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Anti-cholesterol antibody levels in hereditary angioedema

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Abstract

Hereditary angioedema (HAE) is a rare disorder caused by the deficiency of the C1-inhibitor gene (*C1INH*) and characterized by recurrent bouts of angioedema. Autoimmune disorders frequently occur in HAE. Previously we found, that danazol has an adverse effect on serum lipid profile: reduced high-density lipoprotein (HDL) and elevated low-density lipoprotein (LDL) cholesterol levels are associated with long-term prophylactic use, whereas total cholesterol levels are unchanged. Our aim was to study the anti-cholesterol antibody (ACHA) production in HAE patients and compare it with those of healthy blood donors, and to investigate the possible associations between ACHA levels and serum lipid profile alterations caused by danazol. Anti-cholesterol IgG levels were measured by ELISA and their correlation with serum concentrations of total cholesterol, HDL, LDL, triglycerides was determined in HAE patients receiving/not receiving danazol. Serum ACHA levels were significantly higher in HAE patients, compared to healthy blood donors ($P < 0.0001$). Long-term danazol prophylaxis had no effect on serum ACHA levels in HAE patients. However, we found a significant, negative correlation between ACHA levels and serum total cholesterol ($r = –0.4033$, $P = 0.0200$), LDL ($r = –0.4565$, $P = 0.0076$) and triglyceride ($r = –0.4230$, $P = 0.0121$) levels only in danazol-treated patients, but not in HAE patients who did not receive long-term prophylaxis. Patients with HAE have higher baseline ACHA levels compared to healthy subjects, and this might reflect polyclonal B-cell activation. The latter would be a potential explanation for the lack of an increased incidence of infectious diseases in HAE patients, but might lead to increased autoimmunity.

Keywords: hereditary angioedema • anti-cholesterol antibodies (ACHA) • cholesterol • atherosclerosis • humoral immune response

Introduction

Hereditary angioedema (HAE) is a rare, but potentially life-threatening condition. It is manifested by acute attacks of facial, laryngeal, genital or peripheral swelling, as well as by abdominal pain secondary to intra-abdominal edema. It is caused by the functional deficiency of the C1 inhibitor protein (C1-INH or C1INH; approved gene symbol SERPING1) [1–4]. Treatment involves prophylaxis to prevent, and emergency therapy to manage acute HAE attacks. For long-term prophylaxis, attenuated androgens (danazol or stanazolol) or anti-fibrinolytic agents (tranexamic acid or e-aminocaproic acid) are used, whereas C1-INH concentrate is the drug of choice for emergencie [5]. Danazol is effective for the prophylaxis of

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recurrent attacks of mucocutaneous and visceral swelling that occur in patients with HAE. Danazol apparently increases the synthesis of C1-INH, which is partially deficient in these heterozygous individuals [6, 7]. Our previous findings indicated that the long-term use of danazol has an adverse effect on serum lipid profile: serum concentrations of high-density lipoprotein (HDL) cholesterol and apolipoprotein A-I were significantly lower, whereas low-density lipoprotein (LDL) cholesterol and apolipoprotein B-100 were higher in danazol-treated patients, compared with untreated HAE controls [8]. Danazol treatment was associated with a highly proatherogenic lipid profile in patients with HAE [8].

Naturally occurring autoantibodies against cholesterol are present in the sera of almost all healthy individuals [9, 10]. The protective role of these antibodies against atherosclerosis, induced by cholesterol-rich diet, was demonstrated in rabbit models [11, 12]. According to Alving & Wassef [13], anti-cholesterol antibodies (ACHA) present in normal human plasma also contribute to LDL cholesterol turnover by opsonizing LDL for removal by complement receptors. Previously, we have measured ACHA levels in patients with various vascular disorders of atherosclerotic origin [14, 15] to ascertain that our findings are at variance with the assumption of Alving *et al.* [13]. As expected, ACHA levels were high in patients with coronary heart disease. In patients with stroke or peripheral artery disease, however, they were as low as in age-matched healthy controls. Additionally, in a substudy of the prospective HOPE (Heart Outcomes Prevention Efforts) trial, high ACHA levels were found to confer protection against the development of stroke during the follow-up period [16]. Recently, significantly lower ACHA levels were found in patients with severe carotid atherosclerosis, compared to age-matched healthy subjects. Reduced baseline levels of ACHA returned to normal within a year following the surgical removal of atherosclerotic plaques from carotid arteries [15].

