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Hereditary complement C7 deficiency in nine families: Subtotal C7 deficiency revisited

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Deficiencies in terminal complement components, including the component C7, are uncommon and associated with an increased risk of recurrent systemic neisserial infection. A total of 22 molecular defects have been reported in the C7 gene with both complete (C7Q0) and subtotal (C7SD) C7 deficiencies. In this study we report the molecular basis of nine new cases of C7 deficiencies that were characterized by exon-specific sequence analysis. Seven different C7 gene mutations were identified corresponding to small deletions ($n=2$), splice site changes ($n=1$) and single base pair substitutions leading to nonsense ($n=1$) or missense ($n=3$) mutations. Altogether, three changes of the C7 gene (G357R, R499S and 5′ splice donor site of intron 16) account for half of the molecular defects which emphasize that a restricted number of molecular abnormalities are involved in this deficiency. We identified two patients with combined C7Q0/C7SD(R499S) and established the C7SD(R499S) frequency at about 1% in normal Caucasian population. We demonstrated that C7(R499S) mutant protein is retained in the endoplasmic reticulum whereas the wild-type C7 is located in the Golgi apparatus. Our results provide evidence that R499S represents a loss-of-function polymorphism of C7 due to a defective folding of the protein.

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Introduction

As an important component of the innate immune system, complement provides the initial response to prevent infections by pathogenic microorganisms. Complement may be activated *via* the classical, the alternative or the lectin (*i.e.* mannan-binding lectin and ficolins) pathways. All lead to cleavage of C3 into C3a and C3b. Binding of C3b enables a better clearance of pathogens and immune complexes as well as the generation of the lytic membrane attack complex (MAC), C5b-9. The complement component C7 is one of the five plasma proteins that interact sequentially to form the MAC. After cleavage of C5, the terminal components C6, C7, C8 and C9 bind to form a hydrophilic channel through the membrane of the

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Abbreviations: **C6SD:** subtotal C6 deficiency · **C7Q0:** complete C7 deficiency · **C7SD:** subtotal C7 deficiency · **FIMAC:** factor I-membrane attack complex · **MAC:** membrane attack complex · **PDI:** protein disulfure isomerase · **TRITC:** tetramethyl-rhodamine isothyocyanate · **TSP1:** thrombospondin type 1 domain · **WGA:** wheat germ agglutinin

Table 1. Clinical features of meningococcal systemic infection in nine unrelated C7-deficient patients

Patient	Sex	Number of infection	Age at infection (years)[b)]	Serogroup[b)]
I	F	1	18	NT P1-2.5
II	F	2	19 and 22	W135 and N/A
III	M	1[a)]	N/A	N/A
IV	F	2	15 and 18	A and A
V	M	2	5 and 5	B and N/A
VI	F	3	N/A, N/A and 17	B and N/A
VII	F	1 (+2 *H. parainfluenzae* meningitis)	22 (5, 19)	B
VIII	F	3	N/A, 21 and 33	N/A, N/A and Y
IX	F	1[a)]	N/A	N/A

[a)] Patients III and IX presented at least one meningococcal infection but the follow-up was not available.
[b)] N/A: not available.

target cells causing cell death [1]. Other sublytic functions on nucleated cells are described including protection against complement-mediated lysis, procoagulant and proinflammatory effects [2–4].

The C7 gene maps to 5p12-14 at the same location as C6 and C9 genes and is composed of 18 exons [5, 6]. The single polypeptide chain of C7 is composed of 821 amino acid residues and is biochemically and structurally closely related to other terminal complement complex factors, especially C6 [7]. They share the same modular organization including a thrombospondin type 1 (TSP1) domain, an LDL receptor domain, a cysteine-poor region designed as MAC/perforin, an EGF receptor domain, and two pairs of cysteine-rich modules [the short consensus repeats and a factor I-MAC (FIMAC) domain].

