Generation of Multiple Fluid-Phase C3b:Plasma Protein Complexes during Complement Activation: Possible Implications in C3 Glomerulopathies

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Generation of Multiple Fluid-Phase C3b:Plasma Protein Complexes during Complement Activation: Possible Implications in 3 Glomerulopathies

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The complement system is tightly regulated to safeguard against tissue damage that results from unwanted activation. The key step of C3 cleavage to C3b is regulated by multiple mechanisms that control the initiation and extent of activation. This study demonstrated that C3b:plasma protein complexes form in the fluid-phase during complement activation. Several different plasma proteins displayed a discrete high molecular SDS-resistant band when any of the three complement activating pathways were triggered in normal human serum or plasma. Serum depleted of individual complement proteins revealed that C3 and factors B and D were essential for complex formation. Inactivation of the thioester bond in C3 also prevented complex formation. In vitro, complexes could be generated using four purified proteins—C3, factor B, factor D, and target protein—and Mg2+ to allow C3 convertase formation. These studies showed that the complexes consisted of a plasma protein covalently bound to C3b in a 1:1 molar ratio; the C3b portion was rapidly degraded by factors H and I. Analysis of plasma samples from patients with dense deposit disease and C3 glomerulonephritis demonstrated that C3b:protein complexes form spontaneously in the blood of patients with dense deposit disease and, to a lesser extent, in C3 glomerulonephritis patients, but not in healthy controls. This finding supports the underlying hypothesis that these C3 glomerulopathies are diseases of fluid-phase complement dysregulation. These complexes could normally function as a passive mechanism to intercept C3b from depositing on host cells. However, excessive generation and/or defective clearance of fluid-phase C3b:protein complexes may have pathological consequences.

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complexes with C3b that could be degraded by the fluid-phase RCA protein factors H and I. Although it is intuitive that nascent generated C3b should be able to attach to proteins in close proximity, there are very few descriptions of these complexes and the observations that have been reported focus on C3b binding to other complement proteins or activators (16–18). Thus, the existence of fluid-phase C3b:plasma protein complexes generally is not recognized and their physiological significance has not been described. In this study we observed these complexes in plasma samples from DDD patients and, to a lesser extent, in plasma samples from C3GN patients. Their presence further supports the pathophysiological basis of these two C3Gs as fluid-phase dysregulation of the C3 convertase.

Our findings also suggest that in the normal state, covariant attachment of C3b to plasma proteins may be a passive mechanism to minimize host cell deposition at sites of complement activation. Excessive generation or defective clearance of these circulating C3b: plasma protein complexes in the C3Gs may contribute to disease but may also offer therapeutic targets to limit the observed renal damage.

Materials and Methods

Reagents

Purified cobra venom factor (CVF), preactivated zymosan A, and sheep erythrocytes were obtained from Complement Technology (Tyler, TX). Human serum depleted of individual complement components (C3, C4, C5, factor B, properdin, factor H, and factor I) and purified human complement components (C3, C3b, factor B, factor D, factor H, and factor I) were all purchased from Complement Technology. The following purified human proteins were all obtained from Athens Research and Technology (Athens, GA): α-1 proteinase inhibitor (α1PI), α-1 acid glycoprotein (α1AG), IgG, and DBP.

The IgG fraction of goat polyclonal anti-human DBP was purchased from DiaSoTein (Stillwater, MN) and then affinity purified in our laboratory using immobilized DBP. Chicken polyclonal anti-α1PI Ab was obtained from ProSci (Poway, CA). Biotinylated rabbit polyclonal anti-α1AG, goat polyclonal anti-human albumin, and rabbit polyclonal anti-kinogen Abs were all obtained from Abcam (Cambridge, MA). Mouse monoclonal anti-human factor B (clone D33/3) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-human C1 inhibitor Ab was developed in rabbits. Chicken polyclonal anti-human C3 was obtained from Gallus Immunotech (Cary, NC). Rabbit IgG Ab developed against epitopes on human C3d (#A15981) was purchased from Abcam. Mouse monoclonal anti-human factor I neutralization Ab (#A247) was obtained from Quidel (San Diego, CA). Williams plasma (deficient in high and low m.w. kininogen) was a generous gift from Dr. Alvin Schmaier, Case Western Reserve University, Cleveland, OH.

