A Humanized Antibody That Regulates the Alternative Pathway Convertase: Potential for Therapy of Renal Disease Associated with Nephritic Factors

Danielle Paixão-Cavalcante, Eva Torreira, Margaret A. Lindorfer, Santiago Rodriguez de Cordoba, B. Paul Morgan, Ronald P. Taylor, Oscar Llorca and Claire L. Harris

*J Immunol* published online 11 April 2014
http://www.jimmunol.org/content/early/2014/04/11/jimmunol.1303131
A Humanized Antibody That Regulates the Alternative Pathway Convertase: Potential for Therapy of Renal Disease Associated with Nephritic Factors

Danielle Paixão-Cavalcante,† Eva Torreira,† Margaret A. Lindorfer,‡ Santiago Rodríguez de Cordoba,‡ B. Paul Morgan,* Ronald P. Taylor,‡ Oscar Llorca,† and Claire L. Harris*

Dysregulation of the complement alternative pathway can cause disease in various organs that may be life-threatening. Severe alternative pathway dysregulation can be triggered by autoantibodies to the C3 convertase, termed nephritic factors, which cause pathological stabilization of the convertase enzyme and confer resistance to innate control mechanisms; unregulated complement consumption followed by deposition of C3 fragments in tissues ensues. The mAb, 3E7, and its humanized derivative, H17, have been shown previously to specifically bind activated C3 and prevent binding of both the activating protein, factor B, and the inhibitor, factor H, which are opposite effects that complicate its potential for therapy. Using ligand binding assays, functional assays, and electron microscopy, we show that these Abs bind C3b via a site that overlaps the binding site on C3 for the Ba domain within factor B, thereby blocking an interaction essential for convertase formation. Both Abs also bind the preformed convertase, C3bBb, and provide powerful inhibition of complement activation by preventing cleavage of C3. Critically, the Abs also bound and inhibited C3 cleavage by the nephritic factor–stabilized convertase. We suggest that by preventing enzyme formation and/or cleavage of C3 to its active downstream fragments, H17 may be an effective therapy for conditions caused by severe dysregulation of the C3 convertase and, in particular, those that involve nephritic factors, such as dense deposit disease. The Journal of Immunology, 2014, 192: 000–000.

Complement is part of innate immunity, with key roles in defense against pathogens through opsonization and lysis, clearance of apoptotic cells, handling of immune complexes, and modulation of adaptive immune responses (1). Complement can be triggered via three activation pathways: the classical, alternative (AP), and lectin pathways, all leading to the generation of a C3 cleaving enzyme, or convertase, the central and most important step of the activation cascade. Cleavage of C3 generates C3b, which covalently links to target cells, binding factor B (fB) in an Mg2+-dependent manner to form C3bB. This proenzyme is activated by factor D (fD), generating the active C3 convertase, C3bBb. Binding of properdin (P) stabilizes this otherwise labile complex. Each C3 convertase cleaves many C3 to C3b, thus providing exponential amplification of the pathway. Complement activation progresses by formation of the C5 cleaving enzyme, resulting in generation of C5a and C5b. C5a is a proinflammatory peptide with anaphylactic and chemotactic properties, whereas C5b binds the next complement component, C6, marking the start of the terminal pathway that culminates in formation of the cytolytic membrane attack complex (MAC) (2).

The AP “ticks over” constantly in plasma. Spontaneous hydrolysis of C3 generates a C3b-like molecule, C3(H2O), that binds fB, which is then processed by fD to form a fluid-phase enzyme, C3(H2O)Bb, that cleaves C3 to C3b, thus “priming” the AP for immediate activation (3). C3b generated in the fluid phase is rapidly inactivated, thus preventing uncontrolled consumption of C3b, thus providing exponential amplification of the pathway. The structures presented in this article have been submitted to the EMDatabank database (http://www.eMDatabank.org/index.html) under accession number EMD-2553. This work was presented in abstract form at the 23rd International Complement Workshop, August 1–5, 2010, New York, NY.