Terminal complement component deficiencies including C7 deficiency are uncommon. They are associated with an increased risk of recurrent systemic neisserial infection [8]. To date, 22 molecular defects associated to complete (C7Q0) or subtotal (C7SD) C7 deficiencies have been reported. Five cases of C7SD resulting from R499S substitution and one case resulting from R198E, both defined by very low levels of C7, have been described [9–13]. The R499S mutation was reported alone or combined with a subtotal C6 deficiency (C6SD), a mutation located at the 5′ splice donor site of intron 15 of the C6 gene, which also leads to very low levels of C6 [14]. The mechanisms and the functional consequences of the R499S mutation have been poorly investigated.

In the present study, we report the genetic basis of the C7 deficiency in nine patients with a clinical history of *Neisseria meningitidis* infection. We investigated the biological consequences of the R499S mutation with *in vitro* synthesis of recombinant proteins and demonstrated that C7SD(R499S) is secondary to a defective protein folding independently of the C6 gene.

Results

Patients

The clinical history of the nine index cases is summarized in Table 1. All patients were of Caucasian origin. All patients experienced at least one meningococcal systemic infection which led to complement system exploration. These infections were often recurrent. All patients recovered after antibiotic chemotherapy. The infecting serogroup was determined in half of the infectious episodes (three B, two A, one Y, one W135 and one non-serotypeable). Median age of the patients at the time of the first infection was 17.5 years. Three family members also experienced meningococcal disease (the brother of patient III, sister of patient VIII and father of patient II). Other family members were healthy. Patient III and his brother also presented cystinosis, a metabolic affection with end-stage renal failure in childhood.

Complement component assessment

In seven out of the nine cases diagnosed as complete C7 deficiency, the patient's complement profile was characterized by the lack of detectable CH50 activity, lack of detectable C7 at a 1/40 dilution of plasma (<0.01% of normal values), normal levels of C4, C3, C5, C6, C8 and C9, and normal levels of hemolytic C2 activity (Table 2). For patient I, C7 level investigation was realized during acute meningococcal disease and C7 was undetectable at a 1/40 dilution of plasma sample. Nine months later, her C7 level became very low but detectable (0.1% of normal values) and CH50 activity restored at 8% of normal value. The plasma of patient II, the ninth case of our series, conserved a low CH50 activity (23%) and low level of C7 at about 1% of normal value. The daughter of patient VIII, the brother of patient III and the father of

Table 2. Results of complement component assessment

Patient	CH50 (%)	C7 (%)[d)]	C5 (%)[d, e)]	C6 (%)[d, e)]	C7 gene mutation	C6 WT/C6SD[f)]
I[a)]	<10	<0.01	139	80	R499S+G357R	C6 WT/C6SD
I[b)]	8	0.1	N/A	N/A		
II[b)]	23	1	112	120	R499S+IVS16+2	C6 WT/C6 WT
III[b)]	<10	<0.01	129	133	Q681X	C6 WT/C6 WT
III[c)]	83	7	N/A	N/A		
Iv[b)]	<10	<0.01	70	94	G357R+1924delAG	C6 WT/C6 WT
V[b)]	<10	<0.01	136	144	C41W	C6 WT/C6 WT
VI[b)]	<10	<0.01	N/A	N/A	G357R	C6 WT/C6 WT
VII[b)]	<10	<0.01	N/A	N/A	G357R	C6 WT/C6 WT
VIII[b)]	<10	<0.01	128	137	1741delT+IVS16+2	C6 WT/C6 WT
IX[b)]	<10	<0.01	90	120	IVS16+2	C6 WT/C6 WT
Normal values	70–130	80–120	80–120	80–120		

a) At the acute phase.
b) At the time of diagnosis (at distance of the acute phase).
c) Patient affected by cystinosis, results 1 year after renal transplantation.
d) Hemolytic or antigenic test.
e) N/A : not available.
f) IVS15+2 T>C.

patient II also presented a total C7 deficiency. Patient III and his brother were also affected by cystinosis, an inherited metabolic disease that caused renal failure in both children and led to renal transplantation. Preceding transplantation, they had no detectable CH50 activity and no detectable C7 in plasma samples collected over several years. Since renal transplantation, CH50 activity has been restored for both children and their C7 levels remained stable between 7 and 9% of normal value.

