Anticardiolipin Antibodies Are Not an Independent Risk Factor for Stroke
An Incident Case-Referent Study Nested Within the MONICA and Västerbotten Cohort Project

Ejaz Ahmed, MD; Birgitta Stegmayr, MD, PhD; Jasmina Trifunovic, MSc; Lars Weinehall, MD, PhD; Göran Hallmans, MD, PhD; Ann Kari Lefvert, MD, PhD

Background and Purpose—Anticardiolipin antibodies (aCL) have been proposed to be an independent risk factor for stroke. To test this hypothesis, a nested case-control study was performed to compare aCL with the other known risk factors for stroke.

Methods—Within the framework of the World Health Organization Multinational Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA) project and the Västerbotten Intervention Program (VIP) health survey, 44 725 men and women were enrolled and followed up from January 1, 1985, through August 31, 1996. Individuals free from cardiovascular events were followed up, and 123 developed stroke (on average, 34.1 months after blood sampling; 21 cerebral hemorrhage and 102 cerebral infarction); they were compared with 241 age- and sex-matched control subjects from the same population. ELISA was used for the analysis of IgG, IgM, and IgA aCL.

Results—IgM-aCL were present in 11.4% of patients (14/123) who developed stroke and in 4.1% of individuals (10/241) who remained healthy (P=0.013, OR 2.97, 95% CI 1.28 to 6.89). The OR for the levels of IgM-aCL was 1.34 (P=0.01, 95% CI 1.07 to 1.68) without adjustment for other risk factors and 1.24 when adjusted for hypertension, diabetes mellitus, cigarette smoking, and use of smokeless tobacco (P=0.077, 95% CI 0.98 to 1.56). There was no difference between patients and controls for the prevalence or level of IgG-aCL and IgA-aCL and also no difference between patients with cerebral hemorrhage and cerebral infarction for the prevalence of all 3 isotypes of aCL.

Conclusions—We conclude that aCL are associated with future stroke but do not constitute an independent risk factor. (Stroke. 2000;31:1289-1293.)

Key Words: autoantibodies • cardiolipins • cerebrovascular disorders • epidemiology • phospholipids

Anticardiolipin antibodies (aCL) constitute a heterogeneous group of antibodies that are directed against cardiolipin and/or other negatively charged phospholipids. aCL are present in association with vascular diseases such as systemic lupus erythematosus, myocardial infarction, recurrent abortion, and thrombocytopenic purpura, all of which are directly related to thromboembolic macrovascular and microvascular damage. There are several reports regarding aCL and relation to stroke, but the results are conflicting. One possible reason for the conflicting results could be the difference in the study designs. Earlier studies can be divided into the following 2 categories: (1) postevent sampling in unselected or selected populations (most studies), in which blood sampling was done within 48 hours, 72 hours, 5 days, or 7 days after an acute stroke; and (2) prospective, population-based cohort studies, in which sampling was done before the index event. In the first category, a recent study of 524 multiethnic patients with ischemic stroke and 1020 healthy individuals showed aCL of IgG and IgM types to be independent risk factors. Other studies showed IgM-aCL to be associated with ischemic stroke in elderly patients (aged 68±7 years), IgA and IgM-aCL to be lower in young stroke patients than in healthy individuals, and aCL not to be a risk factor for mortality, reinfarction, or nonhemorrhagic stroke. The Antiphospholipid Antibodies in Stroke Study (APASS) showed that aCL was significantly increased and identified as an independent risk factor in 255 patients with a first ischemic stroke. Another study showed that individuals with IgG-aCL of >40 GPL units had more prior strokes and more frequent subsequent thrombo-occlusive events and death, whereas still another study reported that the presence of aCL did not confer an increased risk for subsequent thrombo-occlusive events and death. In a recent study, cerebro-
vascular events were associated with high levels of IgG-aCL (>100 GPL) in relatively young adults.

