Frequencies of Certain Complement Protein Alleles and Serum Levels of Anti–Heat-Shock Protein Antibodies in Cerebrovascular Diseases

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Background and Purpose—A strong correlation exists between the intensity of atherosclerotic alterations in different arteries. Marked differences exist, however, in the age and sex distribution and risk factors for coronary heart disease (CHD) and cerebrovascular disease (CVD). We therefore performed genetic and immunologic studies in patients with CVD.

Methods—We studied 292 patients with CVD (stroke or transient ischemic attack) and as control either 198 healthy blood donors and 485 healthy elderly (aged >60 years) people (genetic study) or 94 blood donors aged 45 to 60 years and 49 healthy elderly (aged >60 years) people (anti–heat-shock protein [hsp] measurements). Allele frequencies of 3 genes (C4A, C4B, and C3) encoding proteins of the complement system were determined by electrophoresis and immunofixation. Serum concentration of autoantibodies against 60-kDa heat-shock protein (anti-hsp60) was measured by the enzyme-linked immunosorbent assay method.

Results—Marked differences were observed between CVD patients and controls in the genetic studies. In the CVD patients aged >60 years, the frequency (11.3%) of the deficient allele of the C4B gene (C4B*Q0) was significantly (*P<0.0003) higher than that of the healthy controls (5.4%). By contrast, in the group aged 45 to 60 years, the frequency of the C4B*Q0 allele was lower in patients than in controls. Serum concentration of anti-hsp60 in the CVD patients did not differ from control values.

Conclusions—In previous studies C4B*Q0 frequency was reported to be higher in CHD patients aged 45 to 60 years than in aged-matched controls. Moreover, high anti-hsp60 levels were found in CHD patients. Therefore, genetic and immunologic factors may at least partly explain the differences between the natural history and risk factors of CHD and CVD. (Stroke. 2000;31:2648-2652.)

Key Words: cerebrovascular disorders ■ genetics ■ heat-shock proteins ■ stroke

It has been demonstrated that atherosclerosis develops and progresses in parallel in different arteries.1–6 Therefore, coronary heart disease (CHD) (myocardial infarction, sudden heart death) is the leading cause of death of patients with cerebrovascular disease (CVD).7

When these observations are considered, strong similarities in the epidemiology of CHD and CVD may be expected. In contrast to this assumption, however, there is a marked quantitative, if not qualitative, difference in their important risk factors.8 Compared with myocardial infarction, stroke patients are at least 10 years older, the incidence in middle-aged men compared with women is not as great, and increasing blood pressure is more strongly associated with stroke.8 Part of the reason is presumably that so many epidemiological studies have considered all strokes together, thus weakening causal associations with ischemic stroke if they are not also associations with hemorrhagic stroke. However, this explanation is necessarily incomplete because most strokes are ischemic.8 According to the recent review article of Warlow,8 there is no doubt that new risk factors (including genetic factors) will emerge, some of which will have causal associations.

Previously we have found a highly significant association between the morbidity and mortality of myocardial infarction and C4B*Q0, the silent allele of 1 of the 2 genes encoding C4, a component of the complement system.9 The C4B*Q0 allele was found with increased frequency among myocardial
infarction patients aged 60 to 79 years compared with age-matched healthy controls.\(^9\) Moreover, in C4B*Q0 carriers myocardial infarction had a significantly higher risk for lethal outcome compared with noncarriers.\(^9\) Recently we also found the frequency of the C4B*Q0 carriers to be significantly increased in Icelandic CHD patients.\(^10\) Therefore, we sought to determine whether the silent C4 allele is associated with CVD as well.

Much data have been accumulated in recent years that indicate the essential role of inflammation in atherogenesis\(^11\) and in the rupture of vulnerable plaques.\(^12\) Immunologic factors may have an important role in triggering and maintaining inflammation in arterial walls. According to the recent hypothesis of Wick et al,\(^13\) different stimuli leading to activation of endothelial cells may increase expression of heat-shock proteins (hsp) in these cells and induce cellular and humoral immune responses against these proteins or enhance preexisting immune responses. Many recent studies\(^14\)\^-\(^16\) demonstrated antibodies in high titers against the 60-kDa family of hsp, such as human hsp60 or mycobacterial hsp65 in CHD patients. Since in CVD patients anti-hsp antibodies have not been measured thus far, in the present study we determined the amounts of the antibodies against human hsp60 and mycobacterial hsp65 in patients with CVD and in healthy control subjects.

