Contact activation of C3 enables tethering between activated platelets and polymorphonuclear leukocytes via CD11b/CD18

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Summary

Complement component C3 has a potential role in thrombotic pathologies. It is transformed, without proteolytic cleavage, into $C3(H_2O)$ upon binding to the surface of activated platelets. We hypothesise that $C3(H_2O)$ bound to activated platelets and to platelet-derived microparticles (PMPs) contributes to platelet-PMN complex (PPC) formation and to the binding of PMPs to PMNs. PAR-1 activation of platelets in human whole blood from normal individuals induced the formation of $CD16^+/CD42a^+$ PPC. The complement inhibitor compstatin and a C5a receptor antagonist inhibited PPC formation by 50%, while monoclonal antibodies to $C3(H_2O)$ or anti-CD11b inhibited PPC formation by 75–100%. Using plasma protein-depleted blood and blood from a C3-deficient patient, we corroborated the dependence on C3, obtaining similar results after reconstitution with purified C3. By analogy with platelets, PMPs isolated from human serum were found to expose $C3(H_2O)$ and bind to PMNs. This interaction was also blocked by the anti-$C3(H_2O)$ and anti-CD11b monoclonal antibodies, indicating that $C3(H_2O)$ and CD11b are involved in tethering PMPs to PMNs. We confirmed the direct interaction between $C3(H_2O)$ and CD11b by quartz crystal microbalance analysis using purified native C3 and recombinant CD11b/CD18 and by flow cytometry using PMP and recombinant CD11b. Transfectants expressing CD11b/CD18 were also shown to specifically adhere to surface-bound $C3(H_2O)$. We have identified contact-activated $C3(H_2O)$ as a novel ligand for CD11b/CD18 that mediates PPC formation and the binding of PMPs to PMNs. Given the various roles of C3 in thrombotic reactions, this finding is likely to have important pathophysiological implications.

Keywords

Complement C3, platelets, microparticles, PMN, platelet-leukocyte complex

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Introduction

There is increasing evidence that the complement system is involved in a number of thrombo-inflammatory pathologies. Direct involvement of complement in such conditions is demonstrated by the generation of complement activation products (e.g. C5a and sC5b-9) in thrombotic diseases such as the phospholipid syndrome, stroke, myocardial infarction, and unstable angina (1–3). Increased levels of C3 and C4, and in particular the ratio of C3 to C4, have also been directly correlated with increased incidence and associated with risk factors for cardiovascular disease (CVD) (4, 5).

Although hampered by reagents that were not fully defined, several reports in the 1970s had already suggested that complement was associated with thrombotic events (6); complement components, particularly C3, were shown to bind to activated platelets and contribute to platelet aggregation. The binding of complement components to platelets was later definitively confirmed (7–10). In addition, in two separate studies using distinctive strains of C3-deficient mice, platelet aggregation was shown to be facilitated by C3 and a much-prolonged bleeding time exhibited by both strains of C3 KO mice was observed (11, 12).

Repair and maintenance of the integrity of the endothelial cell lining after damage has been shown to be dependent on interaction between endothelial cells, platelets and polymorphonuclear leukocytes (PMNs), in which PMNs provide signals via the ATP receptor P2X1 to support generation of the fibrin clot by expression of tissue factor and by inhibition of TF pathway inhibitor by elastase (13–16). This process is further aided by the fact that, in thrombotic states, platelets form complexes with leukocytes (mainly PMNs) in the blood (1, 17–20). This complex formation prepares and enables the platelets and leukocytes to bind to activated endothelial cells, thereby promoting both physiological thromboinflammation and thrombotic reactions (1, 17–20).

Elevated levels of platelet-PMN complexes (PPC) are found in conditions associated with CVD, such as atherosclerosis, unstable angina, and ischemia–reperfusion injury, in which PPC can contribute to both thrombotic and inflammatory processes (1). Also, when platelets are activated, microparticles are generated and, like whole platelets, these CD41$^+$ microparticles can bind to leukocytes. They are carriers of complement components and tissue factor (TF) (21, 22), giving them a potential pathophysiologic role in thrombotic states (22).

The mechanisms by which these cell- and particle-based complexes are formed are not fully understood. *In vitro,* platelet-leukocyte complexes (PLC) are formed at least in part as a result of tethering via platelet-exposed P-selectin and its ligand P-selectin glycoprotein ligand-1 (PSGL-1) on the leukocytes, in a manner resembling the initial phase of leukocyte rolling onto activated endothelial cells. The P-selectin-PSGL-1 interactions constitute a primary attachment of platelets to leukocytes (23), but cell adhesion molecules (CAM) form more stable bonds via integrins at a later stage (24). In the case of PLC formation, blocking experiments using receptor-specific monoclonal antibodies (mAbs) have indicated that the integrin CD11b/CD18 (complement receptor 3 [CR3]; Mac-1) is involved (25, 26). Glycoprotein Ib (GPIb) (25–27), junctional adhesion molecule C (JAM-C) (28), fibrinogen (29), and CD40L (30), among others, have been suggested as counter-ligands of CD11b/CD18 on platelets. However, given that CD11b/CD18 is an important complement receptor, it is possible that platelet-bound C3 acts as a ligand of CD11b/CD18, thereby contributing to the formation of PPCs.

We and others have reported that complement activation can be triggered by platelet activation (7, 9, 31). For instance, the classical pathway of complement can be elicited by chondroitin sulfate released from activated platelets (31). Moreover, the involvement of P-selectin and properdin in triggering alternative pathway activation has also been suggested (7, 10). Binding of complement components such as C1q, C4, C3, or C9 to activated platelets has been shown in a number of studies (7, 9, 32), but we have recently demonstrated that under physiological conditions, this binding is not a result of the proteolytic activation of complement (8). Analyses of the bound C3 molecules by flow cytometry and Western blotting showed that they consist of intact α- and β-chains and that, unlike C3b, the α-chain of C3 still contained the C3a portion of the molecule. However, unlike native C3, the reactivity to conformational epitopes and the cleavage pattern and reactivity to complement receptors indicated that the bound C3 was instead in the form of C3(H$_2$O). C3(H$_2$O) is generated by the hydrolysis of the internal thiol ester bond in native C3 without convertase-elicited proteolytic cleavage of the molecule. Like C3b, C3(H$_2$O) is cleaved by factor I in the α-chain and is inactivated with respect to convertase formation, yielding iC3(H$_2$O). C3(H$_2$O) and iC3(H$_2$O) are known to interact with C3 receptors such as CR1(CD35) (33), CR2 (CD21) (34), and a CR3 (CD11b/CD18)-like molecule from *Candida albicans* (35), and we have confirmed that the platelet-bound C3(H$_2$O)/iC3(H$_2$O) binds to soluble CR1 (CD35) (8).

