Circulating Immune Complexes in 50-Year-Old Men as a Strong and Independent Risk Factor for Myocardial Infarction

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Background—Circulating immune complexes (CICs) and autoantibodies against oxidatively modified LDLs (oxLDLs) and cardiolipin occur in patients with atherosclerosis and myocardial infarction (MI). The ability of such CICs and antibodies to predict myocardial infarction (MI) was investigated in a prospective nested case-control study in which healthy 50-year-old men were followed for 20 years.

Methods and Results—Two hundred fifty-seven men were included in the study, and 119 developed MI (39 died) between 50 and 70 years of age. One hundred thirty-eight randomly chosen men who did not develop MI up to 70 years of age served as controls. The prevalence of elevated levels of CICs and the concentration of CICs in men who developed MI were higher than in those who remained healthy. The concentration of CICs at age 50 was associated with a marked increased risk for MI, and this risk was independent of other conventionally recognized risk factors. There was a positive correlation between the levels of CIC and IgG antibodies to cardiolipin in men who developed MI. The level of IgG antibodies and the prevalence of elevated IgG and IgM antibodies to cardiolipin were higher in those who developed MI and had CICs than in those without CICs. Among men homozygous for C4 null alleles, those who developed MI had higher concentrations of CICs than did those who remained healthy.

Conclusions—This prospective study shows that CICs alone or in combination with autoantibodies against cardiolipin in healthy males at 50 years of age predict subsequent MI between the age of and 70 years. (Circulation. 2000;102:2576-2581.)

Key Words: circulating immune complexes ■ antibodies ■ antigens ■ lipoproteins ■ myocardial infarction

The classic risk factors, hypercholesterolemia, smoking, hypertension, and diabetes, explain only part of the epidemiological features of atherosclerotic vascular disease. Therefore, it is likely that other, hitherto unrecognized, factors exist. In the past decade, there has been an upsurge of interest in the role of immune mechanisms in the development and regulation of atherosclerosis and its complications. Both humoral and cellular mechanisms have been proposed to participate in the onset and/or progression of atherosclerotic lesions, and postulates for the involvement of autoantibodies against oxidatively modified LDL (oxLDL), phospholipids, and circulating immune complexes (CICs) have met with considerable experimental support. Repeated immunization of animals with protein antigens leads to the formation of CICs and acceleration of the diet-induced atherosclerotic process in both rabbits and mice. CICs are present in ~50% of cases with acute myocardial infarction (MI), although persistence of CICs is more rare. Precocious MI occurs in individuals with chronic immune complex formation due to inflammatory disorders, such as systemic lupus erythematosus and rheumatoid arthritis. In an earlier study, we described persisting CICs in 20% of patients with precocious MI 6 months to several years after the acute event. In another study, the presence of lipoprotein-containing CICs promoted the onset and development of atherosclerotic lesions in the vessel wall. Several studies have shown that oxLDLs in combination with antibodies obtained by immunization of animals causes an increased rate of foam cell development in vitro via interaction of immune complexes with IgG Fc receptors. That autoantibodies against cardiolipin and oxLDL can participate in the formation of CICs has been shown by recent studies.

The solubilization and efficient elimination of CICs is dependent on an intact classical pathway of complement activation. Genetic deficiencies of complement proteins are associated with high levels of CICs and immune complex-mediated disease. Our earlier studies have shown that the presence of null alleles of complement factor C4 (C4Q0) is
not related to future MI. 26 However, persons with C4Q0 and past premature MI had higher prevalence and levels of CICs. 16 There was also a relation between C4Q0 and CICs in persons with premature peripheral vascular disease. 27 Thus, C4Q0 might predispose an individual to immune complex formation, which might play a role in vascular inflammation.

In the present investigation, the presence and levels of CICs and their relation to antibodies against cardiolipin and oxidatively modified LDLSs and to C4Q0 were determined in samples from healthy 50-year-old men, who were followed for 20 years for the development of MI. The aim of the present study was to assess whether the presence of high concentrations of CICs was related to future MI.