**Subjects and Methods**

**Patients and Controls**

Two series of patients with CVD admitted for hospital treatment to the Department of Neurology of St Imre Hospital of Budapest, Hungary, were studied. Blood was taken from the patients a median of 4 days (interquartile range, 2 to 6 days) after the clinical onset of stroke or transient ischemic attack (TIA). The first series (series 1), investigated in 1993, consisted of 152 patients with stroke aged 45 to 91 years (median, 63 years; 86 men, 66 women). Only complement allotypes were determined in this group. The second series (series 2), tested in 1997, consisted of 140 patients aged 46 to 92 years (median, 72 years; 68 men, 72 women). Fourteen patients had hemorrhagic stroke, and 126 had ischemic stroke. Of the latter group, 18, 21, 45, 9, and 20 patients had TIA; total anterior circulation infarcts, partial anterior circulation infarcts, posterior circulation infarcts, and lacunar infarcts, respectively.\(^11\) No classification was possible in 13 patients. According to the sonographic examination, no alterations were found in 10 patients. In 24 patients intimal thickness, in 64 patients atherosclerotic plaques, in 9 patients measurable stenosis, in 9 patients significant stenosis, and in 9 patients occlusion of the carotid artery were observed (no ultrasonographic examination was performed in 4 patients). In this series both complement genetic studies and anti-hsp antibody determinations were done.

Sera from 204 blood donors (aged 45 to 60 years; median, 53 years; 99 men, 105 women) as well as those from 485 healthy elderly (aged \(>60\) years) people (195 men, 289 women)\(^9\) were used as controls for complement allotyping. The control group for anti-hsp measurements consisted of 94 blood donors aged 45 to 60 years and 49 healthy elderly (aged \(>60\) years) people. Serum samples from the patients and controls were stored in aliquots at \(-70^\circ\)C and thawed only once immediately before the tests were performed.

**Study of Polymorphisms of the C4A, C4B, and C3 Genes**

C4 typing was performed according to Awdeh and Alper\(^9\) with the modification of Sim and Cross.\(^20\) C3 allotypes were determined by the method of Teisberg.\(^21\)

**Measurement of Antibodies Against Mycobacterial hsp65 and Human hsp60**

The amounts of IgG-type antibodies reacting with proteins of the chaperonin 60 family (recombinant human hsp60, SPP-740, Stress-Gen, Victoria, Canada, and recombinant Mycobacterium bovis hsp65 [batch MA14, GFB, Germany; supported by the United Nations Development Program/World Bank/World Health Organization Special Program for Research and Training in Tropical Diseases]) were assessed by enzyme-linked immunosorbent assay as described previously.\(^22\) In brief, plates were coated with 0.1 \(\mu\)g per well human hsp60 or \(M\) bovis hsp65. After they were washed and blocked (PBS, 0.5% gelatin), wells were incubated with 100 \(\mu\)L of serum samples diluted 1:500 in PBS containing 0.5% gelatin and 0.05% Tween 20. Binding of anti-hsp antibodies was determined with the use of \(\gamma\)-chain specific anti-human IgG peroxidase-labeled antibodies (Sigma) and o-phenylenediamine (Sigma) detection system. The optical density was measured at 490 nm (reference at 620 nm), and means of duplicate wells were calculated. A serial dilution of a control anti-hsp60 rabbit polyclonal antiserum (StressGen SPA-804, also reacting with hsp65) was used as standard. Data obtained as optical density values were calculated to arbitrary unit per milliliter (AU/mL) values related to this standard.

**Statistical Analysis**

Categorical variables were compared with the Fisher’s exact test. Since the variable antibody to hsp was not normally distributed, nonparametric tests were used for group comparisons. Spearman rank correlation coefficients were calculated for estimation of interrelations between hsp antibodies and other variables. The significance level was set at a value of \(P<0.05\). The relationship between the levels of anti-hsp antibodies and the extent of ultrasonographic alterations in carotid arteries was calculated by logistic regression analysis.

