Letter to the Editor

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Interference of C-reactive protein with clotting times

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To the Editor,

C-reactive protein (CRP) is an acute phase protein with a known affinity for phospholipids (PLs). Phosphatidylcholine (PC) is the principal CRP ligand, but CRP also interferes with other PL, e.g., phosphatidylethanolamine (PE) [1–3]. Recently, we illustrated that CRP interferes with activated partial thromboplastin time (aPTT), prolonging clotting times (CT) proportionally with CRP concentration [4] and depending on the type of aPTT reagent [4, 5]. PLs act as catalytic surfaces in clotting assays such as aPTT, hence this phenomenon is most likely PL-dependent, with both concentration and type of PL taking part. To unravel the mechanism, we studied the influence of CRP on different aPTT reagents. To work in PL-controlled conditions, we performed thrombin generation (TG) measurements by calibrated automated thrombinography (CAT®; Thrombinoscope, Maastricht, The Netherlands) [6]. We compared the results with the effect on thrombin time, a PL-independent clotting time (CT). Moreover, we compared the effect of CRP with that of annexin V (AnV), a potent endothelial surface anticoagulant based on its ability to displace PL-dependent coagulation factors by binding to negatively charged PL, including phosphatidylserine (PS) and PE [1, 7]. We attempted to visualize the binding of CRP to the PL surface by flow cytometry. Finally, we measured the PC content and the surface tension of the reagents because differences in interference of CRP may be determined by the accessibility of PL.

Platelet-poor normal pooled plasma (NPP) was spiked with CRP or AnV and tested in duplicate on STAR-Evolution (Diagnostica Stago, Asnières, France) with different aPTT reagents and thrombin time. The reagents are listed in Table 1 in Supplemental Material. Only limited information on the type and concentration of PL in these reagents was provided by the manufacturer.

We used purified CRP prepared as described previously [8] to obtain a dilution series with a final CRP concentration of 0, 25, 50, 100, 150, 200, and 300 mg/L. The final concentration range of AnV (Sigma-Aldrich, St. Louis, MO, USA) (0–0.09–0.18–0.36–0.72–1.14 μM) was based on current literature [7].

TG was triggered in platelet-poor plasma by tissue factor (TF, 5 pM) or silica (a 1/400 diluted aPTT reagent, PTT-A; Diagnostica Stago) and PL (4 μM). The CAT was performed as reported previously [6, 9]. Under these conditions, TG is PL-controlled, allowing to perform the test with different PLs separately: PE, PC, PS in purified linear structure (Sigma-Aldrich) and hexagonal PL (PE) of Staclot-LA (Diagnostica Stago) (HPE). Mixtures of PC and PS were used in a 70/30, 50/50, 30/70, 0/100, and 100/0 proportion. The concentration of HPE in Staclot-LA reagent was not revealed by the manufacturer and was therefore used in different dilutions (1/10, 1/50, and 1/100).

To evaluate the prolongation of the lag time (LT) and the reduction of the peak height (PH), TG parameters were coupled in a PH/LT ratio [9].

Microparticles (MPs) were prepared by activating platelets with calcium ionophore (Sigma-Aldrich). Flow cytometric analysis of MP on FACSCanto II (Becton Dickinson, Erembodegem, Belgium) was performed with AnV

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(eBioscience, San Diego, CA, USA) and CD41 (Analis/Beckman Coulter, Suarlée, Belgium) as described previously [10]. As competition between CRP and AnV for binding to PL was presumed, the strength of AnV staining of the MP in relation to the CRP concentration was studied. Furthermore, in an attempt to visualize MP-bound CRP, CRP was labeled with FITC (Sigma-Aldrich). In addition, FITC-labeled anti-CRP (Abcam, Cambridge, UK) was used in parallel with anti-IgG1-FITC functioning as an isotypic control (Sigma-Aldrich).

Surface tension of the reagents was measured using the Wilhelmy plate method [11]. All measurements were performed in duplicate. PC was measured using the LabAssay™ Phospholipid (Wako, Neuss, Germany), an enzymatic assay using *N*-ethyl-*N*-(2-hydroxy-3-sulfopropy)-3,5-domethoxyaniline [12].