Autoimmune diseases, such as systemic lupus erythematosus (SLE) (or SLE-like syndromes), Sjögren's syndrome, scleroderma, thyroiditis, glomerulonephritis and inflammatory bowel disease have been described in patients suffering from HAE [17]. The immunoreactivity of these patients has also been found to exceed normal [18]. Our recently published data – that is, the occurrence of high titres of IgM type natural autoantibodies against C1-INH is more common in HAE patients, compared to healthy controls [19] – also support the assumption that polyclonal B-cell activation occurs in HAE.

Our aim was to study the ACHA-specific antibody reactivity in HAE, comparing serum anti-cholesterol IgG levels of HAE patients with those of healthy blood donors, and to investigate possible associations between ACHA levels and serum lipid profile alterations caused by danazol treatment.

Materials and methods

Study subjects

Fifty-nine adult patients with HAE (36 women and 23 men; aged 18–71 years) registered with and managed at the Hungarian HAE Center until November 2003 were enrolled into this study [20]. These patients represent a subgroup of 64 adult HAE patients, in whom the influence of danazol treatment on lipid profile was originally described [8]. The reason for including only 59 of these 64 patients (92.2%) was the limited availability of blood samples. The history of sudden oedematous attacks, family history, laboratory findings of low functional activity and antigen concentration of C1-INH, as well as low C4 levels established the tentative diagnosis, which was confirmed by ascertaining the mutation of the *C1INH* gene [21]. In our clinical practice, danazol (prescribed by the Budapest protocol [5]) and tranexamic acid (not available in the United States) are the drugs of choice for long-term prophylaxis, whereas a highly purified, lyophilized, human plasma-derived C1-INH concentrate (Berinert P, CSL Behring GmbH, Marburg Germany) is administered in acute attacks only. In 33 of the 59 patients, long-term danazol prophylaxis was necessary to prevent the recurrence of occasionally life-threatening attacks. The duration of treatment ranged between 5 and 195 months, and danazol was administered in daily doses of 33–200 mg. The other 26 patients, who have never received prolonged prophylactic treatment with danazol, were enrolled as HAE-positive controls. Demographic and clinical data of HAE patients and the properties of the medication used are presented in Table 1. Disease severity was determined according to the criteria developed by experts from the Novel Methods for Predicting, Preventing and Treating Attacks in Patients with Hereditary Angioedema group and as described by Agostoni *et al.* [1] during the first year of treatment (Table 1). Blood samples of patients were collected between December 2000 and November 2003 and stored deep-frozen at –70°C until laboratory testing. Samples were obtained at regular control visits, that is, none of the patients was experiencing an acute

angioedematous attack at the time of blood sampling. All blood samples from patients and controls were obtained in a fasting state. Sixty-six age- and sex-matched healthy Hungarian volunteers (38 women and 28 men; aged 22–72 years) served as HAE-negative controls. All of them were examined and asked about any diseases. Only healthy subjects without clinical sign or suspicion of atherosclerotic vascular diseases were enrolled in this study. None of them had clinical or laboratory signs suggestive of HAE. Some of the patients and controls have been recently described [8]. The study was approved by the institutional review board and all subjects provided informed consent.

Measurement of the serum concentration of ACHA

Serum cholesterol-specific antibodies were measured by solid phase enzyme immunoassay as described previously [14]. Briefly, polystyrene plates (Greiner, Frickenhausen, Germany) coated with 5 μg/well cholesterol dissolved in 100 μl absolute ethanol were incubated at +4°C for 24 hrs. After washing with phosphate buffered saline (PBS) and blocking with 0.1% casein (Reanal, Budapest, Hungary) in PBS, the wells were incubated with 100 μl of serum samples diluted to 1:800 in PBS containing 0.1% casein. Binding of ACHA was detected by anti-human horseradish peroxidase conjugated γ-chain-specific rabbit antibodies (DAKO, Glostrup, Denmark); o-phenylene-diamine (Sigma, St. Louis, USA) and H_2O_2 as substrate. Optical density was measured at 490 nm (reference at 600) and the mean value of duplicates was calculated. Serial dilution of intravenous immunoglobulin (IVIG) (Sandoglobulin, 50 mg/ml) was used as the standard in all experiments. Data obtained as optical density values were expressed in arbitrary units per millilitres (AU/ml) related to the standard curve.