In the second category, there is only 1 previous study. In the Physicians Health Study, 22,071 male physicians were sampled and followed for 60.2 months. In that study, aCL levels >95th percentile were an important risk factor for deep venous thrombosis and pulmonary embolus but not for ischemic stroke.9

The reports on aCL and stroke are thus conflicting. Moreover, only IgG-aCL was analyzed in most investigations; few studies also investigated IgM-aCL. To test the hypothesis of whether aCL was a risk factor for stroke, a nested case-control study was performed in a population-based cohort. No other study has assessed the risk for stroke related to all 3 isotypes of aCL in individuals free from cardiovascular diseases.

Subjects and Methods

Study Populations

The 2 northernmost counties in Sweden, with a total population of 510,000, have constituted since 1985 1 of 39 collaborating centers in the World Health Organization (WHO) Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA) study.16 Population-based surveys were performed in 1986, 1990, and 1994. Two thousand individuals were invited at each survey. In total, 4,725 men and women aged 25 to 64 years participated in the MONICA surveys.

A community intervention program, the Västerbotten Intervention Program (VIP) for cardiovascular disease and diabetes prevention, was performed in 1 of the 2 counties.17 In this program, started in 1985, men and women were asked to participate in a health survey (of the same design as the MONICA population surveys) the year they reached the age of 30, 40, 50, or 60 years. Approximately 40,000 men and women participated in this survey between January 1, 1985, and August 31, 1996. Participants in both the MONICA and the VIP surveys were requested to donate a blood sample (minimum 4-hour fasting time) to be stored at the Northern Sweden Medical Bank for future research.

Case Finding

The case finding of stroke was based mainly on 3 sources: discharge records from hospitals, reports from general practitioners, and death certificates. Clinical information on all subjects in the 25- to 74-year range with International Classification of Diseases (8th Revision, used from 1985 through 1986 [ICD-8]; 9th Revision, used from 1987 to present [ICD-9]) codes 430 to 438 was screened and validated for acute stroke events that met the definition. All cases with acute stroke (group aged 25 to 74 years) and acute myocardial infarction (group aged 25 to 64 years) from the MONICA area have, since 1985, been included in the Northern Sweden MONICA registries.

Stroke cases were registered in a standardized way in accordance with the MONICA manual and were defined by the WHO criteria as “rapidly developing clinical signs of focal (or global) disturbance of cerebral function lasting more than 24 hours (unless interrupted by surgery or death) with no apparent cause other than a vascular origin.”16 This definition excludes transient ischemic attacks. Global clinical signs were accepted only in patients with deep coma or subarachnoid hemorrhage. Subdural hemorrhage, traumatic intracerebral hemorrhage, and lesions caused by brain tumors were also excluded. In this longitudinal project, special emphasis was placed on the use of uniform case ascertainment and diagnostic criteria throughout the study. Case finding was therefore based on clinical presentation, and cases detected by brain imaging but not exhibiting any acute symptoms of stroke were excluded. Information was abstracted from medical records, and the codes were the same throughout the study. Every case was strictly validated according to MONICA criteria before registration.16,18

The subtypes of acute stroke, according to the MONICA manual, were based on the following examinations (ICD-9 codes are in parentheses): subarachnoid hemorrhage (430), bloodstained cerebrospinal fluid and an aneurysm or an arteriovenous malformation found on angiography, or positive finding on CT scan or necropsy; intracerebral hemorrhage (431), positive finding on CT scan or necropsy; brain infarction (434), no signs of hemorrhage on CT scan or at necropsy; and unspecified stroke (436), not investigated by CT scan or necropsy. In this study, 3 of 123 patients had a diagnosis of unspecified stroke, and they were categorized in the analysis as having “ischemic stroke.”

Each case was also described as “definite stroke,” “unclassifiable,” or “not stroke” on the basis of available background information. “Unclassifiable” was mainly used in fatal cases for which acute cerebrovascular disease was given as cause of death in the death certificate but for which clinical information was too limited to classify the event as “definite stroke.” The proportion of “unclassifiable” cases was 7.5% of fatal strokes and 1.4% of all strokes, with no important changes over time.19 Fatal cases were those in which subjects died within 28 days from the onset of the stroke.

