A Novel C3 Mutation Causing Increased Formation of the C3 Convertase in Familial Atypical Hemolytic Uremic Syndrome


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A Novel C3 Mutation Causing Increased Formation of the C3 Convertase in Familial Atypical Hemolytic Uremic Syndrome


Atypical hemolytic uremic syndrome has been associated with dysregulation of the alternative complement pathway. In this study, a novel heterozygous C3 mutation was identified in a factor B-binding region in exon 41, V1636A (4973 T > C). The mutation was found in three family members affected with late-onset atypical hemolytic uremic syndrome and symptoms of glomerulonephritis. All three patients exhibited increased complement activation detected by decreased C3 levels and glomerular C3 deposits. Platelets from two of the patients had C3 and C9 deposits on the cell surface. Patient sera exhibited more C3 cleavage and higher levels of C3a. The C3 mutation resulted in increased C3 binding to factor B and increased net formation of the C3 convertase, even after decay induced by decay-accelerating factor and factor H, as assayed by surface plasmon resonance. Patient sera incubated with washed human platelets induced more C3 and C9 deposition on the cell surface in comparison with normal sera. More C3a was released into serum over time when washed platelets were exposed to patient sera. Results regarding C3 and C9 deposition on washed platelets were confirmed using purified patient C3 in C3-depleted serum. The results indicated enhanced convertase formation leading to increased complement activation on cell surfaces. Previously described C3 mutations showed loss of function with regard to C3 binding to complement regulators. To our knowledge, this study presents the first known C3 mutation inducing increased formation of the C3 convertase, thus explaining enhanced activation of the alternative pathway of complement.


Hemolytic uremic syndrome (HUS) is defined as a triad of nonimmune microangiopathic hemolytic anemia, thrombocytopenia, and renal failure. A subtype of HUS, atypical HUS (aHUS) is, in many cases, associated with activation of the alternative pathway of complement. Activation has been linked to mutations in complement factors in 50–60% of cases (1). Mutations have been identified in regulators of the alternative pathway, such as factor H (CFH) (2), factor I (CFI) (3), and membrane-cofactor protein (MCP/CD46) (4), as well as deletions of factor H-related proteins 1 and 3 (CFHR1/3), with the latter being associated with anti-factor H Abs in some patients (5). In addition, mutations have been demonstrated in complement factors C3 (6, 7) and factor B (CFB) (8), as well as in thrombomodulin (9). Up to 10% of aHUS patients have mutations affecting more than one protein (10, 11).

Studies showed that many of the identified aHUS-associated mutations affect protein function, causing loss-of-function in complement regulators or gain-of-function in CFB, thereby enabling uninhibited complement activation to occur on cell surfaces (1). C3 mutations were described in a cohort of patients with aHUS, and functional studies showed that the mutations resulted in decreased secretion of mutant constructs or in a loss-of-function, demonstrated as decreased C3 binding to MCP or CFI (6). In addition, incubation of C3 mutants with CFI and its cofactor MCP or, to a lesser degree, CFB, exhibited decreased cofactor activity, which would lead to less C3b inactivation. These interactions could explain increased complement activation via the alternative pathway. However, none of the mutations studied exhibited increased binding to CFB and formation of the C3 convertase.

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In addition to aHUS, membranoproliferative glomerulonephritis (MPGN) is associated with aberrations of the alternative pathway of complement, often exhibiting low C3 levels (12). MPGN may present with hematuria, nephrotic-range proteinuria, hypertension, and impaired renal function (13). MPGN is subclassified based on the localization of immune deposits consisting of IgG and/or C3 (12). MPGN types I and III are considered immune-complex diseases, whereas type II, also known as dense deposit disease, is associated with complement activation via the alternative pathway and C3 deposition within the glomerular basement membrane (13). Recently, a heterozygous C3 mutation (923ΔDG) was described in familial dense deposit disease. The mutated C3 exhibited increased resistance to cleavage by C3 convertase; however, once cleaved, the active convertase generated by C3b923DG exhibited increased resistance to decay by CFH, and C3b923DG was resistant to inactivation by CFI in the presence of CFH (14).

