Complement activation on platelets – a mechanism for increased vascular disease in systemic lupus erythematosus?
Abstract

Objective. Patients with systemic lupus erythematosus (SLE) have a markedly increased risk to develop vascular diseases such as stroke, venous and arterial thrombosis and traditional risk factors fail to account for this increased risk. The possible role of platelet activation in development of vascular diseases in SLE has not been determined. Complement fragments have been demonstrated on platelets in SLE and patients with stroke suggesting an association between complement activation on platelets and cardiovascular diseases. Thus, we investigated if complement fragments could deposit on platelets and if this was associated with platelet activation. Methods. Platelets, either whole blood or purified platelets, from healthy volunteers were stimulated with IgA immune complexes or thrombin receptor activating peptide (TRAP) and analyzed for complement deposition and platelet activation markers by flow cytometry. Results. Normal human serum supported C3 fragment deposition on purified platelets, but no further complement deposition was detected upon immune complex stimulation. We also demonstrated that IgA immune complexes could activate platelets and support C1q binding to the platelets. Conclusions. Immune complex-mediated activation of platelets and the subsequent complement deposition on platelets might be an important mechanism for the increased risk for development of cardiovascular diseases in SLE.
Introduction

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a chronic, inflammatory, relapsing, autoimmune disorder. Several different organ systems are affected such as the skin, joints, kidneys, nervous system, heart and lungs. The disease can reach different levels of severity ranging from mild to severe and even fatal with (1) a diversity of symptoms including fatigue, fever, arthritis, rash, vasculitis and sensitivity to sunlight. SLE patients also have an increased risk to develop vascular diseases such as myocardial infarction, stroke and also venous thrombosis (2). This is especially pronounced in younger women with a 50-fold increased risk for myocardial infarction (3). There is a difference in the occurrence between the genders with females being afflicted by SLE 6-8 times more often than men. The onset of the disease usually occurs between the ages 20-50 years with an incidence of 4.8/100 000 per year in southern Sweden (4). Both the innate and the acquired immune systems contribute to the pathology of the disease. To date no Mendelian inheritance has been observed, however several genes involved in the immune response have been identified such as the major histocompatibility complex (MHC), genes of the complement system and Fcγ receptor (FcγR) show strong association with SLE (5). Animal models have shown that several genes and pathological pathways can give rise to Lupus-like diseases (6). There have also been several environmental factors suggested to be involved in the development of the disease such as UV-light and viral infections.

The diagnosis of SLE is based on clinical manifestations together with immunological abnormalities. For case definition in clinical research, classification criteria, American College of Rheumatology (ACR) are used. There are 11 manifestations incorporated in the ACR criteria; malar rash, discoid rash, photosensitivity, oral ulcers, arthritis, serositis, renal disease, neurological disorder, haematological manifestation (leucopenia, lymphopenia and thrombocytopenia), immunological disorder (anti-DNA antibodies, anti-Smith autoantibodies and anti-cardiolipin antibodies (aCL)) and anti-nuclear antibodies. To get the diagnosis of SLE the patient must fulfill 4 or more of the ACR criteria (7).

The role of the complement system in SLE was discovered when researchers found hypo-complementanemia and deposition of complement proteins in the target organs such as kidney, which suggested that complement activation is important for the pathogenesis of SLE (1). During the 1970s it was shown that complement deficiencies were associated with development of SLE (8). This would suggest that impaired complement function could not protect against SLE but instead support development of the disease. Deficiencies in the classical pathway of complement (C1q, C1r, C1s, C2 and C4) are the strongest known susceptibility factors for development of SLE (9).

