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Serum complexes between C1INH and C1INH autoantibodies for the diagnosis of acquired angioedema

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Summary

Acquired angioedema due to C1-inhibitor (C1INH) deficiency (AAE) is caused by secondary C1INH deficiency leading to bradykinin-mediated angioedema episodes. AAE typically presents in adulthood and is associated with B cell lymphoproliferation. Anti-C1INH autoantibodies (antiC1INHAbs) are detectable in a subset of AAE cases and considered a hallmark of the disease. When free antiC1INHAbs and malignant tumors are not detectable, diagnosis relies on the finding of low C1INH levels and/or function, lack of family history and SERPING1 mutations, age at onset and low or undetectable C1q levels, none of which is specific for AAE. We tested the diagnostic value of a novel enzyme-linked immunosorbent assay (ELISA) for the detection of circulating complexes between C1INH and antiC1INHAbs (C1INH–antiC1INHAb) in the serum of 20 European AAE patients characterized on the basis of their complement levels and function. Free antiC1INHAbs were detected in nine of 20 patients [six of immunoglobulin (Ig)G class, two of IgM class and one simultaneously presenting IgG and IgM classes], whereas C1INH–antiC1INHAb complexes were found in 18 of 20 of the AAE cases, regardless of the presence or absence of detectable free anti-C1INHAbs. Of note, nine of 20 patients showed negative free antiC1INHabs, but positive C1INH–antiC1INHAb complexes in their first measurement. In the cohort presented, IgM-class C1INH–antiC1INHAb are specifically and strongly associated with low C1q serum levels. Detection of C1INH–antiC1-INHAbs provides an added value for AAE diagnosis, especially in those cases in whom no free anti-C1INH antibodies are detected. The link between IgM-class C1INH–antiC1INHAb complexes and C1q consumption could have further implications for the development of autoimmune manifestations in AAE.

Keywords: acquired angioedema, autoantibody, diagnosis, ELISA, immune complex

Introduction

Angioedema due to acquired deficiency of C1-inhibitor (C1INH) (AAE) is an ultra-rare disorder leading to bradykinin-triggered angioedema (AE) episodes. Its prevalence is unknown, although it has been estimated to be between 1 : 100 000 and 1 : 500 000 inhabitants [1]. AAE is frequently associated with underlying hematological diseases and often presents with inactivating anti-C1INH autoantibodies (antiC1INHAbs). C1INH belongs to the serpin family of protease inhibitors, which function by forming stable, sodium dodecyl sulfate (SDS)-resistant complexes with their target proteases. It controls the activation of the classical complement pathway by regulating the enzymatic activities of the C1r and C1s proteases. In addition, C1INH regulates activated coagulation factor XII (FXIIa), kallikrein (KK), activated coagulation FXI (FXIa) and tissue plasminogen activator (TPA).

The pathological basis of AAE is similar to the hereditary forms of angioedema due to C1INH deficiency (HAE),

with uncontrolled contact system activation and bradykinin (BK) release due to reduced levels or functional C1INH activity, which results in increased vascular permeability and subcutaneous/submucosal edema formation [2].

Two subtypes of AAE were historically considered [1]. Type I AAE was associated with lymphoproliferation and excessive C1-INH consumption in the absence of detectable autoantibodies against C1INH. In type II AAE, antiC1INHAbs were detected in serum. These autoantibodies turned the serpin C1INH into a substrate rather than an inhibitor, avoiding the formation of serpin–protease stable complexes [3]. In these cases C1INH was mainly detected in a cleaved, inactivated conformation. However, this classification is now considered artificial upon the observation that some AAE patients can simultaneously have lymphoproliferative diseases and antiC1INHAbs [4]. When detectable, antiC1INHAbs are the most robust and straightforward diagnostic feature in AAE patients. B cell proliferation, frequently with clonal characteristics, is associated with acquired C1INH deficiency and can be responsible for the production of the neutralizing antiC1INHAbs, as well as for an increased risk of lymphoproliferative disease [5]. Lymphoproliferation in AAE ranges from monoclonal gammopathy of uncertain significance (MGUS) to non-Hodgkin lymphomas, and it has been suggested that this pathological spectrum may reflect evolutionary stages of the same process, starting from expansion of anti-C1INH autoreactive clones [6,7].

Autoimmune manifestations are also common features of AAE and SLE, immune-mediated pancytopenia, Sjögren's disease, hemolytic anemia and anticardiolipin antibodies have all been documented [8–12]. Moreover, very low or undetectable C1q levels in serum is another hallmark finding in many of these patients which, in the presence of angioedema symptoms, low C4 concentration and C1INH levels and/or function, strongly suggests AAE [13]. C1q consumption is attributed to uncontrolled activation of the complement classical pathway due to high titers of circulating C1INH–antiC1INHAb complexes, eventually causing exhaustion of C1INH inhibitory activities. These complexes are detectable at a 1 : 1 ratio in the serum of AAE patients and their levels increase upon infusion of C1INH concentrate or by *in-vitro* incubation of AAE sera with C1INH [14,15].

In the present study we investigated the biochemical features, clinical presentation and the presence of antiC1INHAbs in the sera of 20 AAE patients and tested the performance of a novel enzyme-linked immunosorbent assay (ELISA) for the detection of C1INH–antiC1INHAb complexes as a diagnostic marker of AAE. Moreover, in two patients initially diagnosed of AAE without detectable free anti-C1INH autoantibodies, long follow-up studies allowed the detailed characterization of their complement profiles and the course of appearance of detectable free and C1INH-complexed autoantibodies.