Address correspondence and reprint requests to Prof. Claire L. Harris, Institute of Infection & Immunity, School of Medicine, Cardiff University, Henry Wellcome Building, Heath Park, Cardiff CF14 4XN, United Kingdom. E-mail address: harrisc1@cardiff.ac.uk

The online version of this article contains supplemental material.

Abbreviations used in this article: aHUS, atypical hemolytic uremic syndrome; AP, alternative pathway; C3NeF, C3 nephritic factor; DAF, decay accelerating factor; DDD, dense deposit disease; E, erythrocyte; EA, Ab-coated E; EM, electron microscopy; fB, factor B; fD, factor D; fH, factor H; HBSP, HEPES-buffered saline supplemented with 0.005% surfactant P20; HBSPMg, HBSP supplemented with 1 mM MgCl2; MAC, membrane attack complex; MG, macroglutamin; P perpropion; PDB, Protein Data Bank; PNH, paroxysmal nocturnal hemoglobinuria; RU, resonance units; sDAF, soluble recombinant DAF comprising domains 1–4; SPR, surface plasmon resonance.

Copyright © 2014 by The American Association of Immunologists, Inc. 0022-1767/14/$16.00

1Institute of Infection & Immunity, School of Medicine, Cardiff University, Cardiff CF14 4XN, United Kingdom; 2Centro de Investigaciones Biologicas, Consejo Superior de Investigaciones Científicas, 28040 Madrid, Spain; and 3Department of Biochemistry and Molecular Genetics, University of Virginia School of Medicine, Charlottesville, VA 22908

†Current address: Cristália Produtos Químicos Farmaceüticos Limitada, Itapira, São Paulo, Brazil.

Reprived for publication November 22, 2013. Accepted for publication March 17, 2014.

This work was supported by Kidneeds, USA (to C.L.H. and B.P.M.), the Medical Research Council (Grant G0701298 to C.L.H. and B.P.M.), the Ramon Areces Foundation (to O.L.), the Autonomous Region of Madrid (Grant S2010/BMD-2316 to S.R.d.C. and O.L.), the Spanish Ministry of Science and Innovation (Grant SAF2011-22988 to O.L. and Grant SAF2011-26583 to S.R.d.C.), a Kidney Research UK Post-Doctoral Fellowship (Grant PDFVS2010 to D.P.-C.), the Red Temática de Investigación Cooperativa en Cáncer from the Instituto de Salud Carlos III (Grant RD06/0020/1001 to O.L.), the Fundación Renal Ilígo Alvarez de Toledo (to S.R.d.C.), the Ciber de Enfermedades Raras (to S.R.d.C.), and the 7th Framework Programme European Union project EURenOmics (to S.R.d.C.).

C.L.H., R.P.T., S.R.d.C., O.L., and B.P.M. conceived and performed the research experiments and analyzed the data; D.P.-C., C.L.H., S.R.d.C., B.P.M., and R.P.T. drafted and edited the manuscript; R.P.T. and M.A.L. contributed vital reagents.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1303131
complement in plasma; however, a proportion binds indiscriminately to any cell in its vicinity and, if not strictly regulated, can drive complement activation and cause damage to host cells. Damage to self is restricted by numerous complement regulatory proteins present in the fluid phase (including factor H [fH]) and on cell membranes including CD55, CD35, and CD46. These regulators act by accelerating natural decay of C3bBb or by acting as cofactors for the proteolytic inactivation of C3b by the plasma protease factor I (4, 5).

In health, complement is in homeostatic balance; activation in plasma occurs at a low level, and regulation prevents significant deposition of the central component, C3b, and limits further activation except on pathogens. The capacity of complement to initiate quickly and amplify efficiently means that any disturbance in homeostasis can be devastating to health (6). “Dysregulation” of the central components of the amplification loop, C3, fB, fD, or the control protein, fH, can cause acute or chronic inflammation and contribute to the pathologies associated with diverse diseases, including rheumatoid arthritis, systemic lupus erythematosus, glomerulonephritis, multiple sclerosis, sepsis, asthma, and ischemia/reperfusion injuries. In each, complement activation drives a “vicious cycle” of inflammation and tissue damage (7).