**Results**

**Determination of Polymorphism of C4A, C4B, and C3 Genes in CVD Patients and Healthy Controls**

Allotypes of the 2 genes encoding the fourth component of complement, C4, and that encoding the third component of complement, C3, were studied in series 1 and series 2 of the CVD patients and in healthy controls. A gradual age-dependent increase in the frequency of the C4B*Q0 allele was observed in the CVD patients. In patients aged 45 to 60 years, 61 to 70 years, and >70 years, frequencies of the allele were 13 of 202 (0.064), 15 of 154 (0.097), and 28 of 228 (0.123). With the use of a multiple comparison method (\(\chi^2\) test for trend), a significant relationship (\(P=0.040\)) between age and C4B*Q0 allele was found. Since previously we found pronounced age-dependent changes in the frequency of the silent allele of C4B, the patients were divided into 2 age groups (45 to 60 years and >60 years) and compared with age-matched controls. For the younger age group of patients, healthy subjects from the present study were used as controls, while the results obtained in the second age group of CVD patients were compared with those of 485 healthy elderly (aged >60 years) people from our previous study.\(^9\) For both age groups of patients, similar frequencies were found for all complement alleles in series 1 and 2. For example, in the younger group the frequencies of C4B*Q0 were 0.0643 and 0.0645 (\(P=0.999\)), respectively, while in the older group in series 1 and 2, frequencies of 0.128 and 0.101 (\(P=0.416\)), respectively, were found.

Frequencies of the complement alleles were compared in 191 CVD patients aged >60 years and in 485 age-matched...
Frequency of Common Alleles of C4A, C4B, and C3 Complement Genes in Patients With CVD and Healthy Controls Aged >60 Years

<table>
<thead>
<tr>
<th>Allele</th>
<th>No. of Alleles/No. of All Alleles Tested</th>
<th>Patients With CVD (n=191)</th>
<th>Healthy Controls (n=485)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4A*Q0</td>
<td></td>
<td>58/382 (0.152)</td>
<td>134/970 (0.139)</td>
<td>NS</td>
</tr>
<tr>
<td>C4A*2</td>
<td></td>
<td>8/382 (0.021)</td>
<td>61/970 (0.063)</td>
<td>0.0009</td>
</tr>
<tr>
<td>C4A*3</td>
<td></td>
<td>276/382 (0.723)</td>
<td>688/970 (0.709)</td>
<td>NS</td>
</tr>
<tr>
<td>C4A*4</td>
<td></td>
<td>32/382 (0.084)</td>
<td>47/970 (0.049)</td>
<td>0.0197</td>
</tr>
<tr>
<td>C4A*6</td>
<td></td>
<td>7/382 (0.018)</td>
<td>14/970 (0.015)</td>
<td>NS</td>
</tr>
<tr>
<td>C4B*Q0</td>
<td></td>
<td>43/382 (0.113)</td>
<td>52/970 (0.054)</td>
<td>0.0003</td>
</tr>
<tr>
<td>C4B*1</td>
<td></td>
<td>299/382 (0.783)</td>
<td>793/970 (0.818)</td>
<td>NS</td>
</tr>
<tr>
<td>C4B*2</td>
<td></td>
<td>38/382 (0.100)</td>
<td>99/970 (0.102)</td>
<td>NS</td>
</tr>
<tr>
<td>Other C4B alleles</td>
<td></td>
<td>2/382 (0.005)</td>
<td>26/970 (0.027)</td>
<td>...</td>
</tr>
<tr>
<td>C3*S</td>
<td></td>
<td>307/370 (0.827)</td>
<td>342/412 (0.830)</td>
<td>NS</td>
</tr>
<tr>
<td>C3*F</td>
<td></td>
<td>63/370 (0.170)</td>
<td>70/412 (0.170)</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Significance of difference between patients and controls, calculated by Fisher’s exact test.

controls (Table ). The frequency of the C4B*Q0 allele was significantly higher in the elderly patients than in the age-matched healthy elderly people. Significant differences between the patients and controls aged >60 years were also found in 2 relatively rare alleles of the C4A gene. In CVD patients the frequency of the C4A*2 allele was relatively low, while that of the C4A*4 allele was relatively high. In sharp contrast to the findings in the older age group, the allele frequency of C4B*Q0 in 101 CVD patients aged 45 to 60 years was significantly (P=0.044) lower, almost one half of the frequency observed in 204 age-matched control subjects. This decrease was compensated with a significantly higher occurrence of the C4B*1 allele in these patients. The frequency of the C4B*Q0 allele was lower in the male (0.059) than in the female (0.085) patients, but the difference was not statistically significant. No significant differences between CVD patients and controls were found in the frequencies of any other alleles tested (data not shown).

