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Original Article

Diminished capacity of opsonization and immune complex solubilization, and detection of anti-C1q antibodies in sera from patients with hereditary angioedema

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Abbreviations:
C1-INH, C1 esterase inhibitor;
CR, Complement receptor; HAE, Hereditary angioedema; IC, Immune complex;
NHPS, Normal human pooled sera;
PBS, Phosphate buffered saline;
PI, Propidium iodide; PMA, Phorbol-12-myristate-13-acetate; PO, Peroxidase;
SD, Standard deviation; SLE, Systemic lupus erythematosus;
TMB, Tetramethylbenzidine;
HUVS, Hypocomplementemic urticarial vasculitis syndrome

ABSTRACT

Background: Hereditary angioedema (HAE) is an autosomal dominant disease caused by deficiency of C1 esterase inhibitor. Symptoms of HAE include edema, which can potentially cause suffocation. Some patients with HAE exhibit immunological abnormalities, which could prevent an accurate diagnosis. Low levels of complement components are characteristic of HAE and in other settings are thought to reduce elimination of apoptotic cells and immune complex (IC). Thus, we aimed to experimentally clarify the mechanism of immunological abnormalities using sera from HAE patients.
Methods: Serum samples from 18 patients with HAE were collected when free from angioedema attack and compared with normal human pooled sera (NHPS) from 20 healthy volunteers. Opsonization was measured as the rate of phagocytosis of apoptotic Jurkat cells by macrophages differentiated from THP-1 cells incubated with serum. IC solubilization in serum was analyzed by quantifying peroxidase released from a synthetic IC composed of peroxidase and anti-peroxidase antibodies. Anti-C1q antibody levels were detected using an enzyme-linked immunosorbent assay.
Results: Serological immunological abnormalities were detected in 12 patients. Opsonization in serum samples from each patient with HAE was lower than that in NHPS (~20% versus 70%, respectively). The rate of IC solubilization was lower in serum from HAE patients than NHPS. Some patients had high serum anti-C1q antibody levels with increased serum IC levels.
Conclusions: Sera from patients with HAE exhibit anti-C1q antibodies, with a lower capacity for opsonization and IC solubilization. This may be associated with immunological abnormalities and should be investigated further to facilitate accurate diagnosis of HAE.
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Introduction

Hereditary angioedema (HAE) is a rare autosomal dominant disease caused by mutations in the C1 esterase inhibitor (C1-INH) gene. These mutations cause deficiencies in the expression (HAE type I) or functional activity (HAE type II) of C1-INH, which can lead to continuous and uncontrolled activation of the complement, contact, blood coagulation, and kallikrein-kinin cascades.[1,2] Consequently, the release of bradykinin, a vasoactive peptide in the kallikrein-kinin system, induces acute episodes of localized subcutaneous or submucosal angioedema in the extremities, limbs, torso, face, gastrointestinal tract, genitals, larynx, and trachea.[1,2] These episodes can be triggered by stress, minor trauma, drugs, surgical procedures and other unknown reasons.[1] A third type of HAE has also recently been reported, where patients exhibit normal C1-INH levels but have mutations in the gene for clotting factor XII.[3,4]

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Although HAE is a rare disease, estimated to affect around 1 in 50,000 individuals, acute attacks of edema are life-threatening and can cause suffocation if they develop in the upper airways.[5] Thus, it is essential that HAE is accurately and rapidly diagnosed and appropriately treated. Plasma-derived human C1-INH was approved for treatment of HAE attacks in Japan in 1990 and has been first-line therapy in Europe and other countries for several decades.[6] Despite this, awareness of HAE is low and in Japan the mean time to diagnosis from first symptoms was reported to be 13.8 years.[7] According to the World Allergy Organization guideline for the management of HAE, patients with suspected HAE should be screened for low serum levels of C4, and then diagnosed by low functional and antigenic levels of C1-INH.[8] However, since it has been reported that a proportion of HAE patients show immunological abnormalities,[9–12] these abnormal findings could mimic other disorders and further delay or prevent an accurate diagnosis of HAE.