In this article, we describe the finding of a novel heterozygous gain-of-function C3 mutation, V1636A, leading to increased C3 binding to CFB and increased formation of the C3 convertase. The gain-of-function C3 mutation, V1636A, leading to increased C3

### Materials and Methods

#### Subjects

One Swedish kindred with three affected individuals was investigated in this study (Patients 1–3). The family pedigree is presented in Fig. 1. All patients presented with clinical manifestations of HUS (nonimmune hemolytic anemia, thrombocytopenia, and acute renal failure) with simultaneous hypertension, hematuria, and nephrotic-range proteinuria. Clinical and histopathological findings in these patients are summarized in Tables I and II. Levels of C3, C4, CFB, CFH, CFI, and MCP are shown in Table III. Biopsies were taken during the acute phase of disease in Patients 1 and 2. In Patient 1, a biopsy was also taken after transplantation because of relapse of HUS. Patient 2 was on dialysis at the time of biopsy. Patient 3 underwent biopsy 8 mo after the onset of symptoms when acute signs of aHUS were no longer evident but chronic glomerular affection was present (hypertension, proteinuria, and renal failure).

DNA extracts were obtained from Patients 1–3 and their family members. DNA extracts from an apparently healthy cohort of adult blood donors (n = 102) were screened as controls. Sera from Patients 2 and 3, as well as two healthy individuals, were used to purify C3b. Sera from the patients and five healthy controls were used for measurement of C3a.

The study was performed with the approval of the Ethics Committee of the Medical Faculty at Lund University (protocol nos. 731-04 and 323-06) and The study was performed with the approval of the Ethics Committee of the Medical Faculty at Lund University (protocol nos. 731-04 and 323-06) and the local medical ethics committee of the participating hospitals.

#### Mutation screening

DNA was extracted from whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), as previously described (15), and from liver tissue (Patient 1) using the QIAamp DNA FFPE tissue kit (Qiagen). Exons of CFH (2), CFI (3), MCP (4), CFB (8), and C3 (primers listed in Supplemental Table I) were bidirectionally sequenced using the Big dye terminator kit (Applied Biosystems, Foster City, CA) and analyzed on an Applied Biosystems DNA Analyzer (model 3730).

#### C3b preparation

C3 was purified from serum, according to a previously described method (18), and degraded to C3b by trypsin (Sigma-Aldrich, St. Louis, MO). C3 (1 mg) was incubated with trypsin (13 μg) in 1 mM HCl with 100 mM iodoacetamide (Sigma-Aldrich) in a total volume of 1 ml for 10 min at room temperature, followed by addition of soybean trypsin inhibitor (65 μg; Sigma-Aldrich) on ice, to stop the reaction. The resulting C3a and C3b fragments were separated by gel filtration on a Superose 6 column (GE Healthcare, Uppsala, Sweden). Gel filtration was performed in PBS (Medicago, Uppsala, Sweden) at 0.16 ml/min. Fractions of 0.5 ml were collected, and absorbance was read at 280 nm. Fractions were analyzed by C3 ELISA (19), pooled, and concentrated. Samples were kept at −80°C until analyzed.

#### Mutant constructs

A plasmid containing C3 cDNA in the psi expression vector was kindly provided by David Isenman (University of Toronto, Toronto, ON, Canada) (20). Mutant constructs were prepared using the QuikChange XL Site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primers were forward, 5′-CTTCACGAGAGCATGGTTGCCTTTGGGTGCC-3′ and reverse, 5′-CTTCACGAGAGCATGGTTGCCTTTGGGCACCGAAGATGCA-3′. The mutated fragment was sequenced, digested, and repositioned in the original vector by enzymatic digestion with Fast-Digest restriction enzymes XhoI/Bsp1407I (Fermentas Life Sciences, Helsingborg, Sweden).