In a previous study, we showed that PPC formation is, to a substantial degree, dependent on platelet-mediated complement activation and C5a receptor stimulation (31), occurring as the result of the up-regulation of CD11b/CD18 on the leukocyte surface. The fact that activated platelets in whole blood also expose an activated form of C3 (i.e. C3(H$_2$O) (8)) suggests that C3 may be directly involved in the formation of PPCs. Our previous studies have indicated that the platelet-bound C3(H$_2$O) is partially cleaved by factor I into iC3(H$_2$O), the equivalent of iC3b, which is a ligand of CR3 (CD11b/CD18) (36). Here, we have identified C3(H$_2$O)/iC3(H$_2$O) as a novel ligand of CD11b/CD18 and have shown that C3 alone, in the absence of any proteolytic activation, can support the formation of PPC. The dependence of PPC formation on C3(H$_2$O)/iC3(H$_2$O) and CD11b/CD18 was corroborated by the robust inhibition achieved with anti-C3a and anti-CD11b mAbs. In addition, platelet microparticles (PMPs) were shown to expose C3(H$_2$O) in a fashion similar to that shown by activated platelets, and the interaction of PMPs with PMNs was found to be similar in nature.

Materials and methods

An extended version of *Materials and methods* is found in the Supplemental Material (available online at www.thrombosis-online.com).

Whole-blood preparations

- *Depletion of plasma proteins:* Freshly drawn whole blood containing 5 mM EDTA from a C3 deficient patient and from normal individuals was centrifuged at 2,500 × g for 10 minutes (min) at room temperature (RT). The plasma was removed, and the blood cells were washed three times with Tyrode´s medium.
- *Depletion of platelets and plasma proteins:* Freshly drawn whole blood containing 5mM EDTA was centrifuged at 150 × g for 15 min at RT. The PRP supernatant (containing platelets) was discarded, and the pellet containing the erythrocytes and leukocytes was washed twice in Tyrode´s medium.

Preparation of platelet microparticles (PMPs)

Serum from nine healthy persons was prepared from freshly drawn blood without additives. The blood was allowed to clot for 1 hour (h), then centrifuged at 2,000 × g for 15 min at RT. The sera were pooled and centrifuged at 17,500 × g for 45 min at RT, and the pellet was washed twice.

Platelet-PMN complex (PPC) formation in whole blood

- *Whole blood:* PPC formation in whole blood was studied according to Hamad et al. (31). Freshly drawn lepirudin-anticoagulated (50 μg/ml) blood was activated by the addition of 33.5 μM thrombin receptor activating peptide-6 (TRAP-6; Sigma-Aldrich) for 30 min at 37 °C in the absence or presence of inhibitors.

- *Whole blood depleted of plasma proteins:* In the whole-blood preparation depleted of plasma proteins, PMNs and platelets were activated (in the presence of inhibitors) in the presence or absence of purified native C3 (0.7 mg/ml) by the addition of 5 nM C5a peptide (Sigma-Aldrich) to activate the PMNs and 33.5 μM TRAP-6 for platelet activation, then incubated for 30 min at 37 °C.

PMN-PMP complex formation

PMPs prepared from 6 ml of serum, resuspended in 500 μl of Tyrode's medium, were added to 400 μl of the blood preparation depleted of platelets and plasma proteins. The blood cells were incubated with inhibitors for 15 min at 37 °C, and the PMNs were then activated with 5 nM C5a peptide and incubated for 30 min at 37 °C.

PMP-bound complement proteins

Flow cytometry was used to monitor the binding of complement proteins to PMPs. PMPs pelleted from 1 ml of serum were resuspended in 100 μl of Tyrode´s medium. Samples were incubated for 1 h at RT with specific antibodies and analysed by flow cytometry.

Binding of CD11b to PMP

In order to study the binding of PMP-bound C3 to recombinant CD11b, isolated and washed PMPs were incubated with 25 μg/ml biotinylated-rCD11b or rMac-1 in the presence or absence of anti-CD11b and anti-C3a.

Western blotting

SDS-PAGE, followed by Western blotting, was performed as in (8).

Quartz crystal microbalance with dissipation (QCM-D)

QCM-D was used to follow the interaction between C3(H$_2$O), generated after the adsorption of C3 to a hydrophobic surface, and CD11b. Polystyrene sensors (QSX 305 PS, 5-MHz frequency) were coated with C3, IgG, and casein (0.2 mg/ml in PBS) at 4 °C overnight. The interaction of rMac-1 (10 μg/ml) with the coated proteins was monitored. In some experiments, adsorbed C3 was preincubated with either anti-CD11b mAb or iC3b.

Adhesion of CHO-cells to surface-immobilised C3

Chinese hamster ovary (CHO) cell lines expressing CD11b/CD18 (CHO-Mac-1) or control resistance vector (CHO-neo) (37) were used to study the adhesion of CHO-Mac-1 or CHO-neo to immobilised C3 or to the positive control iC3b. The cells were stimulated with PMA (200 ng/ml) and allowed to adhere for 30 min at 37 °C to coated plates under serum-free conditions. Images were acquired using an Axiovert microscope, and cells were counted.

Results

Formation of PPC in whole blood

As we previously reported (31), the formation of PPC in whole blood is triggered by thrombin receptor-activated platelets (33.5 μM TRAP-6). Here we show, in blood from a C3-deficient patient, that both CD11b up-regulation on PMNs and the formation of PPC are largely dependent on C3 (►Figure 1). Consistent with our previous data, PPC formation was significantly inhibited by the addition of the complement inhibitors compstatin or C5a receptor antagonist (C5aRA) (►Figure 2A; p< 0.05; n=5). Conjugate formation was also inhibited by anti-C3a (p< 0.0001; n=5), anti-CD11b (p< 0.01; n=5), and anti-CD62P (p< 0.0001; n=5) mAbs. Anti-CD11b inhibited conjugate formation by 65–75 %, whereas anti-C3a and anti-CD62P completely abolished PPC formation. An antibody against activated Mac-1 (CBRM1/5), which inhibited the binding by 85 % (n=3), confirmed the importance for CD11b/CD18 activation for PPC formation (►Figure 2B).

Formation of PPC in the presence of C3

The formation of PPC was further investigated in parallel experiments in which washed blood cells, depleted of plasma proteins and reconstituted with purified native C3, were analysed by flow cytometry. Using this setup, PMNs were activated with 5 nM C5a and platelets with 33.5 μM TRAP-6, in the absence or presence of purified native C3. The C3 preparation that was used consisted of 100 % non-hydrolysed C3(38, 39). The experiments clearly demonstrated that the formation of PPC (►Figure 3A; p< 0.001; n=6) was specifically enhanced in the presence of C3.

Consistent with intact whole blood, addition of mAbs against CD11b, C3a, and P-selectin prevented the formation of PPC (►Figure 3B). The PPC were inhibited by 60–80 % by anti-CD11b (p< 0.001; n= 9) and anti-C3a (4SD17.3; p< 0.001; n=5). Anti-P-selectin mAb reduced the complex formation to background levels (p< 0.001; n=9). The inhibition of PPC formation by the anti-CD11b and anti-C3a mAbs was dependent on the presence of C3, since the antibody had no significant effect on complexes that were formed in the absence of C3 (data not shown). The control Ig (mouse IgG1) had no effect on PPC.

