Cholesterol Content of Circulating Immune Complexes in Patients with Coronary Stenosis and Subjects Without Evidence of Atherosclerosis

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The biological variation factors for cholesterol in circulating immune complexes (CIC-cholesterol) were studied in 941 unselected supposedly healthy volunteers, ages 4 to 78 years. We found a complex effect of age, including the existence of two peaks of CIC-cholesterol, one in males between 11 and 14 years and in females between 11 and 30 years, and in both sexes another peak between 41 and 60 years, and in both sexes a decrease between 31 and 40 years. By use of multiple regression analysis and after adjustment for age, CIC-cholesterol was positively related to plasma cholesterol concentration and leukocyte count, values being lower in females than in males and among subjects taking anti-inflammatory drugs. In addition, CIC-cholesterol was measured in 76 coronary angiography patients and in 100 supposedly healthy controls, ages 30 to 77 years. We noticed a significant increase (P ≤ 0.05) of CIC-cholesterol when patients were affected by coronary stenosis between 20% and 50% (71.8 ± 52.5 μmol/L vs 46.2 ± 45.9 μmol/L in controls), but this was less pronounced in those with >50% of obstruction (58.9 ± 54.3 μmol/L); however, serum total cholesterol was not modified or even surprisingly slightly decreased in the coronary angiography individuals. Nevertheless, an important overlap of values in controls and patients makes questionable the usefulness of this variable in clinical practice.

Indexing Terms: coronary stenosis/apolipoproteins/autoantibodies/variation, source of

The literature now supports the notion that oxidation of low-density lipoproteins (LDL) is important in the pathogenesis of atherosclerosis (reviewed in 1). A part of the atherogenic role of such modified LDL seems to be related to immunological mechanisms. Indeed, oxidative modification of LDL [e.g., fixation of malondialdehyde (MDA), an end product of lipid peroxidation] leads to the generation of autoantibodies against the epitopes created by this phenomenon; thus, autoantibodies specific for MDA-modified or oxidized LDL have been found in the blood of healthy subjects and patients with carotid or coronary atherosclerosis (2–5). The presence of oxidatively modified LDL in the blood, as documented by some authors (6–12), and of antibodies to these LDL (2–5), suggests that they could form circulating immune complexes (CIC). Internalization of LDL included in CIC through receptors for the nonlipoprotein part of the complex seems to lead inevitably to intracellular cholesterol accumulation and foam cell formation (13, 14). Tertov et al. (15, 16) observed a correlation between cholesterol content of CIC and atherogenic properties of sera of patients with coronary heart disease manifested in cell culture and thus suggested that the atherogenic potential of such sera was due to LDL-containing CIC.

CIC including lipoproteins were also studied for their potential interest as peripheral markers of atherosclerosis. Beaumont et al. (17) developed an ELISA involving antibodies to lipoproteins and to various immunoglobulins (Ig) and found that IgM–lipoprotein complexes might be markers for some familial hypercholesterolemia, and IgA–lipoproteins for the risk of atherosclerotic ischemic disease and deposition of lipids in the cornea. More recently, Orekhov et al. (18) attempted to clarify whether the cholesterol content of CIC correlated with the severity of coronary atherosclerosis and examined the diagnostic value of CIC-cholesterol in this field, in comparison with lipid and apolipoprotein measurements classically used; they found that CIC-cholesterol was the most reliable marker of coronary atherosclerosis and was able to diagnose extracoronary atherosclerosis with high accuracy.

Our aim in this work was to identify in an unselected population the factors explaining the interindividual biological variability of this analyte, there being so few data in the literature concerning this variable, and to reexamine the potential diagnostic usefulness of measuring CIC-cholesterol in atherosclerotic disease.

Materials and Methods

**Populations.** For the study of biological variation factors of CIC-cholesterol, the population sample consisted in 941 individuals, 461 males and 480 females, ages 4 to 78 years. These were unselected, supposedly healthy volunteers, attending the Center for Preventive Medicine of Vandoeuvre-lès-Nancy (France) for a health examination between October and December 1992. They were identified from the files of the State’s Health Insurance Fund in Nancy.

For the clinical study, the subjects were 76 patients, 64 men and 12 women ages 30 to 77 years, who were admitted in the Centre Hospitalier Régional de Metz-Thionville, Hôpital Notre Dame de Bon-Secours of

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5 Nonstandard abbreviations: MDA, malondialdehyde; CIC, circulating immune complexes; PEG, polyethylene glycol; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; and apo, apolipoprotein.