It is now established that the prototypic complement dysregulation-associated diseases, dense deposit disease (DDD), atypical hemolytic uremic syndrome (aHUS), and age-related macular degeneration, are each associated with mutations and/or polymorphisms in the components and regulators of the AP C3 convertase (8, 9). Severe dysregulation is also triggered by autoantibodies against complement components, complexes, or regulators. Abs that interfere with function of fH are found in some aHUS and DDD patients. Abs that bind the AP C3 convertase, C3bBb, known as C3 nephritic factors (C3NeF), are present in >80% of patients with DDD (10, 11). Once bound to the C3 convertase, C3NeF stabilizes the C3bBb complex, increasing its half-life and preventing regulation by complement regulatory proteins such as fH (12). This stabilized C3 convertase consumes intact C3, thereby generating large amounts of fluid-phase activated C3 fragments (C3b, iC3b, C3dg), which locate in the kidney. Understanding the mechanisms behind complement-mediated diseases has advanced enormously in recent years, revealing new avenues for development of specific therapies that target different steps within the complement cascade. These include soluble recombinant complement regulatory proteins, such as TP10 (13), and mAbs that block complement proteins, such as eculizumab (anti-C5) (14) and TA106 (anti-fB Fab; Alexion Pharmaceuticals) (15). Small-molecule inhibitors that prevent C3 activation are in trials for disorders such as age-related macular degeneration (compsatin) (16, 17). The most successful anticomplement therapeutic to date is eculizumab, approved by the Food and Drug Administration for treatment of paroxysmal nocturnal hemoglobinuria (PNH) and aHUS, diseases in which dysregulated AP triggers terminal pathway activation, which plays a critical role in pathology. However, not all complement-mediated diseases occur as a consequence of C5a and MAC activities; in DDD, there is marked activation of C3, and fluid-phase C3 activation fragments (primarily iC3b) trapped in the glomerular basement membrane in the kidney are implicated in driving pathology (18).

The mAb, 3E7, and its humanized chimeric counterpart, H17, bind C3b, C3b(H2O), and iC3b, but not native C3 or C4/C4b (19, 20). The mAbs compete with fB and fH for binding to C3b, preventing formation of C3 convertase and generation of iC3b, respectively; however, their precise mechanism of action is unclear (19). In an in vitro model of PNH, they inhibited deposition of C3b and abolished the hemolysis of PNH erythrocytes (E) (21). Inhibition was due to mAb binding to fluid phase C3(H2O), preventing AP tickover and deposition of C3b on the surface of the E. However, it is not clear whether the mAbs bind and influence the AP convertase, which is important particularly in the context of the pathogenic C3NeF-stabilized AP convertase. We describe in this article the mechanism by which 3E7/H17 binding influences AP convertase activity, whether unstabilized or stabilized by C3NeF; these data are supported by structural analysis of the mAb in complex with C3b. The data suggest that mAb H17 may be an effective therapy in disorders mediated by dysregulation of the AP C3 convertase and particularly in C3NeF-associated diseases such as DDD.

Materials and Methods
mAbs and complement components
The mAb 3E7, and its humanized derivative H17, have been described previously (19). Fab fragments of H17 were produced by digestion with papain. C3 was prepared by classical chromatography using an established protocol (22); fB was affinity purified from EDTA plasma on anti-fB (mAb JCl1, in-house). HiTrap column (GE Healthcare). fD was purchased from Complement Technology (Tyler, TX). C3b was generated by AP activation by covalent binding of fB (and fD) to C3b, by flow of mAbs against C3b (23), followed by anion exchange chromatography (Mono Q; GE Healthcare) and size exclusion chromatography (Superdex 200; GE Healthcare). Concentrations of pure proteins were assessed using absorbance at 280 and the following extinction coefficients: fB: 1.43 cm⁻¹(mg/ml)⁻¹; C3: 0.98 cm⁻¹(mg/ml)⁻¹; Ig: 1.4 cm⁻¹(mg/ml)⁻¹. Soluble recombinant decay accelerating factor (DAF) comprising domains 1–4 (sDAF) was gifted by Prof. Margaret Lea (University of Oxford). A strongly C3NeF⁺ Ig preparation was donated by Dr. Margarita López-Trascasa (Hospital Universitario de La Paz, Madrid, Spain).