We have previously reported the binding of C3(H$_2$O) to TRAP-6-activated platelets. To characterise the form of C3 added to the cell suspension and to ensure that no further cleavage of C3 occurred during the activation of the cells, we used Western blot analysis to study C3 associated with free platelets and with PPC. Using a polyclonal anti-C3c antibody (►Figure 3C) specific for the C-terminal end of the α-chain and the β-chain, we were able to detect the α-chain, the β-chain, and the α-chain-derived C-terminal 45-kDa fragment of C3c. With the anti-C3a mAb (►Figure 3D), an intact α-chain was detected. However, we also detected other bands indicating a degradation of native C3, and not C3b, during sample preparation, which could be seen both before and after activation of the cells with C5a and TRAP-6.
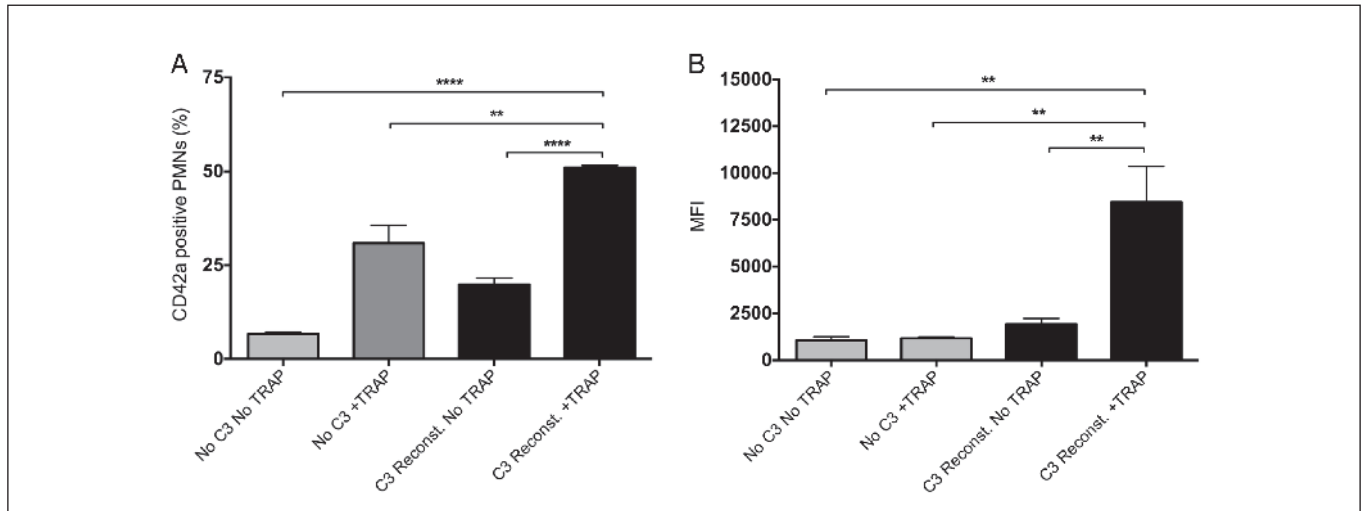
Figure 1: PMN-platelet complex formation in blood from a C3 deficient patient. The formation of PMN (CD16⁺)-platelet (CD42a⁺) complexes (A) and the expression of CD11b as an activation marker on PMNs (B) in whole blood from a C3-deficient patient was investigated by flow cytometry. Complex formation and expression of CD11b in the patient's blood (gray) or blood reconstituted with 0.5 mg/ml of purified native C3 (black) was tested without activation or after activation with 33.5 μM TRAP-6. The results are presented as the percentage of CD42a⁺ PMNs (n=3; mean ± SEM; ** p< 0.01, **** p< 0.0001). Mouse IgG2a was used as a isotype control and CD11b levels are presented as the mean fluorescence intensity (MFI) of CD16⁺ PMNs with isotype control values subtracted. The value of n in this particular experiment indicates the number of individual experiments on one patient's blood.
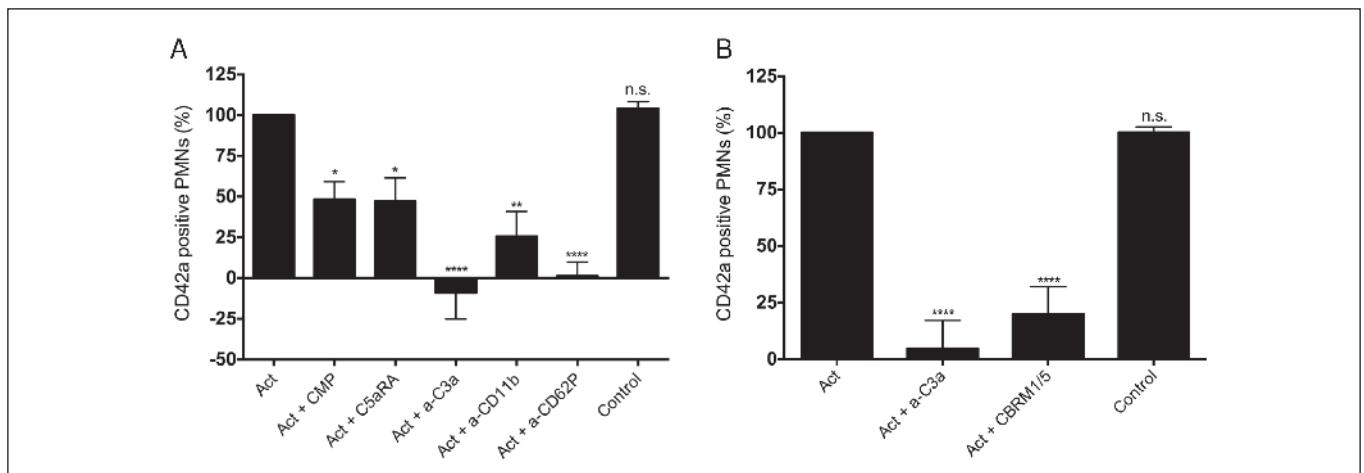

Figure 2: Formation of PMN (CD16+)-platelet (CD42a+) complexes in whole blood after activation with 33.5 μM TRAP-6, as assessed by flow cytometry. A) Data are presented as percentages of CD42a⁺ PMNs of the positive control (TRAP-6 activated blood); the mean value of CD42a⁺ PMNs before normalisation was 29 ± 4.0 for the positive control and 14.2 ± 2.4 for the non-activated control. The activation was inhibited by the addition of the complement inhibitor compstatin (CMP; 50 μM; n = 5) or C5a receptor agonist (C5aRA; 5.5 μM; n = 5). Complex formation was also blocked by using mAbs against C3a (25 μg/ml; n = 5), CD11b (25 μg/ml; n = 5), or P-selectin (25 μg/ml; n = 5). Mouse IgG1 (25 μg/ml) was used as a control. B) The complex formation was blocked by using mAb against C3a (25 μg/ml; n = 3) and the mAb CBRM1/5 specific for activated Mac-1 (25 μg/ml; n = 3). Mouse IgG1 (25 μg/ml; n=3) was used as a control. (All data are shown as means ± SEM; * p< 0.05, ** p< 0.01 and **** p< 0.0001).