Methods

Study Groups
The present study was a prospective nested case-control study 28 composed of 257 individuals: 119 men (mean age 49.63 years) who developed MI between 50 to 70 years of age and 138 men (mean age 49.58 years) who did not develop MI up to 70 years of age. Samples had been collected from 2322 males aged 50 years in Uppsala during 1970 to 1973. This cohort made up 82% of all 50-year-old men living in Uppsala at that time. This cohort was followed for 20 years. Of the 156 men who developed MI, samples were missing or in bad condition for 18 of them, and 19 cases were excluded because of diabetes mellitus. Of the remaining 119 men who developed MI, 39 died. A control group of 156 individuals was selected by simple random selection from those who did not develop MI up to 70 years of age. Samples were missing or in bad condition for 17 of them, and 1 individual was excluded because of diabetes. Thus, the remaining control group consisted of 138 individuals. There were no differences in the conventional risk factors for MI (serum LDL/HDL cholesterol ratio, the ratio between arachidonic acid and dihomo-γ-linoleic acid from serum cholesterol, body mass index, and smoking) between the control group selected in the present study and the rest of the healthy individuals. The mean value of supine blood pressure in the selected control group (130 mm Hg) was lower than that for the rest of the healthy individuals (133 mm Hg). 30 Dososes from hospital records were obtained from the National Central Bureau of Statistics until 1983. From 1983, these data were requested from the hospitals. Information on causes of death was requested from the National Board of Health and Welfare. Only MI as the main diagnosis was used for classification. At each 10-year follow-up, hospital and death records were scrutinized, and the accuracy of the MI diagnosis was assessed. 29 Data from the groups regarding hypertension, dyslipoproteinemia, tobacco use, and other conventionally recognized risk factors were presented earlier. 30

Isolation and Quantitative Determination of Immune Complexes

Ultracentrifugation
Details were as described by us. 29 A sample of 0.5 mL serum was centrifuged for 15.5 hours at 60 000g and 4°C on a continuous sucrose density gradient by using a Beckman L5-65 centrifuge and 12 mL tubes. The gradient was made of 5% to 20% sucrose with 1 mL 40% sucrose as a bottom layer. Fractions of 20 drops were collected from the bottom. Fractions containing protein complexes with a molecular weight >175 kDa were assayed by ELISA for the presence and concentration of immunoglobulins. 29 Pooled purified IgG (Gammaglobulin, Pharmacia-Upjohn) was used as a standard for the determination of IgG, and normal pooled human serum was used as a standard for the determination of IgA and IgM. The protein content in the fractions was measured by the method of Lowry et al. 31 Results were tested for normality of distribution. IgG- and IgA-containing complexes with a molecular weight >200 kDa, in a concentration of >25 mg/mL, by protein determination according to Lowry et al., were taken to be abnormally raised. This value represents the mean ± 3 SD of the values of a control population (350 healthy individuals). 32 The density gradient was standardized by using molecular weight markers according to established criteria. 29 The intraexperimental coefficient of variation between duplicate samples for the ultracentrifugation assay was 14.5%.

Gel Filtration
IgM-containing immune complexes and large IgG- and IgA-containing CICs were isolated from serum by gel filtration with use of a fast performance liquid chromatography system and a Sepharose-6 column (Pharmacia). The buffer used was 0.05 mol/L phosphate, pH 7.0, with 0.15 mol/L NaCl. The fractions were assayed by ELISA for the concentration of immunoglobulins, and the concentration of protein was measured by the method of Lowry et al. 32 Results were tested for normality of distribution. IgM-containing complexes with a molecular weight >900 kDa and a protein concentration >30 mg/L were considered to be abnormally raised. This value represents the mean ± 3 SD of a control population (234 healthy individuals). The intraexperimental coefficient of variation between duplicate samples was 16.3%.

C4 Allotyping
EDTA plasma was kept frozen at −70°C. C4 allotypes were determined by flat-bed agarose gel electrophoresis, followed by immunofixation. 33,34 Briefly, EDTA plasma was treated with neuraminidase and carboxypeptidase B; after dialysis, agarose gel electrophoresis was carried out at pH 8.8, and the C4 bands were detected by immunofixation with use of rabbit anti-human C4 antibody as described previously. 35 After a washing, the gel was dried, and the proteins were stained with Coomassie blue. Standard samples for the C4 allotypes frequently seen in the white population (A2, A3, A6, B1, B2, and B3) were included in each gel. C4 allotypes were assigned according to published criteria. 35 When bands were intermediate between the A and B loci, C4B gene products were distinguished by their greater hemolytic capacity. 34 The density of the C4A and C4B bands was determined by a scanning densitometer. Complete absence of C4A or C4B bands was taken to indicate homozygous deficiency. Heterozygous deficiency was determined by comparison of the densities of the C4A and C4B bands.