The production of autoantibodies are antigen-driven and dependent on T-cell and hyperactive B-cell response which will lead to the formation of circulating immune complexes that will cause damage by deposition in organs and activation of the complement system. These autoantibodies target components of the cellular nucleus including dsDNA, RNA, histones, nucleosomes and small nuclear ribonucleoproteins (snRNPs) (10, 11). To protect against
exposure of these intracellular autoantigens a fast and efficient removal of the dying cells is necessary. Cells that die by apoptosis are normally quickly taken up by neighboring cells or by specific phagocytes, for example macrophages. This uptake of apoptotic cells by the macrophages will lead to the release of anti-inflammatory substances such as IL-10 and transforming growth factor beta (TGF-β) while pro-inflammatory mediators such as IL-2 and TNF-α are suppressed (12, 13). Patients with SLE show an impaired clearance of apoptotic cells (14) and their serum support increased apoptosis (15). The waste and disposal theory of SLE suggests that an inefficient clearance of apoptotic cells and cell debris leads to the initial breakdown in tolerance. The main source of autoantigens in SLE is most likely from apoptotic cell material and this increased amount of potential antigens could prove to be an important disease mechanism (10).

Type I interferons (IFNs) have diverse effects on the immune system and promote an immune response. In SLE, besides the presence of hyperactive B-cell response, autoantibodies against modified nuclear components, increased complement consumption and decreased clearance of apoptotic cells, researchers have also seen an ongoing production of type I IFNs (16). INF-α (a cytokine belonging to the type I IFN family) has been shown to be elevated in serum of SLE patients and these elevated levels correlate with both disease activity and severity (17). Immune complexes containing RNA or DNA may activate plasmacytoid dendritic cells (natural IFN-producing cells) to produce IFN-α. This is mediated by the involvement of Fcγ receptors, TLR-7 (Toll like receptor) or TLR-9 (18, 19). A deficiency of proteins in the classical pathway of the complement system, C1q, C4 and C2 are all associated with the development of SLE (9). In addition, an acquired complement deficiency due to complement activation is also commonly seen in SLE. The classical pathway components are all needed for an efficient phagocytosis of apoptotic cells (20). C1q is also important for the regulation of cytokines and IFN-α production induced by RNA containing immune complexes (18). The complement system has a dual role in the pathogenesis of SLE, complement is needed for an efficient phagocytosis of immune complexes and apoptotic cells but at the same time it mediates inflammation and tissue damage (Figure 1). This is called the Lupus Paradox (21).

Currently there is no cure for SLE, instead treatment aims to relieve symptoms and protect against organ damage by decreasing inflammation in the body. The disease is heterogeneous and varies from person to person as well as in person with periods of flares followed by periods of remission. The drug of choice depends on the patient’s symptoms and which organ systems that are involved and also the severity of the disease. Drugs that can be effective include anti-inflammatory drugs, glucocorticoids and immune suppressive drugs.

The complement system

The complement system consists of approximately 30 proteins that are present in serum or bound to membranes (22). The name complement comes from its ability to complement or in other words, enhance the effect of other components of the immune system such as antibodies. The complement system is a vital part of our innate host defense. It has three main functions: to cause lysis of cells such as bacteria, allografts and tumor cells; to give rise to mediators that participate in the inflammatory response and attract neutrophils; and to opsonize targets thus enhancing phagocytosis. The liver is the main producer of complement proteins but some complement proteins such as C1q are mainly produced by macrophages and dendritic cells (48). Several complement components are proenzymes and need to be cleaved
to form the active enzyme. The sequential activation of complement components happens via one of three pathways: the classical pathway, the lectin pathway and the alternative pathway (Figure 2). All three pathways lead to the generation of C3b which is the central molecule of the complement cascade. C3b on a cell marks it as non-self and targets it for destruction. C3b have two important functions: to join with other complement components to create the C5 convertase, the enzyme that leads to the production of the membrane attack complex (MAC); and to opsonize bacteria for phagocytes that have receptors for C3b on their surface (48).

In the classical pathway an antigen-antibody complex activates C1 to form a protease that cleaves C2 and C4 to form a C4b2a complex. This is the C3 convertase which cleaves C3 into two fragments, C3a and C3b. C3b forms a complex with C4b2a and creates a new enzyme, C5 convertase (C4b2a3b) which cleaves C5 to C5a and C5b. C5b binds to C6 and C7 to form a complex that interacts with C8 and C9 to produce MAC (C5b6789) and this causes cytolysis. In the lectin pathway, mannan-binding lectin (MBL), also known as mannose-binding protein, binds to the surface of microbes that have mannan (a polymer of the sugar mannose). This activates proteases associated with MBL which cleave C2 and C4 and the activation cascade is then similar to the classical pathway. The alternative pathway is activated by cell surface substances, for example bacterial lipopolysaccharides (endotoxin), fungal cell walls and viral envelopes by binding of C3(H2O) to the surface. Factor B, bound to the C3b, is then recognized by the protease factor D which cleaves factor B to produce the alternative pathway C3 convertase (C3bBb) (48).