Complement proteins bound to PMPs

Deposition of complement components on the PMPs was analysed by flow cytometry. Since PMPs express P-selectin, this molecule was used as a positive control in our experiments (►Figure 4A). PMPs were shown to contain C1q, C4, and C3 (►Figure 4B-D). In particular, the binding of C3 to PMP was very intense. In a previous study, we have shown that the C3 bound to TRAP-6 activated platelets is in the form of C3(H₂O), which had been partially cleaved by factor I to iC3(H₂O). In order to characterise the bound C3 in PMPs, we employed two mAbs against C3 (4SD17.3 and C3–9), both of which bind to neoepitopes of activated and conformationally altered C3 products, but not to native C3. Anti-C3–9 is specific for the C-terminal α-chain-derived polypeptide of C3c,
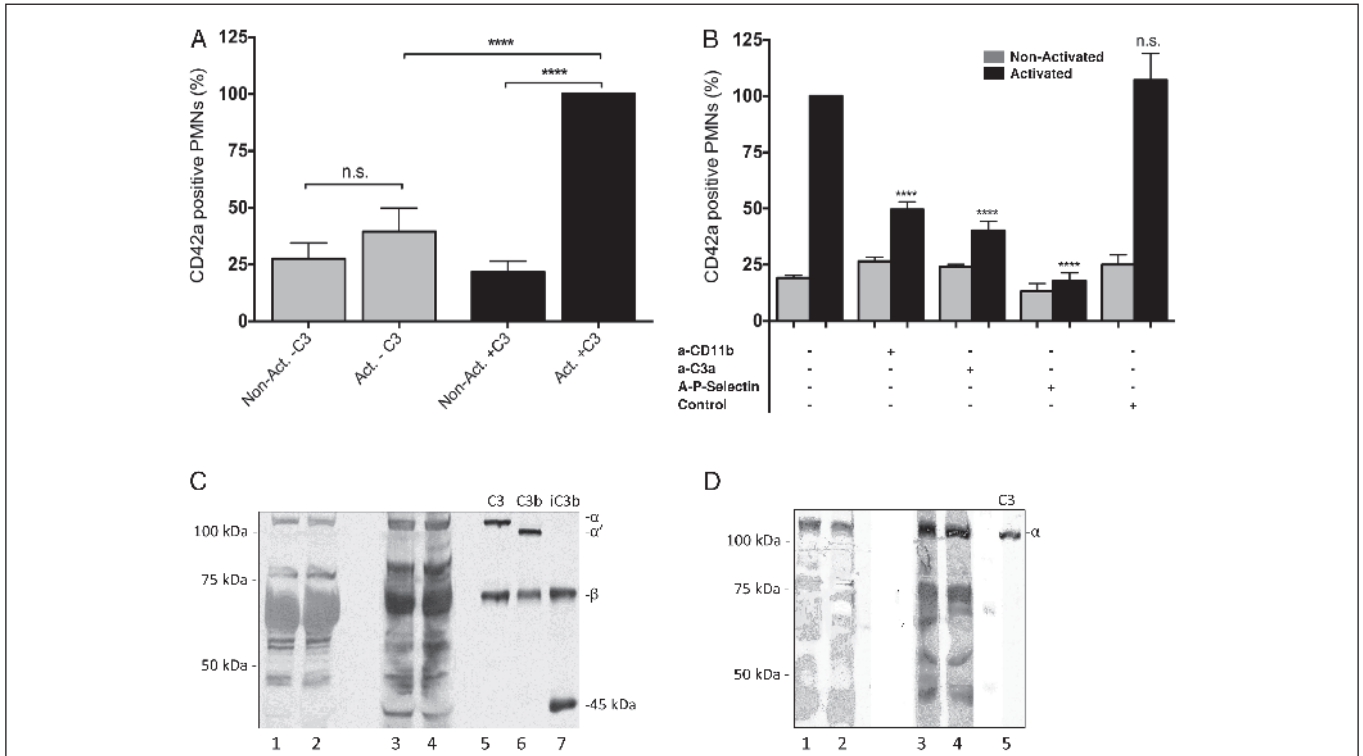
Figure 3: Binding of C3(H2O) to platelets and PMN-platelet complex formation. A) Formation of PMN (CD16⁺)-platelet (CD42a⁺) complexes in blood depleted of plasma proteins, as investigated by flow cytometry. Complex formation in the absence (gray) or presence (black) of purified native C3 was tested without activation (Non-Act) of the cells or after activation of the PMNs with 5 nM C5a and the platelets with 33.5 µM TRAP-6 (Act). The results are presented as the percentage of CD42a⁺ PMNs of the positive control (activated cells in the presence of C3); the mean value of (CD42a⁺ PMNs) before normalisation was 20 ± 3.1 for the positive control and 6.1 ± 1.5 for the non-activated control. Values for complex formation in the control samples were 6.7 ± 0.9 for the C5a-activated PMNs and 6.2 ± 1.0 for the TRAP-activated platelets (n=6; mean ± SEM; **** p<0.0001). B) Inhibition of the formation of PMN-platelet conjugates by anti-CD11b, anti-C3a, and anti-P-selectin mAbs in blood depleted of plasma proteins. Complex formation, in the presence of purified native C3, was tested without activation (gray) or after activation with 5 nM C5a and 33.5 µM TRAP-6 (black) and was analysed by flow cytometry. Mouse IgG1 (25 µg/ml) was used as a control. The results are presented as the percentage of CD42a⁺ PMNs of the positive control (activated cells in the presence of C3) (n=5–9; mean ± SEM; **** p<0.0001). C, D) Conjugate-associated C3 was analysed by Western blotting using polyclonal anti-C3c (C) and monoclonal anti-C3a 4SD17.3 (D) antibodies. Lanes 1–2, C3 associated with free platelets; lanes 3–4, C3 associated with leukocyte-platelet conjugates before (lanes 1,3) and after (lanes 2,4) activation with 5 nM C5a and 33.5 µM TRAP-6, respectively. Lanes 5–7, the C3, C3b, and iC3b controls, respectively, at 3 µg each.

which is exposed in C3b, iC3b, C3c, and C3(H₂O), but not in native C3; 4SD17.3 binds to C3a in both C3(H₂O) and in C3a, but not in native C3. Both antibodies bound to PMPs (▶Figure 4E, F), suggesting that the detected C3 was non-proteolytically activated and still contained the C3a domain. Binding of isotype-matched control antibodies was negligible (▶Figure 4G, H).

These results were confirmed by Western blotting using the polyclonal anti-C3c antibody (see above) (▶Figure 5A). The blots showed intact α- and β-chains and the C-terminal 45-kDa fragment, consistent with the forms of C3(H₂O) previously reported on activated platelets. To further characterise the C3 bound to the PMPs, two different mAbs were used: mAb anti-C3a (▶Figure 5B), recognising the C3a domain of C3; and mAb anti-20 kDa (▶Figure 5C), recognising a 20-kDa fragment of the C3c domain in C3. These antibodies showed the same binding pattern, demonstrating an intact non-proteolytically cleaved convertase cleavage site in the α-chain of C3.