Collection of human blood and in vitro activation of complement

Blood was collected from healthy, medication-free human subjects who gave informed consent using a protocol approved by the Stony Brook University Institutional Review Board. Vacutainer tubes (BD, Franklin Lakes, NJ) containing either 3.2% sodium citrate (for plasma) or a silica clot activator (for serum) were used. Individual serum and plasma samples from at least five subjects were pooled, aliquoted, and frozen at −80°C. Pooled sera or citrated plasma (0.3 ml) was activated either with CVF (416 U/ml), zymosan A (10 mg/ml), or 50 μL heat-aggregated (heated at 63°C for 20 min) human IgG (10 mg/ml) and incubated at 37°C for the time indicated in each experiment. For activation of citrated plasma samples, 2 mM Mg2+ was added to overcome the chelation effect of sodium citrate. Serum depleted of the complement regulatory factors H and I spontaneously activates the alternative pathway and hence was stored in 0.1 mM EDTA. These samples were activated by adding just 0.5 mM Mg2+ and incubating at 37°C. In certain experiments, depleted serum samples were reconstituted by adding back the purified protein to achieve its mean plasma concentration (factor B: 210 μg/ml; factor D: 1 μg/ml; C3: 1.3 mg/ml) along with 0.4 mM Mg2+ and then incubating at 37°C for 15 min.

Human subjects with C3G

A total of 12 patients with biopsy-proven C3G (6 DDD and 6 C3GN) were selected from our C3G registry for inclusion in this study on the basis of histopathological data (light microscopy, immunofluorescence, and electron microscopy) and the availability of sufficient plasma samples to complete all assays multiple times. The human research institutional review board at the University of Iowa approved all procedures, and all patients gave informed consent.

SDS-PAGE and immunoblotting

All samples were separated using 8% polyacrylamide gels with SDS at 80 V for the stacking gel and 100 V for the resolving gel. Resolved gels then were transferred onto an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA) at 100 V for 75 min. The polyvinylidene difluoride membrane was blocked with 5% nonfat dry milk (NFDM) in TBS with 0.1% Tween 20 (TBST) for 30 min, followed by primary and HRP-labeled secondary Ab incubations in 5% NFDM. Finally, blots were developed using HRP:GLO Quick Spray Chemiluminescent Detection Reagent (Denvillie Scientific, Denville, NJ) and x-ray film.

In vitro complex formation and breakdown

To evaluate the role of activated C3 in complex formation, the alternative pathway was assembled in vitro, using the purified proteins C3 (1.3 mg/ml), factor B (200 μg/ml), factor D (1 μg/ml), and DBP (400 μg/ml) or α1AG (1 mg/ml) with 0.5 mM Mg2+ and incubated at 37°C for the specified amount of time. In control experiments, factor B was eliminated from the mixture to prevent activation. The molar ratios of the various components were maintained at physiological levels even when the exact concentrations could not be maintained owing to dilution effects. The roles of regulatory proteins factors H and I in the breakdown of C3 complexes were determined by adding purified factor H alone (340 μg/ml), factor I alone (54 μg/ml), or both together along with C3 (1 mg/ml), factor B (177 μg/ml), factor D (0.76 μg/ml), DBP (307 μg/ml), and 0.5 mM Mg2+ at 37°C along with CVF (416 U/ml) to activate the protein mixture. Complexes were evaluated by SDS-PAGE and immunoblotting.