Clinical laboratory findings from patients with HAE have revealed chronic activation and consumption of the complement system.[2,13] In patients with HAE, low levels of expression or impaired functional activity of C1-INH causes a gradual reduction in serum levels of complement proteins including C4 and C2.[2,13] It is well established that the complement system plays an important role in removal of apoptotic cells and immune complexes (IC), as well as IC solubilization.[14,15] In particular, C1, C3 and C4, which are early complement components of the classical pathway, are required as opsonins for complement-mediated phagocytosis, and C1, C4 and C2 are essential for IC solubilization and clearance.[16,17] Low levels of complement components may reduce the elimination rate of apoptotic cells and IC, which may potentially become sources of autoantigens that could lead to autoimmunity and other immunological abnormalities.[18,19] Genetic deficiencies in complement components such as C1q or C4 have been associated with autoimmune diseases such as systemic lupus erythematosus (SLE).[20,21] In fact, the prevalence of definitive autoimmune disorders in patients with HAE has been reported to range from 0.4 to 12%.[9]

The aim of the present study was to determine the cause of immunological abnormalities in patients with HAE. We hypothesized that low levels of early complement components, which are a characteristic of HAE, may affect opsonization and IC solubilization.

Methods

Patients and blood samples

The study enrolled all patients who had a confirmed genetic diagnosis of HAE at Juntendo University Hospital between November 2013 and October 2014. Study procedures were performed in accordance with the Declaration of Helsinki and the protocol was approved by the Institutional Review Board of Juntendo University (No. 25-325). Written informed consent was obtained from all participants prior to inclusion.

Serum samples were collected from patients with HAE when they were not experiencing angioedema attack. In parallel, serum samples were obtained from 20 healthy volunteers without autoimmune disease or clinical symptoms of angioedema, as described previously[16]; these were used as a control and referred to as normal human pooled sera (NHPS). Serum samples were stored at −80 °C in 500-μL aliquots and each aliquot was only used once.

Laboratory data

Using standard laboratory assays, the following clinical parameters were analyzed in patients with HAE and healthy volunteers: C1-INH activity, serum C1-INH levels, serum C4 and C1q levels, the quantity of IC (C1q-binding assay), and the presence of antinuclear antibodies and/or cryoprecipitate (protein, mostly immune complexes, that become insoluble at reduced temperature and are associated with various diseases) in serum.

Evaluation of opsonization capacity

A human monocytic leukemia THP-1 cell line (Public Health England, London, UK) was cultured in RPMI 1640 medium containing 2 mM L-glutamine (Sigma–Aldrich, St. Louis, USA), supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, USA). Floating, non-dendritic THP-1 cells were differentiated into adherent macrophages by the addition of 100 nM phorbol-12-myristate-13-acetate (PMA) (Abcam, Cambridge, UK), as described previously.[22,23]

Induction of apoptosis in human T-cell lymphoma Jurkat cells (Public Health England) was performed as previously described,[21,24] with minor modifications as follows. Jurkat cells were suspended in RPMI 1640 medium containing 1% penicillin/streptomycin and irradiated with ultraviolet light (8 W, 170 mJ/cm^2) using a FUNA-UV-LINKER (Funakoshi, Tokyo, Japan). Cells were incubated for 4 h or 24 h at 37 °C in 5% CO_2. For detection of apoptosis they were stained using an Annexin V-FITC kit (Beckman Coulter, Brea, CA, USA), in which Annexin V binds to phosphatidyl serine located on the outer cell membrane only during early apoptosis, and propidium iodide (PI) is used to visualize DNA content and integrity. Cells were sorted via flow cytometry using a FACScan system (Salk Institute, San Diego, CA, USA).

Phagocytosis assays were conducted as previously described,[23,24] with minor modifications as follows. Serum samples from patients with HAE were analyzed, using NHPS as a control. Apoptotic cells (1×10^6 cells/mL) and macrophages (1×10^5 cells/mL) were incubated with 30% serum for 1 h at 37 °C in a 25 × 75-mm chamber slide (Thermo Fisher Scientific). After 1 h incubation, the chamber slides were gently washed with phosphate buffered saline (PBS) to remove non-bound apoptotic cells. The chamber slides were air-dried, fixed with methanol for 10 min, and stained with hematoxylin-eosin. The phagocytosis index, as a measure of the opsonization capacity, was defined as the number of macrophages binding to apoptotic cells or undergoing phagocytosis per 100 macrophages. Slides were visualized and scored at ×400 magnification using standard light microscopy. Each experiment was performed in duplicate and the phagocytosis index was expressed as the mean average.