Methods

Patients

Twenty-nine serum samples from 20 patients [12 Spanish (La Paz University Hospital, Madrid, Spain), five Swedish (Clinical Immunology and Transfusion Medicine, Lund, Sweden) and three Danish (Odense University, Odense, Denmark)] with a clinical diagnosis of AAE based on analytical C1INH deficiency and clinical criteria (conforming to Cicardi and Zanichelli [1]) with or without detectable anti-C1INH antibodies were obtained during asymptomatic periods. The three Danish patients were recruited from the Danish National HAE Centre as part of a nationwide study of C1INH deficiency in Denmark, and their clinical features were originally published by Bygum and Vestergaard [16]. Healthy donors with no analytical or clinical evidence of angioedema due to C1INH deficiency were obtained from the Immunology Unit of Hospital La Paz. The Ethics Review Panel of La Paz University Hospital reviewed and approved this study meeting the requirements of the 2013 Helsinki Declaration and its later amendments. All patients and controls included were duly informed and gave written consent to participate and having their samples biobanked.

Complement testing

The levels and function of complement proteins were determined as reported previously [17]. Briefly, C1INH, C4 and C3 serum levels were measured by nephelometry (Siemens, Marburg, Germany). C1INH functionality in plasma was quantified by the Berichrom® chromogenic assay (Siemens Healthcare Diagnostics, Eschborn, Germany) and serum levels of C1q were assessed by radial immunodiffusion (Binarid Radial Immunodiffusion kit; The Binding Site, Birmingham, UK). Anti-C1q antibodies were determined in all samples with the human complement C1q NL Bindarid kit (The Binding Site).

In-house ELISAs for the detection of free antiC1INHAbs and C1INH–antiC1INHAb complexes

Ninety-six-well plates (Costar 3590; Life Technologies SA, Madrid, Spain) were coated with either 1 μg of C1INH (for free antiC1INHAbs detection) (Berinert®, CSL Behring, Marburg, Germany) or 1 μg of polyclonal anti-C1INH IgG (for C1INH–antiC1INHAb complexes detection) (CompTech, Tyler, TX, USA) in carbonate/bicarbonate buffer pH = 9·2 at 4°C overnight and blocked with 3% gelatin Tween-phosphate-buffered saline (PBS) for 1 h at 37°C;

100 µl of 0·1% bovine serum albumin (BSA)–PBS-diluted duplicated serum samples from patients, healthy donors and a positive control were then seeded at convenient dilution factors (50, 200, 800, 1200) for 1 h at 37ºC. Detection was performed with PBS-Tween-diluted horseradish peroxidase (HRP)-labeled antibodies to human IgG (VITRO; Jackson Immuno Research, Madrid, Spain) diluted 1/50 000, human IgM (Nordic MUBio, Cultek, Madrid, Spain) diluted 1/1000 or human IgA (Nordic MUBio) diluted 1/1000. Reactions were developed for 15 min with 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS), stopped with 0·1% azide and read at 405 nm. For the detection of free anti-C1INH antibodies, positivity threshold was defined as threefold of the values obtained with two healthy donor sera at 1/50 dilutions.

Diagnostic performance of C1INH–antiC1INHAb complexes

The diagnostic performance of C1INH–antiC1INHAb complexes for AAE patients was evaluated using a receiver operating characteristic (ROC) analysis. The area under the curve (AUC), sensitivity, specificity and likelihood ratios were calculated for IgG and IgM complexes. Sera from six patients simultaneously presenting both free and complexed anti-C1INH autoantibodies (LP7-3, LP10 and LP11 for IgM- and LP8, OU1 and OU3 for IgG-classes, respectively; Table 1) were included as 'disease population' and 25 healthy donors (HD) were used as 'non-disease controls'. Clinical and biochemical data from patient LP8 can be found in Marbán Bermejo *et al.* [18].

In-vitro assembly of C1INH–antiC1INHAb complexes and neutralization of anti-C1INHAbs

In order to study the *in-vitro* assembly of complexes and neutralization of free anti-C1INHAbs, triplicate 50-µl serum samples LP2, OU1 and OU3 were incubated with 2, 6 and 20 µg of C1INH overnight at room temperature before C1INH-IgG quantification, as described.

Statistical analysis

For the *de-novo* complex assembly assay, the results were analyzed by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. The effects of serum C1INH–antiC1INHAb complexes on C1q and C4 levels were analyzed by Mann–Whitney test and Spearman's rank correlation. Statistical analyses were performed using the statistical package SPSS version 11.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

The ELISAS for detecting free antiC1INHAbs and C1INH–antiC1INHAb complexes were first tested in 20 normal sera stored at –80ºC for periods ranging from 0 to 24 months. Normal sera yielded mostly homogeneous signals, spanning from that of blank control to threefold-blank signal. Storage length at –80ºC did not show a significant effect on immune complex detection, while freeze–thaw cycles proved to increase unspecific detection in control samples (data not shown).

The characteristics of patients are shown in Table 1. All the patients in the cohort presented angioedema symptoms, low C1INH levels and/or function and most low C4 at diagnosis.