C5a sandwich ELISA

A sandwich ELISA to detect human C5a and C5a des-Arg was developed as previously described in detail (19). Briefly, MaxiSorp 96-well plates were coated with 500 ng of mouse monoclonal anti-human C5a (clone 295003; R&D Systems, Minneapolis, MN) capture Ab at 4°C overnight. The coating solution was removed and the wells were blocked with 300 μl blocking solution (3% NFDM in PBS with Tween) for 1 h at room temperature. After three washes, 100 μl standards (78 pg/ml to 5 ng/ml purified natural human C5a; Complement Technology) or experimental samples were added and incubated at room temperature with shaking for 90 min. This was followed by four washes and incubation with 100 ng detection Ab, biotinylated mouse monoclonal anti-human C5a (clone 295009, R&D Systems) for 60 min at room temperature with shaking. Finally, 40 ng of HRP-conjugated streptavidin (KPL, Gaithersburg, MD) was added to each well and incubated at room temperature for 30 min. After five washes, 100 μl HRP substrate solution (KPL) was added until color development, followed by addition of 100 μl stop solution (KPL), and the absorbance was measured at 450 nm using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA).

Hydrolysis of the C3 thioester

The thioester bond in C3 was inactivated by treating 250 μg C3 with 0.5 M hydroxylamine (NH2OH), pH 7.5, for 2 h at 21°C followed by exhaustive dialysis against PBS (1.8 L, 3×) at 4°C for a total of 18 h (20). Inactivation of thioester was confirmed by hemolytic assays using both rabbit erythrocytes for the alternative pathway, and Ab-coated sheep erythrocytes for the classical pathway, as well as a C5a ELISA of the supernatant from the hemolysis assay with Ab-coated sheep erythrocytes. C3-depleted serum alone or C3-depleted serum reconstituted with either native C3 or C3-NH2OH (at 0.5 mg/ml) was taken in a final dilution of 1:100 in GVB++ buffer (gelatin veronal buffer) along with 50 μL Ab-coated sheep erythrocytes (5 × 10^6/ml) and incubated at 37°C for 1 h, after which the cells were centrifuged, supernatant was collected, and C5a levels were quantified.

Ab neutralization of factor I

Mouse monoclonal anti-human factor I, which inhibits the serine protease domain, was used to determine whether enzyme activity of factor I is required to cleave the C3b:plasma protein complexes. Anti-factor I (100 μg/ml) was added to factor H–depleted serum and incubated on ice for 15 min to allow Ab binding, followed by incubation at 37°C for a specified amount of time.
Results
Complement activation correlates with generation of high m.w. SDS-resistant forms of several plasma proteins

Because DBP has been shown to function as a chemotactic cofactor for C5a in vitro, the initial goal of this study was to determine whether complement activation converts DBP into an active chemotactic cofactor (21). Possible structural changes in DBP were investigated in normal human serum (NHS) by treating with three distinct pathway activators and then analyzing the samples by SDS-PAGE and immunoblotting. No diminution in the 56-kDa native DBP band was apparent, and neither were lower m.w. DBP bands detected (Fig. 1 and data not shown). However, Fig. 1 shows that activation of any complement pathway did generate an SDS-resistant DBP band at ~200 kDa that was not observed in untreated serum. Moreover, simultaneous addition of EDTA with CVF to serum inhibited complement activation and prevented formation of this high m.w. band. To determine whether formation of this high m.w. band is specific to DBP, two other plasma proteins, α1PI and α1AG, both of similar size but greater abundance than DBP, were examined in the CVF-activated serum (CVFAS) samples. Fig. 1 shows that activation of any complement pathway also induced formation of high m.w. SDS-resistant bands of α1PI and α1AG; these bands were not observed in untreated NHS or CVFAS treated with EDTA. Factor B cleavage is shown in the bottom panel of Fig. 1 to verify complement activation. Thus, activation of serum complement in vitro correlates with the appearance of high m.w. SDS-resistant bands of DBP, α1PI, and α1AG.