To detect the deposition of complement factors on the cell surface, irradiated apoptotic cells were incubated with 30% NHPS or 30% sera from patients with HAE for 1 h at 37 °C. Each aliquot was incubated with anti-C1q mouse antibody (1:10 dilution; Abcam), anti-C4d rabbit antibody (1:20 dilution; Abcam) or anti-iC3b mouse antibody (1:400 dilution; Genway, San Diego, CA, USA) for 1 h at room temperature after the addition of a human Fc receptor blocking reagent, according to the manufacturer's protocol (MBL, Nagoya, Japan). After several gentle washes with PBS, the cell aliquots were incubated at room temperature for 1 h with secondary goat anti-mouse antibody or donkey anti-rabbit antibody (both 1:100 dilution; Thermo Fisher Scientific). After several further washes, apoptotic cells with complement factors on the cell surface were detected by flow cytometry.

Macrophages and monocytes have been reported to express cell surface complement receptors (CRs), including CR1 (CD35), CR3 (CD11b/CD18) and CR4 (CD11c/CD18).[25] To elucidate the role of cell surface complement receptors in phagocytosis, CR blockade was attempted by incubating macrophages with an anti-CR1 antibody (10 μg/mL), an anti-CR3 antibody (10 μg/mL) and an anti-CR4 antibody (5 μg/mL; all from Abcam), as described previously.[26]

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Evaluation of immune complex solubilization capacity

A simplified and reliable method was used to assess IC solubilization, with horseradish peroxidase (PO; Wako, Osaka, Japan) as an antigen and an anti-PO antibody (Sigma–Aldrich), as described previously.[27] PO and anti-PO antibodies were mixed at varying concentrations, incubated for 1 h at 37 °C, maintained overnight at 4 °C, and then centrifuged to yield the IC.

Solubilization was determined by incubating a fixed quantity of IC (5 μg) over a gradient of serum concentrations for 1 h at 37 °C, as described previously.[27–29] PO concentration in the supernatant was measured by adding tetramethylbenzidine (TMB; Bio Rad, Hercules, CA, USA) and then stopping the reaction with acid. TMB is a substrate for PO which turns yellow upon the addition of acid and absorbs light at 450 nm, and can therefore be used in an assay to estimate PO concentration. The mixture was centrifuged and absorbance was read using a Benchmark Plus Microplate Reader (Bio Rad). The results were calculated as an index of the capacity of IC solubilization. Each experiment was performed in duplicate, and the results expressed as an average.

Detection of anti-C1q antibody

Anti-C1q antibody levels were assessed with a human anti-C1q antibody ELISA kit (Alpha Diagnostic, San Antonio, TX, USA) according to the manufacturer's protocol, using a Benchmark Plus Microplate Reader.

Statistical analysis

GraphPad Prism 6J software for Windows (version 6.01; GraphPad, San Diego, CA, USA) was used for statistical analysis. For the comparison of data a t-test was used, and a two-sided p-value of <0.05 was taken as the level for statistical significance. All data were expressed as mean ± standard deviation (SD).

Results

A total of 18 patients were eligible for the study and were enrolled, including 8 males and 10 females with an average age of 46.7 years.

Clinical background

Several pairs of patients in the study (no. 4 and 5; 12 and 17; 14 and 15, and 16 and 18) were family members. Three patients (no. 4, 14, and 18) were asymptomatic, but were diagnosed with HAE by genetic analysis. The other 15 patients suffered from symptoms associated with HAE, with an age of onset between 10 and 39 years of age. There was an average of 24.3 years (0–61 years) from the onset of symptoms until diagnosis of HAE (Table 1).

Of symptomatic patients, 12 had received on-demand treatment with plasma-derived C1-INH. The majority had experienced no serious effects following acute attacks but 2 patients (no. 6 and 8) had undergone a tracheotomy following suffocation due to attacks affecting the laryngopharynx.

Laboratory findings in serum samples

Clinical laboratory data revealed that both the level and activity of serum C1-INH was reduced in all patients with HAE. The level of serum C4 was reduced in 17/18 patients, although one patient (no. 18) exhibited normal level of serum C4, despite having been genetically diagnosed with HAE. Immunological abnormalities were identified in 12 patients, including increased serum IC levels and the presence of an antinuclear antibody and/or a cryoprecipitate in serum. Two patients (no. 10 and 13) also exhibited extremely low levels of serum C1q, which is a feature of acquired angioedema; however, they had been genetically diagnosed with HAE. All laboratory findings in NHPS were within normal levels (Table 2).