Molecular genetics

All exons of the C7 gene of the nine patients were amplified by PCR and sequenced using forward and reverse primers. Sequence analysis revealed two molecular defects in the C7 gene of each patient (Table 3). Four patients (patients I, II, IV and VIII) were compound-heterozygous and five (patients III, V, VI, VII and IX) carried a homozygous genetic alteration in the C7 gene. We found four mutations previously reported on 13 independent chromosomes. The mutation Gly357Arg and the nucleotide substitution in the splice donor site after exon 16 (c.2350+2 T>C) were found in six and four independent chromosomes, respectively. Patients I and II were heterozygous for the missense mutation Arg499Ser, previously described to be associated with C7SD phenotype. Patient VIII was heterozygous for a small deletion of two nucleotides (c.1924delAG) that would lead to a frameshift and to premature translation termination causing the production of the truncated C7 protein. Two novel mutations and one new deletion were identified. Indeed, patient III and patient V were homozygous for an allele carrying the substitutions Q681X (c.2107 C>T) and C41W (c.189 T>G), respectively. No other abnormalities were detected in patient's C7 exons. Two common polymorphisms, namely M/N polymorphism due to c.1759 A>C substitution in exon 13 [15] and the polymorphic c.1162 G>C substitution in exon 9 [16], were also detected.

Blood samples of some close relatives were available except for patient VI, and genetic analyses were performed in order to detect the mutations found in propositus. The same molecular abnormalities were found in all samples analyzed from the patient's parents. The mutations found in the parents were heterozygous except for the father of patient II, who was homozygous for the 5′ splice donor site mutation in intron 16. This latter case provides an extra independent chromosome that can be included in our analysis of C7 mutations. Six brothers and sisters were analyzed, four were heterozygous for one of the propositus molecular defects, one was homozygous for the same defect and one had no mutation. Both children of patient VIII were heterozygous for their mother's mutation.

Since the R499S mutation in the C7 gene is known to be associated with the C6SD allele, we sequenced the exon 15/intron 15 junction of the C6 gene for all patients. Patient I and her mother were heterozygous

Table 3. Molecular defects of the C7 gene associated with complete C7 deficiency in index cases in nine unrelated families

Molecular defect		Consequences	Number of chromosomes[a)]	Patient[b)]	References
Exon 3	c.189 T>G	C41W	2	V[c)]	
Exon 9	c.1135 G>C	G357R	6	I[d)], IV[d)], Vi[c)], VII[c)]	[12, 43–45]
Exon 11	c. 1561 C>A	R499S	2	I[d)], II[d)]	[13, 14]
Exon 12	c.1741–3delT	Phe569fs 14 missense then stop	1	VIII[d)]	
Exon 14	c.1924delAG	Ser620fs 10 missense then stop	1	IV[d)]	[12, 13]
Exon 15	c.2107 C>T	Q681X	2	III[c)]	
Intron 16	IVS16+2 T>C	Splicing defect	4	II[d)], VIII[d)], IX[c)]	[25]

[a)] Number of independent chromosomes observed with the molecular defect in patients or relatives in our study (alleles observed in blood donors are not reported in this table).
[b)] Patient carrying the mutation.
[c)] Homozygous for the mutation.
[d)] Heterozygous for the mutation.

for C7SD(R499S) and C6SD alleles. None of the other patients presented the C6SD mutation.

C7SD(R499S) prevalence in the normal population

In order to evaluate the frequency of the C1561A substitution associated with the C7SD(R499S) allele in the normal population, 162 DNA samples from unrelated healthy individuals were genotyped by direct sequencing analysis of the exon 11 of the C7 gene. The analysis of this exon revealed the presence of three heterozygous C-to-A transversions at cDNA position 1561, associated in two cases with the heterozygous C6SD allele. Therefore, we estimated the prevalence of the C7SD(R499S) variant of the C7 gene at about 0.93% (3/324) in the Caucasian population.