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Metz (France), for cardiovascular diseases. They had coronary insufficiency and underwent a coronary angiography for diagnostic purposes. Patients who had had a myocardial infarction within the previous 2 months were excluded. Patients were classified into three groups according to the degree of stenosis assessed by coronary angiography: >50% of stenosis in at least one main coronary artery, stenosis between 20% and 50%, and stenosis <20%. The healthy control sample was recruited from subjects who underwent a health check-up at the Center for Preventive Medicine. Inclusion criteria were: age between 30 to 77 years, a normal result for a resting 12-lead electrocardiogram, and the absence of typical pains and symptoms or history of cardiovascular disease, especially angina pectoris, myocardial infarction, or peripheral arterial or cerebrovascular disease. One hundred subjects (77 men and 23 women) fulfilled these inclusion criteria.

Information regarding smoking habits, alcohol consumption, drug intake, and medical history were obtained by questionnaires and interviews by physicians. The procedures followed were in accordance with the ethical standards of the Scientific Committee of the Center for Preventive Medicine and with the Helsinki Declaration of 1975, as revised in 1983.

**Blood samples.** Blood was collected from fasting subjects into heparinized, EDTA-containing, or dry vials (Becton Dickinson, Rutherford, NJ) between 0800 and 0900. The blood was centrifuged at 1000g for 15 min at 4 °C. Assay of CIC-cholesterol was made immediately after sampling.

**Assay of cholesterol in CIC.** To isolate CIC, we combined fresh sera (1 mL) with an equal volume of 50 g/L polyethylene glycol (PEG) 6000 as described (15, 16). After a 24-h incubation at 4 °C, immune complexes were sedimented by centrifugation (15 min, 1000g) and washed thrice with 25 g/L PEG 6000. Precipitates were then dissolved in 0.5 mL of a 9 g/L NaCl aqueous solution. The cholesterol in the solubilized CIC was determined by a manual enzymatic method with kits from Roche (Basel, Switzerland).

**Effect of PEG 6000 on free lipoproteins.** Free lipoproteins were prepared by sequential ultracentrifugation with a tabletop ultracentrifuge (Beckman TL 100 (Beckman, Gagny, France)) of serum from a healthy subject. VLDL, LDL, and HDL solutions (protein concentrations 0.5, 1.5, and 1.8 g/L, respectively) were treated by PEG 6000 to a final concentration of 25 g/L in a way similar to that applied to fresh sera.

**Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis.** The solubilized CIC were dialyzed for 12 h at 4 °C against phosphate-buffered saline (0.15 mol/L NaCl, 0.01 mol/L phosphate buffer, pH 7.4) containing 0.1 mmol/L EDTA. One volume of CIC was mixed with four volumes of a solution containing 10 mmol/L Tris-HCl, 300 mmol/L glycerol, 20 g/L SDS, and 2 mL/L mercaptoethanol. Samples were then heated to 100 °C for 5 min, and 20 μL (corresponding to ~4 μg of protein) was applied to polyacrylamide gradient gels (3–12% or 8–16%). For the standard containing apolipoprotein (apo) A-I, we used Certified Reference Material 391 (Community Bureau of Reference of the Commission of the European Communities, Brussels, Belgium). For apo B, we used lyophilized reference serum for apolipoproteins (ref. 4910005) from Immuno France (Orly, France). After electrophoresis, the proteins were transferred to nylon membranes (0.45-μm pore size; Bioblock, Illkirch, France) and detected by immunoblot analysis as described previously (10). For first antibodies we used rabbit anti-apo A-I (Behring, Marburg, Germany) and goat anti-LDL (Immuno France) antisera diluted to 1/1000 in a buffer, pH 8.5, containing 0.5 mL/L Tween 20, 20 g/L gelatin, 150 mmol/L NaCl, and 10 mmol/L Tris. For the second antibody we used alkaline phosphatase-conjugated anti-IgG antibodies (Sigma Chemical Co., St. Louis, MO), produced in rabbit or goat, diluted to 1/1000 in the same solution.

Similarly, we tested for the presence of immunoglobulins by using goat anti-IgA, goat anti-IgG, or goat anti-IgM antibodies diluted to 1/1000 in the same buffer. The alkaline phosphate-conjugated antibodies were purchased from Sigma.