The temporal correlation between complement activation and generation of these high m.w. bands was investigated in serum and citrated plasma to determine whether the clotting process alters band formation. Samples were blotted for several proteins with different abundance, m.w., and isoelectric points. All proteins examined formed similar SDS-resistant bands upon complement activation in both serum and plasma (Fig. 2A), suggesting that this process is not affected by blood clotting and is not restricted to a certain class of proteins with a common structural motif. The temporal appearance of these SDS-resistant bands correlated with complement activation in both CVFAS and plasma (Fig. 2A). In serum, high m.w. bands begin appearing at the 5-min time point (Fig. 2A), which is the precise time that complement activation products begin to appear in the CVF-treated serum: Bb (Fig. 2A) and C5a generation (Fig. 2B). Peak band formation occurs at ~15 min and then...

FIGURE 1. Complement activation induces formation of high m.w. SDS-resistant complexes with plasma proteins. Pooled NHS was sham treated with PBS (lane 1), or complement was activated by incubating serum at 37°C, using 416 U/ml CVF (lane 2), 10 mg/ml zymosan A (lane 3), or 0.5 mg/300 μl heat-aggregated human IgG (lane 4). As a control to inhibit complement activation, 10 mM EDTA was added to serum prior to the addition of CVF (lane 5). Serum aliquots were separated on an 8% SDS-PAGE and then immunoblotted for DBP, α1PI, or α1AG. Arrows indicate the position of high m.w. SDS-resistant bands formed during complement activation. The same samples also were blotted for factor B (fB) to verify cleavage as an indicator of complement activation (bottom panel).

FIGURE 2. Formation of SDS-resistant bands correlates temporally with complement activation in vitro. Pooled NHS or citrated plasma was activated at 37°C with 416 U/ml CVF for the indicated times. In addition to CVF, 2 mM Mg2+ was added to plasma to overcome the chelation effects of sodium citrate. At each time point, activation was stopped by placing the sample on ice. (A) Aliquots were separated on an 8% SDS-PAGE and then immunoblotted for DBP, α1PI, or α1AG. Samples also were blotted for factor B to verify cleavage as an indicator of complement activation. The 75-kDa β-chain of C3 was used as a loading control. (B) C5a ELISA of complement activation in serum. (C) C5a ELISA of complement activation in citrated plasma.
gradually decreases in intensity (Fig. 2A). In CVF-treated citrated plasma (Fig. 2A), complement activation is delayed owing to chelation of divalent cations by sodium citrate, but bands do consistently appear at the 30-min time point (Fig. 2A), their appearance correlating with factor B cleavage (Fig. 2A) and C5a generation (Fig. 2C). Of interest, complement activation generates a single kininogen band in serum but a doublet in plasma, reflecting the presence of two forms of kininogen in blood, high m.w. kininogen (HK) and low m.w. kininogen (LK). In serum, in contrast, HK is cleaved during the clotting process and only the LK band appears (Fig. 2A). Thus, the formation of high m.w. SDS-resistant bands of several plasma proteins is not unique to specific blood proteins and correlates temporally with in vitro activation of complement.

The role of erythrocytes in the generation of these high m.w. bands was investigated using citrated whole blood. Fig. 3 demonstrates that SDS-resistant bands of α1PI and α1AG form in whole blood during complement activation, indicating that surface expression of RCA proteins on erythrocytes does not prevent high m.w. band formation. Using Williams plasma (deficient in both HK and LK), we also found that these bands form independently of each other. As seen in Fig. 4, although no kininogen bands formed in C-activated Williams plasma, the generation of SDS-resistant bands of DBP, α1PI, and α1AG was not altered.

**FIGURE 3.** High m.w. SDS-resistant bands form in whole blood with complement activation. Citrated whole blood or citrated plasma from the same blood donor was treated with 2 mM Mg2+ and CVF (416 U/ml) for 30 min at 37°C. PBS was added to plasma to compensate for blood cell volume and to equalize the protein concentrations between the plasma and whole-blood samples. After CVF activation, whole blood was centrifuged to pellet the cells, and aliquots of both plasma samples were separated using an 8% SDS-PAGE and immunoblotted for α1PI, α1AG, and factor B.