Laboratory measurements of lipid parameters

Lipid parameters were measured with computerized laboratory analysers: Cobas Integra 400 (Roche, Basel, Switzerland) was used for measuring total cholesterol and triglyceride concentrations, whereas Cobas Mira (Roche) was used for measuring HDL. LDL was calculated according to the Friedewald formula [22]. The ratio of LDL/HDL calculated and used as additional atherogenic index to characterize the abnormality of lipid metabolism. Lipid profiles of the patients were published previously [8].

Table 1 Descriptive statistics of HAE patients

Number of individuals, n	59
Age, years	39.23 (28.55–48.14)
Gender, m/f (%)	23/36 (39.0%/61.0%)
HAE, Type I/Type II (%)	54/5 (91.5%/8.5%)
Severity of HAE	
Class 1 (severe), n (%)	21 (35.6%)
Class 2 (moderate), n (%)	24 (40.7%)
Class 3 (mild), n (%)	2 (3.3%)
Class 4 (minimal), n (%)	7 (11.9%)
Class 5 (asymptomatic), n (%)	5 (8.5%)
Medication	
No medication, n (%)	7 (11.9%)
Danazol, long-term prophylaxis, n (%)	33 (55.9%)
Daily dose, mg/d	100.00 (82.52–173.94)
Danazol, occasionally, n (%)	2 (3.3%)
Dose, mg/occasion	200.00 (200.00–200.00)
TA, long-term prophylaxis, n (%)	5 (8.5%)
Daily dose, g/d	2.00 (1.66–2.00)
TA, occasionally, n (%)	12 (20.3%)
Dose, g/occasion	2.00 (2.00–3.00)

Values presented as absolute numbers (percentages) and median (interquartile range). Abbreviation used: HAE, hereditary angioedema; TA, tranexamic acid.

Statistical analysis

Calculations were performed with SPSS for Windows version 11.5.1 (SPSS Inc, Chicago, IL, USA) and Prism 4.01 (GraphPad Software, San Diego Calif). Because many variables had non-Gaussian distributions, we used the Mann–Whitney non-parametric test for between-group comparisons, the Spearman rho coefficient to calculate correlations, and the chi-square test and chi-square test for trend to compare frequencies. Multiple logistic regression analysis was also performed. All tests were performed

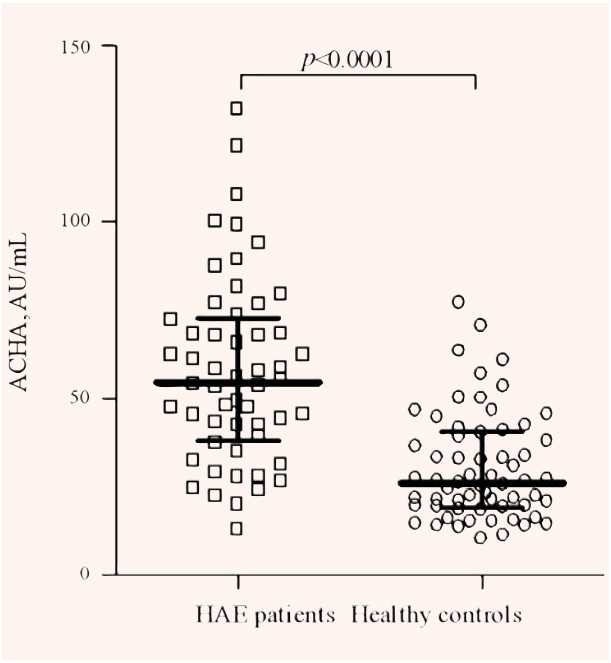

Fig. 1 ACHA titres in HAE patients and healthy controls. Serum ACHA titres were significantly higher in patients with HAE, than in healthy controls. Mann–Whitney's non-parametric test.

two-tailed and $P < 0.05$ was considered as significant. Values shown in the text are presented as medians with interquartile ranges.

Results

ACHA levels of HAE patients and of healthy subjects

Serum ACHA levels of HAE patients were compared to those of healthy controls (Fig. 1). As the two study groups did not differ by age or gender, univariate analysis with Mann–Whitney's non-parametric test was performed. We found that HAE patients had significantly higher ACHA levels compared to healthy controls (54.41 [37.96–72.79] *versus* 25.99 [18.98–40.39] AU/ml; $P < 0.0001$). The frequency of high ACHA levels (defined as ACHA levels exceeding the 90th percentile of controls) was significantly higher in HAE patients: 10/59 HAE patients compared to only 2/66 healthy controls had high ACHA levels ($P = 0.0084$, chi-square test).