**Other laboratory analyses.** Total cholesterol, triglycerides, and glucose were measured with commercially available kits (Merck, Darmstadt, Germany) on an AU 5000 apparatus (Merck). Apolipoproteins A-I, B, and E were determined by nephelometry with the BNA analyzer and reagents from Behring (Rueil-Malmaison, France) or Immuno France. Leukocytes were counted in a Technicon H 6010 analyzer (Bayer Diagnostics, Paris, France).

**Statistics.** Statistical analyses were performed with BMDP® statistical software (Los Angeles, CA).

Statistical significance of CIC-cholesterol variations between classes of age and sex was assessed by the F ratio calculated for log-transformed values. Standard stepwise multiple regression analysis was used to examine the relations between CIC-cholesterol and potential variation factors: gender, age (4–10, 11–14, 15–20, 21–30, 31–40, 41–50, 51–60, 61–70, 71–80 years), systolic blood pressure, Quetelet index (weight/height²), alcohol and tobacco consumption, plasma cholesterol and triglycerides, leukocyte count, and type of drug used (antihypertensive agent, cardiotoxic, hypolipidemic, diuretic, anti-angina, antibiotic, analgesic, anti-inflammatory).

In the clinical study concerning coronary atherosclerosis, the statistical significance of differences between controls and patients was tested with the Mann–Whitney rank sum test for CIC-cholesterol, or with the t-test for unpaired data for the other analytes.

**Results**

Apolipoprotein and immunoglobulin content of CIC. CIC isolated from 12 healthy subjects' sera were submitted to SDS-PAGE; the apolipoproteins were detected after immunoblotting with anti-LDL or anti-apo A-I polyclonal antibodies. Typical patterns are shown in Fig. 1. Fig. 1B gives evidence that apo A-I is present CLINICAL CHEMISTRY, Vol. 41, No. 10, 1995 1527
in CIC (lanes b and c). Similarly, although it appears extensively fragmented, apo B is found in the same CIC (Fig. 1A, lanes b and c). Besides the native apo B (apo B 100) and many minor fragments, a major fragment (apo B 74) with a molecular mass of 74% of that of the native apolipoprotein is present.

In the PEG precipitates we found IgG and IgM, but were not able to detect any IgA (data not shown).

**Effect of PEG 6000 on free lipoproteins.** No significant amounts of cholesterol were precipitated when free VLDL, LDL, or HDL was submitted to a PEG 6000 treatment similar to that applied to fresh sera.

**Analytical variability of the measurements of CIC-cholesterol.** Within-run repeatability was tested on four different sera with concentrations of CIC-cholesterol ranging from 29.8 to 49.4 μmol/L; for each serum the whole procedure, including the precipitation step, was repeated 26 times on the same day; the corresponding CVs were between 7.1% and 5.0%.

**Biological variation factors of CIC-cholesterol in unselected supposedly healthy subjects.** CIC-cholesterol values of the 941 nonselected supposedly healthy subjects ranged from 0 to 326 μmol/L. Arithmetic mean and geometric mean (since the distribution of values was asymmetrical) by gender and age are presented in Table 1.

Table 1. Males had higher concentrations of CIC-cholesterol than females (P ≤ 0.05), and the age-related variations were also statistically significant (P ≤ 0.05). In males, two peaks of CIC-cholesterol concentrations were observed: one between 11 and 14 years old and another between 41 and 60 years old. Age-related variations were relatively similar in women, CIC-cholesterol concentrations reaching maximum values in subjects ages 11–30 years and 51–60 years. In both sexes a decrease was observed between ages 31 and 40 years. Predictors of CIC-cholesterol values were identified by multiple regression analysis; only factors with statistical significance (P ≤ 0.05) are mentioned in Table 2. Five percent of the variance of CIC-cholesterol was explained by six factors: age between 11 and 14 years, plasma cholesterol concentration, and leukocyte count were positively related to its concentration, while ages between 31 and 40 years, female sex, and anti-inflammatory drug use were negatively related to it. The other tested variables, especially smoking and alcohol consumption, had no effect on CIC-cholesterol concentrations.

