematology and platelet activation indices measured on the ADVIA 120 soon after venesection were similar irrespective of whether the two anticoagulants were mixed before or after blood collection. Moreover, the values remained essentially unchanged when also analyzed at 3, 6, and 24 h. Immunofluorescence assays showed that the percentage of leukocytes involved in aggregates with platelets increased slightly (<5% increases at 24 h) in all samples over 24 h (results not shown).

**ADVIA 120 HEMATOLOGY RESULTS IN BLOOD ANTICOAGULATED WITH EDTA, CTAD, OR E/C**

To investigate whether blood anticoagulated with E/C could be used for routine analysis of hematologic indices, the results obtained on the ADVIA 120 from seven control samples anticoagulated with tripotassium EDTA, CTAD, or E/C were compared. There were no significant differences in the values obtained for the white blood cell counts, the red cell counts, and the hemoglobin concentration in blood samples anticoagulated with the three anticoagulants. However, the hematocrit and several platelet indices were significantly different (P < 0.01) in blood anticoagulated with CTAD and E/C compared with that anticoagulated with tripotassium EDTA (Table 2).

**Discussion**

The data presented here clearly show that in blood stored with EDTA, CTAD, or E/C at ambient temperature, the MPV decreased between 0 and 30 min but then increased again. The results for EDTA are largely in agreement with those of previous studies in which MPV was determined by optical procedures [e.g., Ref. (23)] and have been interpreted as representing the initial spherening and subsequent swelling of platelets (19, 20).

Consistent with recent reports, CTAD largely inhibited the increases in CD62P expression that occurred on platelets stored for 180 min at ambient temperature in blood anticoagulated with tripotassium EDTA (1, 2, 35). Furthermore, when blood that had been anticoagulated with tripotassium EDTA was stimulated with thrombin, the subsequent addition of CTAD inhibited platelet degranulation and the increases in CD62P expression that otherwise occurred in blood stored with just tripotassium EDTA. We have previously demonstrated that after in vitro stimulation of tripotassium EDTA-anticoagulated whole blood, increases in CD62P expression are accom-

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**Table 2. Comparison of ADVIA 120 hematology results* in blood anticoagulated with tripotassium EDTA, CTAD, and E/C measured at 30 min after venesection.**