A nested case-referent design was used when the incident cases were definite first-ever stroke events, as classified according to MONICA criteria and identified during the period ranging from January 1, 1985, through August 31, 1996. We identified 166 individuals whom, after participation in either the MONICA or VIP health surveys, suffered from a first-ever ischemic or hemorrhagic stroke before the age of 74. For this study, 123 cases (102 ischemic and 21 hemorrhagic; 77 men and 46 women) remained after the exclusion of individuals with a previous myocardial infarction (n=15) or stroke (n=9) or cancer diagnosis according to the regional cancer registry (n=13); individuals were also excluded if the amount of blood in the sample taken was inadequate for analysis (n=6).

Potential referents for each case were randomly selected among participants in the MONICA or the VIP survey. They were matched for sex, age (±2 years), type of survey (MONICA or VIP), date (±1 year) of health survey, and geographical region. Individuals were excluded if they had died or had moved away from the Northern MONICA region before August 31, 1996. Referents were also excluded if they were known from the Northern Sweden MONICA incidence registry to have had acute myocardial infarction or stroke before the health survey. An additional questionnaire was sent to all referents to further ensure the absence of stroke and/or acute myocardial infarction in their histories. Finally, 2 referents for each case were selected. However, 5 patients had only 1 corresponding matched control.

Smokers were defined as those who reported daily smoking. Ex-smokers and “occasional smokers” were classified as nonsmokers. Smokeless tobacco (oral snuff) users were defined as snuffers if they used snuff daily.

Blood pressure was recorded after 5 minutes of rest. Hypertension was defined as systolic blood pressure of 160 mm Hg and/or diastolic blood pressure of 95 mm Hg and/or use of antihypertensive medication. Total cholesterol was measured by use of a bench-top analyzer (Reflotron, Boehringer Mannheim GmbH Diagnostica) or by an enzymatic method (Boehringer Mannheim GmbH Diagnostica).

Specimen Collection

Blood was drawn by clean venipuncture. Specimens were centrifuged, and plasma was immediately frozen and stored at -80°C until the aCL assay was performed. The Research Ethics Committee of Umeå University approved the study.

Determination of aCL

aCL was measured by ELISA, according to the method of Harris20 with the following modifications. Titertek 96-well polystyrene chloride microplates (Micronic, catalog no. 273-05) were coated with 50 μL of cardiolipin (Sigma; concentration, 50 μg/mL in ice-cold 10% ethanol). The ethanol was allowed to evaporate by leaving the plates
open to air overnight. The plates were washed 3 times with PBS and blocked by 1% BSA in PBS for 2 hours at room temperature in a humid atmosphere. After the plates were washed 3 times with PBS, 50 μL of EDTA plasma samples (dilution for IgG 1/100, IgM 1/50, and Ig A 1/30) diluted in PBS–1% BSA were added in duplicates to each well. The plates were incubated for 3 hours at room temperature in a humid atmosphere and then washed with PBS. Afterward, 50 μL of alkaline phosphatase-labeled, affinity-purified anti-human IgG (Sigma A-3150), IgM (A-3275), IgA (A-3400), dilution 1:1000 in diethanolamine buffer, pH 9.8) was added. Samples that were earlier demonstrated to be clearly positive or negative for aCL were run on each plate. For blank wells, PBS–1% BSA was added instead of plasma sample. The plates were read at 405 nm when the positive control reached an optical density of 1.0, which was 25 minutes of incubation in a humid atmosphere. After the plates were washed 3 times with PBS, 100 μL/well of substrate (p-nitrophenyl phosphate), 1 mg/mL, in substrate buffer (diethanolamine buffer, pH 9.8) was added. Samples that were earlier demonstrated to be clearly positive or negative for aCL were run on each plate. For blank wells, PBS–1% BSA was added instead of plasma sample. The plates were read at 405 nm when the positive control reached an optical density of 1.0 ± 0.1, which was 25 minutes for the IgG assay and 28 minutes for both the IgM and IgA assays. Samples from the patients and the corresponding age- and sex-matched controls were run in the same plate. A known positive sample, a known negative sample, and a blank were run with each plate. Levels of antibodies were expressed in optical density units. Levels of antibodies were expressed in optical density units. The OD indicates optical density.