Formation of PMP/PMN complexes

PMPs isolated from fresh serum were added to blood cell preparations depleted of plasma proteins and with a reduced number of platelets, and the binding of CD41a⁺ PMPs to PMNs was analysed by flow cytometry. As demonstrated in ▶Figure 6A, upon activation with 5 nM C5a, a high percentage of the PMNs bound to CD41a⁺ PMPs. The PMNs bound significantly more microparticles after activation than did activated cells without added PMPs (p<0.001; n=6).

As was true for PPC formation, formation of PMP complexes with PMNs was also inhibited by the anti-CD11b, anti-C3a, and anti-P-selectin mAbs (▶Figure 6B). Anti-CD11b (p<0.01; n=9) and anti-C3a (p<0.05; n=5) significantly reduced the formation of PMP/PMN conjugates (to background levels) when compared to non-activated cells that had been incubated with PMPs. Anti-P-selectin mAb lowered the PMP-PMN complex formation even
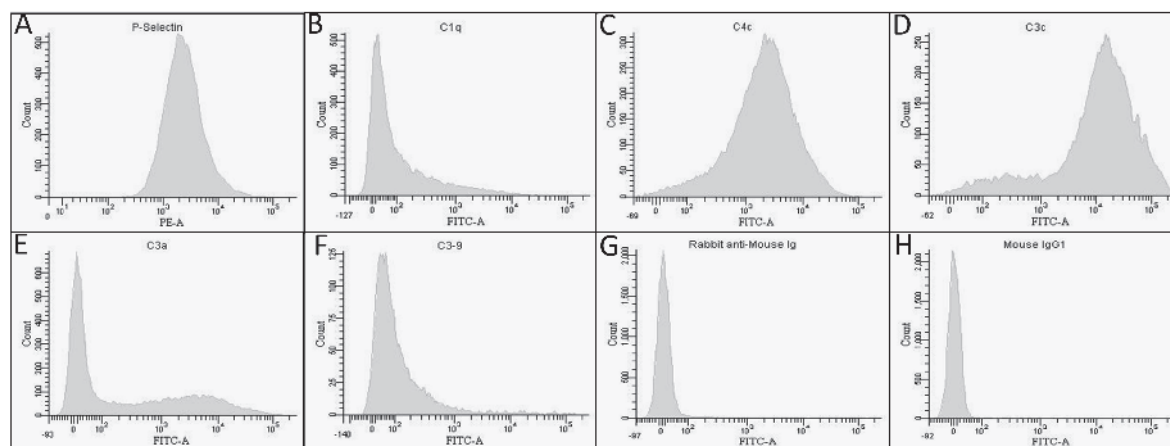
	P-Selectin	C1q	C4	C3	C3a	C3-9	
Specific Ab.	3755 ± 889*	364 ± 90*	3866 ± 1292*	14997 ± 4369*	2583 ± 589*	1328 ± 234*	n=5
Control Ab.	11±3*	7±5*	7±5*	7±5*	11±3*	11±3*	n=5

*Mean MFI values ± SEM from 5 individual experiments.

Figure 4: Binding of Complement components to PMPs. Expression of P-selectin (A) and binding of complement components C1q (B), C4 (C), and C3 (D) to PMPs isolated from fresh human serum (n=5), as detected by flow cytometry. C3 was also detected using mAbs specific for activated and conformationally changed C3. mAb 4SD17.3 (E) is specific for the C3a domain of C3, and C3–9 (F) is specific for the C-terminal α-chain-derived polypeptide of C3c (n=5). Rabbit anti-mouse Ig (G) and mouse IgG1 (H) were used as negative controls. A-H) Representative for five experiments, and the MFI values are summarised and compared to an isotype matched negative control.
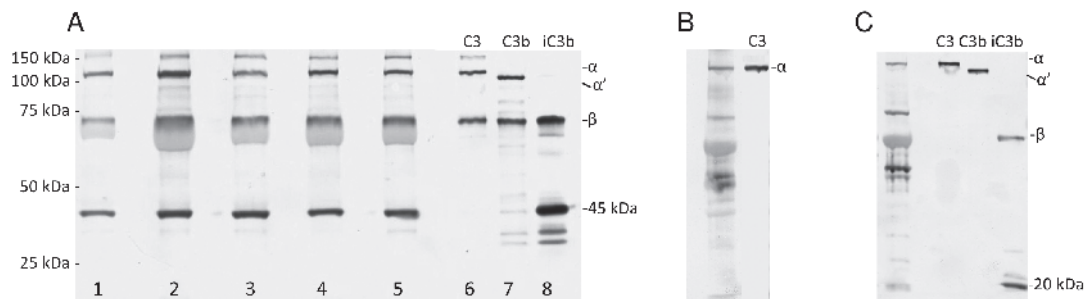

Figure 5: PMP-associated C3, analysed by Western blotting using polyclonal and monoclonal antibodies. A) In lanes 1–5, freshly isolated PMPs from the serum of five individuals are shown, and lanes 6–8 are the C3, C3b, and iC3b controls, respectively, at 3 μg each, as detected by poly-clonal anti-C3c antibody. B) PMP-associated C3 from one representative donor, detected with anti-C3a 4SD17.3 mAb; C3 was used as a control at 3 μg. C) PMP-associated C3 detected with anti-20-kDa mAb (398.1) from one representative donor; C3, C3b and iC3b were used as controls at 3 μg each.

further, to levels comparable to those obtained when no PMPs were added (p< 0.0001; n=9). PMP-PMN complexes were unaffected by the addition of control Ig.

Interaction of various forms of C3(H₂O) and CD11b/CD18

We then used QCM-D to study the interaction between C3 and CD11b, employing purified native C3 and rMac-1 (CD11b/CD18) (►Figure 7A). Native C3 was adsorbed to a hydrophobic polysty-