Table 1
Clinical backgrounds of patients with hereditary angioedema (HAE).

Patient no.	Age at onset (years)	Age at diagnosis (years)	Family members with HAE	On-demand C1-INH therapy upon attack?	Tracheotomy
1	10	63	Mother, daughter, grandchild	Yes	–
2	24	24	Father	Yes	–
3	19	39	Aunt, cousin	Yes	–
4	–	54	Son	–	–
5	22	24	Father	Yes	–
6	20	40	Father	Yes	Yes
7	16	33	Father, aunt, sister	Yes	–
8	39	54	–	Yes	Yes
9	16	35	–	Yes	–
10	25	45	Mother	Yes	–
11	24	38	Aunt, cousin	Yes	–
12	19	35	Father	Yes	–
13	30	57	Mother, sister, nephew	–	–
14	–	46	Mother, aunt, cousin	–	–
15	20	66	Sister, daughter, niece	Yes	–
16	30	64	Son, daughter	–	–
17	10	71	Daughter	–	–
18	–	38	Mother, sister	–	–

Phagocytosis of apoptotic cells

Following induction of apoptosis, incubation for 4 h resulted in 77.0 ± 0.6% (mean ± SD) Jurkat cells being identified as positive for Annexin V but negative for propidium iodide (PI) staining by flow cytometry. After incubation for 24 h, 86.4 ± 1.1% of Jurkat cells were positive for both Annexin V and PI, indicating that the majority of cells were apoptotic.

Phagocytosis of apoptotic cells by macrophages was found to plateau at 30% serum concentration using NHPS (data not shown); therefore, a fixed concentration of 30% serum was used. In NHPS, approximately 70% of macrophages captured apoptotic cells (Fig. 1a), whereas in sera from patients with HAE, fewer macrophages captured apoptotic cells (Fig. 1b). For each individual patient with HAE the rate of phagocytosis after incubation with serum was between 10% and 30%, and overall this was significantly lower than that with NHPS ($p < 0.0001$; Fig. 1c). Addition of 30% NHPS to the serum of HAE patients was able to significantly restore macrophage-induced phagocytosis; on average the phagocytosis index following incubation with NHPS was 70% (Fig. 1c).

Deposition of complement factors on apoptotic cells

Levels of complement factors that promote opsonization of apoptotic cells levels were analyzed in NHPS and sera from patients with HAE to determine if there were any differences. Deposition of C1q, C4d and iC3b was greater with serum than without serum (Fig. 2). Incubation with NHPS or sera from HAE patients produced similar levels of C1q deposition on apoptotic cells (Fig. 2a); however, deposition of C4d and iC3b on apoptotic cells was greater after incubation with NHPS than sera from HAE patients (Fig. 2b,c). Although Figure 2 only represents data from one patient with HAE, pooled results using sera from other patients with HAE were similar (data not shown).

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Table 2
Clinical laboratory findings in serum samples from patients with hereditary angioedema (HAE) taken when not experiencing an HAE attack.

Patient no.	Sex	Age (years)	C1-INH activity (%)	C1-INH (mg/dL)	C4 (mg/dL)	C1q (mg/dL)	IC (μg/mL)	Antinuclear antibody (1:dilution)	Cryo-precipitate
1	F	63	<25	6	3	10	<1.5	20	X
2	M	33	30	6	9	10.4	<1.5	20	X
3	M	41	<25	5	3	8.0	2.3	20	✓
4	M	54	35	10	15	8.0	<1.5	20	X
5	M	24	<25	6	7	8.0	<1.5	20	X
6	M	40	35	5	3	6.9	<1.5	20	X
7	M	33	<25	10	3	7.0	<1.5	20	X
8	F	54	29	8	7	12.5	<1.5	20	X
9	F	35	<25	7	5	6.3	40.8	20	✓
10	F	45	<25	<3	<2	<2	<1.5	20	X
11	F	38	<25	7	4	8.3	3.4	20	✓
12	F	38	29	10	5	8.6	5.8	20	X
13	F	57	<25	7	<2	2.5	44	20	✓
14	F	46	34	15	10	9.5	<1.5	20	X
15	F	66	39	14	10	12.3	<1.5	80	✓
16	F	64	27	10	11	8.7	<1.5	20	X
17	M	71	<25	6	3	11.8	<1.5	20	X
18	M	38	47	12	29	10.5	<1.5	20	X
Local normal range			70–130	21–39	17–45	8.8–15.3	<3.0	<20	

C1-INH, C1 esterase inhibitor; F, female; IC, immune complex; M, male.