In vitro synthesis and sub-cellular localization of the C7(R499S) protein

To determine whether the change Arg 499 to Ser affects C7 secretion, we measured the C7 synthesis in transient transfection assays. We cloned the cDNA of C7 in pcDNA3.1 TOPO V5HIS and introduced the C7(R499S) mutation by site-directed mutagenesis. The level of C7 expression in both supernatants and cell lysates from transiently transfected COS-1 cells was quantified using specific ELISA after 2 days of culture. As shown in Fig. 1, the recombinant C7 protein was secreted in the medium at a level of 2% of normal plasma level. However, COS-1 cells transiently expressing the C7(R499S) protein showed a major decrease of secretion. In three independent experiments, the secretion of C7(R499S) protein was less than 100-fold the secretion of C7 although it was still detectable at very low levels. Both C7 and C7(R499S) protein levels were comparable in the cellular extracts (Fig. 1). In addition, the C7 and C7(R499S) V5-tagged proteins were detected at the same molecular weight in both the supernatant and the cellular extract by Western blotting (Fig. 1). Similar results were obtained 72 h post-transfection (data not shown).

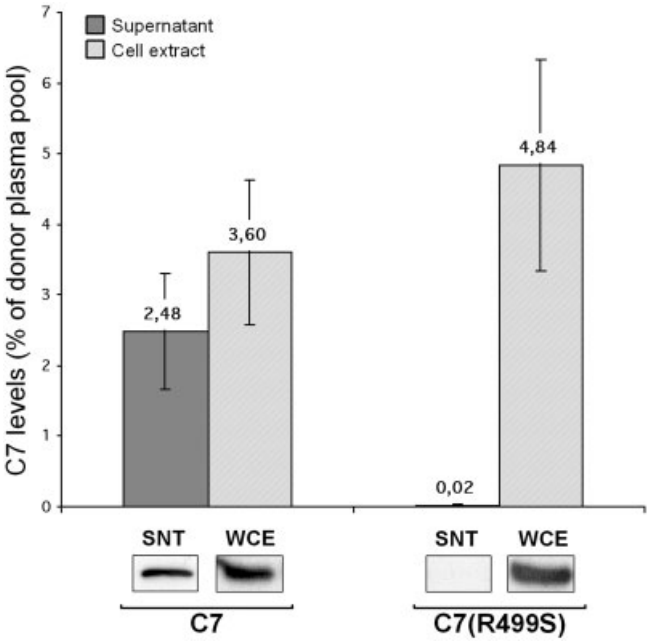

Figure 1. Quantitative and qualitative analysis of V5-tagged C7 and C7(R499S) protein expression in transfected COS-1 cells. Whole-cell extract (WCE) and culture supernatants (SNT) were harvested 48 h post-transfection and measured for C7 by sandwich ELISA and Western blotting with anti-V5 antibody. Data represent the means ± SD of triplicate determinations. C7 levels measured by ELISA are expressed in percentage of human plasma pool.

