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Lubka T. Roumenina, Damien Sène, Maria Radanova, Jacques Blouin, Lise Halbwachs-Mecarelli, Marie-Agnes Dragon-Durey, Wolf H. Fridman and Veronique Fremeaux-Bacchi

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Functional Complement C1q Abnormality Leads to Impaired Immune Complexes and Apoptotic Cell Clearance

Lubka T. Roumenina,^{*,†,‡} Damien Sène,^{†,§} Maria Radanova,[¶] Jacques Blouin,^{||}
Lise Halbwachs-Mecarelli,^{‡,#} Marie-Agnes Dragon-Durey,^{*,†,‡,||} Wolf H. Fridman,^{*,†,‡,||}
and Veronique Fremeaux-Bacchi^{*,||}

C1q plays a key role in apoptotic cell and immune complex removal. Its absence contributes to the loss of tolerance toward self structures and development of autoimmunity. C1q deficiencies are extremely rare and are associated with complete lack of C1q or with secretion of surrogate C1q fragments. To our knowledge, we report the first case of a functional C1q abnormality, associated with the presence of a normal C1q molecule. Homozygous GlyB63Ser mutation was found in a patient suffering from lupus with neurologic manifestations and multiple infections. The GlyB63Ser C1q bound to Igs, pentraxins, LPSs, and apoptotic cells, similarly to C1q from healthy donors. However, the interaction of C1r₂C1s₂ and C1 complex formation was abolished, preventing further complement activation and opsonization by C3. The mutation is located between LysB⁶¹ and LysB⁶⁵ of C1q, suggested to form the C1r binding site. Our data infer that the binding of C1q to apoptotic cells in humans is insufficient to assure self-tolerance. The opsonization capacity of C4 and C3 fragments has to be intact to fight infections and to prevent autoimmunity. *The Journal of Immunology*, 2011, 187: 4369–4373.

Complement is an innate surveillance system, orchestrating elimination of pathogens, as well as immunological and inflammatory processes (1). Finely tuned reactions lead to: pathogen destruction, silent clearance of apoptotic cells and immune complexes, and synapse maturation and tissue regeneration. Complement quantitative deficiencies, particularly of C1q, are associated with autoimmune diseases and recurrent infections. C1q, which is the recognition molecule of the classical complement pathway, is composed of three types of chains—A, B and C, assembled in a collagenous stalk (collagen-like region [CLR])—and six globular head regions (gC1q) (2). The gC1q domains carry the recognition capacity for Igs, pentraxins, apoptotic cells, and LPS, and their binding sites have been mapped (3–6). The stalk carries the binding site for the C1r and C1s serine proteases. Together, C1q, C1r, and C1s form the C1 complex (7).

In this article, we report the molecular characterization and the clinical history of a functional C1q abnormality due to a homo-

zygous mutation. We show that C1q was present in normal amounts in plasma and could bind to its various ligands. Nevertheless, it was unable to form a C1 complex with C1r and C1s and to deposit C3 via the classical pathway.