**CIC-cholesterol in atherosclerosis.** CIC-cholesterol was assayed in coronary angiography patients and in supposedly healthy subjects (control population). The coronary angiography patients were classified into three groups according to degree of coronary stenosis: <20% (group I), between 20% and 50% (group II), and >50% stenosis in at least one main coronary artery.
Table 3. Age, cholesterol content of CIC, and other biological variables in controls and coronary angiography patients.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 100)</th>
<th>Group I (n = 10)</th>
<th>Group II (n = 11)</th>
<th>Group III (n = 55)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>59.3 ± 10.3</td>
<td>54.2 ± 12.5*</td>
<td>58.1 ± 8.3</td>
<td>60.3 ± 10.7</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>6.25 ± 1.07</td>
<td>6.21 ± 0.94</td>
<td>6.26 ± 1.28</td>
<td>5.89 ± 1.34*</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.44 ± 0.63</td>
<td>1.91 ± 0.76†</td>
<td>1.69 ± 0.51*</td>
<td>1.87 ± 0.62*</td>
</tr>
<tr>
<td>Apo A-I, g/L</td>
<td>1.65 ± 0.28</td>
<td>1.60 ± 0.23</td>
<td>1.60 ± 0.34</td>
<td>1.39 ± 0.27*</td>
</tr>
<tr>
<td>Apo B, g/L</td>
<td>1.36 ± 0.29</td>
<td>1.43 ± 0.27</td>
<td>1.45 ± 0.30</td>
<td>1.34 ± 0.34</td>
</tr>
<tr>
<td>Apo E, ng/mL</td>
<td>6.69 ± 1.97</td>
<td>6.70 ± 2.40</td>
<td>6.09 ± 1.44</td>
<td>6.29 ± 1.99</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>5.30 ± 0.97</td>
<td>5.65 ± 1.94</td>
<td>4.99 ± 1.00</td>
<td>5.49 ± 1.73</td>
</tr>
<tr>
<td>Leukocytes × 10⁹/L</td>
<td>7140 ± 2083</td>
<td>7350 ± 2422</td>
<td>7764 ± 1473</td>
<td>8236 ± 2443*</td>
</tr>
<tr>
<td>Cholesterol of CIC, μmol/L*</td>
<td>46.19 ± 45.98</td>
<td>33.75 ± 38.83</td>
<td>71.83 ± 52.56*</td>
<td>58.96 ± 54.34*</td>
</tr>
</tbody>
</table>

* a, b, c Degree of stenosis: * <20% stenosis; † stenosis between 20% and 50%; ‡ >50% stenosis in at least one main coronary artery.

d Log transformation to increase normality.

**a, ‡ Significantly different from controls (unpaired t-test): * P ≤ 0.05, † P ≤ 0.01, ‡ P ≤ 0.001.

(group III). Mean values (±SD) of age, CIC-cholesterol, and various blood analytes in the different groups of subjects are given in Table 3. The values obtained in patients were compared with those of the controls. No variation of plasma total cholesterol was noticed in groups I and II, whereas it was slightly diminished in group III (P ≤ 0.05). Triglycerides were significantly increased in all patient groups. Apo B and apo E were not different in patients and controls. Apo A-I was strongly diminished in group III (P = 0.001). Blood leukocytes were significantly increased in the patients. CIC-cholesterol was lower in patients of group I than in controls, although this difference was not statistically significant. Conversely, CIC-cholesterol was increased in groups II and III (P ≤ 0.05) in comparison with group I (more in group II than in group III).

Figure 2 shows the values of CIC-cholesterol for individuals of the various groups. An important overlapping of the distributions of CIC-cholesterol concentrations in the various groups is noticed; however, 40.0% and 50.0% of the control subjects and patients of group I, respectively, had values <18 μmol/L, whereas 9.1% and 20.0% of patients of groups II and III, respectively, had CIC-cholesterol concentrations under this limit.

Discussion

Oxidation of LDL is thought to play a principal role in the pathogenesis of atherosclerosis (1). In previous years, immunological implications of this phenomenon have been the topic of a number of studies (2, 3, 13–18), and previously, Orekhov et al. (18) proposed as markers of atherosclerosis lipoprotein immune complexes, which were evaluated by the measurement of cholesterol in total CIC. In the present work, we studied the cholesterol content of CIC and its potential factors of variation in supposedly healthy subjects; we also reexamined the possible usefulness of this determination in the diagnosis of coronary atherosclerosis.