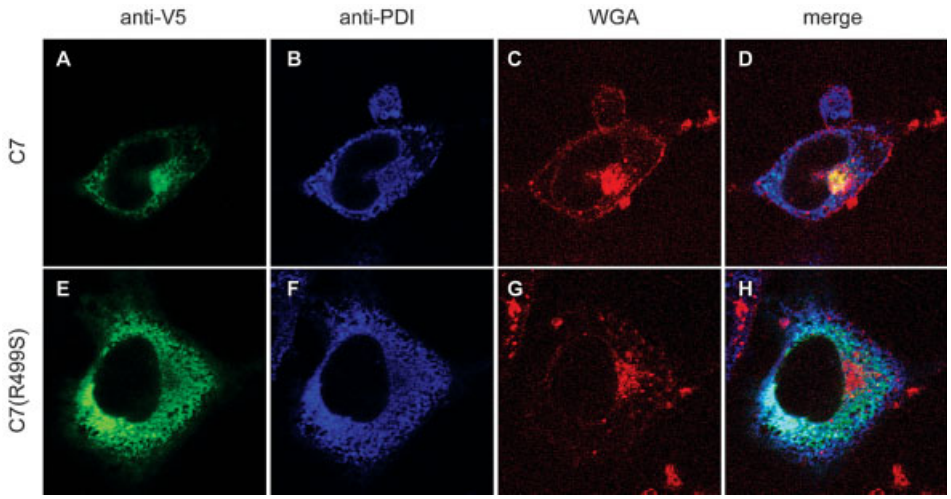

Figure 2. Sub-cellular localization of C7 and C7(R499S) in COS-1 cells. Photomicrographs of confocal immunofluorescent microscope analysis of transfected COS-1 cells expressing either C7-V5 (A–D) or C7(R499S)-V5 (E–H) proteins labeled with anti-V5 antibody (green staining; A, E). ER was stained with anti-PDI (blue staining; B, F) and Golgi apparatus was decorated with WGA (red staining; C, G). PDI localizes primarily in the ER compartment where it catalyses protein disulfide formation, isomerization or reduction, whereas WGA is a lectin specific for N-acetyl glucosaminyl moieties suitable for staining Golgi apparatus and endosomes. Merge of the triple-labeled cells showed the localization of the C7 protein in the Golgi apparatus (yellow staining; D) whereas the C7(R499S) mutant protein was retained in the ER (turquoise staining; H).

To analyze the pattern of sub-cellular distribution of the C7 isoforms and to explore their ability to be secreted, we investigated their localization by confocal microscopy. COS-1 cells were transiently transfected with the corresponding constructs, and the localization of the C7 and C7(R499S) proteins was analyzed by co-immunofluorescence using anti-V5 (Fig. 2A, E) and anti-protein disulfure isomerase (PDI) (Fig. 2B, F) antibodies, and tetramethylrhodamine isothyocyanate (TRITC)-wheat germ agglutinin (WGA) (Fig. 2C, G). In transfected COS-1 cells observed under laser confocal microscopy, C7 co-localized predominantly with WGA, revealing a Golgi apparatus location of the WT protein (Fig. 2D). In contrast, C7(R499S) co-distributed exclusively with PDI, showing that the mutant protein is retained in the ER (Fig. 2H).

Discussion

Molecular characterization of 27 cases of total or subtotal C7 deficiencies have been previously reported and 22 different molecular defects in C7 gene are currently known [9–13, 17]. Molecular mechanisms of nine new cases of C7 deficiency are reported in this study. We identified four known mutations in six patients, suggesting the existence of restricted genetic defects in the C7 gene. Among all known molecular defects, about half correspond to three mutations, namely G357R (25%, 18/72), R499S (15%, 11/72) and splice donor mutations in intron 16 (IVS16+2) (8%, 6/72). The G357R mutation represents a polymorphism with a prevalence of 1.1% in Israeli Moroccan Jewish population [18], but it was not found in 259 healthy Spanish individuals [12].

While defects in the C2 and C6 genes are known to be associated to a few mutation hot spots [19, 20], C7 mutations seem more heterogeneous. In agreement with the relative diversity of molecular defects observed previously carried by the C7 gene, we found three novel mutations expected to be deleterious for C7 protein production or function. Indeed, the single nucleotide deletion at position 1924 and the nucleotide change C2107T create stop codons leading to premature translation termination. The third mutation, T189G, is located in exon 3, which encodes the C-terminal part of the first TSP1 domain of C7. This mutation involves the substitution of a Cys to a Trp residue at position 41. This Cys is highly conserved in terminal complement factors (C6, C8α, C8β and C9) and in most TSP1 domains, where it is involved in a disulfide bound structurally important for domain stability [5, 21]. Its substitution for a large and highly hydrophobic residue is likely to disrupt C7 protein folding, resulting in the undetectable levels of C7 in the plasma.