The effect of freezing/thawing was analyzed and showed that repeated cycles of freezing/thawing affected the intensity of the bands but not the allotyping itself.

Determination of Autoantibodies Against OxLDL and Native LDL
LDL was isolated by ultracentrifugation and was copper-oxidized as described. 3,23,37 Autoantibodies of IgG, IgA, and IgM isotypes against oxLDL and native LDL were determined by ELISA as described earlier. 23,37,38 Briefly, microplates were coated with oxidized or native LDL, diluted serum samples were added, and the plates were incubated for 3 hours at room temperature in a humid atmosphere. After a washing, alkaline phosphatase-labeled second antibody was added to each well, and the plates were further incubated for 2 hours at 37°C. After another washing, the substrate p-nitrophenyl phosphate was added. One standard containing high concentrations of antibodies against oxLDL was used to determine antibodies against oxLDL, 2 samples that were earlier demonstrated to contain antibodies against oxLDL, 2 samples lacking such antibodies, and a blank were run on each plate. For blank wells, phosphate buffer was added instead of serum. The plates were read at 405 nm when the positive control reached an optical density of 1.0±0.1, which took ~30 minutes. All assays were run in duplicate, and observers were blinded to the study protocol. The cumulative interassay coefficient of variation was <10%. The assay has been further validated with the use of F(ab') 2 fragments from purified IgG fractions with high concentrations of specific antibodies.

The cutoff level for the presence of antibodies, as determined by the absorbance units in the ELISA, was the mean±2 SD of that of individuals who remained healthy.
Determination of Autoantibodies Against Cardiolipin

Anticardiolipin antibodies of IgG, IgA, and IgM isotypes were analyzed by ELISA according to the method of Harris et al., with minor modifications. Briefly, polyvinyl chloride microplates were coated with bovine heart cardiolipin and blocked by 1% BSA. Diluted serum samples were added, and the plates were incubated for 3 hours at room temperature in a humid atmosphere. After a washing, alkaline phosphatase–labeled secondary antibody was added to each well, and the plates were further incubated for 2 hours at 37°C. After another washing, the substrate p-nitrophenyl phosphate was added. One standard containing high concentrations of antibodies against cardiolipin, 2 samples that were earlier demonstrated to contain antibodies against cardiolipin, 2 samples lacking such antibodies, and a blank were run on each plate. For blank wells, phosphate buffer with BSA was added instead of serum. 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 30 to 40 minutes for the IgM and IgA assays. The cumulative interassay coefficient of variation was <10%. The cutoff level for the presence of antibodies, as determined by the absorbance units in the ELISA, was the mean±2 SD of that of individuals who remained healthy.

Statistical Evaluation

The nonparametric Mann-Whitney U test and Fisher exact test were performed for group comparisons. A 2-tailed value of P<0.05 was regarded as significant. Simple regression was used to explore the correlation between the concentrations of CICs and levels of antibodies against oxLDL and cardiolipin.

The association between the concentration of CICs and the health status of the men (1 indicates healthy; 2, survival of MI; and 3, death by MI) was tested by Spearman rank correlation analysis. The importance of the concentration of CICs when adjusted for the conventionally recognized risk factors for MI (supine blood pressure, serum LDL/HDL cholesterol ratio, ratio between arachidonic acid and dihomo-γ-linoleic acid from serum cholesterol, body mass index, and smoking) was examined by use of partial Spearman rank correlation analysis. A logistic regression analysis was used to test the predictive importance of the concentration of CICs alone and after adjustment for the above-mentioned risk factors. The statistical package was SAS for Windows, version 6.12.

Results

Conventionally Recognized Risk Factors

Men who developed MI had higher systolic and diastolic blood pressure, body mass index, serum triglycerides, serum cholesterol, LDL/HDL ratio, and ratio between arachidonic acid and dihomo-γ-linoleic acid from serum cholesterol. Persons with diabetes mellitus were excluded from both populations because of the high prevalence of increased levels of CICs and the increased incidence of cardiovascular diseases.