#### Transient transfection

COS-7 cells were cultured in DMEM (Invitrogen, Karlsruhe, Germany), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS (all from Invitrogen) to ~95% confluence, followed by transient transfection with Lipofectamine (Invitrogen), according to the manufacturer's protocol. DNA was extracted from whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), as previously described (15), and from liver tissue (Patient 1) using the QIAamp DNA FFPE tissue kit (Qiagen). Exons of CFH (2), CFI (3), MCP (4), CFB (8), and C3 (primers listed in Supplemental Table I) were bidirectionally sequenced using the Big dye terminator kit (Applied Biosystems, Foster City, CA) and analyzed on an Applied Biosystems DNA Analyzer (model 3730).
facturer’s instructions. A total of 4 µg C3 DNA was added to each well. After 24 h, medium was changed to serum-free medium and cultured for an additional 48 h. Medium was removed, and a protease inhibitor mixture (complete Mini EDTA; Roche Diagnostic, Mannheim, Germany) was used for washing and capping of unreacted carboxymethyl Healthcare) at 25˚C in running buffer consisting of HBSS (PAA, Pashing, Austria), supplemented with 1 mM NaCl (Merck, Darmstadt, Germany). CFB was injected (up to 80 µg/ml in running buffer) at 35 µl/min over the flow cells with different C3b variants, and binding was assessed. In the second experimental setting, a polyclonal rabbit anti-human C3c Ab (Dako, Glostrup, Denmark) was first immobilized on flow cell surfaces. All surfaces were adjusted to bear equal amounts of Ab. This was followed by C3(H2O) injection (wild-type and mutant constructs diluted in running buffer) at 10 µl/min and a subsequent injection of CFB at 35 µl/min. C3 (H2O) contact times were varied to give similar responses of C3(H2O) capture by the Ab between variants. After subtraction of a control flow cell (treated as above but without addition of immobilized protein C3b in the first setting or anti-C3c in the second setting), sensorgrams were created using BIA Evaluation 4.1 software (GE Healthcare). From these sensorgrams, net binding (in RU) was calculated as the increment in RUs after CFB injection compared with baseline values corresponding to bound C3b in the first setting, as well as C3 (H2O) captured on the Ab in the second setting. Formation of the C3 convertase and decay induced by decay-accelerating factor or CFI. C3 convertase was formed on the surface of a Biacore chip by serial injections of purified C3b alternating with CFB and factor D (CFD), as previously described (24). Conversion formation consisted of four serial injets of C3b, followed by CFB and CFD. The binding of CFB to the formed C3 convertase was tested by a subsequent injection of CFB at 35 µl/min, as described above. For decay assays, C3 convertases were formed, as described above, with the exception that the chip surface was obtained by immobilization of the polyclonal rabbit anti-human C3c Ab (as above), followed by serial injections of C3b (patients or control), CFB, and CFD. After the final C3b injection, levels of the conversion were adjusted to equivalent RUs to allow functional comparison. This was followed by injection (10 µl/min) of C3 Variants (patients or control) over their respective C3 convertases. C3 was cleaved to C3b and deposited on the chip surface. A period of spontaneous convertase decay was followed by accelerated decay induced by injection of soluble decay-accelerating factor (DAF; a kind gift from professor Anna Blom, Division of Medical Protein Chemistry, Wallenberg Laboratory Malmö, Lund University) or CFI (Calbiochem, Darmstadt, Germany), both at 0.4 µM (10 µl/min), after which an additional period of time was

### Table II. Pathological findings in patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Light Microscopic Glomerular Pathology</th>
<th>Immunofluorescence/Immunohistochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBM Thickening and Double Contours</td>
<td>Mesangial Cell Proliferation</td>
<td>Lobulation</td>
</tr>
<tr>
<td>1 Pre-Tx</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2 Post-Tx</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**a**Tram-tracks.  
**b**Granular deposition along glomerular capillary walls.  
**c**Mucoid intimal hyperplasia with fibrinoid necrosis was also noted.  
**d**GBM, glomerular basement membrane; NA, not available; Post-Tx, posttransplantation; Pre-Tx, pretransplantation; +, Present; −, absent; ++, present with excess labeling.