Materials and Methods

Patient description

A 32-y-old male patient, born in the Arabic Middle East, was diagnosed with systemic lupus erythematosus with CNS involvement (neurolupus). The neurolupus diagnosis was based on a medical history remarkable for autoimmune polymyositis in 1999 treated by methotrexate and steroids and, since 2002, the occurrence of autoimmune hemolytic anemia and thrombocytopenia, paraplegia, and seizures with brain atrophy and signs of subacute myelitis leading to thoracic spinal cord atrophy, as evidenced by brain and spinal cord magnetic resonance imaging. The baseline immunological findings included positive anti-nuclear Abs (1/640), anti-cardiolipin Abs, and lupus anticoagulant. No familial history of autoimmune disease or immunodeficiency was found in anamnesis. The patient received antiepileptic drugs (valproic acid) and a combination of i.v. cyclophosphamide (monthly pulse) and prednisone for 7 mo. At this time, cyclophosphamide was stopped because of a disseminated *Escherichia coli* infection; prednisone was maintained until the end of 2004. In 2005, the patient was referred to the Internal Medicine Department, Pitié-Salpêtrière Hospital, for evaluation of his neurolupus. The clinical findings were of a persistent paraplegia with neurologic bladder and mild splenomegaly. There was neither arthralgia nor cutaneous eruption. Biological abnormalities included autoimmune hemolytic anemia (10.9 g/dl) and thrombocytopenia (71 g/l), lymphopenia (0.8 g/l), and moderate postrenal insufficiency (creatinine, 134 μmol/l). Immunological findings were consistent with probable neurolupus with the presence of anti-ribosomal P Abs (1200 U/ml), anti-ribonucleoprotein Abs, and anti-cardiolipin Abs without lupus anticoagulant. The patient's medical history was also marked by a high susceptibility to viral and bacterial infections, which were often life threatening: severe EBV primo-infection in 1998, septic shock following *Salmonella typhimurium* infection in 1998, thoracic herpes zoster infection in 1999, disseminated *E. coli* infection in 2002, and acute respiratory distress syndrome in 2003. The last infectious event occurred 4 wk after the patient was admitted to the hospital with bacterial septic shock and multiorgan failure, leading to death.

Complement assays

Complement proteins, complement functional determinations, and gene sequencing were done following the standard procedures of Complement

*Centre de Recherche des Cordeliers, INSERM Unité Mixte de Recherche en Santé 872, 75006 Paris, France; [†]Université Pierre et Marie Curie, 75006 Paris, France; [‡]Université Paris Descartes, 75006 Paris, France; [§]Assistance Publique-Hôpitaux de Paris, Groupe Hospitalier Pitié-Salpêtrière, 75013 Paris, France; [¶]Department of Biochemistry, Molecular Medicine, and Nutrigenomics, Medical University Varna, 9002 Varna, Bulgaria; ^{||}Assistance Publique-Hôpitaux de Paris, Service d'Immunologie Biologique, Hôpital Européen Georges-Pompidou, 75008 Paris Cedex 15, France; and [#]INSERM U845, Hôpital Necker, 75015 Paris Cedex 15, France

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Address correspondence and reprint requests to Dr. Veronique Fremeaux-Bacchi or Dr. Lubka T. Roumenina, Service d'Immunologie Biologique, Hôpital Européen Georges Pompidou, 20-40 Rue Leblanc, 75008 Paris Cedex 15, France (V.F.-B.) or Cordeliers Research Center, INSERM Unité Mixte de Recherche en Santé 872, Team 13, 15 Rue de l'Ecole de Médecine, Entrance E, Floor 3, 75006 Paris, France (L.T.R.). E-mail addresses: veronique.fremeaux-bacchi@egp.aphp.fr (V.F.-B.) or lubka.roumenina@crc.jussieu.fr (L.T.R.)

Abbreviations used in this article: CH50, functional hemolytic test for classical complement pathway activity; CLR, collagen-like region; CRP, C-reactive protein; gC1q, globular domain of C1q; NHS, normal human serum; VBS, Veronal-buffered saline.

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Diagnostic Laboratory, Georges-Pompidou Hospital. Levels of complement proteins C1q, C1s, C5, C6, C7, C8, and C9 were determined by sandwich ELISA. Three polyclonal anti-C1q Abs were used for the detection of C1q: anti-C1q-HRP, chicken anti-C1q (Abcam), and biotinylated IgG, purified in-house from anti-C1q antiserum (Calbiochem). C3 and C4 were measured by nephelometry. Anti-C1q Abs were determinate against the collagen-like region (CLR) of C1q by ELISA, as described (8). Standard haemolytic assays were used to measure the classical complement pathway activity (CH50), the alternative complement pathway activity, the C1 functional activity, and the C2 functional activity.

Genetic analysis

Genomic DNA from the patient was obtained from peripheral blood leukocytes. Coding sequences of the C1q gene cluster were amplified with primers flanking all nine exons (the primer sequences are available from the authors upon request), purified using Multiscreen plates (Millipore), and sequenced with 96 capillary Sequencer 3730 using the dye terminator method (Applied Biosystems).

