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An evaluation of tests used for the diagnosis and monitoring of C1 inhibitor deficiency: normal serum C4 does not exclude hereditary angio-oedema

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Summary

Reduced levels of serum C4 have been considered a ubiquitous finding in hereditary angio-oedema (HAE), and consequently low C4 is often used to 'request manage' access to C1 inhibitor assays in the United Kingdom. However, in our experience normal C4 may occasionally be compatible with HAE. We audited the results of serum C4, C1 inhibitor antigen (C1inhA) and C1 inhibitor function (C1inhF) in 49 HAE patients, compared to a control group of 58 unaffected subjects. The sensitivity of low serum C4 for HAE among untreated patients was 81%; levels of complement C4 were within the normal range on nine separate occasions in five untreated HAE patients. Molecular genetic analysis of these individuals demonstrated novel mutations in the C1 inhibitor gene. The supplied reference ranges for the Quidel C1inhF enzyme-linked immunosorbent assay (ELISA) system appear to be too low, with a sensitivity of just 57% for HAE. Following optimization of the reference ranges using receiver operating characteristic analysis, low C1inhF was found to be 78% sensitive and 100% specific for HAE. The diagnosis of HAE is not excluded by normal levels of complement C4. We conclude that C1 inhibitor studies should be performed regardless of serum C4 where a high index of clinical suspicion exists.

Keywords: angio-oedema, C1 inhibitor deficiency, HAE

Introduction

Hereditary angio-oedema (HAE) is a rare autosomal dominant disorder resulting from quantitative (type I) or qualitative (type II) deficiency of C1 esterase inhibitor [OMIN(606860)] leading to attacks of subcutaneous and/or submucosal swelling [1]. Fortunately, prophylactic therapy is highly effective [2–5], and severe acute attacks are amenable to emergency treatment with C1 inhibitor concentrate [6,7]. Accurate laboratory diagnosis of HAE is therefore essential to facilitate potentially life-saving therapy in affected patients, and conversely so that treatment is avoided where not indicated. Uncontrolled activation of the classical complement pathway leads to C4 consumption and low serum C4, a useful laboratory marker of the disease. However, more definitive diagnosis of HAE requires quantitative and functional assay of C1 inhibitor. C1 inhibitor antigen (C1inhA) levels are low in type I disease but normal or high in type II HAE, while functional C1 inhibitor activity (C1inhF) is low in both type I and type II disease.

A previous study described a combination of low C4 and low C1inhF as 98% specific for the diagnosis of HAE in unselected patients, with a negative predictive value of 96% [8]. Complement C4 values were below the local reference range in all patients with HAE. Indeed, according to a recent publication, serum C4 is 'invariably' low in untreated HAE [9]. Low levels of serum C4 are therefore often used to 'request manage' access to C1 inhibitor assays in the United Kingdom. However, in our unit and elsewhere [10] it has been observed that C4 may occasionally be normal in HAE. In view of the potentially serious consequences of missed diagnoses, it is essential that screening tests have sufficient sensitivity. We sought to characterize further the diagnostic performance of assay of C4, C1inhA and C1inhF for HAE.

Materials and methods

Patients and data collection

Ethics Committee approval was not required for this audit, in accordance with local guidelines. The study group comprised 49 patients with confirmed HAE. Patients with acquired C1 inhibitor deficiency were excluded. Fifty-eight unaffected subjects under investigation for chronic

Table 1. Values for serum C4, C1inhA and C1inhF in control individuals and hereditary angio-oedema (HAE) patients.

	Control	HAE (untreated)	HAE (treated)
C4 (g/l) (median, range), normal range 0·14–0·54 g/l	0·22 (0·09–0·36)	0·06 (0·01–0·23)	0·10 (0·01–0·29)
C1inhA (mg/l) (mean, s.d.), normal range 150–350 mg/l	302 (72)	58 (32)	74 (31)
C1inhF (%) (mean, SD), normal range > 84%	106 (8)	58 (25)	71 (22)

idiopathic urticaria/angio-oedema served as a control group. Clinicians were granted access to C1 inhibitor assays regardless of serum C4. Results were accessed from computerized records and treatment details obtained from the medical notes. Data from patients treated recently with intravenous C1 inhibitor concentrate were excluded, as were C1inhA results from two patients with HAE II. Included in the analysis were numerous paired data from subjects before and after initiation of therapy.