Interestingly about half of the molecular defects described for C7 are located in the four last exons of the C7 gene. These exons encode one short consensus repeat and two FIMAC modules of C7 which are known to be involved in C7 interaction with the C345C domain of complement component C5 [22, 23]. This interaction has been shown to be crucial for MAC assembly and complement lytic activity [24]. Accordingly, we can propose that C7 protein lacking the two terminal FIMAC

domains would not be functional. Since these mutations lead to absence of C7 protein in the plasma, this C-terminal region is also likely to be crucial for C7 protein synthesis, secretion and/or stability [25].

C7SD is characterized by low concentration of C7 protein in plasma. The protein has a normal molecular weight but exhibits an altered isoelectric point. Since C7SD was not found in the normal population by isoelectric focusing, it was considered as a mutation rather than a polymorphism in the C7 gene [9]. In this work we established for the first time a prevalence of the C7SD(R499S) variant in the healthy Caucasian population at approximately 1% by sequencing analysis. The calculated frequency of subjects homozygous for the C7SD(R499S) allele is about 1:12 000. This prevalence is similar to the prevalence of C2 deficiency which is between 1:13 500 and 1:40 000, and was considered as the most frequent defect associated with human complement deficiency in the Caucasian population [26].

Combined C7SD and C6SD in which both proteins are expressed at very low levels, have been observed in homozygous form in two families [14]. In this report, we have also identified two patients and three healthy donors presenting combined C7SD(R499S)/C6SD alleles. A defect at the 5′ splice donor site of intron 15 of the C6 gene explains the low molecular weight of the C6 protein and is responsible for its expression at low concentration [27]. In subjects homozygous for the C6SD mutation, the C6 level is about 1–5% of normal but retains hemolytic and bactericide properties [27, 28]. The C7SD(R499S)/C6SD combined mutation characterizes a set of polymorphic DNA markers in the C6/C7 region, forming a distinct haplotype [29].

In this study, we have established that the mutation R499S does lead to a loss-of-function polymorphism in the C7 gene. We provide evidence that this mutation disrupts C7(R499S) trafficking. Indeed, in transiently transfected cells, WT C7 is found to localize in the Golgi apparatus, which is necessary for efficient protein secretion [30]. In contrast, C7(R499S) protein is expressed but retained in the ER. Consequently, C7(R499S) protein is barely detectable in the corresponding cell supernatant whereas normal C7 is efficiently secreted. Interestingly, traces of C7 were detected in the supernatant *in vitro* and also found in our two patients with the C7Q0/C7SD genotype, suggesting that low amount of C7 protein is secreted. According to the results of patients' CH50, C7(R499S) protein is able to incorporate into terminal complement complex and retains hemolytic activities. These observations lead to the conclusion that the C7SD(R499S) deficiency leads to a block in protein trafficking at the level of the ER and ultimately a decrease in C7 protein secretion.

The influence of sepsis on the C7 concentration in C7SD patients is still debated. A consumption of the very small amount of C7 during sepsis has been suggested [31]. Transition from subtotal to total C7 deficiency has already been reported in a Russian subject with R499S mutation who suffered from chronic otitis media [32]. In the plasma of patient I, which was collected near the acute phase of neisserial infection, the C7 level was nearly undetectable ($<$0.06%). Nine months later, C7 was quantified at very low level (0.12%) with detectable CH50 activity. In this case, the low amount of C7 in plasma could be consumed by generation of C5b-6 as a result of the infection. Therefore, our results support the theory that sepsis leads to consumption of the C7 protein *in vivo*.

C7 synthesis depends on its cellular origin and regulation. Endothelial cells are known to synthetize C7 *in vitro,* but the proportion of this cellular source of circulating C7 *in vivo* remains unknown [33–35]. We report two brothers with C7Q0 due to homozygous nonsense mutation and a genetic kidney disease which leads to an end-stage renal failure. The presence of stable plasmatic C7 level after renal transplantation in both brothers provides evidence of expression and secretion of C7 by the transplanted kidney *in vivo*. Our results emphasize that one kidney is sufficient to maintain C7 plasmatic level at 7–9% of normal values and to restore normal lytic complement function. Since the kidney is largely vascularized, the C7 may be produced by the kidney endothelial cells. Local C7 synthesis could therefore be a modulator of complement terminal pathway activation and might explain the variation of C7 production in patients during infection [36].