Regulation of the complement system

This powerful protective system needs to be regulated to avoid unwanted damage. The first regulatory step in the classical pathway is at the antibody level. The C1 component of the complement system cannot bind to the complement-binding site on the heavy chain of IgM or IgG unless an antigen is already bound to these antibodies. So even though IgM and IgG are present in the blood all the time the complement system is not activated. However when a specific antibody binds to its antigen a conformational shift will occur and the C1 component can bind and activate the complement system.

There are also several serum proteins that regulate the complement system at different stages. The balance between activation and inhibition determines the outcome. C1 inhibitor is an important regulator of both the classical pathway and the lectin pathway and inactivates the protease activity of C1 and by binding to MASP-2. The regulation of the alternative pathway is mediated by the binding of factor H to C3b which then is cleaved by factor I. Another component that enhances activation of the alternative pathway is properdin which stabilizes the C3 convertase. The glycoprotein, decay-accelerating factor (DAF, CD55) is another protective protein that is located on human cells and prevents the assembly of C3 and C5 convertases and thereby the cells from lysis (48).

Biological effects of complement

- Opsonization: various microbes like bacteria and viruses are phagocytozed more efficiently in the presence of C3b by binding to C3b receptors on the surface of many phagocytes.
- Chemotaxis: neutrophils migrate especially well towards C5a which also enhances the adhesiveness of neutrophils to the endothelium.

- Anaphylatoxin: C3a and C5a all cause degranulation of mast cells with the subsequent release of mediators like histamine which leads to an increased vascular permeability and smooth muscle contraction.

- Cytolysis: insertion of the C5b6789 complex into the cell membrane leads to the killing or lysis of many types of cells including erythrocytes, bacteria and tumor cells. The cytolysis is not an enzymatic process but rather the result of a membrane disruption where water and electrolytes can enter the cell due to osmotic balance.

- Binding of C3d to complement receptor 2 on the surface of activated B-cells greatly enhances the antibody production as compared with B-cells that are activated by antigen alone.

C2D

C2 deficiency (C2D) has a calculated prevalence of 1/20000 in people with European decent (23). The structural gene for C2 is located in the MHC class III region together with the genes for C4 and factor B (24). Nearly all of the patients with C2D have a 28-bp deletion in the C2 gene, type I C2D, a mutation associated with HLA-B*18, S042, DRB1*15 haplotype (25, 26). There is also the less common type II C2D where a selective block keeps C2 from being excreted. Deficiencies of C1q, C1r, C1s and C4 have a more diverse genetic background. The role of C2 is to contribute to the catalytic part of the C3 convertase (C4b2a) which can be generated through the classical or the lectin pathway of the complement system. This indicates that abnormal immune functions in C2D could be explained by an impaired activity of the classical and lectin pathways of complement. The development of SLE when deficient in components of the classical pathway is probably a result of autoimmunity caused by impaired complement-dependent elimination of apoptotic cells and immune complexes.

The alternative pathway (factor B, factor D and properdin) is mostly intact in C2D patients and an MBL-dependent activation of C3 and the alternative pathway without involvement of C2 has been reported which could play an important role (27). A lower frequency of severe SLE with complication of the kidneys is seen in C2D patients (1). The general hallmark of C2D is susceptibility to invasive infections and a variety of other immunological diseases but despite this many C2D individuals seem to be healthy (23, 28). C2D is associated with significant disease in about 50% of the cases. C2D patients have a 4-fold increased risk for a first acute myocardial infarction (AMI) (23). The risk to develop SLE is high in C1 (>90%) and C4 (75%) deficient individuals but lower in C2D individuals (~20%) and uncommon in deficiencies of C3 (9).