ELISA for formation of C1 complex

Microtiter wells were coated with purified polyclonal anti-C1q Abs (purified in-house from anti-C1q antiserum; Calbiochem) diluted 1/500 in PBS for 1 h at 37°C. The plates were then blocked with 2% BSA in Veronal-buffered saline (VBS; 5 mM sodium barbital, 145 mM NaCl [pH 7.4]) at 37°C for 30 min and washed with VBS with 0.05% Tween 20 and 1 mM CaCl₂. Wells were incubated with the patient's serum and normal human serum (NHS) and diluted 1/40, 1/80, 1/160, 1/320, 1/640, 1/1280, and 1/2560 in VBS with 0.05% Tween 20 and 1 mM CaCl₂ for 1 h at 37°C. After washing, goat antisera to human C1s (Quidel) was added at 1/500 dilution. Then, HRP-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch) was applied at 1/1000 dilution. After washing, wells were developed with *o*-phenylenediamine. The reaction was stopped with 3 N HCl, and absorbance was measured at 490 nm. Data are given as mean \pm SD from the replicates in one experiment. Three independent experiments were performed.

ELISA for interaction of C1q with target molecules

Microtiter wells were coated with 50 μ g/ml pooled IgG (Endobulin, Baxter), IgM (a kind gift from Dr. Jordan Dimitrov, Paris, France), C-reactive protein (CRP; Calbiochem), or LPS from *E. coli* (Sigma-Aldrich) in PBS for 1 h at 37°C. The plates were then blocked with 2% BSA in PBS at 37°C for 30 min and washed with HEPES (10 mM HEPES, 145 mM NaCl, 5 mM CaCl₂, and 0.05% Tween 20). Wells were incubated with the patient's plasma and pooled plasma from 100 healthy donors and diluted in HEPES, as indicated in each figure legend, for 1 h at 37°C. After washing, anti-C1q Abs biotinylated were added at 1/500 dilution. Then streptavidin-HRP was applied at 1/1000 dilution. Alternatively, anti-C1q-HRP (Abcam) was used to detect bound C1q. After washing, wells were developed with *o*-phenylenediamine. The reaction was stopped with 3 N HCl, and absorbance was measured at 490 nm. Data are given as mean \pm SD from the replicates in one experiment. Each experiment was performed twice with similar results.

Endothelial cell assay

HUVECs were isolated from human umbilical cord veins, as described (9), and cultured in M199 medium (Life Technologies, Paisley, U.K.) with 20% FCS. Third-passage quiescent cells were grown to confluence in T175 flasks. The overnight detached HUVECs were collected and shown to be late apoptotic cells (Annexin V⁺, propidium iodide⁺, trypan blue⁺) (10). Late apoptotic cells were incubated with 100 μ l 1/10 diluted with M199 medium AB group NHS, pooled NHS from five healthy donors, patient serum, or C1q-depleted serum (Quidel). The dilution was set to 1/10 to eliminate the alternative complement pathway. After a 30-min incubation at 37°C, the reaction was stopped with cold PBS-BSA-azide, and the cells were washed by centrifugation. The cells were labeled with anti-C3c or anti-C1q mAbs (Quidel), or a control mouse IgG1, followed by PE-labeled secondary Ab (Beckman Coulter). Cells were analyzed by flow cytometry on a Becton Dickinson FACSCalibur (Mountain View, CA), using CellQuest and FSC Express software. The results are presented as graphs of one representative experiment or as average relative fluorescence intensity (equal to the mean fluorescence intensity of each sample divided by the corresponding isotype control) from four independent experiments. Statistical analyses were performed using GraphPad Prism 5, using the *t* test.