rene-coated QCM sensor surface, after which the binding of rMac-1 (CD11b/CD18) was monitored. IgG and casein served as negative controls. As seen in ►Figure 7A, the values for the mass of rMac-1 bound to the various surfaces clearly demonstrated the binding of rMac-1 to the C3-coated surface, whereas no binding of rMac-1 was seen to casein, and only trace amounts bound to IgG. iC3b was intended to be used as a positive control but binding of rMac-1 to the iC3b coated surface was approximately 60% of that to the C3 coated surface. The incubation of rMac-1 with increasing concentrations of soluble iC3b prior to the addition to the
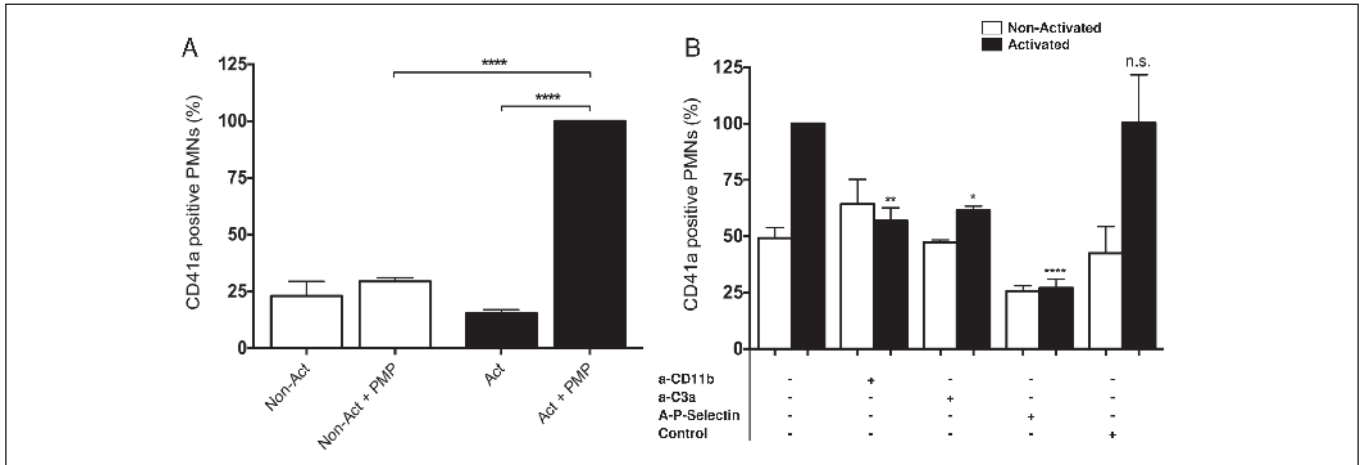
Figure 6: Binding and inhibition of binding of PMPs to PMNs. A) Binding of PMPs (CD41a$^+$) to washed non-activated (gray) and C5a-activated (black) PMNs (CD16$^+$) in blood cells with a reduced number of platelets and depleted of plasma proteins, as investigated by flow cytometry. Complex formation was tested without activation of the cells or after activation of the leukocytes with 5 nM C5a (n=6; mean ± SEM; **** p<0.001). The results are presented as the percentage of CD41a$^+$ PMNs of the positive control (C5a-activated PMNs in the presence of PMPs); the mean value of (CD41a$^+$ PMNs) before normalisation was 7.9 ± 1.4 for the positive control and 1.7 ± 0.35 for the non-activated control. B) Inhibition of the binding of PMPs to granulocytes (PMN) by anti-CD11b, anti-C3a, and anti-P-selectin mAbs in washed blood cells with a reduced number of platelets and depleted of plasma proteins. The complex formation was tested without activation (white) or after activation with 5 nM C5a (black) and analyzed by flow cytometry. The results are presented as the percentage of CD41a$^+$ PMNs of the positive control (C5a-activated PMNs in the presence of PMPs) (n= 5–9; mean ± SEM; * p<0.05, ** p<0.01, **** p<0.0001).

C3-coated surface inhibited the binding of rMac-1 at a 1:1 ratio by 20 %, at 5:1 by 45 % (not shown), and at 10:1 by 60 % (►Figure 7B). Similar experiments employing an anti-CD11b mAb showed an inhibition of binding by 70–80 % (data not shown).

In order to provide further evidence of the direct interaction between CD11b/CD18 and C3, we studied the adhesion of CHO cells expressing Mac-1 (CD11b/CD18) and of control cells (CHO-neo) to immobilised C3 (►Figure 7C). As a positive control we used immobilised iC3b, which is a known ligand of CD11b/CD18. Specific CD11b/CD18-dependent adhesion to both C3 and iC3b was observed, confirming that C3 acts as a ligand of CD11b/CD18 (p< 0.05; n=4). The level of binding of CHO-Mac-1 cells to C3 was identical to that of the positive control, iC3b (p-values 0.0011 and 0.0027, respectively).

We also investigated whether the binding of PMP-bound C3 to PMNs was mediated by CD11b/CD18. For this purpose, we quantified the binding of recombinant CD11b and Mac-1 (CD11b/CD18) proteins to PMPs by flow cytometry. Both proteins bound to PMPs, and the binding was inhibited by the addition of anti-C3a and anti-CD11b mAbs (►Figure 7D). The binding of rCD11b was decreased by ~40–50 % by both antibodies (p< 0.05; n=5), and the binding of CD11b/CD18 was inhibited to an even greater extent by both the anti-C3a (p< 0.0001; n=5) and anti-CD11b (p< 0.001; n=5) mAbs.

Discussion

In our previous studies using whole-blood models, we have demonstrated that PPC formation is dependent on platelet-mediated complement activation and C5a receptor stimulation (31), leading to up-regulation of CD11b on leukocytes. Here we further elucidate the importance of complement in the platelet-PMN interaction, by demonstrating that 1) CD11b up-regulation is absent, and PPC formation is significantly lower in a patient with C3 deficiency; and that 2) contact activation of C3 to C3(H$_2$O) by activated platelets and PMP plays a significant role in PPC formation and the binding of PMP to PMNs is dependent on the binding of C3(H$_2$O)/iC3(H$_2$O) to CD11b/CD18. These findings were corroborated in an *in vitro* human whole-blood model, in which PPC formation was efficiently blocked by receptor CD11b- and ligand C3a- (present in C3(H$_2$O), but not in C3b) specific mAbs. The robust inhibition by the antibodies suggests that the interaction is a major tethering mechanism between platelets and PMNs. In the same experiments, anti-P-selectin mAb reduced the PPC formation to levels below background, confirming previous observations that the initial PPC formation is primarily dependent on P-selectin and its ligand, PSGL-1 (40). Also other receptor-ligand interactions, like GPIb/fibrinogen, JAM-C/CD11b and CD40L/CD40, were most likely involved, since an increased PPC formation was also observed in the absence of C3.