Surface plasmon resonance studies
Surface plasmon resonance (SPR) analyses were conducted at 25°C on a BiACore T100 (GE Healthcare), except for kinetic data, which were performed on a BiACore 3000; kinetic/affinity data were collected at 30 μl/min with a reference flow of 20 μl/min. Proteins were amine-coupled to a CM5 (carboxymethylated dextran) chip (NHS/EDC coupling kit; GE Healthcare). Interactions were analyzed in HEPES-buffered saline (10 mM HEPES pH 7.4, 150 mM NaCl) supplemented with 0.005% surfactant P20 (HBSP) and either 1 mM MgCl2 (HBSPMg) or 1 mM Ni2SO4 (HBSN). Generation was achieved using 10 mM sodium acetate pH 4, 1M NaCl. Data were evaluated using Biacalculator 4.1 (BiACore ROC) and/or BIAevaluation 1.1 software (Biacore T100). Affinity of mAb for C3b was measured by immobilizing the mAb (335–400 resonance units [RU]) and flowing HBSP in concentrations indicated. Data were analyzed using the 1:1 Langmuir binding model. C3b was densely immobilized (1200 RU) and mAb flowed to test avidity effects. mAb (20 μg/ml) was flowed over a C3b surface (1200 RU) in HBSPMg, followed by fB (70 μg/ml), to test whether mAb 3E7 or H17 blocked fB binding to C3b. mAbs (varying concentrations as indicated) were flowed over the C3b-coated surface in HBSPMg before flowing fB (140 μg/ml) and fD (1 μg/ml), to test whether mAb blocked convertase formation. fB (140 μg/ml) and fD (1 μg/ml) were flowed across the C3b surface in HBSPMg, followed by immediate injection of mAb (10 μg/ml), to investigate whether mAb bound preformed convertase. Convertase on the surface was decayed using sDAF (two injections), and residual mAb binding was measured and compared with that bound to C3b in the absence of convertase formation. A high concentration of fB (350 μg/ml) was flowed in HBSN before flowing mAb (20 μg/ml) to test whether fB prevented mAb binding to C3b; binding of mAb to surface with and without fB was compared.

To test whether mAbs affected the capacity of chip-bound convertase to cleave C3 to C3b, we formed C3 convertase by flowing a mix containing fB and native C3, followed by injection of native C3. Cleavage of C3 to nascent C3b resulted in covalent deposition on the chip surface apparent as an increased RU in the sensorgram.

Hemolysis assay
Sheep E were sensitized by incubating 1 vol of 4% E (v/v) with 1 vol of 1:4000 Ambroceptor (Siemens Healthcare, Marburg, Germany) for 30 min at 37°C. Ab-coated E (EA) were washed twice in complement fixation diluent

Downloaded from http://www.jimmunol.org/ by guest on April 9, 2017
containing 20 mM EDTA. Lysis was developed by adding 50 µl of depleted serum diluted 1:25 in PBS/20 mM EDTA. Percent lysis was calculated as described previously.

Electron microscopy of C3b–mAb complexes

C3b (6 µg) was incubated with an approximate 3-fold molar excess of H17 Fab fragments (6 µg) in 25 mM Tris HCl pH 7.4, 150 mM NaCl, and 10 mM DTT for 1 h at room temperature. C3b–Fab complex was purified by gel filtration chromatography using a Superdex 200 PC 3.2/30 column (GE Healthcare). Fractions were analyzed by SDS-PAGE.