### C3 functional assays

#### C3 cleavage assay
C3 cleavage was measured in the fluid phase by crossed immunoelectrophoresis, as previously described (22). Patient serum was combined with normal serum, as a source of C3, for 30 min at 37˚C. The C3-cleavage reaction was terminated by dilution 1:3 in 0.075 M Diemal buffer (Barbitulat Natricum; APL, Gothenburg, Sweden) with EDTA (2 mM [pH 8.6]; Sigma). The resulting degree of C3 cleavage (measured as a decrease in C3 and an increase in cleaved C3 [C3c]) was estimated by the area under the curve, calculated by planimetry and analyzed by Digital board (GTCO; CalComp, Columbia, MD) as a quotient between the area representing C3 and the area under the curve, calculated by planimetry and analyzed by Digital board (GTCO; CalComp, Columbia, MD) as a quotient between the area representing C3c. Results are presented as percentage C3 cleavage after subtraction of C3 cleavage in the control (normal and patient sera, which were first incubated separately for 30 min at 37˚C and then combined). C3 cleavage levels <10% are considered normal (23). In certain samples, IgG was depleted from serum by passage through a Protein G Sepharose column (Amersham Biosciences, Uppsala, Sweden).

#### C3a detection
C3a and C3a(desArg) were detected and quantified in sera using a C3a assay kit (Quidel, San Diego, CA), following the manufacturer’s instructions. Samples were stored at −80˚C until assayed.

#### C3 binding to CFB determined by surface plasmon resonance
C3 binding to CFB (Complement Technology, Tyler, TX) was assayed using two experimental setings. In the first approach, purified C3b (diluted in 10 mM sodium acetate [pH 4]) was immobilized onto a CMS Biacore sensorchip flow cell surface (GE Healthcare) via amine coupling. C3b preparations were immobilized corresponding to 1000 response units (RU) after N-hydroxysuccinimide/N-ethyl-dimethylaminopropyl) carbodiimide (GE Healthcare) activation of the CMS matrix. Ethanolamine (1 M [pH 8]; GE Healthcare) was used for washing and capping of unreacted carboxymethyl groups. Analyses were performed on a BIACore 2000 instrument (GE Healthcare) at 25˚C in running buffer consisting of HBSS (PAA, Pashing, Austria), supplemented with 1 mM NaCl (Merck, Darmstadt, Germany). CFB was injected (up to 80 µg/ml in running buffer) at 35 µl/min over the flow cells with different C3b variants, and binding was assessed. After subtraction of a control flow cell (treated as above but without addition of immobilized protein C3b in the first setting or anti-C3c in the second setting), sensorgrams were created using BIA Evaluation 4.1 software (GE Healthcare). From these sensorgrams, net binding (in RU) was calculated as the increment in RUs after CFB injection compared with baseline values corresponding to bound C3b in the first setting, as well as C3 (H2O) captured on the Ab in the second setting.

Formation of the C3 convertase and decay induced by decay-accelerating factor or CFI. C3 convertase was formed on the surface of a Biacore chip by serial injections of purified C3b alternating with CFB and factor D (CFD), as previously described (24). Conversion formation consisted of four serial injets of C3b, followed by CFB and CFD. The binding of CFB to the formed C3 convertase was tested by a subsequent injection of CFB at 35 µl/min, as described above. For decay assays, C3 convertases were formed, as described above, with the exception that the chip surface was obtained by immobilization of the polyclonal rabbit anti-human C3c Ab (as above), followed by serial injections of C3b (patients or control), CFB, and CFD. After the final C3b injection, levels of the conversion were adjusted to equivalent RUs to allow functional comparison. This was followed by injection (10 µl/min) of C3 Variants (patients or control) over their respective C3 convertases. C3 was cleaved to C3b and deposited on the chip surface. A period of spontaneous convertase decay was followed by accelerated decay induced by injection of soluble decay-accelerating factor (DAF; a kind gift from professor Anna Blom, Division of Medical Protein Chemistry, Wallenberg Laboratory Malmö, Lund University) or CFI (Calbiochem, Darmstadt, Germany), both at 0.4 µM (10 µl/min), after which an additional period of time was