**Activation of the C3 thioester during C-activation causes formation of covalent complexes of C3b with plasma proteins**

The evidence presented above indicates a strong correlation between complement activation and formation of high m.w. SDS-resistant bands of several plasma proteins. To identify the steps involved in the generation of these complexes, sera depleted of different complement components were used. Fig. 5A shows the formation of high m.w. SDS-resistant bands of DBP, α1PI, and α1AG in various depleted serum samples activated with CVF; reconstitution of depleted serum with the purified missing component was performed in samples where depletion abolished band formation. C3-, factor B–, and factor D–depleted sera were unable to generate high m.w. bands upon addition of CVF (Fig. 5A), but formation was restored with addition of the deficient proteins. In contrast, generation was not altered in serum depleted of properdin, C4, or C5, clearly demonstrating that C3 cleavage is essential to this process.

The role of the C3 cleavage product C3b was examined next (Fig. 5B). Purified C3b added to factor B-depleted serum showed no complex formation. Addition of C3b to C3-depleted serum generated a C3 convertase but was not able to induce formation of high m.w. forms of DBP, α1PI, and α1AG, even though robust factor B cleavage could be demonstrated, indicating the requirement for both native C3 and the components needed to form C3 convertase (factors B and D). Because native C3 is essential for this process, the role of the thioester bond was investigated by treating C3 with hydroxylamine (NH2OH) to inactivate this bond and then using this chemically modified C3 to reconstitute C3-depleted serum (Fig. 6). Hydroxylamine-treated C3, like C3b, could not attach to an activating cell surface and generate C5a, indicating effective thioester inactivation (Fig. 6A). This inactivated C3 also failed to induce formation of high m.w. complexes of DBP, α1PI, or α1AG upon addition of CVF (Fig. 6B), indicating that an intact thioester bond is essential for this process. Thus the SDS-resistant high m.w. bands of plasma proteins formed during complement activation are covalent complexes with nascently generated C3b (note high m.w. bands in the C3 blot of Fig. 6B). Consistent with this mechanism, multiple high m.w. C3 bands were observed when the samples described in Figs. 1 and 2 were blotted for C3. Fig. 6C shows immunoreactive C3 bands above the 110-kDa C3 α-chain in the samples from Fig. 1. Furthermore, the temporal appearance and intensity of these multiple C3 bands correlate precisely with the individual proteins detected in CVFAS and plasma in Fig. 2 (shown in Fig. 6D). Moreover, Fig. 6C and 6D show that the appearance of high m.w. C3 bands correlate with a reduction in the intensity of the native C3 α-chain (110 kDa) that contains the thioester bond.

**Factors H and I degrade the C3b:plasma protein complexes**

The time course of complex formation (Fig. 2A) shows that band intensity decreases over time, consistent with the breakdown of these complexes. Because C3b is cleaved by factors H and I, their
possible role in this process was examined. Factor H–depleted and factor I–depleted sera were incubated at 37˚C for 15 min to allow for C3b:protein complex formation, following which breakdown was monitored for 16 h, with or without the addition of purified factor H or factor I (Fig. 7A). No breakdown was observed in the factor I–depleted serum, but addition of purified factor I induced complete degradation of each complex, consistent with the known role of this protease (Fig. 7A). In factor H–depleted serum, there was significant breakdown of C3b:protein complexes that was only marginally increased by the addition of purified factor H (Fig. 7A). Next, factor H–depleted serum was examined at very early time points to assess its role in complex degradation. DBP, α1PI, and α1AG complexes spontaneously formed in factor H–depleted serum at 37˚C, but were all cleaved to the lower m.w. form by 5 min (Fig. 7B). No doublets were observed in normal serum even 1 min after CVF was added (data not shown), indicating that complex cleavage is delayed in the absence of factor H, further confirming that, although factor H facilitates complex breakdown, other serum cofactors (factor H–like 1, factor H–related proteins) can substitute in the absence of factor H (22–24). To confirm the role of factor I in the initial rapid
cleavage reaction, purified factor I was added to factor I–depleted serum. As expected, reconstitution of factor I to the depleted serum restored the initial cleavage (Fig. 7C). Furthermore, an Ab that specifically blocks the serine protease domain in factor I also inhibits complex cleavage (Fig. 7D). These results indicate that factor I is essential for the initial cleavage and subsequent breakdown of these complexes, a process that can be facilitated by the cofactor activity of factor H. C3b:protein complexes are initially generated as higher m.w. forms, and factor I along with cofactors mediates the cleavage of C3b to iC3b and smaller C3b cleavage products. This degradation pattern is readily observed on a C3 blot of CVFAS versus factor I–depleted serum (Fig. 7E), where in CVFAS there is a distinct 67-kDa iC3b product of the α-chain, whereas in factor I–depleted serum the prominent C3 cleavage product is the 100-kDa α-prime chain of C3b.