It is remarkable that only seven of 20 patients showed free IgG or IgM antiC1INHAbs in the first measurement, whereas the only two patients from whom we had several samples developed them in subsequent measurements. No patient showed free IgA antiC1INHAb. Also, 16 of 20 patients had positive C1INH–antiC1INHAb complexes in their first measurement, whereas the two patients with several samples developed them in subsequent samples. It is of note that nine of 20 patients showed negative free antiC1INHabs, but positive C1INH–antiC1INHAb complexes in their first measurement. Interestingly, patient LP7 first presented IgM C1INH–antiC1INHAb complexes and later free IgM antiC1INHAb, and patient LP12 first had detectable free IgG antiC1INHAb and later IgG C1INH-antiC1INHAbs. There were two patients with both negative free antiC1INHAb and C1INH–antiC1INHAb complexes (LP9 and LU4).

All the patients positive for free C1INHautoAbs of a given immunoglobulin class were also positive for the corresponding circulating complexes (nine of nine). One of these patients (LP10) showed both free IgM and IgG antiC1INHAbs, but only IgM C1INH-antiC1INHAbs.

Only two patients, LP9 and LU4, were considered negative for both assays despite having low C1INH levels or function and remarkable angioedema symptoms in both patients and lymphoproliferation in patient LP9.

A ROC curve analysis was performed with sera from patients simultaneously presenting both free antiC1INHAbs and C1INH–antiC1INHAb complexes and healthy donors, obtaining 0·96 [95% confidence interval (CI) = 0·9–1·02, P = 0·0013] and 0·94 (95% CI = 0·83–1·05, P = 0·0009) as values of AUC for the IgM and IgG ELISAs respectively. Cut-off values of 0·31 absorbance units (AU) (likelihood ratio 7·33) and 0·27AU (likelihood ratio 10·0) were established for IgM and IgG C1INH–antiC1INHAb complexes, respectively (Fig. 1).

Conserved C4 was detected in all the LP12 samples and one LP7 sample. In addition, conserved C1q concentrations were observed in all the samples from patients LP2, LP4, LP8, LP12 and OU3, who were positive for free IgG antiC1INHAbs and/or C1INH–antiC1INHAb complexes (patient OU3 was also slightly positive for IgM C1INH–antiC1INHAbs). Conversely, all the patients with