The vascular damage seen in C2D individuals is probably a more direct consequence of complement deficiency. A lack of MBL has been linked to coronary artery disease (29) and the vascular damage has been shown to be enhanced in genetically engineered C3D mice (30). Altogether this suggests that the development of cardiovascular disease in C2D is a consequence of the impaired function of the classical and lectin pathway of complement.
Jönsson et al found high concentrations of aCL and anti-C1q antibodies in patients with C2D (28). The reason for this is not known but could be related to the importance of complement for elimination of auto-reactive lymphocytes and potential auto-antigens. Observations that patients with aCL antibodies had higher frequency of anti-C1qCLR antibodies than patients without aCL support this hypothesis. aCL have also been reported to play a part in the development of atherosclerosis and can also contribute to the cardiovascular events in C2D. The anti-phospholipid antibody (aPL) syndrome (APS) is characterized by aCL antibodies and vascular disease such as venous thrombosis. In SLE, there is an association between aCL antibodies and venous thrombosis. In C2D, however, there are high levels of aCL antibodies but not increased frequency of venous thrombosis suggesting that C2D could protect against some of the manifestations of this syndrome.

Coagulation

The circulatory systems developed early in animal evolution and with them evolved mechanisms for stopping leaks which could be fatal. The body has three different hemostatic mechanisms: vascular spasm, platelet plug formation and blood clotting (coagulation). In all these mechanisms platelets play a vital role. The main goal of the coagulation system is to convert the plasma protein fibrinogen into fibrin, a sticky protein that attaches to the walls of the vessel. As blood cells and platelets approach they are caught in the sticky web that fibrin has created. The result is a mass of fibrin, blood cells and platelets that seal the break in the blood vessel.

There are two pathways to coagulation; the extrinsic and the intrinsic pathway (Figure 3). In the extrinsic pathway damaged blood vessels and perivascular tissue release clotting factors that initiate the reaction. In most cases of bleeding, both the extrinsic and the intrinsic pathway work together to sustain homeostasis. When one factor is activated the procoagulants activate the next procoagulant in the reaction cascade and amplify the response. Many of the clotting factors are named with roman nomenclature which indicates the order in which they were discovered and not their activation order in the cascade. The last four procoagulants are called platelet factors (PF1 to PF4) to highlight the fact that they are being produced by platelets (49).

The extrinsic pathway, damaged blood vessels and perivascular tissue release a lipoprotein mixture called tissue thromboplastin (factor III) that combines with factor VII to form a complex that (in the presence of Ca^{2+}) activates factor X. Once after factor X has been activated the intrinsic and extrinsic pathways proceed through the same steps. The intrinsic pathway is dependent on platelets that release factor XII (Hageman factor named after the patient in whom it was discovered) upon degranulation. A cascade of reactions lead to the activation of factor XI, IX and VIII where each factor serves as an enzyme that catalyzes the next step through to factor X. After factor X has been created both pathways proceed in the following steps: factor X combines with factor III and V in the presence of Ca^{2+} and PF3 to produce prothrombin activator. This enzyme works on a globulin called prothrombin (factor II) and converts it to the enzyme thrombin. Thrombin then proceeds by cutting fibrinogen into smaller strands of fibrin. Factor XIII then cross-links these fibrin strands to create a dense aggregation called fibrin polymer which forms the structural framework of the blood cloth.
Once a clot has formed it initiates a self accelerating positive feedback loop that seals off the leak faster. Thrombin works together with factor V to accelerate the production of prothrombin activator that leads to a higher production of thrombin.

Platelets

Platelets are not cells but rather small fragments of bone marrow cells called megakaryocytes. Platelets are the second most abundant formed elements after erythrocytes in the blood. Normal platelet count in blood ranges from 130 000 to 400 000 platelets/µl but can vary greatly under different physiological conditions. Despite being present in such large numbers, platelets contribute less than white blood cells to the blood volume. The internal structure of platelets is similar to regular eukaryotic cells with lysosomes, mitochondria, microtubules and microfilaments and granules filled with platelet proteins, but platelets have no nucleus. When platelets get activated they form pseudopods and are capable of ambient movement. They have many different functions including secreting vasoconstrictors, chemicals that cause spasmodic constrictions of broken vessels and thus help reduce blood loss. They stick together to form temporary platelet plugs that seal small breaks in injured blood vessels. Platelets secrete procoagulants or clotting factors which promotes blood clotting. They also initiate the formation of a clot-dissolving enzyme that dissolves blood clots that have served their purpose. Platelets also secrete chemicals that attract neutrophils and monocytes to the site of inflammation which could kill and phagocytose the bacteria. Another function of the platelets is to secrete growth factors that stimulate mitosis in fibroblasts and smooth muscle, which help to maintain and repair blood vessels (49).