In vitro generation of C3b:DBP complexes using purified proteins

The preceding data demonstrate that formation of C3b:protein complexes requires native C3, a C3 convertase, and a target plasma protein. To examine whether this system can be assembled in vitro, purified C3, factor B, factor D, and a target protein of interest (DBP) were mixed at physiological ratios in the presence of Mg2+. Fig. 8A shows that a C3b:DBP complex was formed upon incubation at 37˚C for either 15 or 30 min. This complex was the same size as the uncleaved complex in factor I–depleted serum (Fig. 8A). Moreover, hydroxylamine-treated C3 was capable of activating the alternative pathway, as evidenced by factor B cleavage, but, in contrast to native C3, it could not form a complex with DBP (Fig. 8B). These results confirm that only four components are necessary and sufficient for C3b:protein complex formation.

Because the use of purified proteins provides a well-defined in vitro model, the breakdown of C3b:protein complexes was investigated using purified factors H and I. Fig. 9A shows C3b:DBP complexes after 15 min, 2 h, or 16 h with no regulators, factor H alone, factor I alone, or both factors H and I. Only the combination of both factor H and factor I could initiate cleavage and breakdown of the C3b:DBP complex. These results using purified proteins confirm that C3b:DBP (or other plasma proteins) are initially generated as higher m.w. complexes that are rapidly cleaved by factor I with the help of a cofactor to a lower m.w. form, and that this complex represents the relatively stable SDS-resistant complex observed in serum and plasma following complement activation (Figs. 1–5). Although the iC3b:DBP band completely disappeared after a 16-h incubation, an intermediate cleavage product, which could potentially be C3dg:DBP, appeared at 15 min and was not further degraded even after 16 h (Fig. 9B). These results were confirmed using purified α1AG as the target protein, where a prominent band at an approximate m.w. corresponding to C3dg:α1AG also appeared at 15 min and was present at 16 h (Fig. 9C, center panel). Similar results were also observed using purified α1PI (data not shown). This putative C3dg:protein

FIGURE 7. C3b:protein complexes are cleaved in serum by factor I with the help of a cofactor. (A) Factor I–depleted serum and factor H–depleted serum were reconstituted with 0.5 mM Mg2+ and incubated at 37˚C for 15 min, to allow for complex formation, followed by addition of 10 mM EDTA to stop the reaction. The samples were then reconstituted with either PBS or the purified depleted component (factor I or factor H) and incubated for 16 h at 37˚C. Samples were separated using 8% SDS-PAGE and immunoblotted for DBP, α1PI, and α1AG. (B) Factor H–depleted serum was reconstituted with 0.5 mM Mg2+ and incubated at 37˚C for the indicated times. Serum aliquots were separated using 8% SDS-PAGE and immunoblotted for DBP, α1PI, α1AG, and factor B. (C) Factor I–depleted serum alone or reconstituted with 25% (0.25×) or 100% (1×) of the plasma concentration of factor I (70 μg/ml) was activated with CVF (416 U/ml) for 15 min at 37˚C. CVF-activated NHS was included as a positive control for complex formation. Serum aliquots were separated using 8% SDS-PAGE and immunoblotted for DBP, α1PI, and α1AG. (D) Factor H–depleted serum was treated with 100 μg/ml of either an irrelevant mouse IgG or an Ab that neutralizes the serine protease domain of factor I for 15 min on ice. Serum samples then were incubated with 0.5 mM Mg2+ at 37˚C for the indicated times. Immunoblots for DBP and α1PI complex formation are shown. (E) Pooled NHS and C3-depleted serum were activated with CVF for 15 min, and factor I–depleted serum was incubated at 37˚C for 15 min. The samples were separated using 8% SDS-PAGE and immunoblotted for C3.
C3b:plasma protein complexes form spontaneously in patients with DDD