Immediately after gel filtration, 5–10 µl of the peak fraction containing the C3b-H17 Fab complex was diluted to 10 µg/ml in the same buffer and adsorbed to glow-discharged, carbon-coated copper grids and stained with 0.2% uranyl formate. Grids were imaged on a JEOL JEM-1230 electron microscope at 100 kV. Micrographs were collected under low-dose conditions (∼10e−/Å² per exposure) using a 4k × 4k TemCam-F416 camera (TVIPS) at 2.28 Å/pixel. A total of 7227 images of C3b-H17 was selected and binned to 4.56 Å/pixel after contrast transfer function correction. Particles were subjected to reference-free alignment and classification, and were refined using angular refinement methods in EMAN (25). An ab initio model for refinement was obtained using the random conical tilt method applied to those averages where the Fab was evident (Supplemental Fig. 1). The high correlation between those averages obtained after angular refinement and the reference-free averages supported the correctness of the final structure. The resolution of the structure (30 Å) was estimated using the Fourier Shell Correlation method and a 0.5 correlation coefficient. A pseudomolecular atom model was obtained by fitting the atomic structure of C3b (Protein Data Bank [PDB] ID 2I07) and a Fab (PDB ID 1H0D; chains A and B) obtained from the PDB within the electron microscopy (EM) density of C3b-H17 using Chimera (26). The top fitting solution showed 0.89 and 0.93 cross-correlation coefficients between the x-ray and the EM structures for C3b and the Fab, respectively.

EM database

The structure of the C3b–H17 complex has been deposited in the EMDatadbank database (http://www.emdatabank.org/index.html) under accession number EMD-2553.

Results

mAbs 3E7 and H17 bind with high affinity to C3b, free, and in the AP convertase

Previous studies have shown that mAb 3E7 binds to C3b and C3(H2O). Affinity of the interaction was investigated in this study using SPR; 3E7 (Fig. 1A) was immobilized on the surface of a CM5 sensor chip, and C3b was flowed across. Affinity (Kd) was 35 nM as determined using the Langmuir 1:1 binding model (χ² < 1.0, indicating a good fit to the model with little heterogeneity). H17 is a humanized, chimeric form of mAb 3E7 in which the

![Figure 1](https://via.placeholder.com/150)

**FIGURE 1.** Kinetics of interaction of 3E7/H17 with C3b. (A) 3E7 or (B) H17 was immobilized on the surface of a CM5 chip, and C3b flowed across at indicated concentrations (1:2 serial dilution). Binding was monitored and kinetics evaluated using the 1:1 Langmuir binding model; Kd (KD) of 3E7 was 35 nM and of H17 was 28 nM (mean of two different experiments using multiple concentrations and global fitting). (C) C3b (1200 RU) was immobilized on the chip and 3E7 was flowed across to investigate how mAbs would bind to a C3b-coated surface; avidity effects markedly alter the binding kinetics, indicating the binding interaction that may take place in vivo on a tissue attacked by complement and coated in C3b.

![Figure 2](https://via.placeholder.com/150)

**FIGURE 2.** 3E7/H17 bind to the preformed convertase, but not the proenzyme. (A) C3 convertase was formed by flowing a mix containing fB and fD over immobilized C3b (1200 RU); 3E7/H17 was immediately flowed across the convertase, followed by sDAF (two injections) to remove Bb from C3b (black line). 3E7/H17 was also flowed in the absence of preformed convertase (gray line), and the two mAb injects were aligned. The amount of mAb bound was the same irrespective of whether convertase had been preformed on the surface. (B) Binding to proenzyme was assessed by flowing high concentration of fB in Ni²⁺-containing buffer (to stabilize) across C3b to form proenzyme followed by mAb. Binding of mAb was compared with that achieved in the absence of fB. Solid black line represents convertase followed by mAb; gray line represents mAb on bare C3b surface; dashed line represents convertase only to illustrate proenzyme decay curve.
mouse Fc region and CH1 domain is replaced with human IgG1 constant regions (19). Affinity of H17 for C3b was similar to that of the parent Ab (28 nM; Fig. 1B). The interaction was reversed and 3E7 was flowed across a high-density C3b surface (1200 RU). Affinity cannot be accurately measured in this latter orientation because of avidity effects and cross-linking of C3b via both Fab arms of 3E7; however, the data illustrate that 3E7 binds tightly to C3b-opsonised surfaces (Fig. 1C).