Statistical Analysis

The SAS Statistical Software Package (SAS Institute Inc., 1995) was used for data analysis. Conditional logistic regression analyses were performed to determine the most powerful predicting factors with respect to the outcome measure. For each predicting factor (explanatory variable), the 95% confidence limits for the conditional risk ratio (eβ) was calculated. The conditional logistic regression is a stratified analysis in which each matched set is a stratum. The Fisher exact test was used for the comparison of categorical variables, and a t test was used for the comparison of continuous variables.

Results

Demographics

Table 1 summarizes the demographic characteristics and stroke risk factors in individuals who developed stroke and remained healthy. Persons who developed stroke more often had hypertension (51.7% versus 26.9%, P<0.0001) and diabetes (10.1% versus 0.8%, P<0.0001). There were no differences in current cigarette smoking, use of smokeless tobacco, age, or sex. There were also no differences in body mass index or levels of serum cholesterol and high-density lipoproteins. However, persons who developed stroke had higher levels of serum triglycerides (P<0.01).

Prevalence of aCL

The prevalence and levels of aCL in persons who developed stroke and those who remained healthy are summarized in Table 2. IgM-aCL were present in 11.4% (14/123) of those who developed stroke and in 4.1% (10/241) of those who remained healthy. Persons who developed stroke more often had hypertension (51.7% versus 26.9%, P<0.0001) and diabetes (10.1% versus 0.8%, P<0.0001). There were no differences in current cigarette smoking, use of smokeless tobacco, age, or sex. There were also no differences in body mass index or levels of serum cholesterol and high-density lipoproteins. However, persons who developed stroke had higher levels of serum triglycerides (P<0.01).

Levels of aCL

The levels of IgM-aCL were higher in individuals who developed stroke (P=0.026). The OR for IgM-aCL when not adjusted for other risk factors for stroke was 1.34 (P=0.011, 95% CI 1.07 to 1.68). The ORs adjusted for other classic risk factors for stroke are summarized in Table 3. The OR for

| Table 1. Clinical Characteristics of Patients Who Developed Stroke and Control Subjects |
|----------------------------------|-----------------|-----------------|-----------------|
| Characteristic                   | Patients (n=123) | Controls (n=241) | P               |
| Mean age (SD), y                 | 55.2 (7.6)       | 55.1 (7.6)       |                 |
| Sex, % female                    | 37.3 (46/123)    | 36.9 (89/241)    |                 |
| Hypertension, %                  | 51.7 (58/112)    | 26.9 (61/226)    | <0.0001         |
| Diabetes mellitus, %             | 10.1 (12/118)    | 0.8 (2/241)      | <0.0001         |
| Cigarette smoking, %             | 25.8 (31/120)    | 17 (14/214)      | 0.05            |
| Smokeless tobacco, %             | 7 (8/113)        | 12 (28/233)      | NS              |
| Other characteristics, mean (SD)* |                  |                 |                 |
| Body mass index                  | 26.9 (4.2)       | 26.3 (3.9)       | NS              |
| Serum cholesterol, mmol/L        | 6.4 (1.3)        | 6.2 (1.4)        | NS              |
| High density lipoprotein         | 1.2 (3.3)        | 1.3 (3.3)        | NS              |
| Serum triglycerides, mmol/L      | 1.8 (1)          | 1.4 (6)          | 0.006           |

*Information not known for all patients or controls.