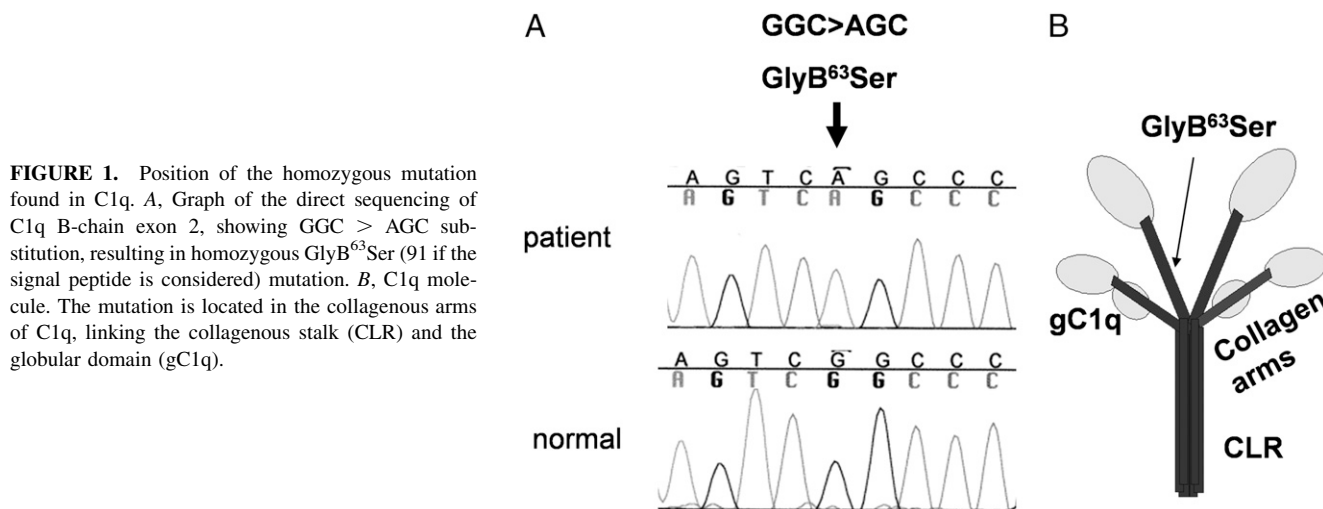
Results

Assessment of complement and mutation identification

The patient's complement proteins levels (C1–C9) were normal, including C1q. Nevertheless, CH50 and C1 hemolytic activities were undetectable. C2 hemolytic activity, as well as the alternative pathway hemolytic activity, was normal. No anti-C1q Abs were detected. CH50 remained undetectable when patient plasma was mixed with C1q-deficient plasma. Lysis was restored when patient plasma was added to C1r- or C1s-deficient plasma or when purified C1q was applied. A functional abnormality of C1q was hypothesized. Sequencing of the C1q gene cluster in the patient revealed a novel homozygous missense mutation in exon 3 at codon 48 in the B-chain that changed GGC to AGC and caused a glycine-to-serine exchange at position 63 (numbering without signal peptide) or position 91 (with signal peptide) in the C1q B-chain (Fig. 1A). This exchange was localized in collagen-like arms of C1q (Fig. 1B). The silent polymorphism in codon 276 in exon 3 of the A-chain (rs172378), corresponding to GlyA70, was homozygous for the A allele. GlyB63Ser C1q was recognized by three polyclonal anti-C1q Abs in the same manner as the one from normal plasma (data not shown).

C1q from the patient's serum can bind to its target molecules but cannot make a C1 complex

Binding activities of GlyB63Ser C1q toward IgG, IgM, CRP, and LPS were analyzed and compared with the binding from