To test whether 3E7/H17 bound C3b in the AP convertase, C3bBb, binding of mAb was compared before and after convertase formation on the surface of the BIACore chip. To enable accurate quantitation of mAb binding to C3b in the convertase, we removed Bb bound to C3b after flowing mAb using sDAF. Both mAbs bound equally well to C3b and C3bBb on the surface, demonstrating that mAb binding to C3b is not hampered by the presence of Bb in the complex (Fig. 2A). High concentration of fB was flowed over the C3b surface in nickel-containing buffer (to stabilize the C3bB proenzyme complex) to investigate whether the Ba domain in intact fB interfered with mAb binding. The amount of 3E7 or H17 bound to the C3b surface in the absence or presence of fB was compared; data in Fig. 2B illustrate that intact fB blocked binding of either mAb.

mAbs 3E7 and H17 prevent AP C3 convertase formation by blocking binding of fB

The earlier data demonstrate that the mAbs bound C3b in the AP convertase, but not the preenzyme, implying that the mAbs and the Ba domain share a binding site on C3b. It remained important to assess whether the mAbs inhibited convertase formation. The surface was saturated with mAbs before flowing fB to analyze the effect of 3E7/H17 on fB binding to immobilized C3b. Binding of mAbs to C3b completely prevented subsequent attachment of fB and formation of the proenzyme, C3bBb (Fig. 3A). Both mAbs also prevented convertase formation in a dose-dependent manner (Fig. 3B).

mAb 3E7 inhibits lysis of target cells bearing preformed C3 convertase

The capacity of 3E7 to inhibit lysis of cells targeted by the AP was investigated by coating sheep E with AP convertase, C3bBb. Cells were incubated with increasing concentrations of 3E7 before developing lysis with plasma/EDTA (depleted of fH). Data in Fig. 4 demonstrate that 3E7 blocks AP-mediated lysis of E. Because of limited amounts of material, H17 was not tested in this assay, but DiLillo (19) reported that mAbs 3E7 and H17 were equally effective in blocking AP-mediated hemolysis of rabbit E.

mAbs 3E7 and H17 block C3-cleaving activity of both normal and C3NeF-stabilized AP convertase

Cleavage of C3 by the convertase was assessed in real time by SPR to test whether mAb binding affects C3 convertase activity. Convertase was formed by flowing fB and fD over immobilized C3b, then mAb was flowed across to bind to free C3b and C3bBb. C3 convertase activity was determined by flowing the substrate, C3, over the surface to generate nascent C3b that binds the chip surface through its exposed thioester. In the absence of mAb, convertase-generated C3b deposition was evident on the chip surface (Fig. 5A); however, preincubation with mAb prevented C3b deposition, demonstrating that mAb blocked interaction of the convertase with C3. To test the influence of C3NeF, we...
carried out a similar experiment where C3 convertase was formed in the presence of C3NeF-containing IgG; in this case, all normal (nonstabilized) convertase was removed using sDAF before flowing C3. Incubation of the highly active, C3NeF-stabilized convertase with 3E7 or H17 blocked C3 cleavage (Fig. 5B).

**FIGURE 5.** 3E7/H17 inhibit both normal and C3NeF-stabilized convertase by preventing C3 cleavage. (A) C3 convertase was formed on the surface of a CM5 chip by flowing fB and fD across immobilized C3b. Either 3E7 (gray sensorgram) or buffer (black sensorgram) was flowed across the surface followed by convertase substrate, C3. Binding of mAb blocked C3b deposition by the convertase, C3bBb. (B) C3NeF-containing C3 convertase was formed on the surface of the chip by flowing fB and fD across immobilized C3b in the presence of Ig-containing C3NeF. Two injections of sDAF decayed any normal convertase (nonstabilized) and ensured that only NeF-stabilized convertase remained. Either 3E7/H17 mAb (gray sensorgram) or buffer (black sensorgram) was flowed across the surface followed by convertase substrate, C3. Only a short pulse of C3 was passed over the surface because of the high activity of the C3NeF-stabilized enzyme. Binding of 3E7/ H17 blocked C3b deposition.