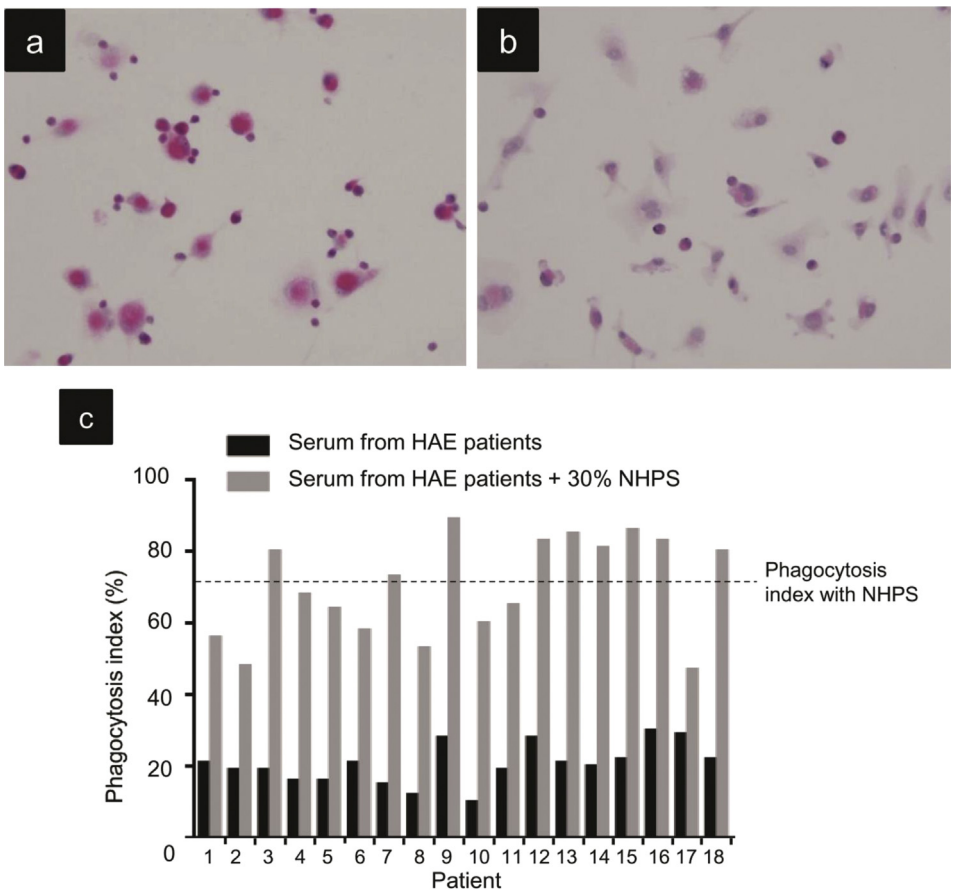

Fig. 1. Phagocytosis of apoptotic cells by macrophages incubated with normal human pooled serum (NHPS) or sera from patients with hereditary angioedema (HAE). **(a)** Phagocytosis of apoptotic cells by macrophages incubated with 30% normal human pooled sera (NHPS) with hematoxylin-eosin staining. Large cells with a red nucleus and some dendrites were identified as macrophages, and small cells with purple nuclei were apoptotic cells. **(b)** Phagocytosis of apoptotic cells by macrophages incubated with 30% serum from patients with HAE, with hematoxylin-eosin staining. **(c)** Phagocytosis index after incubation with sera from patients with HAE, before (black) and after (grey) the addition of NHPS.

Blockade of complement receptors on macrophages

Immunofluorescence demonstrated that macrophages differentiated from THP-1 cells expressed CR1, CR3 and CR4 (data not shown). Antibodies to CR1, CR3 and CR4 were utilized to assess the effect on phagocytosis of blocking these receptors. Figure 3 shows that blocking each receptor resulted in a significant decrease ($p < 0.005$) in the phagocytosis index.