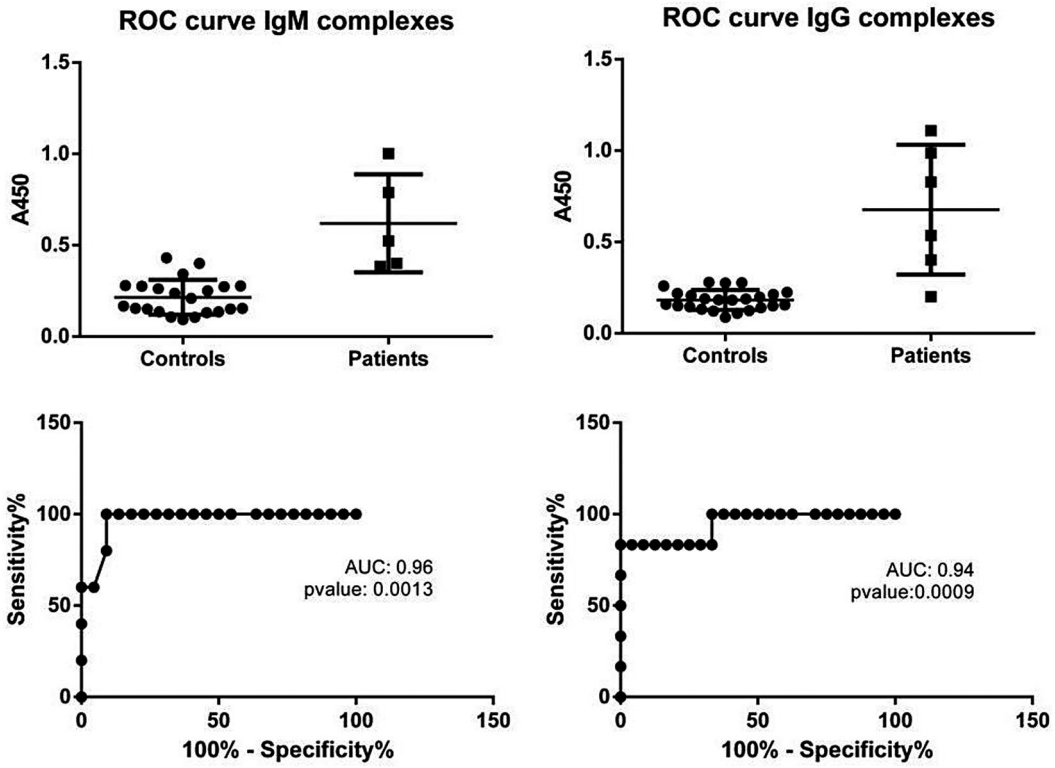
Table 1. Patient cohort

Sample code	Age	[C3] mg/dl	[C4] mg/dl	[C1INH] mg/dl	C1INH func (%)	[C1q] μg/ml	Anti-C1q	Free anti-C1INH	C1INH–IgM A_{405} units	C1INH–IgG A_{405} units	Diagnosis
	–	75–135	14–60	16–33	>50	56–276	–	–	>0·31‡	>0·27‡	–
LP1	74	93	1·33	16·5	22·2	0	–	–	0·46 (+)	0·27 (+)	SLE
LP2	75	80	8·8	34·2	14	180	–	G	0·203	0·677 (++)	No
LP3	83	110	1·4	3·04	12·45	0	–	–	0·753 (+++)	0·247	ANA/ENA/Ro/Jo+
LP4	69	37	<1·6	7·13	20	103	–	G	0·112	0·458 (+)	No
LP5	81	97	<1·6	7·84	8·39	0	n.d.	–	0·43 (+)	0·178	Leukemia
LP6	78	88,1	<1·6	8·17	12·96	0	–	–	0·597 (++)	0·143	SMZL
LP7-1, March 11	48	86	8·4	17·9	14	120	n.d.	–	0·208	0·157	No
LP7-2, September 11	49	95	16	12	10·9	0	–	–	0·742 (++)	0·148	No
LP7-3, April 13	51	141	<1·6	5·13	40	0	–	M	0·837 (+++)	0·132	CLL
LP8‡	40	110	2·69	11	19	180	n.d.	G	0·18	1·109 (+++)	No
LP9	45	117	1·56	15	6	0	–	–	0·117	0·219	MLUS IgA–κ
LP10†	79	111	1·33	7·6	8	0	–	M·G	0·4 (+)	0·198	BGUS IgG–κ and IgA–κ
LP11	45	147	1·56	5·82	10·77	0	+	M	1·002 (+++)	0·536 (++)	No
LP12-1, April 10	43	118	35·9	13	21·6	233	n.d.	–	0·167	0·127	No
LP12-2, November 13	46	128	25	14·8	51·4	328	n.d.	–	0·19	0·033	No
LP12-3, May 14	47	155	59·3	20·5	24·7	228	n.d.	–	0·13	0·07	No
LP12-4, June 15	48	128	28	13·6	27·9	191	n.d.	–	0·163	0·047	No
LP12-5, December 15	48	117	18·4	9·3	25·3	181	n.d.	G	0·211	0·085	No
LP12-6, May 16	49	145	25·9	15·5	35·9	240	n.d.	G	0·165	0·317 (+)	No
LP12-7, September 16	49	130	24·8	13·4	36·7	205	n.d.	G	0·132	0·557 (++)	No
LP12-8, March 17	49	n.d.	23·6	14·3	34·4	207	n.d.	G	0·139	0·455 (+)	No
LU1	64	n.d.	<2	5%	2	11%*	–	–	0·376 (+)	0·198	Lymphoma, M component and cured colon cancer
LU2	57	n.d.	5	15%*	n.d.	53%*	–	–	0·163	0·424 (+)	No
LU3	67	n.d.	< 2	12%*	n.d.	19%*	–	–	0·121	0·64 (++)	PR3–ANCA+ without vasculitis
LU4	45	n.d.	<2	30%*	n.d.	60%*	–	–	0·155	0·085	No
LU5	55	n.d.	<2	22%*	9	<6%*	–	–	0·313 (+)	0·087	No
OU1†	37	n.d.	<0·2	11	5	21	–	G	0·251	0·828 (+++)	No
OU2	70	n.d.	0·4	5	<20	<4	–	–	0·271	0·356 (+)	Waldenström/ MGUS, IgM κ M–component
OU3	78	n.d.	0·3	8	22	84	–	G	0·392 (+)	0·987 (+++)	CLL

Patient cohort. Twenty-nine samples obtained from 20 European patients with a clinical diagnosis of acquired angioedema (AAE) were included in the cohort. Twelve patients (LP) were followed at University Hospital La Paz (Madrid, Spain), five were referred to Clinical Immunology and Transfusion Medicine (Lund, Sweden) and three to Odense University Hospital (Odense, Denmark). †Cut-off values were determined by receiver operating characteristic (ROC) curve analysis, positivity is indicated by +, ++ and +++ signs according to an arbitrary scale based on the maximum/minimum A_{405} absorbance values obtained in the cohort. SLE = systemic lupus erythematosus; ANCA = anti-neutrophil cytoplasmic antibodies; ANA = anti-nuclear antibodies; ENA = extractable nuclear antigen autoantibodies; Ro = anti-Ro60 autoantibodies; Jo = anti-histidyl-tRNA synthetase autoantibodies; SMZL = splenic marginal zone lymphoma; SLL = small lymphocytic lymphoma; BGUS = bi-clonal gammopathies of undetermined significance; MBL = monoclonal B lymphocytosis; MLUS = monoclonal B lymphocytosis of undetermined significance; CLL = chronic lymphocytic leukemia; AHT = arterial hypertension; n.d. = not determined.

*Values presented as % of healthy donor plasma. Values < 6% are considered as out of the detection range; β, patient LP10 was previously described in [17]; †Patient LP8 was previously described in [18]; ‡Patient OU1 was positive for immunoglobulin (Ig)G class immune complexes.

free IgM antiC1INHAbs and/or C1INH–antiC1INHAb complexes showed low serum C1q. Moreover, one sample from patient LP7 first had normal serum C1q levels, when both free and complexed antiC1INHAbs were negative (LP7 1, March 11), but then decreased to undetectable levels when the patient developed free and/or complexed IgM C1INH autoantibodies (LP7, 2 September 11; LP7, 3 April 13).