The data presented above show that C3b:protein complexes are generated by complement activation and then degraded by the actions of factors H and I. Defective regulation may allow excessive generation of C3b:plasma protein complexes in the circulation. The C3Gs are a type of ultra-rare renal diseases characterized by fluid-phase dysregulation of the alternative pathway of complement. Aliquots of EDTA plasma from six C3GN and six DDD patients were analyzed by SDS-PAGE and immunoblotting for C3b:protein complexes, but no sample showed high m.w. SDS-resistant complexes when blotted for C3 and α1AG (Fig. 10A). To determine whether complexes could form in these patients’ plasmas, samples were incubated at 37°C for 60 min in the presence of 7.5 mM Mg²⁺ to overcome the EDTA chelation, and in three samples (DDD patient samples 1, 4 and 12) complexes were then observed (Fig. 10B). No complex formation was observed in normal controls and C3GN samples, although multiple high m.w. bands that did not correspond to complexes on the α1PI and α1AG blots were present on the C3 blot in C3GN sample 5 (Fig. 10B).

Because many of the samples from patients were significantly depleted of C3, purified C3 (1.3 mg/ml) and 7.5 mM Mg²⁺ were added to all samples from patients, followed by 37°C incubation for 30 min or 60 min. Five of six DDD samples (1, 4, 6, 11, 12) formed complexes (Fig. 10C). Sample 8, the only DDD sample that showed no complex formation, was also the only DDD sample negative for C3Nefs. C3GN sample 7 formed a clear complex with α1AG, whereas samples 2 and 9 formed weak complexes and samples 3, 5, and 10 were essentially negative. Of interest, the bands formed in the plasma samples from patients corresponded to the approximate size of bands in factor H-depleted serum (Fig. 10C). The presence of C3b:protein complexes in the blood of patients with DDD and, to a lesser extent, in C3GN patients, but not in plasma samples from healthy controls, indicates that there is dysregulation of the C3 convertase.

Discussion

Excessive activation and/or poor regulation of the complement system can trigger significant host cell damage, the possibility of which is minimized by multiple levels of regulation that control both the initiation and the extent of complement activation. Among the key steps in complement activation is the cleavage of C3 to C3b, which exposes the labile thioester bond in the TED of C3b (27). The extremely rapid ability of the thioester to react with available nucleophile acceptor groups (amino or hydroxyl) covalently attaches C3b to the surface of cells or tissue debris. This essential step of C3b tagging marks foreign targets for destruction and/or removal by phagocytes (28). However, the C3b thioester also reacts with H₂O in plasma, resulting in fluid-phase hydrolysis. In this article, we demonstrate that upon complement activation in serum (or plasma), C3b also forms transient covalent complexes with ≥ 10 different plasma proteins of various sizes, isoelectric points, and abundance, and that these C3b:protein complexes are subsequently degraded by factors H and I. Covalent binding of C3b to IgG (18), C4 (16), and properdin (17) has been reported previously, but these studies were examining formation of C3 convertases or immune complex clearance, and not the role of plasma proteins in binding and neutralizing C3b. Given our current knowledge of the reactivity of the thioester in C3, this process of generating C3b:plasma protein complexes is predictable but, surprisingly, is neither well recognized nor documented.