### Table III. Complement levels in Patients 1–3

<table>
<thead>
<tr>
<th>Patient</th>
<th>C3</th>
<th>C4</th>
<th>CFB</th>
<th>CFH</th>
<th>CFI</th>
<th>MCP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(770–1380 mg/l)</td>
<td>(120–330 mg/l)</td>
<td>(59–154%)</td>
<td>(69–154%)</td>
<td>(60–152%)</td>
<td>(500–1000 MFI)</td>
</tr>
<tr>
<td>1</td>
<td>670</td>
<td>328</td>
<td>117</td>
<td>140</td>
<td>103</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>470</td>
<td>220</td>
<td>113</td>
<td>105</td>
<td>70</td>
<td>719</td>
</tr>
<tr>
<td>3</td>
<td>590</td>
<td>140</td>
<td>68</td>
<td>95</td>
<td>67</td>
<td>NA</td>
</tr>
</tbody>
</table>

**a**Normal range.  
**b**Mean fluorescence intensity (MFI) analyzed by flow cytometry, as described (4).  
**c**NA, Not analyzed.
C3 or C9 deposition on washed platelets was detected by incubation with chicken anti-human C3:FITC (1:2000; Diapensia, Linköping, Sweden) or mouse anti-human C9 neoantigen (1:100; Hycult Biotechnology, Uden, The Netherlands) for 10 min. Chicken anti-human insulin:FITC (1:2000; Diapensia) and mouse IgG1 (1:100; Hycult Biotechnology) were used as the irrelevant Abs, respectively. Goat anti-mouse:FITC (1:700; Dako) was the secondary Ab.

**Acquisition and interpretation of flow cytometry.** Platelets were analyzed using a BD FACSCantoII cytometer and FACSDiva software (Becton Dickinson Immunocytometry Systems, San Jose, CA), as previously described (17).

**Results**

**C3 mutation**

A novel heterozygous missense C3 mutation V1636A was found in Patients 1–3 (described in Tables I–III). The mutation is located in exon 41, in the α-chain of C3 in one of three CFB-binding regions of C3b (25). This mutation was not found in 102 DNA samples from controls. The phenotypic consequences of this mutation were studied, as described below. In addition, rare polymorphisms were found in the MCP gene, A304V and CFI gene, IVS12+5G>T (Patient 2), and in the CFH gene, Q950H (Patient 3). The C3 mutation was the only mutation common to all affected family members. The mutation and polymorphisms are presented in Fig. 1 and Table IV.

**Functional assays**

**C3 cleavage in patient sera.** C3 cleavage was assayed in Patients 2 and 3. In serum from Patient 2, the C3-cleaving activity was elevated at 16%; in serum from Patient 3, the level was 20% (reference value <10%). IgG was depleted from the serum of Patient 3, and the C3-cleaving activity was unaltered (20%), indicating that cleaving activity was not due to the presence of an Ab (such as C3 nephritic factor).

**Serum C3a levels.** Patients 2 and 3 exhibited elevated levels of C3a in serum (1700 and 1600 ng/ml, respectively). Control sera (n = 5) had values of 350, 480, 510, 520, and 1000 ng/ml. Normal values of serum C3a are 71–589 ng/ml, according to the manufacturer of the C3a assay kit.

**C3 binding to CFB determined by surface plasmon resonance.** Surface plasmon resonance analysis of purified C3b from Patients 2 and 3, as well as two healthy controls, showed increased binding of patient C3b to CFB in comparison with normal C3b (Fig. 2A).