Individuals with deficiencies in terminal complement proteins have a high risk of contracting meningococcal infection due to the particular importance of cytolytic functions on the host defense against *Neisseria* [37]. The risk of neisserial infection in cases of C7SD remains debated. At this time no cases of neisserial infection have been reported in patients with a homozygous C7SD. Two subjects homozygous for combined C6SD and C7SD genes have also never experienced systemic meningococcal disease [38, 39]. However, cases of this disease have been seen in subjects compound-heterozygous for the C7SD and C7Q0 alleles [14, 40]. Thus patients with combined C7Q0/C7SD(R499S) may have insufficient C7 production, leading to an increased risk for systemic neisserial infection. Since the frequency of C7SD mutation in patients with meningococcal disease remains to be determined, we recommend the measurement of plasma C7 in patients with neisserial infection. We suggest that C7SD(R499S) polymorphisms results in impaired protection against *N. meningitidis* and may predispose to the risk of recurrent episodes of meningococcal disease.

Materials and methods

Patients

Patients included in this report were recruited from departments of internal medicine from European academic hospitals between 1989 and 2003. Biological investigations to support the diagnosis were carried out centrally in the Immunology Laboratory of Hôpital Européen Georges Pompidou (Paris, France), which is the reference laboratory for the investigation of the complement system in France. Blood samples were collected from patients and family members to extract DNA following informed consent. EDTA plasma samples were stored at –70°C. Clinical and biological evaluations of clinical disorders were carried out in clinical investigator's centers.

Complement assays

CH50 and C2 hemolytic activities were determined according to standard procedures [41]. Results were expressed as a percentage of the CH50 and C2 activities of a reference plasma pool obtained from 100 healthy blood donors. Plasma concentrations of C4 and C3 were measured by nephelometry (Dade Behring). Normal values established with pooled plasma from 100 healthy blood donors ranged between 220±120 mg/L and 1200±250 mg/L (mean ± 2 SD) for C4 and C3, respectively.

Concentration of C7 in plasma was established by means of a double-ligand ELISA. Briefly, Nunc MaxiSorp ELISA plates were coated with goat anti-human C7 IgG (Calbiochem). After washing and blocking of free reactive site with PBS containing 1% BSA, the 1/2000 diluted plasma was added for 1 h. After washing, the plates were incubated with in-house biotinylated goat anti-human C7 Ig prior to the addition of streptavidin-biotinylated horseradish peroxidase and further incubation for 30 min at 37°C. Enzymatic activity was revealed using orthophenyldiamine substrate. The same method was used to determine the C7 concentration in cellular extract and supernatant. The C5, C6, C8 and C9 concentrations in the samples were determined with analog ELISA using corresponding polyclonal antibodies (Calbiochem).

Genomic C7 DNA sequencing

Genomic DNA was extracted from whole-blood samples according to standard methods [42]. Exon-specific PCR was carried out with Taq DNA polymerase (Roche Molecular Biochemicals) for the 18 exons of C7 together with their flanking introns using specific primers as previously described in [25], or newly designed: 5′-CTATAACTTCCAAACAGTCCC and 5′-CAGTTCAGCCAAAGTGAATTC for exon 0, 5′-CCTGAT--CCTGATGATATTTGGGGGAAT for exon 3, 5′-GAGCCAAT-GAGCCAATGAATAATCTTGCC for exon 4, 5′-TTGCTTTG-TGCTTTGTGCCAATGAAGAG for exon 6, 5′-CCACAGTAGCTA-CCACAGTAGCTATAATCTGGA for exon 10, 5′-TACTGTG-TACTGTGCAAATGCATTGCAG for exon 15, 5′-AGGTCAGTG-AGGTCAGTGCATGTCAAATCA for exon 16, 5′-CTTTGGAGGT-CTTTGGAGGTGATGTTCTTTG and 5′-TTGGGAGAACAAAG-TGGGAGAACAAAGGATTGTG for exon 17, 5′-ACTTAGAATC-ACTTAGAATCCATGGTGTGCA and 5′-AAAGCTCAGCTCTTC-AGCTCAGCTCTTCCATCAA for C6 exon 15. The purified PCR products (Multiscreen® plates; Millipore) were sequenced by the Dye terminator cycle sequencing method (Applied Biosystems). Sequence analysis was performed using the Sequence Navigator® software. Each exon of C7 was analyzed for every index case. Mutated exons were sequenced in available family member's blood samples.