Ig class	Cut-off (A_{450nm} units)	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	Likehood ratio
Ig M	0.31	100% (57 – 100%)	86% (65 – 97%)	0.96 (0.9-1.02)	7.33
Ig G	0.27	83% (45-99%)	91% (73-98%)	0.94 (0.83-1.05)	10.0

Fig. 1. Receiver operating characteristic (ROC) curve analysis. A ROC curve was developed using sera from 25 healthy donors and seven acquired angioedema (AAE) patients simultaneously presenting both free and complexed anti-C1-inhibitor (C1INH) antibodies of the immunoglobulin (Ig)M (LP7, LP10, LP11) or IgG class (LP2, LP8, OU1, OU3). For IgM C1INH–anti-C1INH antibody (C1INH–antiC1INHab) complexes, a cut-off value of 0·31 A_{405nm} units provided 100% sensitivity, 86% specificity and a likelihood ratio of 7·33. For IgG C1INH–antiC1INHAb complexes, a cut-off value of 0·27 A_{405nm} units yielded 83% sensitivity, 91% specificity and a likelihood ratio of 10.

Only one patient (LP1) diagnosed with systemic lupus erythematosus presented anti-C1q antibodies at low titer (Table 1). In our series, the presence of detectable IgM class C1INH–antiC1INHAb complexes was associated with signs of more marked complement activation (low C4 and C1q serum levels). All our patients positive for IgM-class complexes except patient OU3 presented low or undetectable C1q levels while those with IgG-class C1INH–antiC1INHAb complexes tended to have normal or slightly reduced C1q (median = 136 μg/ml; IQ range = 153·5). C4 levels, although also lower in patients with IgM C1INHAb, did not show significant differences between the two groups (Fig. 2). Interestingly, it is only IgM C1INH–antiC1INHAb complexes and not free IgM, free IgG or IgG C1INH–antiC1INHAb complexes that associate with very low C1q levels, which is exemplified by samples LP1, LP3, LP5, LP6, LP7.2 and LU1, presenting only IgM C1INH–antiC1INHAb complexes but not detectable free autoantibodies (Table 1).

Two different courses of appearance for C1INH–antiC1INHAbs, either preceding or following free anti-C1INH detection, could be documented in patients LP7 and LP12. LP7 had been followed-up over 3 years at the immunology unit in La Paz Hospital. The first sample, obtained in March 2011, was taken at angioedema onset and showed low C4 and C1INH functional levels, absence of detectable – free