Significant differences (P=0.001) were found between patients aged ≤60 and >60 years in the proportion of those with TIA and ischemic stroke as well: TIA was observed in 37% and 14% of the patients, respectively. CHD was found in 35% and 43% of the patients, respectively; the difference between the 2 groups, however, was not significant. We performed a logistic regression analysis and found significant differences between the 2 age groups in the frequency of C4B*Q0 carriers even after adjustment for the TIA/stroke proportion, occurrence of CHD, and age.

When the series 2 patients with hemorrhagic stroke and TIA were excluded from the evaluation, ie, the analysis was restricted to the patients with ischemic stroke, the frequencies of the different alleles were very similar to those obtained in the whole group of CVD patients. For example, in the 99 patients aged >60 years with ischemic stroke, the C4B*Q0 frequency was 21 of 198 (0.106), very close (P=0.889) to the frequency of 0.113 in the Table.

The limited number of patients tested did not allow a precise analysis of the possible connection between clinical subgroups of the patients and the complement allotypes.

Measurement of Antibodies Against hsp in CVD Patients and Healthy Controls

Serum concentrations of the antibodies against human hsp60 (Figure, panel A) and mycobacterial hsp65 (Figure, panel B) were determined in healthy controls and in series 2 of CVD patients. No significant differences were found between patients and controls in the case of either anti-hsp60 antibodies (P=0.21) or anti-hsp65 antibodies (P=0.71).

We found a significant positive correlation between age and anti-hsp65 antibodies in both controls (R=0.26, P<0.0001) and CVD patients (R=0.13, P=0.03). Anti-hsp60 antibodies correlated with age only in the control group (R=0.15, P=0.01).

No differences in serum concentrations of anti-hsp60 and anti-hsp65 antibodies were observed in the comparison of patients with hemorrhagic and ischemic stroke (data not shown). Levels of anti-hsp60 and hsp65 antibodies were about the same in 20 patients with TIA and 98 with ischemic stroke: 56.9 (25th to 75th percentile, 20.6 to 91.1) and 56.2 (25th to 75th percentile, 36.7 to 89.8) AU/mL, respectively, for anti-hsp60 antibodies, and 6.08 (25th to 75th percentile, 2.95 to 9.35) and 8.86 (25th to 75th percentile, 4.80 to 15.72) AU/mL, respectively, for anti-hsp65 antibodies. As calculated by nonparametric ANOVA (Kruskal-Wallis test), there were no differences in serum concentrations of anti-hsp60 or anti-hsp65 antibodies between different subgroups of ischemic stroke (data not shown).

The serum concentration of anti-hsp65 antibodies (but not of anti-hsp60 antibodies) was found to be higher in CVD patients with major ultrasonographic alterations (median, 9.2 AU/mL; 25th to 75th percentile, 4.7 to 16.4 AU/mL) than in those with no or only minor alterations (median, 5.7 AU/mL; 25th to 75th percentile, 3.7 to 8.9 AU/mL). The difference was of borderline significance (P=0.049). However, when the data were analyzed by the multiple regression method, it was determined that the difference was only an apparent one because the average age of the former group was significantly (P=0.0007) higher than that of the latter group.
Lack of Relationship Between C4B*Q0 Allele and Serum Concentration of Anti-hsp Antibodies

In the carriers and noncarriers of the C4B*Q0 allele, the levels of antibodies against hsp did not significantly differ. Median and interquartile range values of the anti-hsp60 antibodies were 78.8 (34.4 to 103.9) and 51.5 (30.5 to 82.2) AU/mL, respectively ($P=0.151$). In the case of anti-hsp65 antibodies, values were 7.2 (5.1 to 12.3) and 8.6 (4.5 to 15.7) AU/mL ($P=0.934$).