Our spiking experiments confirmed that prolongation of aPTT through CRP is dose- and reagent-dependent [4, 5]. CTs were prolonged with all aPTT reagents (mean prolongation 64.1%, range 33.2%–99.2%) with increasing CRP concentrations but not with thrombin time (see Table 1). The two silica-based reagents frequently used in lupus anticoagulant (LAC) detection showed high sensitivity for CRP interference: we observed an aPTT prolongation of more than 20% from 25 to 50 mg/L on for Staclot-LA PTT-LS (buffer) and PTT-LA, respectively. Staclot-LA PTT-LS with excess of HPE used as a confirmation test in LAC did not show a prolongation, thus matching with the mechanism of aPTT prolongation by LAC due to competition of coagulation factors on negatively charged PL [4, 13, 14]. Cephascreen showed prolongation from 50 mg/L on and showed the most strongly prolonged aPTT (up to 120% for 175 mg/L CRP). All other reagents showed prolongation from 100 mg/L CRP on. As expected, thrombin time, a PL-independent coagulation test, was not influenced by CRP. A similar pattern of prolongation was observed for increasing concentrations of AnV (Table 2), supporting the idea that prolongation of CT in PL-dependent assays by CRP is based on binding to negatively charged PL.

To evaluate the influence on TG parameters, coefficients of variation (CVs, %) were calculated for NPP not spiked with CRP in all methodological conditions. Based on the maximal between-run CVs for PH/LT in test methods with PC, PS, and PE with silica and TF (28.5% and 17.6%, respectively) and in the test methods with HPE with silica and TF (55.6% and 22.2%, respectively), cutoffs for normalized PH/LT were set at 0.72, 0.82, 0.44, and 0.78, respectively. TG performed with PC, PS, PE, and variable PC/PS ratios (all 4 μM final concentration), and both reaction triggers were not significantly decreased with increasing CRP concentrations (0–300 mg/L CRP) (see Figure 1A and B). TG performed with HPE was more

Table 1 Results of the CTs of NPP spiked with increasing CRP concentrations.

Reagent	CRP, mg/L						
	0.0[a] (1.0)[b]	25.0 (38.4)[b]	50.0 (78.7)[b]	100.0 (165.2)[b]	150.0 (251.7)[b]	175.0 (302.2)[b]	200.0 (323.2)[b]
PTT-A	1.0	1.1	1.1	1.5	1.6	1.7	1.6
C.K. Prest	1.0	1.0	1.1	1.5	1.7	1.7	1.6
Cephascreen	1.0	1.1	1.3	1.9	2.1	2.2	2.0
Actin	1.0	1.0	1.1	1.4	1.6	1.7	1.9
Actin FS	1.0	1.1	1.1	1.6	1.8	1.9	1.9
Actin FSL	1.0	1.0	1.1	1.2	1.4	1.4	1.5
Pathromtin SL	1.0	1.0	1.1	1.5	1.6	1.6	1.6
SynthASil	1.0	1.0	1.0	1.2	1.4	1.3	1.3
APTT-SP	1.0	1.0	1.0	1.6	1.7	1.6	1.7
PTT-LA	1.0	1.1	1.2	1.4	1.5	1.5	1.6
Staclot LA (PTT-LS)							
With excess HPE[c]	1.0	1.0	1.0	1.0	1.0	1.1	1.1
With buffer	1.0	1.2	1.3	1.4	1.5	1.5	1.5
Thrombin time	1.0	1.0	1.0	1.1	1.1	1.1	–

[a]The sample with 0 concentration was prepared by adding an equal volume of the native elution fluid to the NPP. Addition of a small volume of solution (<10% of total volume) was allowed without influence on CT by dilution effect. [b]Final CRP concentration measured in the spiked NPP through a particle enhanced immunoturbidimetric assay (CRPLX; Roche Diagnostics, Mannheim, Germany). [c]Test principle of Staclot-LA – a LAC-sensitive aPTT reagent (PTT-LS) is used, and aPTT is performed twice, with addition of buffer (neutral) and with addition of excess of HPE. The experiments were performed with two different lots and all measurements were performed in duplicate. Only data for one lot of reagent are shown. The results are expressed as normalized ratios: CT of NPP spiked with CRP divided by the CT of the NPP without added CRP.

Table 2 Results of the CTs of NPP spiked with increasing AnV concentrations.