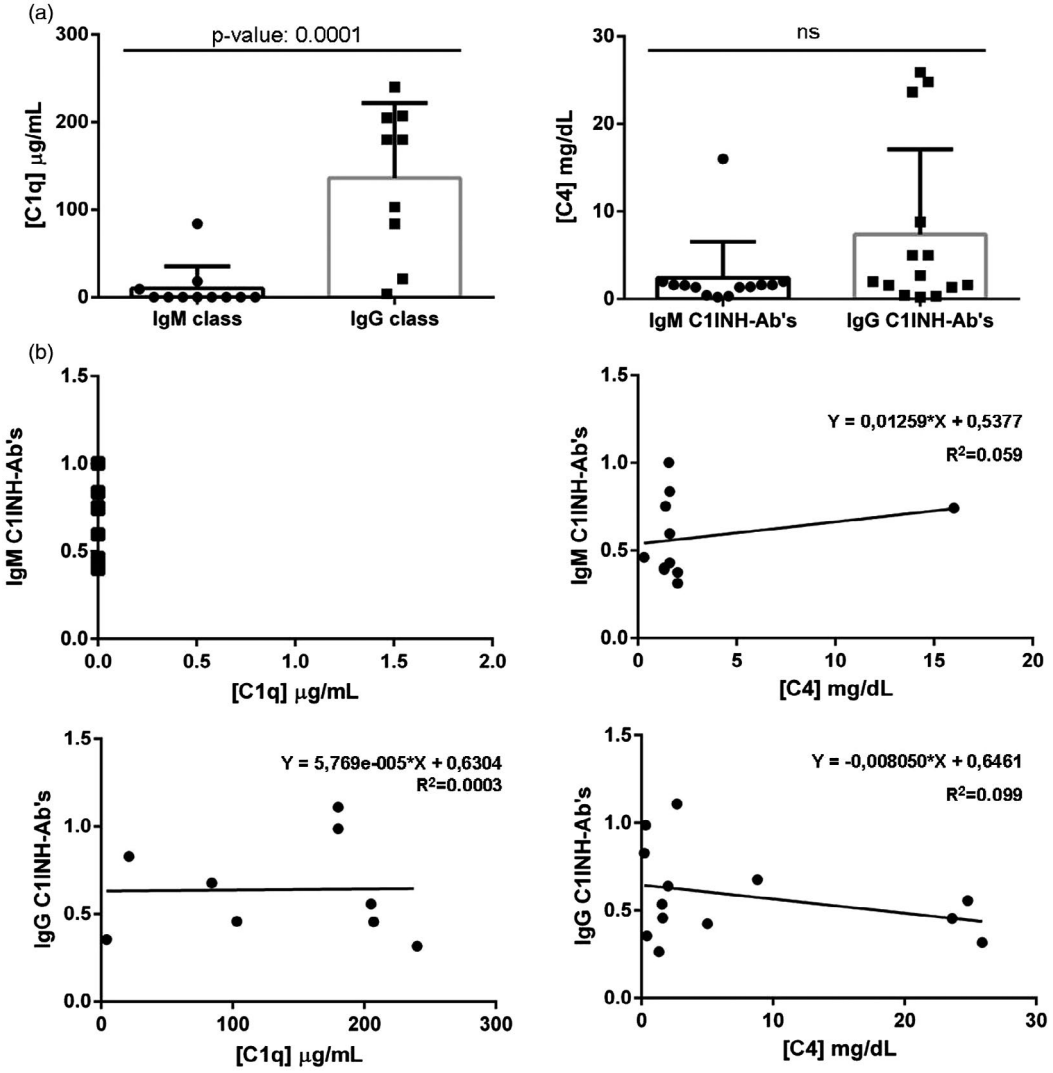


Fig. 2. Effect of serum C1-inhibitor–anti-C1INH antibody (C1INH–antiC1INHab) complexes on C1q and C4 levels. (a) Acquired angioedema (AAE) patients can be clustered into two groups according to their serum C1q and C1INH–antiC1INHAb levels. All except one of the patients presenting C1INH–antiC1INHAb of the immunoglobulin (Ig)M class had very low or undetectable C1q ($P < 0{\cdot}0001$) and tended to show lower C4 values ($P = 0{\cdot}1428$), while those with IgG-class C1INH–antiC1INHAb and free IgM-class or IgG-class antiC1INHab had normal or slightly low C1q (median = 136 μg/ml). No correlation was found between C1q or C4 concentrations and the absorbance values of IgM (b) or IgG-class (c) C1INH–antiC1INHAb. Statistical analysis was performed with Mann–Withney test (a) and Spearman's rank correlation (b,c).

or complexed – anti-C1INH autoantibodies, normal C1q concentration and no signs of lymphoproliferation. In the second sample, obtained in September 2011, low C1INH levels and function, undetectable C1q and IgM class C1INH–antiC1INHAb complexes (but no IgM or IgG free antiC1INH) were detected. Finally, in the last sample from April 2013, with undetectable C1q and C4, low C1INH levels and function and detectable free and C1INH-complexed IgM autoantibodies, a monoclonal IgM band was identified and the patient was diagnosed with chronic lymphocytic leukemia (CLL). Follow-up data of LP7 exemplify the strong association between the appearance of IgM-class C1INHAbs and C1q consumption in our series.

Patient LP12 was diagnosed in 2010 at 43 years of age presenting with angioedema symptoms, low C1INH function and slightly diminished C1INH levels. During follow-up from 2007 to 2015, four serum samples showed the same complement profile and remained negative for free autoantibodies and C1INH–antiC1INHAbs. The patient first became positive for free anti-C1INH IgG in December 2015 followed, 1 year later, by positive free and C1INH-complexed IgG (Table 1 and Fig. 3).

Further investigation on the factors contributing to the appearance of C1INH–antiC1INHAb complexes in AAE patients was made by studying the neutralization of free anti-C1INHautoAbs in overnight incubations with

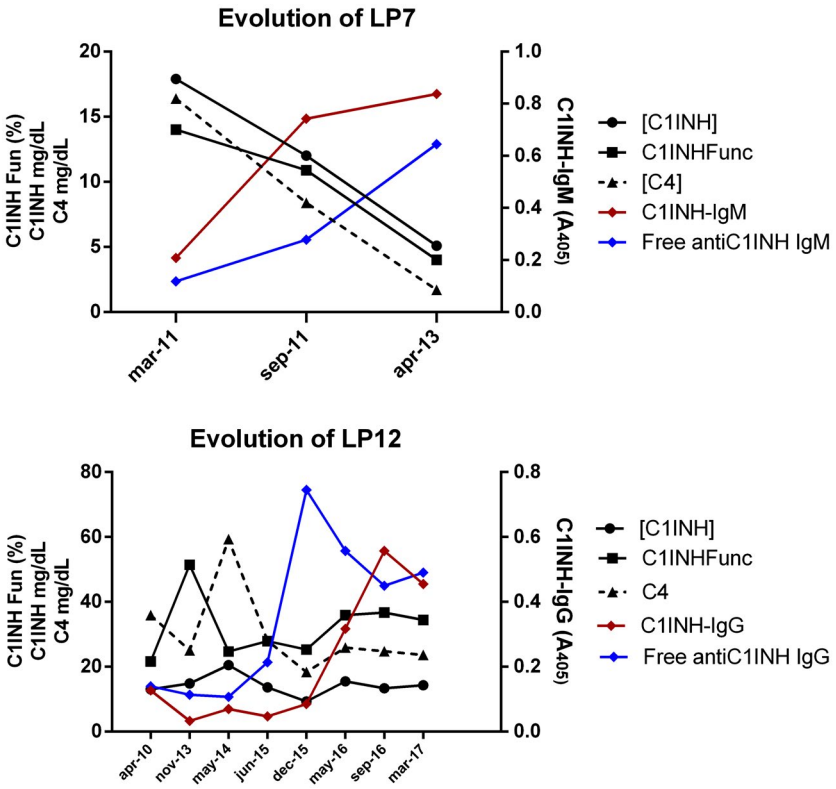


Fig. 3. Follow-up of patients LP7 and LP12. Patients LP7 and LP12 were followed-up for 3 and 7 years, respectively. In LP7, C1-inhibitor immunoglobulin (C1INH–IgM) detection preceded free anti-C1INH–IgM and coincided with a marked reduction of C1q, C4, C1INH levels and C1INH function. In the case of LP12, free anti-C1INH IgG were detectable 1 year before the onset of C1INH-IgG.