| Table 2. Level and Prevalence of aCL in Patients Who Developed Stroke (n=123) and Control Subjects (n=241) |
|----------------------------------|-----------------|-----------------|-----------------|
| Isotype                          | Levels of aCL (mean±SD) | OD Value | Prevalence of aCL, % |
|                                  | Patients (n=123) | Controls (n=241) | P               |                  |
| IgG                              | 0.317±0.218       | 0.299±0.184       | NS              |                  |
| IgM                              | 0.223±0.217       | 0.181±0.108       | 0.02            |                  |
| IgA                              | 0.236±0.114       | 0.238±0.107       | NS              |                  |

| Table 3. Levels of aCL in Patients Who Developed Stroke: ORs Adjusted for Hypertension, Diabetes Mellitus, Cholesterol, and Cigarette Smoking |
|----------------------------------|-----------------|-----------------|-----------------|
|                                 | OR   | z     | P     | Lower CI | Upper CI |
| Hypertension                    | 3.01 | 4.429 | <0.0005 | 1.85     | 4.90     |
| Diabetes                        | 8.57 | 3.147 | 0.002  | 2.25     | 32.66    |
| Cholesterol                     | 1.09 | 0.648 | NS    | 0.84     | 1.42     |
| Smoking                         | 1.78 | 1.839 | 0.066  | 0.96     | 3.28     |
| IgA-aCL                          | 1.05 | 0.510 | NS    | 0.88     | 1.24     |
| IgM-aCL                          | 1    | −0.02 | NS    | 0.79     | 1.26     |
| IgM-aCL                          | 1.24 | 1.769 | 0.013  | 0.98     | 1.56     |

The SAS Statistical Software Package (SAS Institute Inc., 1995) was used for data analysis. Conditional logistic regression analyses were performed to determine the most powerful predicting factors with respect to the outcome measure. For each predicting factor (explanatory variable), the 95% confidence limits for the conditional risk ratio (eβ) was calculated. The conditional logistic regression is a stratified analysis in which each matched set is a stratum. The Fisher exact test was used for the comparison of categorical variables, and a t test was used for the comparison of continuous variables.
IgM-aCL when adjusted for hypertension, diabetes mellitus, current cigarette smoking, and smokeless tobacco was 1.24 (P=0.077, 95% CI 0.98 to 1.56).

In the subgroup analysis, the levels of IgA-aCL were significantly higher in persons who developed stroke and were current smokers than in those who did not smoke (P<0.05), whereas those using smokeless tobacco had lower levels of IgM-aCL than both future stroke patients and healthy individuals who did not (P<0.05).

There was no difference in aCL between persons who developed stroke and had hypertension and those with normal blood pressure. Persons who developed stroke and had diabetes had lower levels of IgG-aCL than those without diabetes (P<0.05).

Women who developed stroke had higher levels of IgM-aCL (P<0.01) and serum triglycerides (P<0.05) than men and the corresponding female control subjects. Males who developed stroke had higher systolic and diastolic blood pressures than the corresponding male controls (P<0.001), and females who developed stroke had higher systolic blood pressure than the corresponding female controls.

There was no difference in the levels and prevalence of aCL between persons who developed cerebral hemorrhage and those with cerebral infarction. There were also no differences in body mass index, high-density lipoproteins, serum cholesterol, serum triglycerides, systolic and diastolic blood pressures, and blood sugar levels between those who developed cerebral hemorrhage and those who developed cerebral infarction.