Reagent	AnV, μM					
	0.00	0.09	0.18	0.36	0.72	1.44
PTT-A	1.0	1.0	1.0	1.1	1.3	1.6
PTT-LA	0.9	0.9	1.0	1.1	1.7	3.4
SynthASil	0.9	0.9	1.0	1.4	3.2	4.0
Staclot LA (PTT-LS)						
With excess HPE	1.0	1.0	1.0	1.0	1.0	1.0
With buffer	1.0	1.0	1.1	1.2	1.6	2.3

The results are expressed as normalized ratios: CT of NPP spiked with AnV divided by the CT of the NPP without added AnV.

sensitive to interference of CRP, at least at higher concentrations. A slight decrease (normalized ratio PH/LT 0.78) was observed from a CRP concentration of 150 mg/L on for TG triggered with TF and PE. TG with HPE diluted 1/10 showed a reduced PH/LT from 25 mg/L CRP on. CRP did not affect TG with higher dilution of HPE (1/50 and 1/100) (see Figure 1C and D). Corresponding with former findings, TG with HPE showed a decreased normalized PH/LT ratio starting from 24 mg/L CRP on, whereas for all other PL tested, TG showed no decrease in normalized PH/LT ratio at any CRP concentration [4]. These TG experiments suggest that not the type of PL but the conformation (linear vs. hexagonal structure) plays a role in interference by CRP, supporting the complex mechanism of CRP and PL interaction that can be influenced by the conformation of the PL [15]. Examples of TG curves are shown in Figure 1 in the Supplemental Material.

No competition of binding of AnV with CRP could be demonstrated by flow cytometric experiments. Binding of CRP to the MP could not be objectified as a reduced AnV-CD41-intensity (see Supplemental Material, Figure 2). AnV expression on MP did not decrease in the presence of increasing concentrations of CRP. Probably, AnV binds more strongly, compared with CRP, to the MP surface. By labeling CRP with FITC, no FITC staining of the MP was observed, nor could binding of CRP to the MP be demonstrated using FITC-labeled anti-CRP (data not shown).

Clotting assay reagents are mixtures of activators, PL, and non-specified additives. The large CRP molecule (105 kDa) may be hampered for binding on the PL depending