Background to the present investigation

The complement and coagulation systems have evolved independently of each other from a common protease ancestor but there is still cross-talk between them (31). Activated coagulation factors like thrombin, plasmin and factor XIIa can cleave complement components (32, 33). The MAC complex generates platelet microparticles, and C5a initiates the expression of tissue factor (TF) in endothelial cells and granulocytes (34-36). Furthermore, complement activation seems to be necessary for aPL-mediated thrombosis. Animals deficient in complement components are protected against aPL-mediated thrombosis (37, 38) and C2D individuals also seem to be protected against development of aPL-mediated venous thrombosis (28).

In SLE, our group has demonstrated that the platelets are more activated with increased expression of P-selectin, phosphatidylserine and CD69 as well as increased levels of platelet-monocyte complexes. Upon platelet activation they expose chondroitin sulfate (CS) on the surface which could initiate complement activation through binding of C1q (39). C4d deposition on platelets has been demonstrated in SLE as well as in patients with stroke (40, 41). Unpublished data from our group suggest that not only C4d but also other complement factors of the classical pathway such as C1q and C3d are increased on platelets from SLE patients. Interestingly, patients with a previous history of vascular disease showed increased complement deposition on the platelets as compared to SLE patients without any vascular disease. Furthermore, there was a statistical correlation between complement deposition and
platelet activation suggesting involvement of chondroitin sulfate (CS). The authors also found statistical correlations between circulating immune complexes, platelet activation and complement deposition indicating involvement of immune complexes either in the activation of the platelets or in the complement deposition.

With this in mind, it would be interesting to investigate if immune complexes, which are frequently seen in SLE, could activate platelets and increase the expression of P-selectin. Furthermore, since platelet activation has been demonstrated to be associated with platelet complement deposition it would be interesting to measure C1q, C4 and C3 deposition on platelets upon platelet activation. It should also be important to determine whether the complement deposition on platelets were due to complement activation through the classical or lectin pathway. If dependent on C2, this could be a mechanism of how C2D individuals are protected against aCL-mediated venous thrombosis (Figure 4).
Materials and Method

Preparation of immune complexes

Heat-aggregated IgG (HIgG) was made by incubating human IgG (1mg/ml) at 56°C for 30 minutes. Human serum albumine immune complexes (HSA IC) and immune complexes purified from SLE patients’ sera (SLE IC) were also used. In some experiments, anti-IgA antibodies (Dako, Glostrup, Denmark) were used to form immune complexes with IgA in serum from a normal healthy volunteer.

Platelet purification

Blood was collected in sodium-citrate tubes (BD Biosciences Pharmingen, San Diego, CA, USA) and used within 15 min. Whole blood was centrifuged for 10 minutes at 280 g. The platelet rich plasma (PRP) was kept and mixed with 500 µl PBS pH 7.2 (PBS). The PRP was then centrifuged at 1125 g for 10 minutes and resuspended in 500 µl PBS.

Complement deposition on purified platelets

To detect C3d deposition on platelets, purified platelets (2 µl) were added to every sample together with 20 µl normal human serum, stimuli (5 µl HSA ICs, 10 µl HIgG, 7 µl a-IgA or 7 µl SLE ICs) and 30 µl veronal buffered saline with 0.15 mM Ca$^{2+}$ and 0.5 mM Mg$^{2+}$ (VBSCaMg). The samples were incubated for 30 minutes at 37°C followed by the addition of 500 µl 10 mM EDTA. PBS was added to the platelets and the samples were centrifuged for 10 minutes at 1125 g and then the platelets were resuspended in 500 µl PBS together with 0.5 µl C3d antibodies (Quidel, San Diego, CA, USA). The samples were then incubated for 30 minutes at 4°C. After the incubation, PBS was added to the platelets and the samples were washed for 10 minutes at 1125 g and the platelets were resuspended in 500 µl PBS together with 3 µl FITC-conjugated rabbit-anti-mouse antibodies (Dako) and incubated at 4°C for 30 minutes. After the incubation PBS was added to the samples and the platelets were washed twice at 1125 g for 10 minutes. Then the platelets were resuspended in 500 µl PBS and 3 µl PE-conjugated CD42a (BD Biosciences Pharmingen) antibodies and incubated for 30 minutes at 4°C. After incubation PBS was added to the platelets and the samples were centrifuged at 1125 g for 10 minutes and then analyzed by flow cytometry (Epics XL-MCL, Beckman-Coulter, Fullerton, CA, USA).