Discussion

Previously, we found marked age-dependent changes in the occurrence of the C4B*Q0 allele in healthy people: the frequency of the allele was found to drop with increasing age. Frequency values were 0.161 and 0.054 in 252 healthy subjects aged <45 years and 485 healthy subjects aged >60 years, respectively. To explain this dramatic change, we have assumed that the C4B*Q0 allele is a negative selection factor for health and survival: carriers of C4B*Q0 are continuously selected from the healthy population because of their increased susceptibility for life-threatening and/or chronic diseases.

The leading cause of death in Hungary, as in many other parts of the developed world, is CHD and its consequence, myocardial infarction. In populations of Hungary and Iceland, we have obtained much evidence indicating that the morbidity in CHD and myocardial infarction is significantly higher in C4B*Q0 carriers than in noncarriers. The difference in frequency between these patients and age-matched healthy controls was highest in elderly subjects aged >60 years. In the present study we found the frequency of the C4B*Q0 allele in CVD patients aged >60 years to be 0.113, which is more than twice that obtained in our previous study in healthy subjects of the same age (0.054). Therefore, the frequency of C4B*Q0 in CVD patients aged >60 years is comparable to that in CHD patients aged >60 years and in patients with myocardial infarction aged 60 to 70 years. Furthermore, C4B*Q0 frequency was highest in CVD patients aged >70 years. Therefore, our present findings indicate that carriers of the C4B*Q0 allele are more susceptible not only to CHD but to CVD, but in the case of CVD the disease is manifested primarily in the elderly.

In sharp contrast to the elderly patients, in CVD patients aged 45 to 60 years the frequency of the C4B*Q0 allele (0.064) was lower than in age-matched healthy individuals. If this observation can be repeated in a larger cohort of young CVD patients, it indicates a different genetic background for the 2 disease entities of atherosclerotic vascular diseases when they develop in middle-aged subjects. This finding is in agreement with some data in the literature.

In the second part of the present study we compared the serum concentration of the antibodies against human hsp60 or mycobacterial hsp65 in patients and controls. These antibodies were shown to be a marker of cardiovascular disease and carotid atherosclerosis by several groups. No similar studies in CVD patients have been reported, however.

Our present findings indicate that there is no difference in the average serum levels of either anti-hsp60 or anti-hsp65 antibodies between CVD patients and age-matched healthy subjects. The only clinical association we found was a correlation between the severity of ultrasonographic alterations in carotid arteries and anti-hsp65 titers. When the difference was analyzed by a multiple regression test, however, we found that the difference was due to the higher average age of the patients with severe carotid alterations compared with those with mild ones. Previously, Xu et al also found an age-dependent increase of anti-hsp65 antibody levels in healthy inhabitants of the southern Tirol region with carotid atherosclerosis compared with those with no alterations. Since the prevalence of carotid alterations increased with age, similar to our present findings, they also found an increase in the anti-hsp65 antibody titers. The lack of elevated serum concentrations of anti-hsp60 and hsp65 antibodies in CVD patients is in sharp contrast to findings obtained in CHD, in which the levels of anti-hsp antibodies were found to be high. The overexpression of hsp in endothelial cells is an initiating event of local immune complex formation and complement activation that may significantly contribute to the development of atherosclerosis.

Thus, our present findings indicate marked differences between CHD and CVD in one of the immune mechanisms that may contribute to atherogenesis. We have found an even more pronounced difference in another immune mechanism, namely, the formation of anticholesterol antibodies. Compared with age-matched healthy subjects, serum concentration of anticholesterol antibodies was significantly lower in CVD patients but significantly higher in CHD patients.

When these differences in immune mechanisms as well as the difference between CHD and CVD patients in the frequency of the C4B*Q0 complement allele are taken into account, it seems that there are marked differences in genetic background and immunopathological factors that contribute of the pathomechanism of CHD and ischemic stroke. The nonidentity of these factors may at least partly explain the differences between the natural history and risk factors of CHD and CVD.

Acknowledgments

This work was supported by the OTKA T17740, T032661, and F029030 grants of the Hungarian Research Fund, by the FKFP 0084/1997 grant of the Ministry of Education, and by the 430/1996 ETT grant of the Ministry of Health. Dr Prohászka is a Bolyai János research fellow.

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*Stroke*. 2000;31:2648-2652
doi: 10.1161/01.STR.31.11.2648

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

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