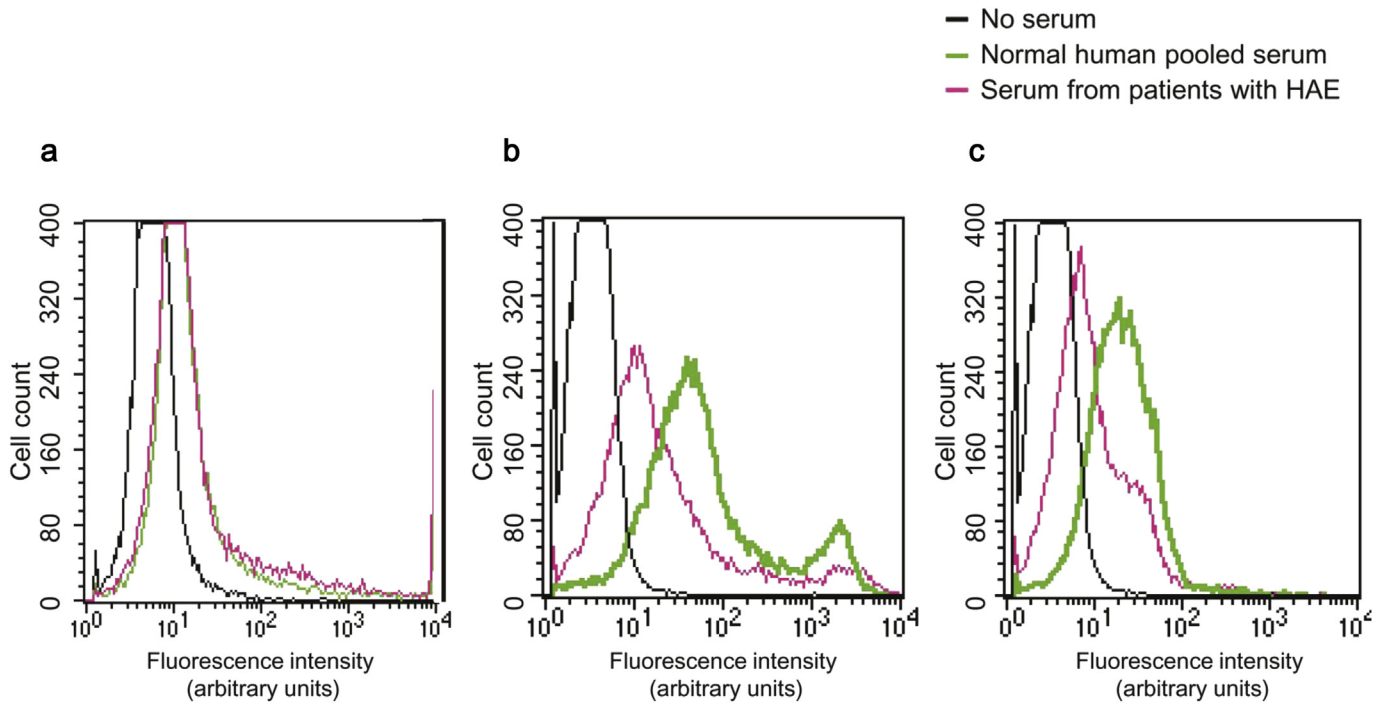

Fig. 2. Detection of complement factors C1q **(a)**, C4d **(b)** and iC3b **(c)** on apoptotic cells by flow cytometry, following incubation with no serum, normal human pooled serum (NHPS) or sera from patients with hereditary angioedema (HAE).

Capacity for immune complex solubilization

A titration demonstrated that the addition of 250 μL 80% serum to 5 μL IC was sufficient for IC solubilization (data not shown). The capacity of serum from patients with HAE to solubilize the IC was lower overall than that of NHPS ($p < 0.05$); IC solubilization capacity was reduced in 17 of the 18 patients with HAE (Fig. 4).

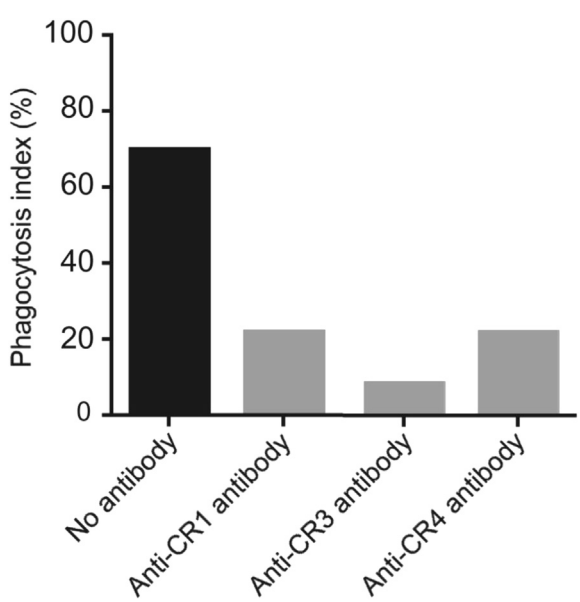

Fig. 3. Phagocytosis index after incubation with normal human pooled serum (NHPS) and complement receptor blockade with antibodies targeting complement receptors CR1, CR3 and CR4.