**C3 convertase formation.** The C3 convertase was formed on a Biacore sensor chip by serial injections of purified C3b from Patients 2 or 3 or from one control. This was followed by alternating injections of CFB and CFD. Formation of the C3 convertase is shown in Fig. 2B. After formation of the C3 convertase, CFB was injected, showing increased binding to the convertase formed with patient C3b compared with control C3b (Fig. 2B, inset).

**Table IV. Molecular characteristics of genetic alterations**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Complement Protein</th>
<th>Mutation or Polymorphism</th>
<th>Codon</th>
<th>Protein</th>
<th>Phenotype</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C3</td>
<td>V1636A</td>
<td>4973 T&gt;C</td>
<td>Val1636Ala</td>
<td>Increased affinity for CFB and C3 convertase</td>
<td>This study</td>
</tr>
<tr>
<td>2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C3</td>
<td>V1636A</td>
<td>4973 T&gt;C</td>
<td>Val1636Ala</td>
<td>Intrinsic</td>
<td>(26)</td>
</tr>
<tr>
<td></td>
<td>CFI</td>
<td>IVS1245&lt;sup&gt;b&lt;/sup&gt;</td>
<td>IVS1245 G&gt;T</td>
<td>Val1636Ala</td>
<td>No documented abnormality and normal CFI serum levels</td>
<td>(27)</td>
</tr>
<tr>
<td></td>
<td>MCP</td>
<td>A304V&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1058 C&gt;T</td>
<td>Ala304Val</td>
<td>Increased activation of alternative pathway on cell surface</td>
<td></td>
</tr>
<tr>
<td>3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>C3</td>
<td>V1636A</td>
<td>4973 T&gt;C</td>
<td>Val1636Ala</td>
<td>Gln950His</td>
<td>No documented abnormality</td>
</tr>
<tr>
<td></td>
<td>CFH</td>
<td>Q950H&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2850 G&gt;T</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All genetic alterations were heterozygous.

<sup>a</sup>Tested and found not to have serum anti-CFH Abs.

<sup>b</sup>These genetic alterations have also been detected in the healthy population, indicating they are rare polymorphisms.
Binding of C3 constructs to CFB. Wild-type and mutant C3 constructs were produced to study the V1636A mutation. The wild-type construct corresponded to V1636 (identical to the cDNA sequence). The heterozygote construct was composed of 50% wild-type V1636 and 50% mutant 1636A and was comparable with the C3 genotype of Patients 1–3. When CFB was injected over C3 (H2O) constructs bound to the C3c Ab-coated surface, a higher degree of binding of CFB to the C3 V1636A construct was detected compared with wild-type V1636 (Fig. 2C).

Net formation of the C3 convertase after spontaneous and DAF- or CFH-induced decay. The C3 convertase was formed by serial injections of C3b from each patient or control, CFB, and CFD, followed by the respective C3, which deposited on the chip as cleaved C3b. This was followed by a period of spontaneous decay (80 s), after which CFB displacement was induced by DAF or CFH. The results showed increased formation of the C3 convertase using C3b, followed by C3 from Patients 2 and 3, compared with the control. Increased convertase formation persisted, even after spontaneous and accelerated decay (Fig. 3). Measuring ΔRU from the start of accelerated decay for a period of 200 s showed that, after addition of DAF, the control curve decreased by 23 RU compared with 5 RU for Patient 2 and 3 RU for Patient 3. This pattern was not the same after addition of CFH: the control curve decreased by 11 RU, Patient 2 decreased by 24 RU, and Patient 3 decreased by 20 RU. These results indicated that the mutant C3 mediated excess formation of the C3 convertase but not excess
resistance to decay by DAF and CFH. Control cells (without addition of anti-C3c or with anti-C3c onto which DAF or CFH was injected) did not exhibit binding.

To confirm that differences in decay were not due to altered binding affinity for CFH, equimolar amounts of CFH were injected over bound C3b from patients and controls; there was no difference in binding affinity (depicted as changes in RUs after addition of CFH; ΔRU in Fig. 4). No binding was detected when CFH was injected over the anti-C3c Ab-coated chip.