**FIGURE 6.** Three-dimensional structure of C3b-H17 Fab. (A) The C3b–H17 complex was purified by gel filtration chromatography. Fractions were analyzed by SDS-PAGE (bottom panel), revealing two peaks, the first corresponding to the complex and the second to the free excess H17. Peak fraction 15 was selected for its observation by EM. (B) EM of C3b-H17. The peak fraction was observed in the microscope revealing several views of the complex, including side views with the typical structural features of C3b being evident, and tilted views where density for H17 could be visualized. A two-dimensional average of single-molecule images corresponding to each type of view is shown. Scale bar, 10 nm. (C) Structure of C3b-H17. Two views of the pseudomolecular model of C3b-H17 with the EM density represented as a white transparent density where the atomic structures of C3b (red color; PDB ID 2I07) and Fab (blue color) have been fitted. The location of H17 on C3b clashes with the binding of the Ba fragment from fB, as determined by the location of the three SCRs in the crystal structure of C3Bb (32). Scale bar, 3 nm.
Structural basis for complement inhibition by mAbs 3E7 and H17

The C3b–H17 Fab complex was isolated by size-exclusion chromatography (Fig. 6A) and analyzed by EM to determine the molecular basis for the observed effects of 3E7 and H17 (Supplemental Fig. 1). Images of individual molecules of the complex were collected, and reference-free two-dimensional averages of this data set revealed that complexes were interacting with the support film in several distinct orientations (Fig. 6B). Strikingly, those views where the triangular shape of C3b was more evident (“side view”) revealed no apparent density that could be assigned to H17. This suggested that H17 projected perpendicular to C3b, and thus was not obvious in this orientation (Fig. 6B). In contrast, “tilted views” of C3b–H17 revealed a density absent in EM images of C3b alone (27), corresponding to H17 Fab.

A 30-Å resolution structure of the C3b–H17 Fab complex was obtained by angular refinement of the images collected (Fig. 6C). C3b–H17 revealed two distinct regions of density that could be readily assigned as C3b and H17 after computational fitting of the atomic structures of C3b (PDB ID 2I07) (28) and a Fab (PDB ID 1H0D) within the density of the EM structure (Fig. 6C). The crystal structures of C3b and Fab perfectly matched within the EM structure (cross-correlation coefficients of 0.89 and 0.93 for C3b and Fab, respectively), thus annotating every domain in the complex. The exact epitope mapped by the Fab could not be determined at this resolution, but the pseudoatomic model of C3b–H17 Fab revealed that H17 bound a site placed within the C3b macroglobulin 6 (MG6)-MG7 region, which is exposed as a consequence of the C3 to C3b conformational change; this site overlapped with the Ba binding site (Fig. 7). As predicted from the single-molecule images (Fig. 6B, Supplemental Fig. 1), H17 projects outward and perpendicular to C3b (Fig. 6C).

Discussion

The AP C3 convertase, C3bBb, is the key enzyme of the complement system, delivering tickover activation and amplification that are essential to roles in immune defense and waste disposal. AP activation and dysregulation also contribute to many disease processes (7); this can be caused by polymorphisms and mutations in AP proteins or by autoantibodies against AP components (6). C3NeF, autoantibodies that bind the AP C3 convertase, cause AP dysregulation by inhibiting both spontaneous and accelerated decay (12). To date, no specific therapies to counter the effects of C3NeF have been developed.

The mAb 3E7 and the humanized derivative, H17, were previously shown to bind C3b, iC3b, and C3(H2O), but not native C3 (19). In this study, we confirmed that binding to C3b was high affinity and stable. Binding of the mAb to C3b prevented the binding of Bb to form the proenzyme, C3bBb, and thus stopped further AP activation. The mAb also efficiently bound preformed C3bBb, implying that the mAb binding site overlapped the Ba-binding region on C3b. The importance of Ba binding in formation of the proenzyme has been emphasized in several earlier studies (27, 29, 30). Importantly, we also found that 3E7/H17 binding to the C3 convertase abrogated its C3 cleaving capacity and prevented further complement activation. The mAbs therefore have multiple effects as inhibitors of AP convertase formation and blockers of AP convertase C3-cleaving capacity. The compound effect is powerful inhibition of AP convertase formation and function, raising the possibility that the mAbs could play roles in therapy of AP-driven diseases. Although binding of 3E7/H17 prevents fH binding (data not shown), this will have no pathogenic effect, as we show that the mAb-bound convertase is nonfunctional.