Detection of anti-C1q antibodies

Finally, the relationship between serum anti-C1q antibody levels and serum IC was evaluated. As shown in Figure 5, some patients with HAE had high serum anti-C1q antibody levels, in particular, patients 9, 12 and 13; these patients also exhibited increased levels of IC.

Discussion

Our present study demonstrated that serum from patients with HAE had a lower capacity for opsonization and IC solubilization as compared to serum from healthy individuals. Reduced clearance of apoptotic cells is thought to be a potential pathophysiological mechanism for the induction of autoimmune disease.[30] Moreover, inadequate IC solubilization due to reduced levels of early complement components in patients with HAE has been proposed as another factor that may contribute to autoimmunity.[9] Although in this study none of the enrolled patients had a confirmed diagnosis of definite autoimmune disease, some experimental data exists to show the dysregulation of immunity in patients with HAE.[9–12] In the present study, laboratory analyses showed that cryoprecipitate (which is thought to be composed of some immune complexes) was present in the serum of five patients with HAE. We suggest that cryoprecipitate in some patients with HAE may be due to the reduced capacity of IC solubilization, as demonstrated by the findings in this study.

The prevalence of autoantibodies in patients with HAE is reported to be 0.4–12%,[9] and SLE is an autoimmune disorder associated with the production of a variety of autoantibodies. We suggest that the distinction between HAE and SLE might be derived from the pathogenetical difference between these conditions. The major difference is that late complement components and complement regulatory factors of the complement system are normal in HAE. For example, the serum level of C3 in patients with SLE is

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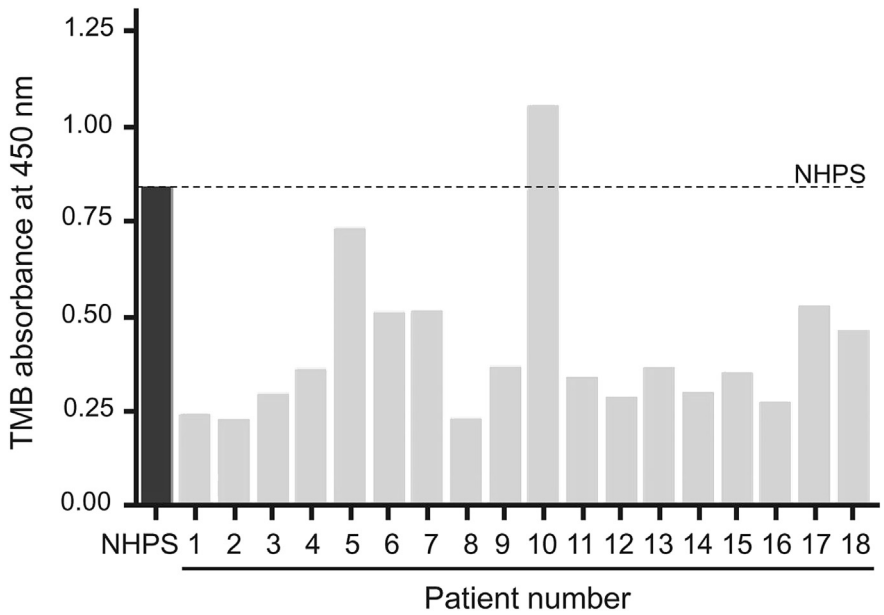

Fig. 4. Immune complex (IC) solubilization capacity in sera from patients with hereditary angioedema (HAE) versus normal human pooled serum (NHPS). Solubilization was measured of a synthetic IC composed of peroxidase and anti-peroxidase antibodies. Peroxidase released from the IC was measured by tetramethylbenzidine (TMB) assay and absorbance at 450 nm.

decreased, but is within the normal range in patients with HAE. C3 (as well as C1 and C4) is an opsonin strongly associated with phagocytosis.[16] Thus, due to the lack of phagocytosis in SLE, antigenic apoptotic cells may produce various autoantibodies against cellular components, in addition to serum antigenic components. These may explain why immunological abnormalities in HAE are not more frequently associated with development of autoimmune diseases than in SLE.