We further dissected this reaction by using another human whole-blood model from which all the plasma proteins had been depleted and replaced with purified native C3 alone. Activation of the platelets with TRAP-6 (which stimulates PAR-1) and the PMNs with C5a (which stimulates the C5a receptor and up-regulates surface CD11b) triggered formation of PPC. The formation of PPC was specifically dependent on purified C3 in this experimental system, since no significant amounts of PPC were formed without the addition of native C3. Blocking experiments using
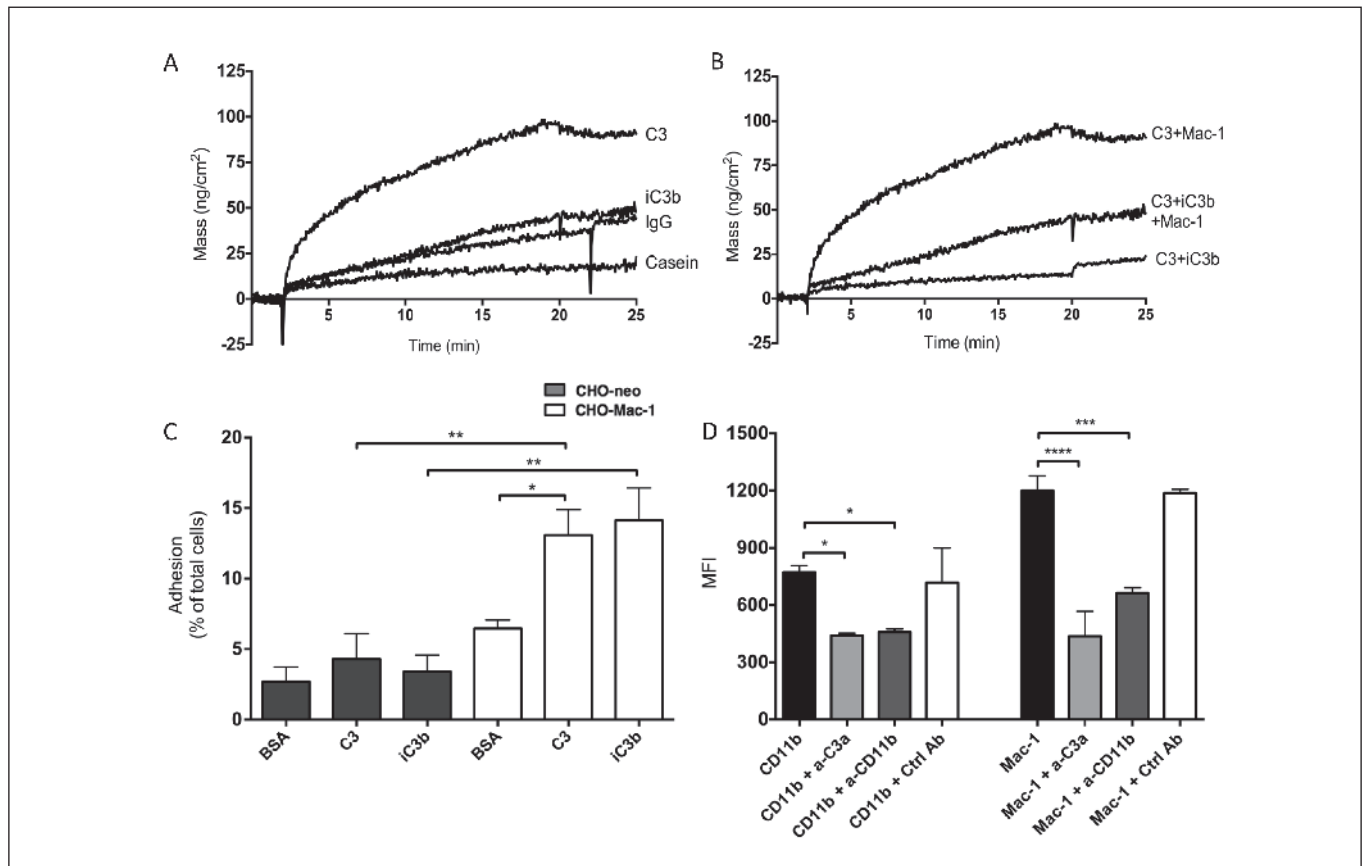
Figure 7: C3 – CD11b interactions measured with different reagents and with several techniques. A) The interaction between C3 and CD11b, as studied by QCM-D, with polystyrene sensors coated with purified native C3. The sensors were coated with 200 µg/ml C3 and 10 µg/ml rMac-1, then added to the sensor chip. iC3b-, IgG-, and casein-coated polystyrene sensors were used as control surfaces. As shown in the figure, 100 ng/cm^2 of rMac-1 bound to the C3-coated surface, whereas 40 ng/cm^2 of rMac-1 bound to iC3b and IgG and only 20 ng/cm^2 to casein. Data are representative of four independent experiments. B) The interaction between C3 and CD11b was partially counteracted by preincubation of rMac-1 with iC3b at a 1:1 molar ratio, prior to the addition of C3-coated polystyrene sensors. Measurement of the mass uptake of Mac-1 (upper trace), Mac-1 pre-incubated with iC3b (middle) and iC3b only (lower trace). C) Binding of CHO-neo (vector-transfected control cells) and CHO-Mac-1 was studied in microtiter plates coated with BSA, C3, or iC3b. Binding of CHO cells to C3 was found to be dependent on the expression of Mac-1 (n= 4; mean ± SEM; * p< 0.05, ** p< 0.01). D) Binding of recombinant CD11b and Mac-1 to isolated PMPs was investigated by flow cytometry using biotinylated proteins and streptavidin-FITC. The results are presented as the mean fluorescence intensity (MFI) of FITC-positive CD41a$^+$ labelled PMPs. Binding of both rCD11b and rMac-1 was blocked by the anti-C3a and anti-CD11b mAbs (25 µg/ml). Mouse IgG1 (25 µg/ml) was used as a control (n= 5; mean ± SEM; * p< 0.05, *** p< 0.001, **** p< 0.0001).

anti-P-selectin mAb reduced the PPC formation to levels below background. CD11b was up-regulated on PMNs after C5a stimulation, and this receptor was clearly linked to complex formation in the experiments, since the C3-dependent complex formation was blocked by receptor CD11b- and ligand C3a-specific mAbs, similar to the one in the intact whole blood model. Neither antibody had any significant effect on complex formation in the absence of C3 (data not shown).

In the present study, we extended our observations concerning C3(H$_2$O) to PMP. By using Western blotting and flow cytometry with polypeptide-specific mAbs, we were able to show that in PMPs produced by activated platelets in clotting blood, C3(H$_2$O) and iC3(H$_2$O) were present on the microparticles and resembled the C3 cleavage products found on the activated platelets. Confirming the data of Yin et al. (22), we also found that the exposure of other complement components, such as C1q, and C4 on the surface of microparticles was similar to that on activated platelets. These results suggest that, in analogy with the conditions on activated platelets, C3 is not activated by any of the three activation pathways of complement but is instead transformed to C3(H$_2$O) upon contact with the PMP surface. Studies of the binding of PMPs to PMNs showed a binding pattern similar to that seen for platelets. This binding was blocked by the same antibodies that abrogated PPC formation, demonstrating that the tethering of PMP to PMNs was also dependent on CD11b/CD18 and the C3(H$_2$O) exposed on PMPs.

In a number of test systems, we confirmed a specific binding between CD11b/CD18 and the mixture of C3(H$_2$O) and iC3(H$_2$O). In QCM-D experiments, surface-bound C3(H$_2$O) (native C3 adsorbed to polystyrene) specifically bound soluble

recombinant CD11b/CD18. This binding was inhibited by a blocking anti-CD11b mAb or by soluble iC3b, corroborating the specificity of the interaction and suggesting that C3(H$_2$O)/iC3(H$_2$O), similar to iC3b, bind to the I domain of Mac-1 (41). Also, CHO cells expressing CD11b/CD18, but not those without the receptor, adhered to surface-bound C3(H$_2$O), and we also demonstrated the binding of recombinant CD11b and CD11b/CD18 to PMP-bound C3(H$_2$O)/iC3(H$_2$O). These data further suggest that the C3(H$_2$O)/CD11b interaction is an important component in the tethering between platelets/PMP and PMNs.