Laboratory measurements

Complement C4 was measured by nephelometry (Dade Behring, Milton Keynes, UK). C1inhA was measured by radial immunodiffusion (The Binding Site, Birmingham, UK). C1inhF was assayed by functional enzyme-linked immunosorbent assay (ELISA) (Quidel, San Diego, CA, USA). Selected samples were re-analysed for C1inhF at the Protein Reference Unit, St Georges' Hospital, Tooting by the Technoclone Technochrom assay (Technoclone, Dorking, UK).

Genetic techniques

DNA was extracted by standard desalting procedures. All eight exons and parts of the adjacent introns were amplified with specific intronic primers, and mutation screening was performed by direct sequencing of the exon-specific polymerase chain reaction (PCR) products. For recombinant expression, a C1inh cDNA construct in vector pCEP4 was transfected into HEK293 cells. Two days after transfection, C1inh activity was measured in the supernatant by the Technochrom C1inhF assay (Technoclone). Activity of the wild-type cDNA was set to 100%. Mutations were introduced into the cDNA by the pCR2·1-Cloning kit (Invitrogen, Paisley, UK) as described previously (T. Foerster, Molekulargenetik des hereditaeren Angiooedems, PhD Thesis, University of Wuerzburg, Germany, 2007).

Statistical analysis

Genetic data were evaluated with the CEQ™ Analysis System (Beckman Coulter, High Wycombe, UK). Other statistical analysis was performed with PRISM 3·0 and SPSS 10. Normality was assessed by the Kolmogorov–Smirnov test. For normally distributed data values were expressed as means, and significance tested by the unpaired *t*-test. For nonparametrically distributed data, values were expressed as medians, and significance tested by the Mann–Whitney test.

Results

Study group

Data were analysed for 194 test requests from 58 control subjects and 424 test requests from 49 HAE patients. The median age of the HAE patients was 32 (range 3–78), comprising 21 male and 28 female patients. At the time of analysis, 27 patients required no maintenance treatment, and the remainder were taking attenuated androgens ($n = 12$), tranexamic acid ($n = 9$) or both ($n = 1$); however, the majority of treated patients had results available prior to beginning therapy.

Serum C4, C1inhA and C1inhF

Values for complement C4, C1inhA and C1inhF were distributed normally in control subjects. In both treated and untreated HAE patients, values for C1inhA and C1inhF were distributed normally, but values for complement C4 failed the test of normality. Table 1 summarizes results of C4, C1inhA and C1inhF levels in control subjects and untreated/treated HAE patients. Compared to the untreated group, values for C4, C1inhA and C1inhF were significantly greater ($P < 0·01$) for HAE patients taking prophylaxis, consistent with previous observations [2].

Patients with normal C4 values

Levels of complement C4 were within the normal range ($\geq 0·14$ g/l) in five untreated HAE patients on nine separate occasions (Table 2), all of which were routine clinic visits when the patients were free of symptoms. Low C4 values were obtained on four occasions in the same five patients. Four of these five patients had normal C1inhF on one or more occasion. As this questions the diagnosis of HAE, frozen sera were re-analysed for C1inhF by chromogenic assay, confirming low C1inhF in all cases.

Genetic analysis

Genetic analysis was restricted to these five patients with normal C4. Patients 1, 2 and 3 have a missense mutation in exon 7 of the C1 inhibitor gene (c.1109T > G, Met348Arg). The activity of the Met348Arg mutation in a recombinant expression system was 5% of normal, indicating a severe impairment of protein function. Small deletions of one base in exon 5 of the C1 inhibitor gene were found in patient 4

Table 2. Characteristics of untreated hereditary angio-oedema (HAE) patients with normal serum C4.

Patient	Age (years)	Sex	Normal C4 values (g/l) (≥ 0·14)	Clinical features	Low C4 (g/l) values (≥ 0·14)	C1inhA (mg/l) (> 150)	C1inhF by Quidel (%) (> 84)	C1inhF by Technochrom (units/ml) (> 0·7)
1	69	F	0·15, 0·19	Peripheral swellings	None	78	69, 93, 86, 51	0·6
2	20	M	0·16	Peripheral swellings	0·11	100	91,93, 81	< 0·3
3	11	M	0·23, 0·19	None (affected mother)	0·10	122	81, 84	< 0·3
4	36	M	0·16, 0·15, 0·15	Peripheral/facial swelling	0·11	108	81,69	0·4
5	75	M	0·21	Peripheral/facial swelling	0·13	83	37	Not performed

(c.727delC) and patient 5 (c.804delG). These two deletion mutations cause a translational frame shift leading to nonsense-mediated mRNA decay and/or premature termination of protein synthesis.