C7 cloning by RT-PCR and constructs

Total hepatic RNA was reverse-transcribed using High-Capacity cDNA Archive Kit (Applied Biosystems). The full-length human C7 cDNA was amplified by PCR using Pfu DNA polymerase (Stratagene) combined with Taq DNA polymerase (Roche Molecular Biochemicals) and specific primers (5′-TAC--TACTAACTCGAGCCCTGAATGTTTTCCCAAACA and 5′-CTG-CTGGGTTTCCGCAGCACAAGG). The PCR product was purified using the QIAQuick Gel Extraction kit (Qiagen) and cloned into the pcDNA3.1/V5-His TOPO TA expression vector (Invitrogen). The C7(R499S) mutation was introduced by site-directed mutagenesis (QuickChange® Site-Directed Mutagenesis kit; Stratagene). A stop codon was then inserted at the 3′ end of both C7 and C7(R499S) cDNA by site-directed mutagenesis on the previous constructs to allow the production of untagged proteins. The four different constructs were verified by sequencing.

Transfection and C7 analysis

COS-1 cells (ATCC) were plated (3×10^5 per well) on six-well tissue culture plaque (Falcon) and transfected with 2 μg of pcDNA3.1/V5-His-C7 using the FuGENE transfection kit (Roche Molecular Biochemicals) in 5% fetal calf serum-supplemented Dulbecco's medium. At different periods of time, supernatants were collected and cells were harvested, washed in ice-cold PBS and resuspended in 100 mL of lysis buffer (10 mM Tris-HCl pH 7.4, 50 mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 25 mM sodium pyrophosphate, 5 mM EDTA, 10 μg/mL pepstatin and protease inhibitors (Complete Protease Inhibitor Cocktail Tablets; Roche Molecular Biochemicals). After three freeze-thaw cycles in liquid nitrogen, the resulting cell lysates were cleared by centrifugation. Cellular extracts and supernatants were frozen at –20°C.

Cellular extracts and supernatants were analyzed by PAGE and the C7 and C7(R499S) V5-tagged proteins were detected by Western blotting with mouse monoclonal anti-V5 antibody (Invitrogen). ELISA was performed to determine C7 concentration.

Immunofluorescence

COS-1 cells were transfected as described above and grown 48 h on microscope cover slips in culture medium. After fixation in PBS/4% PFA for 15 min, cells were permeabilized for 5 min in PBS with 0.1% Triton X-100 and washed twice 5 min in PBS containing 1% FCS. The cells were incubated for 45 min at room temperature with mouse monoclonal anti-PDI antibody (1/300, Hybridome RL-77; Abcam) in PBS/1%FCS. Following washes, cells were incubated with Cyanin 5-

conjugated anti mouse IgG (1/200, donkey $F(ab')_2$ anti-mouse IgG-Cyanin 5; Jackson ImmunoResearch) and TRITC-conjugated WGA (1/200; Sigma) for 45 min at room temperature, then washed and incubated with monoclonal mouse FITC-conjugated anti-V5 antibody (1/1000; Invitrogen) for 45 min at room temperature. Slides were mounted with Vectashield (AbCys) and analyzed by laser-scanning confocal microscopy using Zeiss LSM 510.

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