Deposition of C3 and C9 on patient platelets. Washed platelets (from EDTA tubes) from Patient 2 and 3 displayed increased levels of C3 (24 and 16%, respectively) and C9 (15% and 13%, respectively) compared with washed platelets from healthy controls (C3: median 8%, range 6–11%; C9: median 7%, range 5–12%, n = 5).

Serum from patients induced increased C3 and C9 deposition on washed platelets. Incubation of washed platelets with patient serum induced an increase in surface-bound C3 and C9 compared with control serum (Fig. 5). Similarly, incubation of washed platelets with C3-depleted serum, to which purified C3 from patient 2 or 3 was added, increased surface-bound C3 and C9 compared with purified C3 from the control (Fig. 6).

Patient serum exposed to washed platelets induced C3a release. Levels of C3a were measured in the sera exposed to washed platelets. Patient sera induced an increase in C3a concentration over time (Fig. 7) compared with sera from healthy controls (n = 3).

Discussion

This study presents a novel C3 mutation, V1636A, in three family members with aHUS. The mutation is located in exon 41 in one of the three CFB-binding sites and induces enhanced C3 binding to CFB, thereby increasing formation of the C3 convertase. Increased formation persisted, even after spontaneous and accelerated decay induced by DAF and CFH. This mutation would be expected to enhance activation of the alternative pathway of complement and, indeed, all three patients exhibited clinical features of complement activation, such as decreased C3 levels in serum, increased C3a, increased C3 cleavage, and presence of C3 deposits on platelets and in glomerular capillary walls. Patient sera induced increased C3 and C9 deposition on normal washed human platelets over time and a parallel increase in C3a release. Previously described mutations in C3 exhibited loss-of-function with regard to the interaction between C3 and complement regulators, thus leading to activation of the alternative pathway. The V1636A would lead to a direct gain-of-function with regard to formation of the C3 convertase.

Serum from aHUS patients with CFH mutations localized at the C-terminal enabled increased C3 and C9 deposition on washed human platelets (17). The effect was specifically related to mutated CFH, as shown by combining washed platelets in CFH-depleted serum with purified CFH (normal or mutant variant). Mutant, but not normal, CFH allowed excess complement activation to occur on the cell surface. Addition of normal CFH to patient sera inhibited complement activation. In the current study,
excess complement activation on the cell surface was demonstrated using patient platelets, as well as normal washed platelets exposed to patient sera. However, the patients had other mutations/rare polymorphisms in CFH, CFI, or MCP, which also might have contributed to complement deposition on the platelet surface. C3-depleted serum was therefore used to specifically analyze whether the mutant variant of C3 induced complement activation on the platelet surface. The mutant variant of C3 resulted in excess C3 and C9 deposition on platelets.

Experiments showing excess C3 and C9 deposition on the surface of washed platelets were carried out over a period of 30 min, indicating an increase in C3 and C9 deposition, as well as C3a release, over time. This suggested a prolonged half-life of the C3 convertase. To more specifically address this issue, binding experiments were carried out, showing that the mutant variant of C3 exhibited a higher affinity for CFB and more C3 convertase formation. The convertase formed from the mutant C3 variant persisted during spontaneous and DAF- or CFB-induced decay over time, indicating a prolonged half-life. DAF (41 kDa) or CFH (150 kDa) displace CFBb (60 kDa) bound to C3b in the C3bBb convertase. Surface plasmon resonance will not detect major changes in the curve when one molecule is replaced by another, particularly when the molecules have a similar molecular mass. A total decay of C3bBb was not visualized, rather there was a replacement of CFBb by DAF or CFH; therefore, a major change in the curve was not demonstrated. The results indicated that the mutant C3 most probably did not influence accelerated decay.