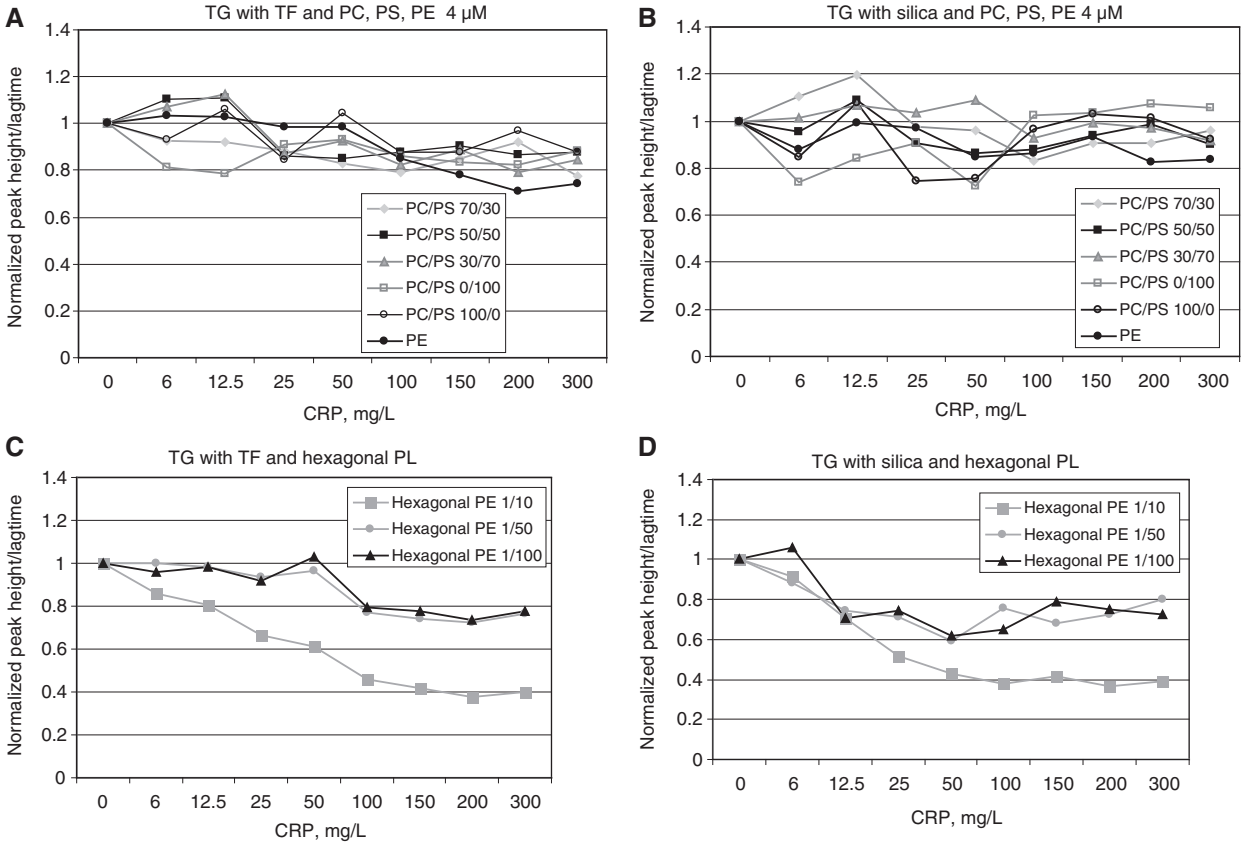

Figure 1 Relation between normalized PH/LT and concentration of CRP.
TG parameters were normalized by dividing the results for the samples spiked with CRP and those measured for the NPP not enriched with CRP.

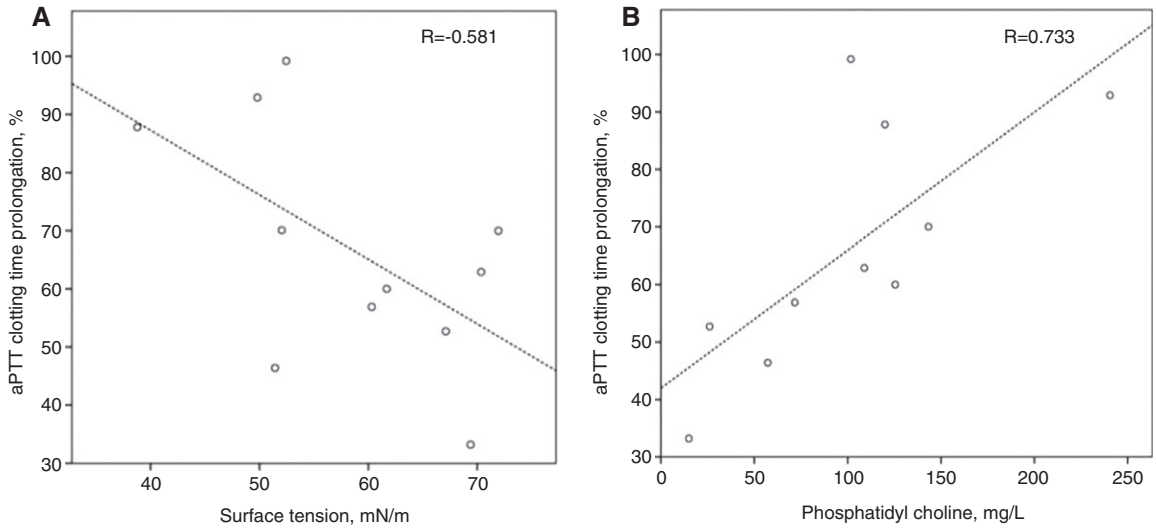

Figure 2 Correlation of aPTT-CT prolongation with the surface tension (A) and phosphatidyl choline concentration (B).
The percentage of aPTT-CT prolongation is expressed as a ratio of aPTT with the highest concentration of CRP to aPTT without CRP. (A) A moderate correlation exists between the surface tension of the reagents and the aPTT correlation. (B) A good correlation exists between the PL content of the reagent and the aPTT prolongation.

on the surface tension of the liquid. The surface tension for the aPTT reagents ranged from 38.8 to 71.9 mN/m. A moderate correlation (Pearson correlation coefficient R=−0,58) was found between the surface tension and the extent of CT prolongation (see Figure 2A): the higher the surface tension, the less the CRP-dependent prolongation of the CT, suggestive of a steric hindrance of the large CRP molecule toward binding to the PL in the reagent.

We found a good correlation (R=0.73) between the aPTT prolongation and the PL content of the reagents (see Figure 2B). The PC concentration ranged from 150 to 241 mg/dL. The high value measured in the CK Prest reagent (2331 mg/L) was excluded.

In conclusion, the in vitro interaction of CRP with PL-dependent CTs is complex. Nonetheless, we demonstrated through the similar action of AnV compared with CRP that the mechanism of prolongation of the PL-dependent CT is based on binding to negatively charged PL. The interaction is not only influenced by the type of PL but rather by the conformation and the concentration of PL. Moreover, the variation in surface tension suggests that the overall constitution of the reagent plays a role in the CRP-dependent prolongation of the CT.

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