<table>
<thead>
<tr>
<th>Index</th>
<th>K₃EDTA*</th>
<th>CTAD*</th>
<th>E/C</th>
<th>E/C at 4 °C*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLTs, 10⁹/L</td>
<td>246 ± 14</td>
<td>229 ± 14</td>
<td>238 ± 14</td>
<td>242 ± 14</td>
</tr>
<tr>
<td>MPV, fl</td>
<td>7.7 ± 0.27</td>
<td>8.4 ± 0.24*</td>
<td>8.6 ± 0.32*</td>
<td>7.6 ± 0.27</td>
</tr>
<tr>
<td>PDW, %</td>
<td>58.9 ± 3.24</td>
<td>53.8 ± 2.40*</td>
<td>55.6 ± 2.24</td>
<td>62.6 ± 3.24</td>
</tr>
<tr>
<td>PCT, %</td>
<td>0.19 ± 0.01</td>
<td>0.17 ± 0.01*</td>
<td>0.18 ± 0.01</td>
<td>0.20 ± 0.14</td>
</tr>
<tr>
<td>MPC, g/dL</td>
<td>28.6 ± 0.52</td>
<td>27.1 ± 0.40*</td>
<td>26.6 ± 0.66*</td>
<td>26.2 ± 0.46*</td>
</tr>
<tr>
<td>PCDW, g/dL</td>
<td>5.00 ± 0.09</td>
<td>7.2 ± 0.13*</td>
<td>6.9 ± 0.38*</td>
<td>4.8 ± 0.13</td>
</tr>
<tr>
<td>WBCs, 10⁹/L</td>
<td>5.35 ± 0.40</td>
<td>5.27 ± 0.42</td>
<td>5.36 ± 0.40</td>
<td>5.90 ± 0.46</td>
</tr>
<tr>
<td>Neutrophils, 10⁹/L</td>
<td>3.13 ± 0.24</td>
<td>3.07 ± 0.24</td>
<td>3.10 ± 0.22</td>
<td>3.20 ± 0.12</td>
</tr>
<tr>
<td>Lymphocytes, 10⁹/L</td>
<td>1.61 ± 0.20</td>
<td>1.65 ± 0.23</td>
<td>1.69 ± 0.24</td>
<td>1.66 ± 0.14</td>
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<tr>
<td>Monocytes, 10⁹/L</td>
<td>0.32 ± 0.04</td>
<td>0.28 ± 0.03</td>
<td>0.30 ± 0.04</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td>Eosinophils, 10⁹/L</td>
<td>0.11 ± 0.04</td>
<td>0.12 ± 0.04</td>
<td>0.11 ± 0.03</td>
<td>0.13 ± 0.03</td>
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<tr>
<td>Basophils, 10⁹/L</td>
<td>0.06 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>RBCs, 10¹²/L</td>
<td>4.77 ± 0.20</td>
<td>4.82 ± 0.21</td>
<td>4.87 ± 0.21</td>
<td>4.71 ± 0.16</td>
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<tr>
<td>HGB, g/dL</td>
<td>14.7 ± 0.40</td>
<td>14.8 ± 0.40</td>
<td>15.1 ± 0.44</td>
<td>14.2 ± 0.37</td>
</tr>
<tr>
<td>HCT, L/L</td>
<td>0.421 ± 0.01</td>
<td>0.434 ± 0.01*</td>
<td>0.438 ± 0.01*</td>
<td>0.426 ± 0.01</td>
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<tr>
<td>MCV, fl</td>
<td>88.7 ± 1.82</td>
<td>90.5 ± 1.89*</td>
<td>90.4 ± 1.93*</td>
<td>90.9 ± 1.82*</td>
</tr>
<tr>
<td>MCH, pg</td>
<td>30.9 ± 0.56</td>
<td>30.9 ± 0.63</td>
<td>31.1 ± 0.56*</td>
<td>30.3 ± 0.66</td>
</tr>
<tr>
<td>MCHC, g/dL</td>
<td>34.9 ± 0.20</td>
<td>34.2 ± 0.20*</td>
<td>34.4 ± 0.23*</td>
<td>33.3 ± 0.27*</td>
</tr>
<tr>
<td>RDW, %</td>
<td>12.5 ± 0.10</td>
<td>12.6 ± 0.11</td>
<td>12.6 ± 0.12</td>
<td>12.9 ± 0.13</td>
</tr>
</tbody>
</table>

* Mean (± SE).
* K₃EDTA, tripotassium EDTA; PLT, platelet; PDW, platelet distribution width; PCT, platelet crit; PCDW, platelet component distribution width; WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red blood cell distribution width.
* Values corrected for increased dilution (dilution factor, 1.11).
* Values corrected for increased dilution (dilution factor, 1.125).
* Significantly different (P < 0.01) from result obtained with tripotassium EDTA.
panied by a concurrent decrease in MPC (1). We now show that at ambient temperature, the MPC decreases spontaneously more in tripotassium EDTA than in CTAD or in E/C, consistent with the notion that EDTA causes platelet activation. In blood anticoagulated with CTAD or E/C and stored at ambient temperature, MPC values were largely similar over 180 min and were significantly lower than those in blood stored in tripotassium EDTA. Taken together, the changes in MPV and MPC values at ambient temperature suggest that in blood anticoagulated with E/C, the platelets become spherical by 30 min without degranulating. This was confirmed by the low percentage of platelets expressing CD62P in E/C-anticoagulated blood, which was comparable to that found in blood anticoagulated with CTAD alone.