Gain-of-function mutations affecting CFB have been described (8, 30). CFB mutations found in aHUS patients resulted in a high-affinity binding site to C3 and led to a hyperfunctional C3 convertase. Certain mutations led to resistance to decay by DAF and/or CFH (8, 30). In addition, mutant CFB had the capacity to bind to iC3b, forming a C3 convertase from inactive C3b (30). Thus, mutations in both C3 and in CFB may lead to increased affinity between C3 and CFB.

Interestingly, all three patients had normal levels of CFB. Increased formation of the C3 convertase with a prolonged half-life leads to more cleavage of C3 to C3b and consumption of C3, thus reducing serum levels. CFB levels do not necessarily decrease in parallel, even in patients with hypocomplementemia secondary to MPGN and C3 nephritic factor (23). Prolonged C3 convertase half-life, whether due to C3 nephritic factor or to the C3 mutant, V1636A, leads to excess C3 cleavage and, thus, lower C3, but not CFB, levels in serum.

The family members described in this article exhibited a unique phenotype of aHUS. All affected individuals exhibited late onset of disease. In addition to the triad of HUS (DAT-negative hemolytic anemia, thrombocytopenia, and acute renal failure), these patients presented simultaneously with acute glomerulonephritis, as indicated by concurrent hematuria, hypertension, and nephrotic-range proteinuria. In all three individuals, pathological findings suggested the presence of thrombotic microangiopathy, as well as membranoproliferative features, such as glomerular lobulation, mesangial proliferation, tram-tracking of the glomerular basement membrane, and C3 deposits in the glomeruli. All of these light microscopy features may be associated with chronic thrombotic microangiopathy and with MPGN (31). The similarity in clinical and pathological presentation in the three patients suggested that the common C3 mutation was an underlying cause of the mutual unique phenotype. We speculated that increased formation of the C3 convertase may give rise to a mixed phenotype combining features of aHUS and glomerulonephritis resembling MPGN, al-

FIGURE 5. C3 and C9 binding to normal washed platelets exposed to serum over time. Normal washed platelets were incubated with serum from Patient 2 (●), Patient 3 (▲), or controls (○). Patient sera induced increased C3 and C9 binding to normal platelets compared with normal sera. More C3 and C9 deposited over time.

FIGURE 6. Binding of C3 and C9 to washed platelets exposed to purified C3 added to C3-depleted serum. Normal washed platelets were incubated with C3-depleted serum, to which purified C3 from Patient 2 (●; 0.3 mg/ml final concentration), Patient 3 (▲; 0.7 mg/ml final concentration), or one control (○; 0.3 mg/ml or ◦; 0.7 mg/ml final concentration) was added. Purified C3 from patients induced increased C3 and C9 binding compared with the control.

FIGURE 7. C3a release over time in sera exposed to washed platelets. C3a was measured in sera exposed to washed platelets over a period of 30 min. All sera C3a levels were adjusted to a baseline “0” level at the exposure to serum. Sera from Patient 2 (▲) and Patient 3 (●) exhibited steadily increasing levels, whereas sera from three controls (○) showed persistently low levels.

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though ultramorphology in Patient 3 did not exhibit features of MPGN. In MPGN, the C3 nephritic factor stabilizes the C3 convertase, whereas in the patients described in this article, the C3 mutation had a similar effect, which may explain the phenotypic overlap resembling MPGN.

In summary, this study presents a novel C3 mutation, leading to increased formation of the C3 convertase, in three related individuals affected with aHUS. This C3 mutation promotes uncontrolled complement activation, as demonstrated in the patients by decreased serum C3 levels and C3 deposits in renal tissue. To our knowledge, this is the first C3 mutation to be described leading to a direct gain-of-function, and we suggest that its functional consequences may lead to increased activation of the alternative pathway.

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Disclosures
K.N.-E. holds a patent related to the modification of biomaterials, VF-B. was consultant for Alexion Pharmaceuticals, and D.K. was the national coordinator in Sweden for the Eclizumab trial (Alexion Pharmaceuticals) in patients with aHUS.

References