ACHA levels in HAE patients taking danazol, compared to HAE patients not receiving danazol prophylaxis

HAE patients were classified into two groups according to the use of long-term danazol prophylaxis. We did not find any differences in age, gender or HAE type between the two patient groups (Table 2). However–as expected, patients receiving long-term danazol had more severe disease (classified as severe [Class 1] or moderate [Class 2]) in comparison to the HAE control group ($P < 0.0001$, chi-square test for trend), where disease severity ranged from asymptomatic (Class 5) to severe (Class 1). We found that serum ACHA levels did not differ in the two patient groups: danazol-treated patients had ACHA levels similar to those of HAE patients who did not receive long-term prophylaxis (Table 2). However, danazol treatment is associated with decreased HDL and elevated LDL levels, as well as with a remarkable increase in the LDL/HDL ratio.

Correlations between ACHA levels and serum lipid parameters

Next, we calculated correlations between ACHA levels and lipid parameters (Table 3). We found significant negative correlations between ACHA levels and total cholesterol, LDL, LDL/HDL ratio and triglycerides, respectively, in HAE patients (Table 3, panel B), but not in healthy controls (Table 3, panel A). After stratifying patients according to the use of danazol prophylaxis, we found that these correlations were significant only in HAE patients receiving long-term danazol prophylaxis (Table 3, panel C), but not in patients who were not taking danazol (Table 3, panel D).

Discussion

The principal finding of the present study is that compared to healthy individuals, naturally occurring autoantibodies against cholesterol are present in the blood of HAE patients in higher titres: patients had more than twice higher ACHA IgG levels, than controls.

The possible explanation for the high ACHA levels of HAE patients is polyclonal B-cell activation, result-

Table 2 Serum ACHA titres, lipid concentrations and complement values in HAE patients on long-term danazol prophylaxis, compared to HAE patients not receiving danazol

	Patients taking danazol	Patients not taking danazol	P*
Number of individuals, n	33	26	-
Age, years	39.6 (32.2–47.7)	35.1 (20.4–46.7)	0.3361
Gender, m/f (%)	15/18 (45.5%/54.5%)	8/18 (30.8%/69.2%)	0.2918**
HAE Type I/Type II, n	30/3 (90.9%/9.1%)	24/2 (92.3%/7.7%)	1.0000**
HAE severity, class	2.0 (1.00–2.0)	3.0 (1.0-4.0)	*P*<0.0001***
ACHA, AU/Ml	53.56 (38.40–71.11)	55.83 (31.63–82.95)	0.8486
Total cholesterol, mmol/l	5.23 (4.37–6.25)	5.03 (4.04–5.64)	0.3323
HDL cholesterol, mmol/l	1.08 (0.91–1.38)	1.38 (1.26–1.75)	0.0003
LDL-cholesterol, mmol/l	3.44 (2.93–3.94)	2.72 (2.06–3.38)	0.0176
LDL/HDL, ratio	2.98 (2.10–4.32)	2.04 (1.34–2.67)	0.0006
Triglycerides	1.22 (0.92–1.55)	0.89 (0.70–1.66)	0.1512

Values presented as absolute numbers (percentages) and median (interquartile range). *P* values were calculated with the *Mann–Whitney's non-parametric test, **Fisher's Exact test, ***chi-square test for trend. Abbreviations used: ACHA, anti-cholesterol autoantibodies; HAE, hereditary angioedema; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Table 3 Correlations between serum ACHA titres and lipid parameters