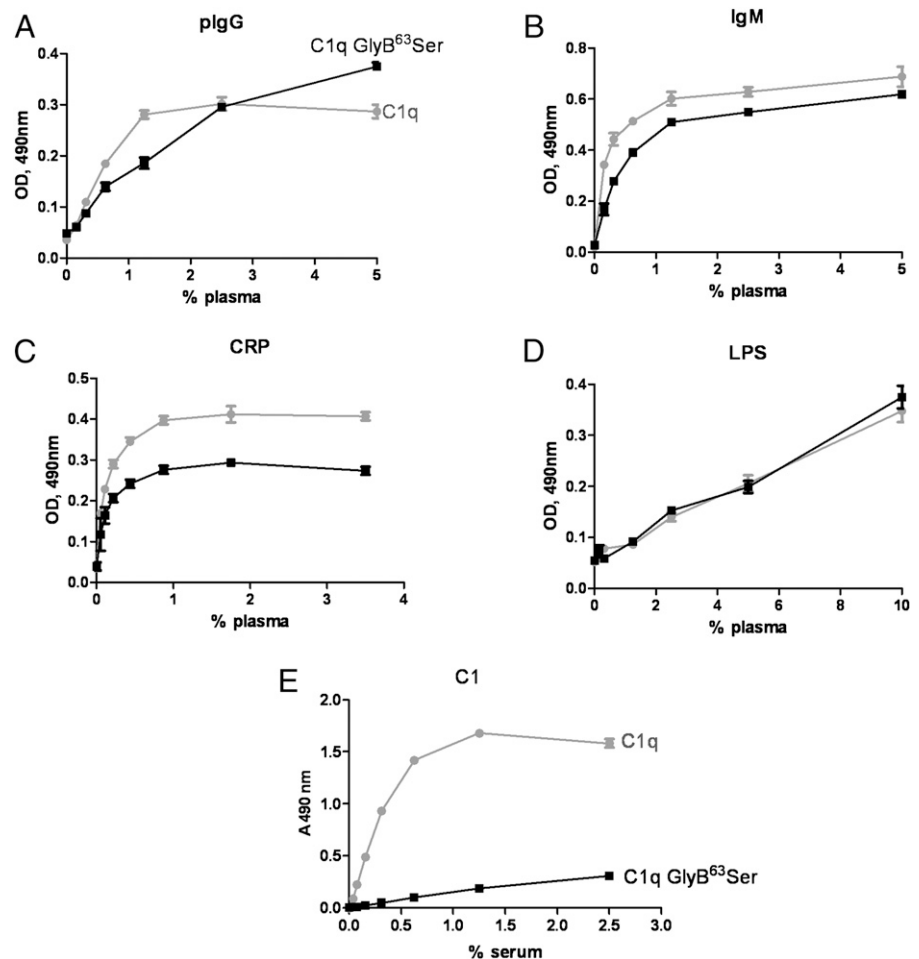


FIGURE 2. Binding of GlyB⁶³Ser C1q to target molecules. The capacity of C1q, present in the patient's serum, to bind to its target molecules was assessed by specific ELISAs. *A*, Pooled IgG. *B*, IgM. *C*, CRP. *D*, LPS from *E. coli*. The amount of bound C1q was revealed by anti-C1q-HRP. *E*, Formation of C1 complex in the patient's serum. Anti-C1q Abs were coated on a microtiter plate and incubated with different dilutions of normal and the patient's sera. C1 complex was developed by anti-C1s Abs, demonstrating absence of C1 in the patient's serum. The ability of mutant C1q to interact with different target molecules was assessed when each molecule was coated on the plate and further incubated with the patient's and normal sera.

normal plasma (Fig. 2*A–D*). No major difference was observed for LPS, IgG, or IgM (Fig. 2*A*, 2*B*, 2*D*). The binding of patient C1q to CRP was decreased 30% compared with normal plasma (Fig. 2*C*).

The ability of C1 subcomponents to form intact C1 complex was assessed in NHS and patient serum using this ELISA approach. C1q-associated C1s was readily detectable in NHS, but the signal was not observed using the patient's serum (Fig. 2*E*).

Mutant C1q binds more strongly to late apoptotic cells but does not lead to classical pathway-mediated opsonization by C3 fragments

To assess the ability of C1q to bind to late apoptotic primary HUVECs (90% Annexin V⁺ propidium iodide⁺), these cells were incubated with 1/10 diluted NHS, the patient's serum, or C1q-immunodepleted serum (Fig. 3*A*). Binding from the patient serum was markedly increased, exceeding the level seen in NHS