Blood anticoagulated with CTAD contained higher numbers of platelet-leukocyte aggregates than did blood that had been anticoagulated with tripotassium EDTA or E/C (Fig. 5). The reasons for this are not immediately apparent, but seem dependent on the presence of EDTA. Somewhat paradoxically, EDTA has been shown to affect platelet membrane-bound receptors in a way that enhances, rather than diminishes, granule secretion and aggregation (10). However, external Ca$^{2+}$ is required for aggregation, and because EDTA is a better chelator of Ca$^{2+}$ than citrate, it may have a greater inhibitory effect in these respects. If this explanation is true, then it is possible that CTAD might not have completely inhibited increases in CD62P expression and that activated platelets were present but remained uncounted because they were attached to leukocytes. These results suggest that platelet-leukocyte aggregate formation should be monitored during studies of platelet activation in whole blood and also highlight the fact that the formation of platelet-leukocyte aggregates ex vivo is anticoagulant dependent. It has previously been shown that when blood anticoagulated with either sodium citrate or ACD is cooled to $\approx 20^\circ$C, the platelets undergo granule secretion and reversibly develop a spherical morphology with pseudopodia (38, 39). This low-temperature-induced activation was shown to be dependent on release of intracellular free calcium. We have shown (unpublished data) in blood anticoagulated with sodium citrate incubated at $4^\circ$C that there is a time-dependent increase in CD62P expression on platelets and a significant increase in platelet-leukocyte aggregate formation. Presumably the inhibitory effects of CTAD on intracellular calcium mobilization prevent granule release and subsequent platelet-leukocyte aggregate formation.

The crucial finding of this study is that E/C effectively spheres platelets in 60 min at $4^\circ$C without simultaneously causing degranulation, or in 30 min at ambient temperature with only minimal activation, thereby allowing the accurate measurement of MPC on the ADVIA 120. Moreover, if blood anticoagulated with E/C is stored at $4^\circ$C, there are only minimal changes in all indicators of platelet activation for at least 180 min. This finding will be important for clinical studies because 30 min to $3\,\text{h}$ is usually sufficient time for a sample collected in a ward or clinic to reach the laboratory for analysis. In fact, preliminary studies (unpublished results) indicate that the time period at $4^\circ$C could probably be safely extended to 6 h. It is known that the inhibitory effects of the platelet antagonists in CTAD begin to dissipate at 3–4 h when blood is stored at ambient temperature and that, if necessary, storage times could probably be prolonged by increasing their concentrations (2). Before MPC values can be used diagnostically, it seems essential to (a) have a set of reference reagents for calibrating the MPC scale on the ADVIA 120 so that interlaboratory comparisons can be made and (b) to determine reference intervals for this marker in healthy control populations. To this end, reference reagents are currently being developed and reference intervals are being established by the Bayer Corporation. It is also worth noting that the temperature ($4^\circ$C or ambient) at which blood that had been anticoagulated with E/C was stored markedly affected the MPV and MPC values that were subsequently obtained. These effects may be caused by the actin-mediated shape change (from smooth discs into spiny spheres with irregular projections) that platelets undergo at temperatures <15$^\circ$C (40).

The ability of E/C to inhibit platelet-leukocyte aggregate formation ex vivo indicates that this combined anticoagulant is also suitable for the investigation of these interactions in clinical studies. Indeed, with this anticoagulant, significantly greater numbers of platelet-leukocyte aggregates ($5.16 \pm 1.48$) were found in the blood of patients ($n = 62$) with inflammatory bowel disease than in healthy controls ($n = 20$; mean $\pm$ SE, $3.43 \pm 0.82\); $P = 0.03$; unpublished data, an example of which is illustrated in Fig. 1). A recent preliminary study (41) has shown that CTAD is suitable for the analysis of routine hematology indices on the CELL DYN 4000 (Abbott) and SE-9000 (Sysmex Corporation), although the results for platelet count and MPV were $\sim$10% lower than in dipotassium EDTA irrespective of which analyzer was used. Our preliminary results (Table 2) suggest that E/C is probably a satisfactory anticoagulant for routine analysis on the ADVIA 120, although certain algorithms may need to be altered. Additional studies are needed to confirm this point. From a clinical viewpoint, it is not practical to collect blood into one Vacutainer and then pour it into a second or to mix the contents of two Vacutainers. For this reason, we are currently undertaking studies, in association with BD Biosciences and the Bayer Corporation, to develop tubes containing both anticoagulants.

References