increasing amounts of C1INH. Shorter incubation times with C1INH ranging from 30 min to 2 h showed little or no effect (data not shown), which is in agreement with the reported low affinity of antiC1INHAbs [19]. Incubation of sera from LP2, OU1 and OU3 with increasing amounts of C1INH showed great differences in their neutralization dynamics. While LP2 and OU1 did not reach complete autoantibody neutralization upon incubation with 20 μg of C1INH, OU3 complex formation became saturated with as little as 2 μg of C1INH, which may be attributed to variability in autoantibody concentrations, clonalities and affinities for C1INH among AAE patients (Fig. 4). It is also of note that the absorbance values for C1INH–antiC1INHAb complexes obtained in this assay upon overnight incubation with no added C1INH of samples from patients LP2 and OU1 but not OU3 were still positive, but significantly lower than those obtained during their diagnosis using fresh serum samples. We hypothesize that this discrepancy could be due to the activation of proteolytic pathways in serum during incubation. However, the fact that the sample from OU3 was not significantly affected could also reflect the detection of neutralizing rather than reactive center loop (RCL)-directed autoantibodies in OU3. Unlike RCL-directed autoantibodies which, according to the mechanism established by Alsenz and others [14,15], would be released upon protease attack of the reactive center, the binding of those targeting epitopes elsewhere in the molecule would not be altered by the proteolysis of the RCL.

Discussion

Free C1INH from the serum of AAE patients predominantly circulates in its cleaved/inactivated 96-kDa form. Zuraw and Curd attributed this fact to an autoimmune mechanism, and found that patients' autoantibodies promoted the cleavage of normal C1INH to the 96-kDa species when incubated with Clr, C1s or plasmin. Moreover, they also showed *in vitro* that the autoantibody reduced the amounts of stable serpin–protease complexes formed and that this facilitated cleavage of C1INH occurred at the same time or subsequent to the serpin–protease complex formation, thus proposing that autoantibodies mediate their effect by binding to normal C1INH, altering its conformation and destabilizing the complexes formed

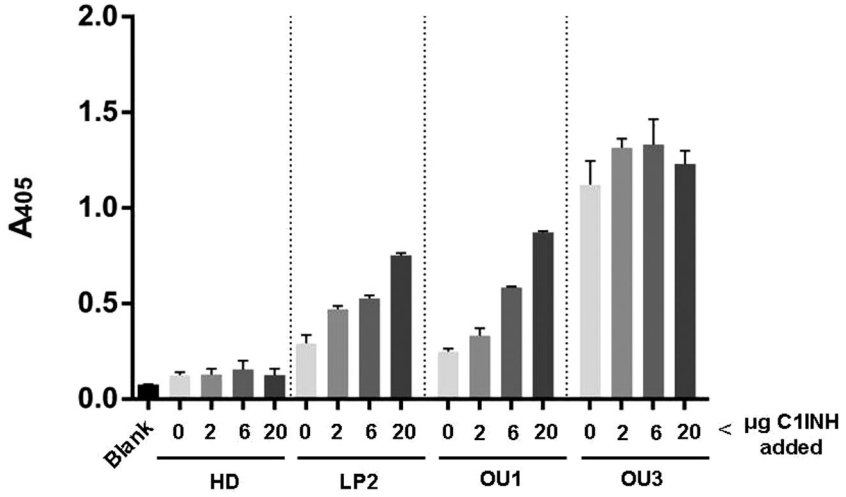


Fig. 4. *In-vitro* complex assembly assays. Sera from patients LP2, OU1 and OU3 were incubated overnight with increasing C1-inhibitor (C1INH) amounts. LP2 and OU1 showed a constant increase of C1INH–immunoglobulin (IgG) complexes detection in the range of 2–20 µg C1INH, while OU3 complexes rapidly saturated upon incubation with 2 µg C1INH. The mean ± standard error of the mean (s.e.m.) from three independent experiments is presented. Statistical analyses were performed by two-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. HD = healthy donor serum.

between C1INH and its target proteases [20]. Shortly after, this mechanism was demonstrated by using different target proteases and showing that the autoantibody was able to interact with normal, uncleaved C1INH but not with the cleaved and protease-complexed forms [3]. Moreover, Alsenz *et al*. also showed that autoantibodies prevented the formation of a stable C1INH-C1s complexes (*in vitro*), the addition of active C1s induced the appearance of the 96-kDa form (*in vitro*) [14] and that infusion of C1INH concentrate in AAE patients induced an increase of C1INH–antiC1INHAb complexes *in vivo* [15]. Finally, using synthetic peptides, He *et al*. mapped the epitopes bound by autoantibodies obtained from six AAE patients to residues around the reactive center of C1INH [21].

According to this mechanism, AAE sera present two C1INH subpopulations, antibody-bound and free C1INH, and the chance for spontaneous activated target proteases to interact with either population will depend on their relative abundances. Moreover, cleavage of the reactive center of C1INH by proteases erases the autoepitope and releases free autoantibody for further interaction with native C1INH molecules [21] Consequently, the complexes detected in our ELISAs represent the pool of C1INH–antiC1INHAb complexes that had not yet interacted with proteases, which is expected to be variable in time for a given individual and dependent on the spontaneous activation of serum proteases. Plasma samples taken during asymptomatic periods or after receiving C1INH infusions are likely to preserve a higher proportion of intact circulating C1INH–antiC1INHAb complexes than those obtained during angioedema attacks or from contact system-activated plasmas. However, we could not test this hypothesis due to the lack of during-attack samples, which is an evident limitation of the present study.