C3NeF stabilize the AP convertase and are likely pathogenic because the convertase continues to cleave C3, causing unregulated activation of the AP. To test whether 3E7/H17 could be used as therapy in diseases associated with C3NeF, the C3NeF-stabilized convertase was generated using IgG isolated from the serum of a patient with a proven high-titer pathological C3NeF. C3 was added as substrate to test whether bound 3E7/H17 blocked C3-cleaving activity; the mAb completely blocked C3 cleavage by the C3NeF-stabilized enzyme. The mAb will therefore inhibit the activity of C3NeF in two ways: first, by preventing the formation of C3Bb by binding nascent C3b; and second, by binding to the preformed NeF-stabilized convertase and inhibiting C3-cleaving activity. Notably, although all C3NeF stabilize the convertase and most prevent accelerated decay, they are heterogeneous between, and possibly within, patients (12). So far, the inhibitory effects of 3E7/H17 have only been demonstrated for one C3NeF. It is entirely possible that some C3NeF-stabilized C3 convertases will be resistant to mAb inhibition; however, even in this eventuality, the mAb will still inhibit AP activation by preventing Bb binding to C3b, and thus convertase formation.

To shed further light on the mechanism by which 3E7/H17 affected convertase formation and activity, we analyzed complexes of C3b and a Fab fragment of H17 using high-resolution EM. The components of the complex were obvious in the images obtained with the Fab binding in the vicinity of the MG6 and MG7 domains of C3b. The transition from C3 to C3b is accompanied by major structural rearrangements that include a substantial shift of
the MG7 domain, likely explaining the finding that the mAb binds C3b, iC3b, and C3(H2O), but not native C3 (28). The structure of the proenzyme C3Bb (PDB 2XWJ) shows that the three SCRs that comprise Ba interact with the α’NT and CUB domains of C3b and occlude binding sites for decay accelerators, fH and DAF (31, 32). These Ba-binding domains are immediately adjacent to MG6 and MG7; indeed, α’NT and MG6 form one continuous exposed region that includes binding sites for fB, fH, and CR1 (32, 33). Binding of the Fab thus likely impedes subsequent binding of fB by occluding the Ba binding site and, when bound to the convertase, directly interferes with cleavage of substrate. Binding of the mAb could cause distortion of the MG ring in C3b, thus preventing C3 binding; alternatively, it might displace the Bb serine protease domain away from the substrate (C3), thus preventing cleavage (Fig. 7). It is also possible that, through binding to MG7, the Ab prevents the substrate, C3, from binding to the convertase. The crystal structure of cobra venom factor with C5 implicates this domain in intermolecular interactions, and a disease-associated 2-aa deletion within the MG7 domain has been shown to prevent substrate binding (33, 34). The binding site(s) of C3NeF on the C3 convertase has yet to be mapped; our data demonstrate that, at least for the C3NeF tested, these are distinct from the mAb and substrate binding domains described earlier.

Ab-based therapeutics represent one of the fastest growing sectors of the pharmaceutical industry and, with the rise of the anti-C5 mAb eculizumab, are now impacting on complement-driven diseases (35, 36). Different diseases may require that different steps of the complement cascade are blocked by targeting a specific component (37, 38). Eculizumab abolishes the generation of C5a and MAC formation, thereby reducing tissue damage caused by activation of the terminal pathway. Additional strategies for treatment include upstream inhibition of C3 activation, which can be mediated by 3E7/H17, as well as the newly developed CR2-fH chimeric construct, TT30 (21, 39). Several other mAb targeting AP components (fD, fB) are in development (37), although none has demonstrated that, at least for the C3NeF tested, these are distinct from the mAb and substrate binding domains described earlier.

Acknowledgments

We thank Dr. Leslie Casey and EluSys Therapeutics for generously providing the mAb H17.

Disclosures

R.P.T. owns stock in EluSys, a start-up company that is not publicly traded. S.R.d.C. has provided consultation to Alexion Pharmaceuticals. B.P.M. has provided consultation to Baxter. C.L.H. has an employment contract with GlaxoSmithKline; the entire study was completed and the manuscript assembles before this employment. All other authors declare no competing financial interests.

References