Platelet activation and complement deposition in whole blood

For platelet activation, 5 µl of whole blood were incubated with different stimuli: either 7 µl anti-IgA or 5 µl thrombin receptor-activating peptide (TRAP, Sigma, St. Louis, MO, USA) in 45 µl VBSCaMg for 15 minutes at room temperature. Antibodies against P-selectin, CD42a (both from BD Biosciences Pharmingen) or C1q (Dako) were added and the incubation continued for 30 minutes at room temperature. The activation was stopped by the addition of 0.2% paraformaldehyde. The samples were diluted 1/20 in PBS before analyzed by flow cytometry.
Results

C3d deposition on purified platelets

Previous unpublished data from our group suggested an association between the level of circulating immune complexes and complement deposition on platelets. Based on this, we investigated if immune complexes could support an increased complement deposition on purified platelets. When incubating the platelets in normal human serum they increased their C3 fragment deposition as compared to when no serum was added (Table 1). However, none of the tested immune complexes could increase the C3 fragment deposition as compared to when only adding normal human serum. This suggested that the immune complexes were not able to activate the complement system on the platelets using this method. However, during the purification process, platelets are activated and this could lead to complement deposition through the previously described chondroitin sulfate (39).

Increased platelet activation upon immune complex stimulation

Since immune complexes could not increase complement deposition on purified platelets we investigated if immune complexes instead could activate platelets with subsequent increased complement deposition. Unstimulated platelets had very low levels of the activation marker P-selectin (2.25%) whereas addition of anti-IgA antibodies could activate the platelets markedly (23.9%, Figure 5). As a positive control we used the synthetic molecule TRAP which increased the activation of the platelets even further. Several other immune complexes (including HSA IC and HIgG) were tested but none of these could activate the platelets. Altogether, this demonstrated that immune complexes could activate the platelets.

Increased complement deposition upon platelet activation

Previous studies have demonstrated complement deposition on platelets in association with platelet activation. Since immune complexes could activate the platelets, we measured complement deposition on the platelets upon immune complex activation. Platelets stimulated with anti-IgA antibodies had an increased deposition of complement component C1q (30.1%) as compared with the unstimulated sample (23.8%, Figure 6). TRAP stimulation increased the deposition of C1q even further (40.9%). For detection of further complement activation we measured deposition of C4d, C3a and C3d fragments. The C3a antibody recognises the naïve non-activated form of C3 while the C3d and C4d antibodies recognise the activated fragments of the respective complement component. However, we could not detect any increase in either C4 or C3 fragments on the platelet even though increased C1q levels were detected (data not shown). In summary, we could demonstrate that C1q could deposit on platelets and this was associated with platelet activation.
Discussion

SLE is an autoimmune disease characterized by the involvement of several organ systems such as kidney and skin, but SLE is also associated with increased risk for the development of vascular diseases (VD), especially at a younger age (3). The increased risk for VD is not solely explained by traditional risk factors but clearly non-traditional risk factors seem to contribute (42-45). There is also a substantially increased risk for venous thrombosis in SLE, often associated with aPL antibodies (46). In SLE, as well as in cardiovascular diseases as stroke, complement fragments have been found on platelets suggesting an important role for the complement system in platelet activation and cardiovascular diseases (40, 41). The importance of complement activation has also been implicated in animal models where complement deficiencies are protective against aPL mediated thrombosis (37, 38). Furthermore, in humans, C2 deficiency seems to protect against development of aPL mediated venous thrombosis (28).