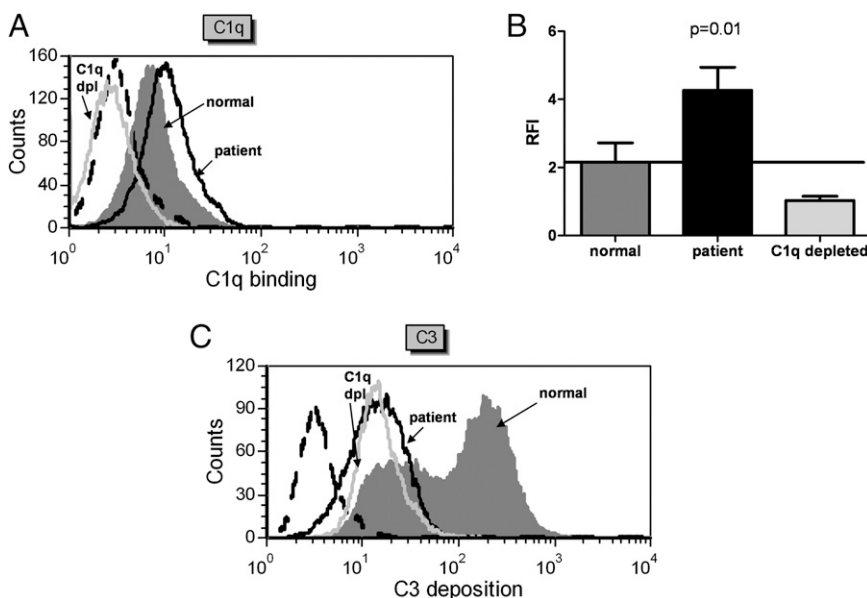


FIGURE 3. C1q binding and C3 deposition on late apoptotic cells. Late apoptotic HUVECs were incubated with 1/10 diluted pooled NHS, C1q-immunodepleted serum, or the patient's serum. Anti-C1q and anti-C3c mAbs were used to probe bound C1q and C3 by flow cytometry. *A*, A typical graph for C1q binding. Dashed line represents the isotype control, the shaded gray graph is pooled NHS, the light gray line is C1q-immunodepleted serum, and the black line corresponds to the patient's serum. *B*, C1q binding, expressed as mean relative fluorescence intensity (mean fluorescence intensity divided by the corresponding isotype), from four independent experiments using the patient's serum was compared with normal serum or C1q-immunodepleted serum. *C*, Typical graph for C3 deposition. Dashed line represents the isotype control, the shaded gray graph is pooled NHS, the light gray line is C1q-immunodepleted serum, and the black line corresponds to the patient's serum.

by >2-fold (Fig. 3B). C1q-immunodepleted serum showed no detectable C1q binding. Strong C3 deposition was detected from NHS (pooled and from individual donors). The level of C3 binding from the patient's serum was minimal and similar to that observed for C1q-immunodepleted serum, despite the increased C1q binding (Fig. 3C).

Discussion

C1q plays a key role in apoptotic cell and immune complex removal, and its absence contributes to the breaking of the tolerance toward self structures and development of autoimmunity (11–13). In this article, we reported, to our knowledge, the first case of a functional C1q abnormality. The patient presented with neurolupus and multiple infections. The patient's C1q had preserved integrity, because it was recognized normally by three anti-C1q polyclonal Abs. The existence of dysfunctional C1q in lupus patients was suggested previously but, in all cases, only surrogate C1q fragments or low m.w. C1q were detected in low concentrations and unable to assemble the entire molecule of C1q (14). This situation may be considered a quantitative deficiency and is different in nature from the mutant C1q molecule reported in this article, which retains its normal structure but is incapable of performing part of its functions. Preserved binding to target molecules via gC1q suggested maintenance of overall integrity of the globular domains. Although the patient's C1q was able to bind to the apoptotic cells, protein, and bacterial ligands, these interactions were not followed by classical pathway activation and opsonization with C3 fragments. These phenomena were due to GlyB⁶³Ser mutation in the collagenous arms of the C1q B-chain, which impaired C1q binding of the C1r₂C1s₂ complex. The mutation is located between LysB⁶¹ and LysB⁶⁵ of C1q, which were suggested to form the C1r binding site (7, 15). Obtained results validated the structural prediction of the importance of this region for the formation of the C1 complex and explained the observed lack of classical pathway activation and C3b deposition on activating surfaces. C3b and its degradation products are of importance for the proper clearance of the apoptotic cells via interaction with CR3 on monocytes, macrophages, and dendritic and microglial cells (12, 16). Therefore, GlyB⁶³Ser could be classified as a loss of function in terms of complement activation and apoptotic cell opsonization.