Our results showed that some patients with HAE exhibited high levels of anti-C1q antibodies, which was associated with increased levels of IC. It has been reported previously that the prevalence of anti-C1q antibodies in patients with HAE was higher than those with angioedema without complement deficiency (6.2% and 2.3%, respectively).[31] Although anti-C1q antibodies are frequently found in patients with lupus nephritis and hypocomplementemic urticarial vasculitis syndrome (HUVS),[32,33] the pathogenesis of these disorders is vasculitis. In contrast, tissue samples from patients with HAE never have vasculitis but are associated with edematous change. With regards to the etiology of the occurrence of anti-C1q antibodies, we suggest that C1q bound to apoptotic cells or IC for phagocytosis may become antigenic itself by reducing clearance of apoptotic cells and IC due to low complement components. This could give rise to the production of anti-C1q antibodies, which can lead serum C1q deficiency associated with the development of autoimmune diseases such as SLE and IC-type glomerulonephritis.[34–38] However, the difference in the function of late complement components between HAE, SLE and HUVS might influence the prevalence of anti-C1q antibodies between these conditions, although comprehensive analyses of the presence or levels of anti-C1q antibodies in patients with HAE have not been published. Interestingly, while serum levels of IC and anti-C1q antibodies appeared to be associated in this study, serum C1q levels were not strongly associated with either parameter. Since patients with low serum C1q levels and/or high anti-C1q antibody levels have been reported to more susceptible to immunological abnormalities,[20,35] it may be beneficial to monitor serum C1q and anti-C1q antibody levels in patients with HAE to determine the risk and/or progression of immunological abnormalities.

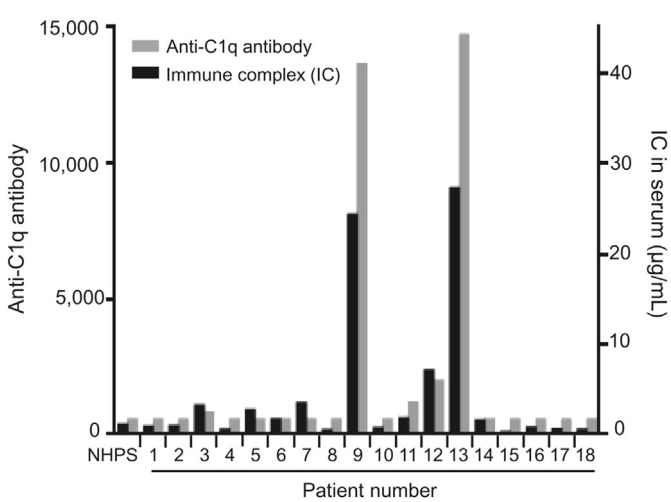

Fig. 5. Anti-C1q antibody levels and immune complex (IC) in sera from patients with hereditary angioedema.

Some limitations of our study should be mentioned. This study was an *in-vitro* investigation using samples from a small population of patients. Serum contains a multitude of potential mediators and other molecules relevant to HAE that might be responsible for immunological abnormalities, which may not have been identified in our study. Further studies in this field are necessary to facilitate accurate diagnosis of HAE and to fully understand pathogenesis of the disease. Furthermore the use of *in-vivo* assays to measure IC solubilization in patients with HAE would be desirable to corroborate findings from *in-vitro* assays used within this study. In conclusion, immunological abnormalities in patients with HAE may be caused by reduced levels of early complement components, which give rise to a lower capacity for opsonization and IC solubilization, and production of anti-C1q antibodies.

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Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

DH conceived the study, participated in the study design, carried out the basic experimental analysis and data interpretation, performed the statistical analysis, drafted the manuscript, and critically revised of the article for important intellectual content. IO conceived the study, participated in the study design, and contributed to data interpretation and drafting of the manuscript. NS, HI and SM were responsible for collection of blood samples and assembly of clinical data, and were involved in some of the basic experimental analyses. YT and YS conceived and participated in the design of the study, and performed critical revision of the manuscript. The final version of the manuscript was approved by all authors.

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