Immune complexes were isolated by a classical method involving PEG 6000 precipitation (19). Confirming previous results of Tertov et al. (15), we demonstrated that PEG 6000 at a final concentration of 25 g/L does not precipitate free lipoproteins. Study of the apolipoprotein content of total CIC by electrophoresis and immunoblot techniques shows that both HDL and LDL are present in the isolated CIC. This is not in agreement with the statements of Tertov et al. (15), who did not find appreciable amounts of apo A-I in their PEG precipitates. We have no clear explanation concerning this discrepancy. However, the presence of HDL in CIC, beside that of LDL, is not surprising, given that HDL may undergo oxidation (20–23) that could provoke genesis of autoantibodies directed to the corresponding structural modifications. Besides, as has been previously shown, oxidative modification of HDL may stimulate development of atherosclerosis by limiting efflux of cholesterol from foam cells (24). Why apo B in CIC appeared fragmented is not known with certainty, although the role of oxidation phenomena may be hypothesized (25). Such a fragmentation of apo

![Fig. 2. Distribution of CIC-cholesterol in different groups of subjects. Controls, supposedly healthy subjects; Groups I, II, III, coronarographied patients with coronary stenosis <20%, between 20% and 50%, and <50%, respectively.](image-url)
B was observed in a previous report (10) in LDL isolated from the serum of atherosclerotic patients.

To our knowledge, there are no published data concerning CIC-cholesterol in a large population of healthy subjects. Multiple regression analysis allowed us to explain only ~5% of the biological variance of CIC-cholesterol in such a population. Beside a complex effect of age, a state of inflammation, as approximated by leukocyte count, seems to increase CIC-cholesterol; this hypothesis is confirmed by the effect of anti-inflammatory drugs, which clearly decrease its concentration. Note that CIC-cholesterol and serum total cholesterol were correlated in the supposedly healthy subjects, whereas the study conducted in patients hospitalized for cardiovascular disease demonstrated no clear association between these two variables (see below). Indeed, CIC-cholesterol was measured in patients who underwent coronary angiography, and in supposedly healthy subjects of the same age class (controls). CIC-cholesterol was lower in patients with stenosis <20% (Group I) than in controls, but the difference was not statistically significant. This fact must be analyzed carefully since the size of the group I is limited (10 subjects); in addition, it cannot be excluded that the control group, supposedly healthy individuals showing no evidence of risk of coronary disease who did not undergo the angiography, might include nevertheless a few subjects with some degree of coronary stenosis. Conversely, we found a significant increase of CIC-cholesterol in patients with >20% of coronary stenosis, which was surprisingly less pronounced in those with >50% obstruction. These results are consistent with those obtained by Orekhov et al. (18) in similar populations.

A large interindividual variability for CIC-cholesterol noticed in all the examined groups, as shown in Fig. 2, leads to an important overlap of values between controls and patients. Therefore, it is difficult to establish a cutoff value suitable for diagnostic purpose. Thus the concentration of 15 mg/L (39 µmol/L) proposed by Orekhov et al. (18) as the highest value of normal CIC-cholesterol appears inadequate for use in the present study: 40% of our control population and only 54.5% of patients with coronary stenosis >20% have a value beyond this threshold. Although the cutoff value of 18 µmol/L increases the sensitivity of the test, this does not allow us to propose the use of CIC-cholesterol determination in clinical diagnosis. CIC-cholesterol should be rather a marker of evolution or progression of atherosclerosis than an indicator of the size of the atheromatous plaques, at the time of serum sampling; this could explain the existence of high concentrations of CIC-cholesterol in some control subjects and of low concentrations of this analyte in patients with severe coronary atherosclerosis but perhaps without evolving lesions. As Salonen et al. (3) showed, the amount of autoantibody to oxidized LDL was a predictor of the progression of carotid atherosclerosis and was not a simple reflection of lesion size.

In this study, contrary to CIC-cholesterol, total cholesterol and triglyceride concentrations are not related to the coronary angiography criteria. Apo A-I appears diminished only in patients with coronary stenosis >50%, whereas apo B and apo E do not vary significantly in the different groups; however, most of the patients were receiving drug treatments for their cardiovascular disease, which may influence the circulating concentrations of lipids and apolipoproteins. Thus, the fact that most of the patients were treated by hypolipidemic agents could explain that, contrary to the current dogma, total cholesterol concentrations were identical or even slightly diminished in the patients, compared with those measured in the control subjects.

In conclusion, the origin of the interindividual biological variability of CIC-cholesterol is not completely elucidated and has to take into account the role of the immune system in atherogenesis (26). Nonetheless, this analyte appears related to heart vessel lesions as assessed by coronary angiography, contrary to the more classical measurements of cholesterol, triglycerides, apo A-I, and apo B. Unfortunately, the large overlapping of distributions of values of CIC-cholesterol in healthy subjects and patients with atherosclerosis makes questionable the usefulness of this variable in clinical practice.

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