Since immune complexes might be involved in the complement activation on platelets, platelets were stimulated with different immune complexes and the complement deposition measured by flow cytometry. Using different immune complexes we observed that these could not increase the platelet C3d deposition to a greater extent than the C3d deposition on unstimulated platelets. This could be due to the purification process which activates the platelets up-regulating P-selectin on their surface. To avoid pre-stimulation due to purification whole blood was used in the following experiments. Others have previously shown that IgG surfaces could activate platelets (47) and we could also demonstrate that our IgA immune complexes were able to activate platelets with an increased P-selectin expression. However, none of the other investigated immune complexes were able to activate the platelets by unknown reasons. Most likely the inability to activate the platelets were dependent on the size and amount of the immune complexes.

Interestingly we observed hemolysis when activating the platelets with IgA immune complexes suggesting complement activation and subsequent destruction of the erythrocytes. Our interpretation was that there ought to be complement deposition on the platelets as well. We measured complement deposition on IgA immune complex activated platelets and found deposition of C1q but unexpectedly not C3 or C4 fragments on these platelets. This could be in accordance with the previously reported binding of C1q to chondroitin sulfate on activated platelets (39). The lack of C3 deposition on platelets when using whole blood could be due to the presence of erythrocytes since our previous results with purified platelets indicated C3 deposition. Furthermore, unpublished results from our group demonstrate a statistical significant correlation between the immune complex levels and complement deposition on the platelets further strengthening the hypothesis of an immune complex-mediated complement deposition on platelets.

Altogether, our results suggest that immune complexes could activate platelets and thereby the complement cascade with subsequent complement deposition on the platelets. This could be an important mechanism in cardiovascular diseases but more experiments are needed to elucidate the exact functions for the complement system in the platelet activation and development of cardiovascular diseases.
References


Table 1. C3d deposition on purified platelets upon immune complex stimulation.

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<th>C3 fragment positive platelets (%)</th>
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<tr>
<td>Negative control</td>
<td>4.88</td>
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<tr>
<td>Normal human serum</td>
<td>19.2</td>
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<tr>
<td>Anti-IgA antibodies</td>
<td>18.8</td>
</tr>
<tr>
<td>SLE immune complexes</td>
<td>18.2</td>
</tr>
<tr>
<td>HSA immune complexes</td>
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<td>HIgG</td>
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Figure 1. An impaired complement function leads to a decreased clearance of apoptotic cells which gives rise to an exposure of autoantigens, such as modified nuclear components and formation of immune complexes causing complement activation, inflammation and organ damage. UV-light and virus infection can cause increased apoptosis.

Figure 2. Activation of the complement cascade via the classical (A), lectin (B) or alternative (C) pathway results in the initiation of the terminal complement pathway (D), leading to the formation of membrane attack complexes.

Figure 3. The coagulation pathway.

Figure 4. Illustration of a hypothetical way to deposit complement fragments on platelets. Immune complexes bind to platelet FcγRIIA and allow the activation of the complement cascade in the proximity of the platelet and the subsequent deposition of complement fragments on the immune complex and on the platelets. C2 deficiency (C2D), however, might protect against complement activation on platelets.

Figure 5. Platelet activation upon immune complex stimulation. Platelets were gated on size, granularity and specific platelet markers (CD42a) before analyzed for P-selectin expression. The flow cytometry plots illustrate unstimulated, anti-IgA and TRAP-stimulated platelets and are representative of three experiments.

Figure 6. C1q deposition on platelets upon platelet activation. Platelets were gated on size, granularity and specific platelet markers (CD42a) before analyzing the C1q deposition. The flow cytometry plots illustrate unstimulated, anti-IgA and TRAP-stimulated platelets.
Figure 1.
A  Classical
  C1q binding to:
  - antibody-antigen complex
  - certain virions/infected cells

B  Lectin
  MBL-binding to:
  - oligosaccharides on certain virions/infected cells

C  Alternative
  Spontaneous breakdown of C3 in serum

D  C3 convertases

C5 convertases (C4b2a3b & C3bBb3b)

Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.

unstimulated

a-IgA

TRAP