	Total cholesterol, mmol/l	HDL-cholesterol, mmol/l	LDL-cholesterol, mmol/L	LDL/HDL, ratio	Triglycerides, mmol/l
A. Healthy controls (n = 66)					
ACHA, AU/ml	0.0282 (*P* = 0.8219)	0.0396 (*P* = 0.7524)	–0.0535 (*P* = 0.6694)	–0.0669 (*P* = 0.5937)	0.0168 (*P* = 0.8936)
B. All HAE patients (n = 59)					
ACHA, AU/mL	–0.2965 (*P* = 0.0226)	0.0671 (*P* = 0.6135)	–0.3244 (*P* = 0.0122)	–0.2947 (*P* = 0.0235)	–0.3403 (*P* = 0.0084)
C. HAE patients taking danazol (n = 33)					
ACHA, AU/mL	–0.4033 (*P* = 0.0200)	0.0731 (*P* = 0.6861)	–0.4565 (*P* = 0.0076)	–0.3723 (*P* = 0.0329)	–0.4320 (*P* = 0.0121)
D. HAE patients not taking danazol (n = 26)					
ACHA, AU/ml	–0.1737 (*P* = 0.3961)	0.0325 (*P* = 0.8748)	–0.2264 (*P* = 0.2661)	–0.2280 (*P* = 0.2625)	–0.2103 (0.3024)

Values presented as Spearman's rho (P-value). Abbreviations used: ACHA, anti-cholesterol autoantibodies; HAE, hereditary angioedema; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

ing from the regulatory defect of C1-INH. As the complement system plays an essential role in controlling the adaptive immune system [23] as well as in B-cell regulation [24], it is possible that complement activation during HAE attacks might lead to B-cell activation. It has been shown earlier that hypergammaglobulinaemia is more common in HAE [17]. Recently, we have measured total concentrations of different Ig classes in HAE patients and found significantly higher levels of IgM, IgG1 and IgG3 in patients, than in

healthy controls (Varga *et al.*, manuscript in preparation). In agreement with probable B-cell activation, ACHA titres are elevated in these patients; however, no association with long-term danazol treatment could be found. Danazol is a 17α-alkylated attenuated androgen derivate, which increases the synthesis of certain complement components (such as C1-INH and C4) in the liver and thereby decreases the frequency of HAE attacks in patients with C1-INH deficiencies [6, 7, 25]. Nevertheless, patients receiving continuous danazol prophylaxis suffer from various side effects [26], most frequently weight gain, acne, hirsuitism, menstrual abnormalities and elevation of liver enzyme activity. Earlier, we described that danazol affects plasma lipid profiles in these patients [8]. In that study, we established that patients with long-term danazol prophylaxis have significantly lower HDL and apolipoprotein A-I levels (the latter is the main structural lipoprotein of HDL), whereas they have higher LDL and apolipoprotein B-100 (the main lipoprotein component of LDL) levels. Thus, we concluded that the long-term use of danazol results in a highly atherogenic lipid profile, whereas corresponding clinical data on the frequent manifestations of atherosclerosis in these patients [8] were lacking. Despite the high atherosclerotic risk profile, we found no association between danazol treatment and accelerated atherosclerosis in HAE by determining the prevalence of vascular disorders among the patients and measuring carotid intima-media thickness (Szegedi et al. manuscript in preparation).

In view of this substantial impact of danazol on lipid metabolism, the present study also investigated the effect of long-term danazol treatment on serum ACHA levels. Two groups of patients, concerning long-term danazol treatment, varied in disease severity. The reason is that the indication for continuous danazol therapy is dependent on disease severity (*i.e.* patients with more severe disease need long-term treatment with danazol) and is therefore a major determinant in the classification of severity. Thus, adjustment according to disease severity was not done when comparing patient subgroups. Interestingly however, we did not find any significant difference in the titres of ACHA in patients with danazol prophylaxis compared to other subjects. However, significant inverse correlations were present between ACHA titres and total cholesterol, LDL and triglyceride levels, as well as the LDL/HDL ratio, in patients receiving long-term danazol prophylaxis. These associations were absent both in HAE patients without danazol treatment and in healthy controls. Thus, low ACHA titres might reflect high LDL levels in the long-term prophylaxis group and could be a secondary consequence, similarly to that we found in patients with severe carotid atherosclerosis before the surgical removal of the plaques [15].

In conclusion, we described that patients with HAE have higher baseline ACHA levels compared to healthy subjects, and this might reflect polyclonal B-cell activation in these patients. The latter might result in an enhanced humoral immune response in HAE, a mechanism compensating for the defect caused by the inappropriate regulation of C1-INH on the classical and mannose-binding lectin pathways of the complement system. On one hand, this enhanced antibody response against infectious agents might explain the lack of an increased incidence of infectious disease in patients with HAE. On the other hand, however, polyclonal B-cell activation might lead to increased autoimmunity.

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