C1q also binds directly to apoptotic cells and participates in their opsonization (17–19). The exact ligands of C1q on dying cells are not well studied, but include cell-derived molecules, like phosphatidyl serine and DNA (20–22), or blood-borne factors, such as CRP, serum amyloid P, pentraxin 3, and natural IgM (23–27). All of these molecules are recognized by the globular domain of C1q (3, 19, 28–30). Failure of C1q to opsonize apoptotic cells results in defective phagocytosis by monocytes (12), decreased threshold of dendritic cell activation (31), and, hence active immune response, generating autoantibodies, such as in lupus (11). Because the C1r₂C1s₂ binding site was suggested to overlap with the one for the C1q receptor calreticulin, involved in the phagocytosis of C1q-covered targets (32), the mutation may affect this function. Strikingly, GlyB⁶³Ser C1q bound more strongly to the late apoptotic cells' surface. This increased binding was not due to enhanced interaction with IgM or CRP, because the binding to IgM was unaffected by the mutation and the binding to CRP was moderately decreased.

Another hallmark of autoimmune diseases, such as lupus, is the inefficient clearance of immune complexes via C1q binding and classical complement pathway activation. C1q deficiency or anti-C1q autoantibodies hamper this process (33). The patient described

in this study was negative for anti-C1q IgG, and the binding of GlyB⁶³Ser C1q to IgG and IgM was normal. Nevertheless, the classical pathway was not activated on immune complexes (IgG-bearing sheep erythrocytes in hemolytic tests) because of the inability to form a C1 complex and, hence, to cleave C4 and C3. Lack of classical-pathway activation is characteristic of C1q, C1r, C1s, and C4 deficiencies, which are associated with autoimmune manifestation in >80% of cases (11, 34). C4 and C3 opsonization is required for the clearance of immune complexes via erythrocyte CR1, and the mutant C1q's inability to activate the classical pathway C3-convertase probably results in inefficient immune complex clearance.

C1q-deficient patients suffer from severe infections, including septicemia in >35% of cases (34, 35). The patient described in this article experienced multiple infections and died from septic shock, although the GlyB⁶³Ser C1q bound normally to LPS. C1q binding alone may not be sufficient to eliminate the pathogens. Its inability to deposit C3 resulted in defective host protection.

Recently, C1q was found to participate in synapses remodeling and in the clearance of damaged CNS neurons after neuronal injury and neurodegenerative disorders (36–38). Again, both C1q and C3 binding to damaged neurons is required for efficient silent phagocytosis of apoptotic cells (16). Eighteen percent of the C1q-deficient patients presented with neurologic manifestations, as did the patient described in this article (35, 39), but no neurolupus was found in C1r-, C1s-, or C4-deficient patients (14). Therefore, the peculiar pattern of opsonization by GlyB⁶³Ser C1q and, potentially, the impaired interaction with calreticulin, may affect neuron clearance, leading to neurolupus.

In conclusion, we report, to our knowledge, the first case of a functional C1q abnormality associated with normal C1q levels in plasma. The clinical features and the experimental findings for GlyB⁶³Ser C1q indicated that the abnormal function of C1q is associated with repetitive infections and autoimmunity, particularly with neurologic manifestations. In GlyB⁶³Ser C1q, the target molecules' binding sites were intact, allowing interaction with apoptotic cells, immune complexes, and pathogens, but no further signal could be transmitted because the mutation destroyed the reaction site where C1r₂C1s₂ and the C1q receptor bound. The clinical history of the patient further demonstrated that opsonization of the apoptotic cells by C4 and C3 fragments is required to maintain self-tolerance.

Disclosures

The authors have no financial conflicts of interest.

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