Receiver operating characteristic (ROC) analysis

ROC analysis was applied to the data from untreated patients to determine the diagnostic value of the tests employed (Table 3). The manufacturer's normal range for C1inhF appears to be too low; optimizing the lower limit of normal to 84% improved the sensitivity to 78% without compromising the specificity.

Discussion

A previous study confirmed the accuracy of the laboratory diagnosis of HAE [8] and supports the practice of 'request managing' access to C1 inhibitor assays on the basis of low C4 [9]. However, Karim and colleagues reported a 10-year-old girl with confirmed HAE despite consistently normal C4 levels [10]. In our series, the sensitivity of low C4 for the diagnosis of HAE was just 81%, insufficient for a screening test. Clearly, the validity of the diagnosis in these individuals must be defended: patients 1–4 have a strong family history of HAE type I; all but one have typical (although mild) symptoms – an 11-year-old boy with an affected parent is asymptomatic, but because the first clinical symptoms usually appear during the second decade of life, this does not exclude the diagnosis [11]; all five patients have low levels of C1inhA, and four of five have low serum C4 on occasion (clearly, C4 results during disease exacerbation would be interesting); although C1inhF was sometimes normal by the Quidel assay in patients 1–4, low C1inhF was confirmed by chromogenic assay. Furthermore, novel mutations in the C1 inhibitor gene, which we believe are disease-causing, were described in all five individuals. HAE is a genetically heterogeneous disease, with more than 100 C1 inhibitor gene mutations described to date, spanning all the exons and exon/intron boundaries. Functional studies of these mutations have generally not yet been performed [12]. Patients 1, 2 and 3 belong to a large family with numerous affected members, and they share a missense mutation in exon 7 of the C1 inhibitor gene (Met348Arg). The mutation segregates with the disease in this family and the resulting protein, when expressed *in vitro*, shows only 5% residual activity. Patient 4 has a small deletion of one base in exon 5 of the C1 inhibitor gene (c.727delC), which destroys the open reading frame and leads to the generation of a new stop codon in exon 5; the same mutation was present in two affected siblings (whose C4 is low). If any protein translation were to occur from this allele parts of the protein would be missing, including the reactive centre loop encoded in exon 8. Patient 5 has a small deletion of one base in exon 5 (c.804delG) which destroys the open reading frame, generating a new stop codon, and is therefore likely to be disease-causing.

In all these cases, a clinician experienced in the management of HAE would have been alerted by aspects of the history. However, patients with HAE frequently present to a non-specialist setting; as an illustration, diagnosis in patient 5 was delayed to old age following normal C4 assays at various hospitals. His condition is much improved following appropriate therapy.

Two methods are in routine commercial use for assay of C1inhF in the United Kingdom: the Quidel functional ELISA

Table 3. Diagnostic performance of low C4, low C1inhA/C1inhF for hereditary angio-oedema (HAE) in untreated patients.

	Low C4 (< 0·14 g/l)	C1inhA (< 150 mg/l)	C1inhF (m = manufacturers' range, < 68%)	C1inhF (optimized range, < 84%)	Low C4 and low C1inhF (optimized range)
Sensitivity	81%	97%*	57%	78%	78%*
Specificity	85%	100%*	100%	100%	100%*

*Refers to HAE type I.

technique and two chromogenic assays, the Technochrom assay (Technoclone) and the Berichrom assay (Dade-Behring). Returns from the UK National External Quality Assurance Scheme C1 inhibitor pilot show a bimodal distribution for the two techniques, with the Quidel system reading higher than the chromogenic assays. However, a recent head-to-head comparison of the Quidel ELISA and Technoclone chromogenic techniques [13] demonstrated good correlation between the assays when the samples were correctly stored and transported, suggesting that the colorimetric technique is sensitive to sample degradation. In practical terms, this may be a major problem, as C1inhF assays are performed in reference laboratories. In our series, the diagnostic performance of the Quidel assay was optimized by increasing the lower limit of normal to 84%, highlighting the importance of determining local reference ranges. However, even with this revised range, in our hands the sensitivity of the assay is 78%.

In summary, we confirm that the laboratory diagnosis of HAE using a combination of low C4 and C1 inhibitor studies is extremely accurate. However, normal serum C4 may occasionally be compatible with the diagnosis of HAE. We propose that assay of C1 inhibitor antigen and function should be performed regardless of serum C4 levels when clinical suspicion is high.

Declaration of interests

The Department of Immunology at St Bartholomew's Hospital is currently involved in Clinical Trials of icatibant, C1 inhibitor concentrate and recombinant C1 inhibitor concentrate for the management of HAE. H. L. is a member of the DX88 advisory committee for the Dyax Corporation and the international advisory board for icatibant.

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