Prevalence of High CICs and Concentration of CICs

The prevalence of elevated levels of CICs and the concentration of CICs in men who developed MI compared with those who remained healthy are shown in Table 1.

The prevalence of elevated levels of CICs was 36% (43 of 119 individuals) in those who developed MI and 13% (18 of 138 individuals) in those who remained healthy (P<0.0001). The concentration of CICs was higher in those who developed MI (P<0.0001).

<table>
<thead>
<tr>
<th>TABLE 1. Prevalence and Concentration of CICs in Men Who Developed MI Compared With Men Who Remained Healthy</th>
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<tbody>
<tr>
<td>CICs</td>
</tr>
<tr>
<td>Prevalence, %</td>
</tr>
<tr>
<td>Concentration (mean±SD), protein mg/L</td>
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Statistical significance of group differences was determined by Fisher exact test.

Association of CICs With Other Risk Factors for MI

The association of CICs with other risk factors for MI is shown in Table 2. The concentration of CICs at age 50 years was associated with an increased risk for future MI, with an uncorrected odds ratio of 3.17 (95% CI 1.68 to 5.95, P<0.0001). The odds ratio corrected for the conventional risk factors (supine blood pressure, serum LDL/HDL cholesterol ratio, ratio between arachidonic acid and dihomo-γ-linoleic acid from serum cholesterol, body mass index, and smoking) was 2.95 (95% CI 1.52 to 5.75, P<0.0001).

The results from the Spearman rank correlation analysis (Table 3) show that the concentration of CICs at age 50 years was related not only to future MI but also to death by MI.

Levels of CIC and Antibodies to OxLDL and Cardiolipin in Men Who Developed MI

There was a positive correlation between the levels of CICs and IgG antibodies to cardiolipin (r=0.4, P=0.0001) in men who developed MI. There was no correlation between levels of CICs and IgA or IgM antibodies to cardiolipin or to levels of antibodies against oxLDL. The levels of IgG antibodies against cardiolipin was higher in men who developed MI and had CICs compared with men without CICs (P<0.0001) (Figure). The prevalence of elevated levels of IgG and IgM antibodies against cardiolipin was higher in patients who developed MI and had CICs compared with those without CICs (P<0.0001 and P<0.02, respectively) (Table 4).

Association of CICs With C4 Null Alleles in MI

The prevalence of elevated levels of CICs was higher in men who developed MI and were homozygous for C4Q0 (C4A*Q0 or C4B*Q0) than in men who developed MI and were heterozygous for C4Q0 (C4A*Q0 or C4 B*Q0) (P<0.03, Table 5). Among individuals homozygous for C4Q0, men who developed MI had higher concentrations of CICs (P<0.01) than did those who remained healthy.

<table>
<thead>
<tr>
<th>TABLE 2. Association of CICs With Other Risk Factors for MI</th>
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<tbody>
<tr>
<td>Parameter</td>
</tr>
<tr>
<td>Sensitivity</td>
</tr>
<tr>
<td>Specificity</td>
</tr>
<tr>
<td>Positive predictive value</td>
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<tr>
<td>Negative predictive value</td>
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Conventional risk factors for MI are supine blood pressure, serum LDL/HDL cholesterol ratio, ratio between arachidonic acid and dithomo-γ-linoleic acid from serum cholesterol, body mass index, and smoking.
Discussion

This prospective study shows CICs to be a strong independent risk factor for the development of future MI. Moreover, because there was a positive correlation between elevated levels of CICs and IgG antibodies to cardiolipin, the combination of CICs with autoantibodies to cardiolipin might further increase the pathogenic potential of these humoral factors.

A major obstacle in understanding the pathogenetic significance of CICs in MI has been that most earlier studies have been retrospective, and the formation of CICs could be related to the event. We have previously described a high prevalence of persisting CICs after premature MI. However, our present prospective study has shown that CICs are present before the MI and thus are likely to be more directly related to the event. What induces the formation of CICs in these cases is mostly unknown.

Several studies have shown that autoantibodies to epitopes of oxLDL exist in human serum, are present as part of immune complexes with oxLDL, and can recognize material in atherosclerotic lesions of rabbits and humans. However, we did not find a correlation between elevated levels of antibodies against oxLDL and CICs in the present study.