Discussion
In our prospective, nested case-control study, the presence of IgM-aCL was associated with stroke but did not constitute an independent risk factor. Furthermore, our study supports the strength of an association between stroke and traditional risk factors such as hypertension22 and diabetes mellitus.23,24 In the only prospective study published earlier,1 IgG-aCL was not a risk factor for ischemic stroke in healthy adult men; our results thus confirm these findings and further show the importance of including autoantibodies of all isotypes in studies. The evidence for an association between the presence of aCL and stroke has been derived mostly from retrospective studies.5–8 In one such study, an evaluation of 255 patients with first-ever ischemic stroke and 255 age- and sex-matched hospitalized nonstroke patients showed aCL to be an independent risk factor for first-ever ischemic stroke.6 The limitation of such retrospective studies is mainly the possibility that the vascular damage caused by a stroke may promote the development of aCL. In our present study, blood was drawn on average 3 years before the first stroke, at a time when the individuals were healthy. Because the levels of aCL, like all autoantibodies, are known to fluctuate over time, it is not possible to state that the aCL were present also at the time of stroke. Such variations in aCL concentration should, however, have affected in the same way both individuals who developed stroke and those who remained healthy.

The precise mechanism that initiates the formation of aCL is not known, and the mechanism of action is poorly understood. Autoantigens and/or exogenous antigens may induce the immune response against cardiolipin. Certain drugs may affect the levels of aCL,25 and many microorganisms have phospholipids on their surface and are known to induce an immune response against cardiolipin.26,27 It is hypothesized that endothelial cell injury, perhaps caused by other risk factors for stroke such as hypertension, may lead to the exposure of antigens that are normally secluded within the phospholipid bilayer of the cell membrane, thus eliciting an aCL response.28 aCL can bind to endothelial cells,29 and higher IgG binding to human brain endothelium has been observed in patients with antiphospholipid antibodies and ischemic stroke.30 aCL can also bind to the phospholipid epitopes on platelet membranes,4 causing platelet activation and aggregation. These studies were performed with IgG-aCL, but IgM-aCL should be even more effective because of the higher avidity of IgM antibodies.

Complement activation occurs in patients with stroke and was measured as the terminal components of the complement cascade, C5b-9, in the plasma of patients with cerebral ischemia.31 In that study, C5b-9, also known as the membrane attack complex, was increased in patients with cerebral ischemia and antiphospholipid antibodies. Because IgM antibodies have a high capacity to activate complement, such activation and its consequences may also be involved in our patients who developed stroke.

Pathogenic autoantibodies are rarely of IgM class, although we have earlier described32 increased levels of IgM-aCL in patients with type I diabetes mellitus, particularly in those with vascular complications. Such increased levels and prevalence of IgM-aCL might indicate an ongoing immune response. It has been described that IgM-aCL differs from IgG antibodies in having less cross-reaction with other phospholipids,29 although the implications of such a restricted reactivity are not known. That smoking patients had increased IgA-aCL could be due to an induction of these antibodies at the mucosal level in lungs, possibly caused by frequent infections. The decreased IgG-aCL in persons who developed stroke and had diabetes might well be secondary to the formation of immune complexes containing aCL that are common in diabetes and have been previously described by us.32

The plasma cofactor β2-glycoprotein I (β2-GPI) is required for optimal antibody-phospholipid interaction, and it is hypothesized that antiphospholipid antibodies recognize the β2-GPI–phospholipid complex rather than isolated phospholipid, thus promoting thrombosis via modulation of events mediated by β2-GPI.33 Although the pathogenesis of hemorrhagic stroke differs from that of ischemic stroke, and aCL have been implicated more in thrombosis, aCL levels in this study did not differ between persons who developed cerebral hemorrhage and those who developed ischemic stroke. It has been assumed that aCL primarily causes thrombosis, but reports of homeostatic regulation in association with aCL are inconsistent.34 In our assay, antibodies against cofactor β2-GPI were not primarily detected, although the added plasma should add enough cofactors able to react with cardiolipin to facilitate binding of aCL. Most studies (including this one) have examined aCL, but other antiphospholipid antibodies could certainly be of interest.35
In conclusion, an increased proportion of patients who developed stroke had aCL before the event. The presence of such antibodies constituted a risk factor that was not independent of other conventionally recognized risk factors, such as hypertension, diabetes mellitus, and current cigarette smoking. We have earlier shown that aCL constitute a strong and independent predictor for MI. The role of these antibodies for the development of stroke is not clear and remains a question for further investigation.

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