The experiments performed for the *in-vitro* assembly of C1INH–antiC1INHAb complexes by addition of purified C1INH show a great variability in the neutralization dynamics of free autoantibody (Fig. 4), which is in agreement with the initial reports by Alsenz [15]. Interestingly, the observation that long incubation times were needed to obtain significant increases in the detection of complexes, in agreement with the low affinity reported for anti-C1INH autoantibodies [19], suggests that perhaps a proportion of free anti-C1INH autoantibodies are escaping from detection in the conventional diagnostic ELISA assays due to the short incubation times normally used.

Extended follow-up periods, such as those of patients LP7 and LP12, demonstrate variability in the course of autoantibody appearance. Whereas, in LP7, C1INH–antiC1INHAb complexes preceded the appearance of free autoantibodies, in the case of LP12, C1INH–antiC1INHAb complexes became positive shortly after the detection of free anti-C1INH (Fig. 3).

Little is known about the cellular events triggering anti-C1INH production in AAE, although the latest evidence suggests that antigenic stimulation of B cells, either from persistent microbial infections or from autoantigens, plays a prominent role as indicated by over-representation of

the IGHV1-2*04 immunoglobulin allele in those AAE patients with detectable free autoantibodies [7]. How autoreactive B cells modulate anti-C1INH levels is not known. Interestingly, initial reports showed that the molar concentrations of the autoantibodies were similar to that of C1INH in serum, thus suggesting a tuning that could explain those samples in our cohort where only C1INH-complexed (but no free) autoantibodies were found (10 of 29) [19]. It is likely that such a balance between C1INH and autoantibody molecules would be eventually disturbed by increasing autoantibody production or serum proteases activation turning free autoantibodies detectable by conventional assays. The study of additional cases through extended follow-up periods will help to characterize more clearly the dynamics and impact of the detected C1INH–antiC1INHAb complexes.

According to the literature, most of the anti-C1INH autoantibodies reported to date seem to act in the manner described above [14,15,22–25] by destabilizing the C1-INH–protease complex and/or enhancing the susceptibility of C1-INH to its cleavage by target proteases. However, considering that antiC1INHAbs can present in a polyclonal or oligoclonal nature, also binding of the autoantibodies to other parts of the C1INH molecule is a possibility not discarded by experimental results, according to which they would act by neutralization of the native and/or cleaved molecule, thus reducing the availability of C1INH without facilitating protease cleavage. As commented in the Results, the presence of neutralizing autoantibodies is a possibility also suggested by the *in-vitro* assembly of C1INH–antiC1INHAb complexes in patients LP2 and OU1, although this mechanism has not been explored at the molecular level in this report.

We did not investigate in depth the potential impact of the C1INH–antiC1INHAb complexes on complement dysregulation as classical pathway-activating immune complexes. As commented above, low C4 and C1q concentrations in AAE patients are mainly attributed to an autoantibody-mediated loss of C1INH regulation. However, the role of circulating immune complexes as primary classical pathway activators has been less studied. In our data, the strong correlation between the presence of IgM-class C1INH–antiC1INHAb complexes (but not free anti-C1INH IgM) and extremely low or undetectable C1q (Fig. 2a) is explained by the significantly higher complement-triggering activity of IgM compared to IgG when forming immune complexes. Chronicity for C1q deficiency and B cell dysregulation are both possibly involved in the higher risk of autoimmunity that characterizes AAE pathology [26].

Conversely, despite the great differences in C1q levels observed between patients with IgM- or IgG-class C1INH–antiC1INHAbs, we could not find a significant correlation between IgM–C1INH–antiC1INHAb complexes absorbance values and C1q or C4 concentrations (Fig. 2b,2). This apparent discrepancy can be due to the lack of a reference curve in the C1INH–antiC1INHAb complexes assays. Although all the serum samples were tested in a single ELISA assay under controlled conditions and using AAE sera as internal positive controls for IgM or IgG complexes, ELISA results can be affected by a number of factors that lead to a lack of linearity, especially for samples yielding extreme values. However, considering the large differences in Ig classes, clonalities and affinities expected among AAE patients, we could not define a suitable standard curve and therefore both assays are to be interpreted as semiquantitative.

To summarize, the recognition of C1INH–antiC1INHAb complexes in most (18 of 20) of the AAE cases, even when no free anti-C1INHAb is found, makes C1INH–antiC1INHAb complexes quantification a useful and novel diagnostic tool. Moreover, the follow-up data from some patients presented here suggest that detection of complexes can precede the detection of free anti-C1INHAb and the onset of lymphoproliferative disease in a subset of AAE patients. Finally, we observed a strong association between the presence of IgM-class C1INH–antiC1INHAb complexes and a severe reduction of serum C1q, which might have further implications for the development of autoimmune manifestation in AAE patients.

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Author contributions

A. L.-L. and M. L. T. conceived and designed the study. A. L.-L. and S. G. performed the experimental work. P. N., T. C., M. L.-T., L. S. and A. B. obtained the samples and clinical data. A. L.-L. wrote the manuscript draft and subsequent revisions.

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