Several independent groups reported that LDL-containing immune complexes prepared in vitro or isolated from patients’ sera could induce intracellular accumulation of cholesterol esters. High levels of oxLDL-containing

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unadjusted</th>
<th>Adjusted (Partial)</th>
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<tbody>
<tr>
<td>CICs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>0.282</td>
<td>0.252</td>
</tr>
<tr>
<td>P</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Adjusted values were adjusted for presence of following risk factors: supine blood pressure, serum LDL/HDL cholesterol ratio, ratio between arachidonic acid and dihomo-γ-linoleic acid from serum cholesterol, body mass index, and smoking.

###TABLE 3. Spearman Rank Correlation Analysis of CIC Concentrations at 50 Years of Age (n=235) With Health Status of Individuals (Healthy, Survived MI, Death by MI) at 70 Years of Age

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unadjusted</th>
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<tr>
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Adjusted values were adjusted for presence of following risk factors: supine blood pressure, serum LDL/HDL cholesterol ratio, ratio between arachidonic acid and dihomo-γ-linoleic acid from serum cholesterol, body mass index, and smoking.

###TABLE 4. Percentage and Prevalence of Elevated Levels of Anti-CL, LDL, and oxLDL Antibodies in Men Who Developed MI and Had CICs Compared With Men Without CICs

<table>
<thead>
<tr>
<th>Elevated Levels, % (n)</th>
<th>MI With CICs (n=43)</th>
<th>MI Without CICs (n=76)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA-CL</td>
<td>14 (6)</td>
<td>5 (4)</td>
<td>NS</td>
</tr>
<tr>
<td>IgG-CL</td>
<td>21 (9)</td>
<td>3 (2)</td>
<td>0.001</td>
</tr>
<tr>
<td>IgM-CL</td>
<td>14 (6)</td>
<td>3 (2)</td>
<td>0.02</td>
</tr>
<tr>
<td>IgA-LDL</td>
<td>9 (4)</td>
<td>7 (5)</td>
<td>NS</td>
</tr>
<tr>
<td>IgG-LDL</td>
<td>7 (3)</td>
<td>11 (8)</td>
<td>NS</td>
</tr>
<tr>
<td>IgM-LDL</td>
<td>2 (1)</td>
<td>0 (0)</td>
<td>NS</td>
</tr>
<tr>
<td>IgA-oxLDL</td>
<td>14 (6)</td>
<td>7 (5)</td>
<td>NS</td>
</tr>
<tr>
<td>IgG-oxLDL</td>
<td>9 (4)</td>
<td>5 (4)</td>
<td>NS</td>
</tr>
<tr>
<td>IgM-oxLDL</td>
<td>2 (1)</td>
<td>1 (1)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Statistical significance of group differences was determined by Fisher exact test. Values within parenthesis are absolute number of individuals. CL indicates cardiolipin; NS, not significant.
immune complexes have been suggested to be a risk factor for the development of coronary arterial disease in patients with insulin-dependent diabetes mellitus, a fact that could not be deduced from the measurement of free oxLDL antibody concentrations and the interference of circulating oxLDL-containing immune complexes with the assay of free oxLDL antibodies. Thus, one pathogenic effect of autoantibodies to oxLDL might be participation in CIC formation. However, we did not analyze the constituent of CICs found in the present study.

In one previous report, CICs present in postinfarction patients were found to contain lipopolysaccharides from Chlamydia species. We have observed high persistent concentrations of CICs containing alimentary proteins in patients with precocious MI before 45 years of age (authors’ unpublished data, 2000).

The positive correlation between levels of antibodies to cardiolipin and levels of CICs would indicate that these autoantibodies are involved in the formation of CICs. Our study, such a defect was indeed accompanied by higher prevalence of CICs. Thus, as found in our earlier studies, this investigation also identified C4Q0 as one factor that predisposes to CICs.

In conclusion, increased prevalence and concentrations of CICs alone or in combination with raised levels of autoantibodies against cardiolipin in a cohort of healthy 50-year-old Swedish men predicted subsequent MI. The predictive power was strong and independent of that of other risk factors.

### Acknowledgments

This study was supported by grants from the Swedish Heart-Lung Foundation and the King Gustaf V:s 80-Years Foundation.

### References

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