The amount of C3b:plasma protein complexes generated in complement activated normal serum, and particularly in factor I-depleted serum, is substantial, accounting for a large percentage of the total immunoreactive C3 bands (Figs. 6C, 6D, 7E). Complexes form despite the fact that 91.5% of plasma is water (55 M) and only 7.5% is protein. Given that water reacts with the thioester bond and is in great molar excess compared with plasma proteins, it is noteworthy that C3b:protein complexes form and can be detected...
FIGURE 9. The breakdown of C3b-plasma protein complexes requires cleavage by factor I in the presence of factor H. (A) Purified C3, fB, fD, and DBP were mixed with factor H alone, factor I alone, or a combination of factors H and factor I at physiological ratios and then activated with CVF in the presence of 0.5 mM Mg\(^{2+}\) for the indicated times. Samples were immunoblotted for DBP to assess complex formation and factor B to verify complement activation. C3 β-chain was used as a loading control. (B) The un-cropped DBP immunoblot of the last six lanes of (A) shows intermediate cleavage products. (C) Purified C3, fB, fD, and α\(_1\)AG were mixed with either factor I alone or a combination of factors H and factor I at physiological ratios and then activated with CVF in the presence of 0.5 mM Mg\(^{2+}\) for the indicated times. Samples were immunoblotted for α\(_1\)AG (center panel) or C3d (right panel) to assess complex formation and breakdown. The positions of C3b, iC3b, and C3dg complexes with α\(_1\)AG are indicated. To compare complex degradation in serum with the purified protein system, factor I–depleted serum alone, or factor I–depleted serum reconstituted with factor I and activated with CVF for 15 min, was separated and immunoblotted for α\(_1\)AG (left panel).
stimulate phagocytosis and link the innate and adaptive immune responses. Therefore, defective opsonization makes factor I–deficient patients susceptible to recurrent pyogenic infections and aseptic meningitis (31–34). What is notable, however, is that although renal disease has been described in these patients, it is very uncommon and is associated with deposition of immune complexes, suggesting activation of the classical pathway (35, 36). Thus the clinical picture of factor I deficiency suggests that C3b:protein complexes alone do not promote C3G and that functional factor I is required for the disease to develop, perhaps by generating iC3b and C3dg fragments of the C3b:plasma protein complexes.

Of note, factor I is also required for the development of C3G-like disease in mice. Factor H–deficient mice develop kidney disease that is similar to human DDD, whereas factor I–deficient mice, as well as factors H and I double knockout animals, do not develop kidney disease (37). The authors conclude that to develop DDD, factor I is required to generate C3 fragments in the circulation of factor H–deficient mice (37). This study also showed that plasma samples from factor I–deficient as well as H and I double knockout mice, but not factor H–deficient mice, showed multiple high m.w. SDS-resistant forms of C3 that correspond to the uncleaved C3b: plasma protein complexes reported in this article. However, these bands were noted but were thought to be C3b oligomers (37). These in vivo findings are consistent with our results and support the premise that C3b:protein complexes can be detected in the circulation. Moreover, a follow-up study analyzed the glomerular C3 deposits (obtained by laser capture microdissection) in factor I–deficient mouse kidneys (38). Analysis by SDS-PAGE showed no C3a-chain band (which contains the thioester bond), but a prominent C3b-chain, in factor I–deficient mouse kidneys. However, a higher SDS-resistant band was observed, indicating that the C3 α-chain was covalently linked to other molecules (38). Another study analyzed glomerular dense deposits by mass spectroscopy isolated by laser capture microdissection from patients with DDD and showed that deposits contain various complement components and other plasma proteins such as ApoE, fibronectin, and fibrinogen.


31. Vise, T. J., J. P. Spath, K. A. Davies, B. J. Morley, P. Philippe, A. Thaissianni,