The high degree of inhibition of both PPC formation and the binding of PMP to PMNs that was produced by the anti-C3a mAb clearly support the contention that C3(H$_2$O)/iC3(H$_2$O) is involved in the interaction, since the epitope is available on both these ligands. As confirmed by SDS-PAGE/Western blotting, no generation of C3a and C3b occurred, and no further cleavages beyond the C3(H$_2$O)/iC3(H$_2$O) stage happened during the whole-blood experiments, indicating that the CD11b binding site was selectively present on C3(H$_2$O)/iC3(H$_2$O), since in both test systems the proteolytic fragment C3a is not generated. Further supporting the hypothesis that the C3a fragment is not involved in PPC formation is the fact that the anti-C3a antibody, because of its specificity, cannot block the C-terminal active receptor site on the anaphylatoxin C3a. Also, the involvement of C3a receptors exposed on the platelets is less likely because the C3a receptor binding site on C3a is not available in the C3a domain of C3(H$_2$O)/iC3(H$_2$O). Other sites within C3a that interact with the C3a receptor and could mediate binding between platelets and PMNs have not yet been defined. Thus, it is likely that the interaction between C3(H$_2$O)/iC3(H$_2$O) and CD11b/CD18 is affected by the specific binding of the anti-C3a mAb to the C3a domain of C3(H$_2$O)/iC3(H$_2$O), allowing the mAb bound to this part of the molecule to hinder, either sterically or via a conformational change, the interaction with the receptor.

The mechanism by which C3 binds and is transformed into C3(H$_2$O) on the platelet and PMP surfaces is not clear. The generation of C3(H$_2$O) seems to occur when C3 adsorbs to various hydrophobic surfaces, such as gas bubbles, plastic surfaces, or phospholipid membranes (reviewed in [42]). We recently investigated complement activation triggered by liposomes composed of neutral phospholipids and, by analogy with platelets, we found that these biosurfaces also induced the formation of C3(H$_2$O) in the presence of blood plasma or purified native C3 (43). The transition from native C3 to C3(H$_2$O), which represents a substantial conformational change in the molecule, can be induced by low concentrations of detergents such as sodium dodecyl sulfate, suggesting that hydrophobic sites exist in the molecule that may induce the transformation to C3(H$_2$O) (44).

Recently, Saggu et al. (10) reported that alternative pathway complement activation on activated platelets can occur as a result of the binding of properdin to the surface of the activated platelets, which causes recruitment of C3b or C3(H$_2$O). The generation and binding of C3(H$_2$O) to activated platelets in our experimental models is, however, unlikely to be the result of this mechanism. In our previous and present study, C3(H$_2$O) was detected on acti-

vated platelets in blood in which complement activation had been inhibited with compstatin and in blood chelated with EDTA (8). We also added purified native C3 (38, 39) (free of C3(H$_2$O)) to washed platelets and were still able to detect the binding of C3(H$_2$O) to the TRAP-6-activated platelets (8). Additional support for this notion is that the anti-C3a mAb used in this study did not inhibit the binding of properdin to C3(H$_2$O), as assessed by QCM-D (Suppl. Figure 1, available online at www.thrombosis-online.com).

The functional consequences of the binding of C3(H$_2$O)/iC3(H$_2$O) to activated platelets and PMPs are likely to have an impact on thromboinflammatory reactions. The engagement of C3(H$_2$O)/iC3(H$_2$O) in the tethering of platelets to PMNs would facilitate the formation of circulating PPCs. Recent studies have emphasized the importance of PPC formation to help maintaining the integrity of damaged endothelial linings (13, 14). The fact that C3 KO mice show a much-prolonged bleeding time corroborates this notion (11, 12). C3 would also be able to participate in the cross-talk between platelets, leukocytes, and endothelial cells during thrombotic reactions, by facilitating PPC formation. Moreover, PMP formation would increase thrombotic reactions, since PMNs have been suggested to acquire TF by binding platelet- and monocyte-derived TF-containing microparticles (45). Elevated levels of

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What is known about this topic?

- Platelet-leukocyte complexes (PLC) are formed in response to inflammatory, thrombotic and infectious events. PLC formation plays a role in haemostasis and seems to be involved in the pathophysiology of cardio- and cerebrovascular diseases.
- When platelets are activated, microparticles (PMPs) are generated that have the capacity to bind to leukocytes. These PMPs have previously been shown to bind and expose complement components and tissue factor (TF), which after complex formation with leukocytes can be exposed on the leukocytes.
- The PLC formation involves an integrin-dependent step (CD11b/CD18) in which several ligands, such as fibrinogen, CD40, and GPIb have been suggested as counter-ligands.

What does this paper add?

- We describe a fundamentally new activation mechanism of complement component C3 in thromboinflammation, which does not involve any other complement components or proteolytic cleavages by other proteases.
- We demonstrate that native C3 is prone to transform into C3(H$_2$O) upon contact with a surface cf. Factor XII to Factor XIIa in the contact system.
- We have identified platelet and microparticle-bound contact-activated C3(H$_2$O) as a ligand for CD11b/CD18 promoting formation of platelet-PMN complexes and the binding of platelet-derived microparticles to leukocytes.
- These observations have strong pathophysiological implications in cardiovascular diseases and thrombo-inflammatory processes.

Abbreviations

C5aRA – C5a receptor antagonist; CAM – Cell adhesion molecule; CR – Complement receptor; GPIb – Glycoprotein I beta ; JAM-C – Junction adhesion molecule-C; mAb – Monoclonal antibody ; Mac-1 – Macrophage-1 antigen; PAR-1 – Protease activated receptor-1; PLC-Platelet-leukocyte complexes; PMNs – Polymorphonuclear leukocytes; PMP – Platelet-derived microparticles; PPC – Platelet-PMN complex ; PRP – Platelet-rich plasma; PSGL-1 – P-selectin glycoprotein ligand-1; TF – Tissue factor; TRAP-6 – Thrombin receptor activating peptide-6.

plasma C3 are associated with thrombotic events (4), and we can speculate that in the context of the present study, increased plasma levels of C3 would increase the transfer of TF-bearing PMPs to leukocytes, resulting in an increased procoagulatory state in patients at risk.

Together with our previous results, our experiments presented here reveal a novel activation mechanism for C3, involving contact with biosurfaces such as those on activated platelets and PMNs. We demonstrate that this platelet-mediated complement activation is an important component of the tethering between platelets/PMPs and PMNs involving platelet-bound C3(H$_2$O)/iC3(H$_2$O). (This and other known interactions between ligands and CD11b are summarised in Suppl. Figure 2, available online at www.thrombosis-online.com). The finding that anti-C3a (i. e. C3(H$_2$O)) can efficiently inhibit these interactions underscores our contention that the tethering of C3(H$_2$O)/iC3(H$_2$O) to CD11b/CD18 is of great importance for complex formation between platelets and PMNs. It also suggests that the antibody could be used in future *in vivo* studies to further explore the functional consequences of this interaction and that its epitope could be a potential target of pharmaceutical intervention in thromboinflammatory conditions.

Conflicts of interest
None declared.

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